Gut microbiome and innate immune response patterns in IgE-associated eczema

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Abstract

Background: Gut microbiome patterns have been associated with predisposition to eczema potentially through modulation of innate immune signaling.

Objective: We examined gut microbiome development in the first year of life in relation to innate immune responses and onset of IgE-associated eczema over the first 2.5 years in predisposed children due to maternal atopy [www.anzctr.org.au, trial ID ACTRN12606000280505].

Methods: Microbial composition and diversity were analyzed with barcoded 16S rRNA 454 pyrosequencing in stool samples in pregnancy and at ages 1 week, 1 month and 12 months in infants (n=10) who developed IgE-associated eczema and infants who remained free of any allergic symptoms at 2.5 years of age (n=10). Microbiome data at 1 week and 1 month were analyzed in relation to previously assessed immune responses to TLR 2 and 4 ligands at 6 months of age.

Results: The relative abundance of Gram-positive *Ruminococcaceae* was lower at 1 week of age in infants developing IgE-associated eczema, compared with controls (p=0.0047). At that age, the relative abundance of *Ruminococcus* was inversely associated with TLR2 induced IL-6 (-0.567, p=0.042) and TNF-α (-0.597, p=0.032); there was also an inverse association between the abundance of Proteobacteria (comprising Gram-negative taxa) and TLR4 induced TNF-α (rs= -0.629, p=0.024). This relationship persisted at 1 month, with inverse associations between the relative abundance of *Enterobacteriaceae* (within the Protebacteria phylum) and TLR4 induced TNF-α (rs=-0.697, p=0.038) and *Enterobacteriaceae* and IL-6 (rs=-0.709, p=0.035). Mothers whose infants developed IgE-associated eczema had lower α-diversity of Bacteroidetes (p=0.04) although this was not seen later in their infants. At 1 year, α-diversity of Actinobacteria was lower in infants with IgE-associated eczema compared with controls (p=0.002).

Conclusion and clinical relevance: Our findings suggest that reduced relative abundance of potentially immunomodulatory gut bacteria is associated with exaggerated inflammatory cytokine responses to TLR ligands and subsequent development of IgE-associated eczema.

Background

The environmental changes responsible for the sharp rise in the prevalence of allergic disease remain to be clearly defined, but one now almost unassailable hypothesis has been the loss of "old friends" based on the observed alterations of early gut microbiota and lower biodiversity in children subsequently developing allergic disease (1-3). The steep maturation of both the innate and adaptive immune systems in the postnatal period (4), occurs in tandem with progressive changes in the gut microbiome during this early period (5). Compelling evidence from animal models show that microbial exposure is key for normal immune development (6). In humans, neonatal responses also undergo environmental-driven Th1 maturation in the early postnatal period (7, 8). We have previously proposed that such exposures might be even more important to counter allergic propensity in predisposed subjects (7). Our more recent studies provide emerging evidence that children with allergic disease have presymptomatic altered differences in their innate responses to microbial components, and that these dysfunctional patterns of response are associated with aberrant immune maturation of adaptive T cell responses to allergens (9, 10).

The Toll-like receptors (TLR) are key microbial pattern-recognition receptors (11). TLR-mediated innate immune response pathways are also thought to be critical for the induction of regulatory pathways that suppress immune responses in allergic disease. Recently, regulatory T cells (Tregs) have been shown to express TLRs and TLR activation can increase or decrease their suppressor activity, thus providing an important link between innate and adaptive immunity. Genetic variations in innate immunity genes such as TLR2 and TLR4 have been reported to be associated with a range of Th2-mediated inflammatory disorders including atopic eczema (12, 13), and asthma (13). In our previous reports, we have shown that normal children show progressive maturation of their TLR mediated (TNF-α and IL-6) microbial

responses, in parallel with Th1- maturation, consistent with the hypothesis that this is driven by early microbial exposure (10). Curiously, we found that allergic children instead have exaggerated inflammatory microbial responses in the neonatal period (9, 10), which wane with age and fail to induce the level of robust Th1 responses seen in non-allergic children (10). We speculate that neonatal differences in immune function may be at least in part related to differences in maternal bacterial exposures *in utero* (1, 14), with subsequent difference in infant colonization thereafter- both contributing to the differences in postnatal immune maturation. Providing some support for this we have seen that neonates born in high microbial traditional environments have well modulated, relatively quiescent innate inflammatory responses (also in cord blood) compared with those born in a low microbial Western environment (15). This suggests a role of early microbial exposure in both the set-point for innate immune function as well as the maturation thereafter. However, none of these previous studies provided a direct measure of microbial colonization.

In the present study we have taken the unique opportunity of exploring innate immune development in relationship to longitudinal intestinal colonization in a subgroup of children in a previous prospective cohort who had stool samples collected prospectively (16). Using a case-control study of infants predisposed by maternal atopy, we aimed at determining the early establishment of bacteria and microbial diversity in stool in relation to development of IgE-associated eczema in the first 2.5 years of life. For this, we used barcoded 16S rRNA 454 pyrosequencing which allows detection of microbiota to the genus level (17). We also analyzed the relationship between early gut microbiota establishment and previously assessed innate immune responses following activation with specific microbial ligands for TLR2 and TLR4, as the principal pathways of microbial recognition for Gram-positive (TLR2) and Gram-negative bacteria (TLR4), respectively (18).

Methods

Study population and clinical assessments

We randomly selected 10 children with IgE-associated eczema and 10 non-allergic children that were matched for delivery mode and intervention (probiotic/placebo) from a randomized placebo-controlled trial investigating the effects of probiotics in primary prevention of allergic disease. As previously described (16) 231 atopic, pregnant women were recruited and their infants were given $3x10^9$ CFU *Lactobacillus acidophilus* (LAFTI L10® /LAVRI-A1), Probiomics, Australia or a placebo supplement daily for their first 6 months of life. Hundred and seventy-eight infants completed the intervention phase. Ethical approval for the study was granted by Princess Margaret hospital for children (PMH), King Edward Memorial Hospital (KEMH), St John of God Hospital (SJOG) and Mercy Hospital Ethics committees, and all women gave informed written consent. The study was registered at www.anzctr.org.au, trial ID ACTRN12606000280505.

Children were clinically assessed at 6 and 12 months and 2.5 years of age by one pediatric allergist (Prof. S.L. Prescott) (16, 19). The assessment included a detailed history, allergy skin prick testing (SPT) and physical examinations. All children included in this sub-study were clinically assessed at 6 and 12 months, and all but two non-allergic children attended the follow-up visit at 2.5 years. However, these two children were clinically assessed at 5 years (including detailed history, SPT and physical examinations) and their non-allergic status was confirmed (20). A child was classified as having 'allergic disease' if he/she had a physician diagnosis of IgE-mediated food allergy, eczema, asthma or allergic rhinitis at these assessments. As previously described (16, 19), the diagnosis of IgE-mediated food allergy included a history of immediate (within 60 minutes) symptoms after contact with and/or ingestion of food and a positive skin prick test to the implicated food. Infants with typical skin

lesions responsive to topical steroids were diagnosed with eczema; the severity determined using the SCORAD index (21). Asthma was diagnosed if the child had recurrent wheeze (at least 3 episodes) and responded to bronchodilator therapy. Allergic rhinitis was diagnosed if the child had a clinical history suggestive of allergic rhinitis with evidence of sensitization to inhalants. Sensitization was determined by the presence of a positive SPT response to allergen extracts; cow's milk, peanut, house dust mite, cat, grass, mold (Hollister-Stier Laboratories) and whole egg. Histamine and glycerine served as positive and negative control, respectively. A wheal diameter of ≥ 3 mm was regarded positive. Parents recorded data on other symptoms and environmental exposures on diary cards (16, 19).

Blood sampling and innate immune function

Venous blood was drawn to assess functional TLR responses as previously described (18). Briefly, the pattern and magnitude of cytokine responses following activation with specific microbial ligands for TLR2 (Pansorbin) and TLR4/CD14 [lipopolysaccaride (LPS)] were assessed. PBMC ($1\mathbf{x}10^6$ /mL) were cultured in duplicate in 96-well round-bottomed plates in RPMI plus 10% fetal calf serum (not heat inactivated) (Australian Biosearch, Perth, Australia) either alone or with optimized doses of Pansorbin (0.1%; Calbiochem**s** , San Diego, CA, USA) or LPS (Escherichia coli LPS, Sigma, Castle Hill, Australia; 10 ng/mL) for 24 h at 37º C with 5% CO2 . After 24 h, the supernatants were collected and stored at **-**20º C for cytokine analysis by ELISA or time-resolved fluorometry (TRF) as previously described (18).

Stool sampling procedures

Stool samples were collected from the pregnant mother at inclusion and the infant at 1 week, 1 month, and 12 months of age. All fecal samples were collected within 5-10 minutes of a

bowel motion, placed in sterile containers and frozen immediately before they were transported on dry ice to the hospital. Fecal samples were stored frozen at -20°C.

DNA extraction

DNA from fecal samples from participants was extracted with Ultra clean fecal DNA isolation (MoBio, Naxo ltd, California, USA) from 100 mg of each fecal sample according to the manufacturer's instructions. Final elution was made with 2x50 µl elution buffer (solution S5).

PCR and primers

For each sample, three 50 µl PCR mixes were prepared containing 1X PCR buffer, 200 µM dNTP PurePeak DNA polymerase (Pierce Nucleic Acid Technologies, Milwaukee, USA), 50 mM of each primer, 0.5 U Phusion F-530L enzyme (Finnzyme, Massachusetts, USA) and 1 µL template DNA for the fecal samples.

Primer pairs used amplify the hypervariable 16 rRNA regions V3-V4 were 341f (5'CCTACGGGNGGCWGCAG) with adaptor B and 805r, (5' GACTACHVGGGTATCTA ATCC) with adaptor A, and a sample specific sequence tag of 7 nucleotides as a development of previous study conducted by Andersson et al (17). A PCR negative template control was also made for each primer pair. The PCR conditions used were 95°C for 5 minutes, followed by 25 cycles of 95°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min, followed by a final extension of 72°C for 7 min for the fecal samples.

Purification, quality control and pyrosequencing

The samples and the negative template control were then run on an agarose gel (1% w/v in TBE buffer) for quality control. The three PCR reactions were pooled and for the fecal samples 45 µl of each pooled PCR reaction was purified using Agencourt AMPure beads

(Beckman Coulter, California, USA) according to manufacturer's instructions with final elution in 15 µl 1X TE buffer. The purified PCR products were diluted to a concentration of approximately 3 ng/µl before pooling them together for the multiplexed pyrosequencing. All concentrations were determined using the Qubit system (Invitrogen, California, USA). The DNA pools were finally sequenced on the 454-FLX GS-100 using Titanium kit (Roche 454 Life Sciences, Branford, CT, USA) (22).

Sequence processing, classification and data analysis with Ampliconnoise-pipeline

After splitting raw sequence files into individual samples, sequences were denoised using the AmpliconNoise tools for 454 and PCR artifacts as well as PCR chimaeras (23). This resulted in 225 652 high-quality reads, with 1169-15324 (mean 3526) reads per sample. Subsequently, sequences from all samples were coclustered using FCluster in the AmpliconNoise distribution with complete linkage clustering to identify 937 universal operational taxonomic units (OTUs) at the 97% identity threshold. The taxonomic identity of each OTU was estimated by aligning representative sequences against the SILVA database (24) using the SINA aligner (25), parameters set at default values.

Statistics

The relative abundance of different taxa (RA), the Shannon diversity index, the Pielou's evenness index and the Bray-Curtis dissimilarity index were calculated for all samples based on the observed OTUs **(Supporting information 1)** (26-28). Due to small group sizes, data were not subsampled before calculating the Shannon diversity index and RA. Since the Pielou's evenness index and the Bray-Curtis index require equal number of reads, data were subsampled without replacement to compensate for unequal sequencing depth. All samples were rarefied to 881 sequence reads that were used to calculate the Pielou's index and the Bray-Curtis index. For each considered taxa the relative abundance was estimated with the

number of reads taxonomically classified to the taxa divided with the sample's total number of reads. Differences in microbiome patterns between groups of individuals, i.e. IgE-associated eczema versus controls, as well as vaginally delivered versus caesarean section (CS) born infants, were studied using non-parametric techniques including Wilcoxon's rank sum test with a two-sided alternative hypothesis. For the Bray-Curtis index a modified t-test was used (29). Correlations between cytokine responses and the relative abundance were estimated using Spearman's rank correlation. Principal component analysis (PCA) was calculated based on relative abundance at the phylum level to assess separation between IgE-associated eczema and controls. All statistical analyses were done using R software version 3.0.2 including the R package vegan, except for baseline demographics that were assessed by Fisher's exact test and Student's t-test as appropriate, using SPSS version 21 for Macintosh. A p-value< 0.05 was regarded statistically significant.

Results

Demographic characteristics

Demographic characteristics are displayed in **Table 1**. Children with IgE-associated eczema had a doctor's diagnosis of eczema at 1 or more of the study time points with evidence of sensitization (positive SPT response) whereas the non-allergic control group had no allergic disease and no sensitization. The majority (n=7) was diagnosed with eczema already at the 6 months study visit, and 5 children also had a diagnosis of IgE-associated food allergy **(Table 1)**. Only one child was also diagnosed with allergic rhinitis and asthma. Blood samples had been obtained from all children at 6 months of age for innate immune function studies (18). Stool samples that yielded microbiome data were available from 19 mothers and from 14, 11 and 20 children at 1 week, 1 month and 1 year of age, respectively (**Table 2).**

Development of the gut microbiome during the first year

We followed gut microbiome development from 1 week to 1 year of age in relation to development of IgE-associated eczema during the first 2.5 years of life. Generally, there was a high inter-individual variability in colonization and acquisition of specific taxa during the first year of life (**Table 2**). At 1 week of age, infants who subsequently developed IgE-associated eczema showed a pattern of lower abundance of Proteobacteria, and within this phylum specifically lower abundance of *Enterobacteriaceae* and *Escherichia-Shigella* compared with controls, although this difference did not reach statistical significance (**Table 2**). However, there was a statistically significantly lower relative abundance of *Ruminococcaceae* (p=0.0047) at 1 week of age in infants subsequently developing IgE-associated eczema, compared with controls (**Table 2**). None of the infants who developed IgE-associated eczema had detectable *Ruminococcaceae* in stool at that age, whereas this taxon was present in 7/8

(88%) of the infants that did not develop any allergic manifestations (p=0.06, Fisher's exact test).

By 1 month, Actinobacteria was the most abundant phylum in both groups, with a predominance of *Bifidobacterium* (**Table 2**). All but one infant was breastfed at that age, which is likely to explain this predominance (30). The relative abundance of Bacteroidetes remained low in both groups throughout the first year of life (**Table 2**) with no differences between the groups.

Gut microbiome pattern in pregnant atopic mothers

The predominant phylum in mothers was Firmicutes (**Table 2**). Mothers whose infants subsequently developed IgE-associated eczema had a higher relative abundance of the Bacilli class (p=0.022) and the genus *Streptococcus* (0.043) compared with mothers whose infants remained non-allergic (**Table 2**). They also had lower α-diversity of Bacteroidetes as assessed by the Shannon diversity index, compared with mothers whose infants remained non-allergic **(Table 3)**.

Correlations between the relative abundance and innate immune responses in infants

To investigate the impact of early colonization patterns in relation to development of innate immunity we explored associations between the relative abundance of specific taxa at 1 week and 1 month, and innate immune responses at 6 months of age (18) in the combined study population. First, we explored associations between the relative abundance at the phylum level and cytokine patterns. At 1 week of age, there was an inverse association between the abundance of Proteobacteria (comprising Gram-negative taxa) and TLR4 induced TNF-α (rs= -0.629, p=0.024). This relationship persisted at 1 month of age, with inverse associations

between the relative abundance of *Enterobacteriaceae* (within the Protebacteria phylum) and TNF-α (rs=-0.697, p=0.038) and *Enterobacteriaceae* and IL-6 (rs=-0.709, p=0.035).

At the genus level there were inverse associations between Gram-positive *Ruminococcus* at 1 week of age and TLR-2 induced IL-6 (-0.567, p=0.042) and TNF-α (-0.597, p=0.032). At that age, there was also an inverse association between the lactic acid bacterium (LAB) *Leuconostoc* and TLR-2 induced TNF-α (-0.547, p=0.049). At 1 month, the relative abundance of *Enterococcus* was inversely associated with TLR-2 induced IL-10 (-0.669, p=0.047) whereas the relative abundance of *Actinomyces* was associated with TLR-2 induced TNF-α (rs=0.701, p=0.037).

Next we explored associations between "protective" genera such as *Bifidobacterium* and *Lactobacillus* and TLR-2 induced cytokine production, and potentially "harmful" genera such as *Clostridium* and *Staphylococcus* and TLR-2 induced cytokine production, but no evident associations emerged (data not shown).

Diversity, evenness and development of IgE-associated eczema

We analyzed α-diversity by the Shannon diversity index at each respective age. At 1 year of age, the α-diversity of Actinobacteria was lower in infants with IgE-associated eczema compared with controls (p=0.002) **(Table 3).** As expected, the overall α-diversity increased with age in both groups of infants, but still remained lower at 1 year of age compared with their mothers. The overall α-diversity was lower at 1 week of age in infants developing IgE-associated eczema, but this difference did not reach statistical significance **(Table 3).** We also analyzed evenness by the Pielou's evenness index, although our small sample size is a limitation and warrants conservative interpretation. However, this revealed a similar pattern

for Actinobacteria, with lower evenness in infants developing IgE-associated eczema ($p=0.038$) at 1 year of age **(Supporting information, Fig S1)**. ß-diversity was then analyzed using the Bray-Curtis index; no differences were observed when comparing the average ß-diversity within the IgE-associated eczema group and the control group **(Supporting information, Fig S2).** Next, we performed a PCA analysis but no evident separations according to IgE-associated eczema occurred **(Supporting information, Fig S3)**.

Effects of delivery mode on gut microbiome establishment

Since delivery mode has been reported to impact intestinal colonization (31-33) we also examined the gut microbiome in relation to delivery mode, revealing a number of significant differences even in this small sample size **(Fig 1).** Early in the neonatal period (at 1 week) the most significant compositional differences seen for CS born children were greater relative abundance of Proteobacteria ($p=0.041$) and lower relative abundance of Bacteroidetes compared with VD infants. Bacteroidetes remained in lower abundance throughout the first year of life in CS infants (**Fig 1**); statistically significantly so at 1 week ($p=0.024$) with the same trend at 1 month of age ($p=0.092$). Within this phylum, the relative abundance of the genus *Bacteroides* was lower in CS infants at 1 week ($p=0.024$) than in VD infants (data not shown). Compared with VD infants, the overall α-diversity was lower in CS infants in the first year of life although this difference did not reach statistical significance **(Fig 1)**. However, α-diversity of Bacteroidetes was lower at 1 week (=0.031) and 1 month ($p=0.015$) in CS infants **(Fig 1).** Evenness assessed by Pielou's index revealed a similar pattern, although the differences did not reach statistical significance **(Supporting information, Fig S4).** No difference in ß-diversity was observed when comparing the VD and CS groups (data not shown).

Discussion

We have previously demonstrated dysregulation of neonatal innate immune responses in infants who subsequently developed allergic disease, with exaggerated inflammatory responses to to TLR-ligands (9, 10). In contrast, we have seen that neonates born in a high microbial environment show more quiescent innate inflammatory responses than neonates born in a low microbial environment (15), suggesting modulation of these responses by microbial exposure. Consistent with these observations, here we show that reduced relative abundance of a number of potentially immunomodulatory gut bacteria are associated with exaggerated inflammatory cytokine reponses to TLR ligands. Specifically, we saw that a low abundance of *Enterobacteriaceae* at 1 month of age is associated with exaggerated TLR4 induced TNF-α and IL-6 responses at 6 months. These findings are further supported by a pattern of a lower relative abundance of *Enterobacteriaceae* and *Escherichia-Shigella* already at 1 week of age in infants subsequently developing the allergic phenotype. At that age, a low relative abundance of Proteobacteria (comprising *Enterobacteriaceae)* was associated with high TLR4 induced TNF-α responses a 6 months. Abrahamsson et al (34) previously reported lower α-diversity of Proteobacteria at 1 year in infants developing IgE-associated eczema, which could also be consistent with our findings.

Classical studies using culture methods (35), have shown that facultative anaerobic bacteria e.g. *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Enterobacteriaceae* reach high counts within the first days of life thereby creating an environment allowing the gradual establishment of obligate anaerobes. Reduced enterobacterial colonization in infancy has been reported in Western societies and is suggested to be a consequence of an overly hygienic lifestyle (36). *Enterobacteriaceae* are a large family of Gram-negative bacteria, including *Escherichia coli (E. coli)* which is specialized in colonizing the human intestine (35). In

contrast to adults, other members of the *Enterobacteriaceae* e.g. *Klebsiella* and *Enterobacter* are common colonizers of the infant gut (35), especially in CS infants (36). As assessed by 454 pyrosequencing, a lower relative abundance of *Enterobacteriaceae* was reported in non-eczema infants compared with eczema infants, but only if they were CS born (37), suggesting that the impact might be variable according to delivery mode. This was not further investigated in our study because of small group sizes. Early *Klebsiella* and *Enterobacter* colonization have not been extensively studied in the context of allergic disease, and the role of *E.coli* colonization is conflicting. Using quantitative PCR (q-PCR), (38) the presence of *E.coli* in stool samples at 1 month of age was associated with increased risk of developing eczema whereas another prospective cohort study using culture-based methods did not observe such an association (39). Then again, early *E.coli* colonization (assessed by q-PCR) was associated with reduced risk of sensitization, but not eczema (40). There is also emerging interest in specific strains of *E.coli* for allergy prevention. In experimental models, the *E.coli* Nissle 1917 strain (EcN) prevented allergic skin disease (41) and induced a pro-Th1/Treg adjuvant profile which was reported to be dependent on the TLR4 signaling pathway (42). Here, we show that the relative abundance of *Enterobacteriaceae* at 1 month of age is inversely associated with TLR4 induced TNF-α and IL-6 responses at 6 months, thereby suggesting a modulating effect by early, postnatal *Enterobacteriaceae* colonization on innate immune response patterns. However, to what extent other biodiversity exposures (2), including LPS in the surroundings (43) modulated these patterns were not monitored. Our results also need to be interpreted in light of the potential effect-modifying role of genetic variations in the relationship between the intestinal microbiome and allergy development (44), and may therefore not be fully generalizable.

We also observed a low relative abundance of *Ruminococcaceae* at 1 week of age in infants that developed IgE-associated eczema, and that a low abundance of the genus *Ruminococcus* at that age was associated with exaggerated TLR-2 induced IL-6 and TNF-α responses at 6 months. *Ruminococcaceae* is together with *Lachnospiraceae*, the most abundant Firmicute families in the adult mammalian gut (45), and have been associated with the maintenance of gut health. There is emerging interest in the role of *Ruminococcus* colonization in infancy (46). A possible health benefit is the production of ruminococcins, such as ruminococcin A, which is a bacteriocin that can inhibit the development of *Clostridium* species (47). In animal models, oligosaccharides increased intestinal colonization with ruminococci (48). Breast milk is plentiful in human milk oligosaccharides (49) and may promote *Ruminococcus* colonization, but since all but one infant was breastfed at 1 week of age, differences in feeding mode are not likely to explain our finding.

There was reduced α-diversity and evenness of Actinobacteria (comprising *Bifidobacterium*) at 1 year of age in infants developing IgE-associated eczema. More infants with IgE-associated eczema were still breastfed at that age (although this difference was not statistically significantly different). To what extent breastfeeding influenced this pattern was not examined due to small group sizes, but could be consistent with previous findings of reduced microbiome richness in breastfed compared with formula-fed 4-months-old infants as recently reported by Azad et al (31). Since high bifidobacterial diversity assessed by q-PCR in early life has been reported to augment mucosal SIgA maturation (50), and SIgA appears to be protective against development of allergic disease (1), our findings warrant further study in larger cohorts that can examine this in relation to environmental exposures such as infant feeding.

CS delivery was associated with delayed Bacteroidetes colonization, and reduced α-diversity of Bacteroidetes at 1 week and 1 month of age. This is in agreement with recent findings from other studies using 454 pyrosequencing (31, 33). Low intestinal microbial diversity have also been reported before onset of atopic eczema (34, 51, 52), asthma (53), sensitization, allergic rhinitis and eosinophilia (54). Abrahamsson et al (34) reported lower α-diversity of Bacteroidetes at 1 month of age in infants that were later diagnosed with atopic eczema compared with infants that remained healthy. Compared with pregnant mothers of healthy infants, we observed lower α-diversity of Bacteroidetes in stool samples of mothers whose infants developed IgE-associated eczema, although this was not seen later in their infants. However, the higher frequency of CS deliveries in both the group of infants that developed IgE-associated eczema and controls in our study, is likely to have impacted diversity measures (33) and might have levelled a potential difference. In comparison with previous studies including adults (5, 55), the relative abundance of Bacteroidetes in the atopic, pregnant mothers in this study was low. A reduced relative abundance of Bacteroidetes and a reduced Bacteroidetes/Firmicutes ratio have been reported to be associated with other inflammatory non-communicable diseases (56) and it is plausible that the mothers' atopic status could have contributed to this finding.

In stool samples collected in the third trimester, we also found that mothers whose infants subsequently developed IgE-associated eczema had a higher relative abundance of the Bacilli class and the genus *Streptococcus* compared with mothers whose infants remained non-allergic. In addition to other compositional shifts, enrichment of *Streptococcus* in the third trimester and postpartum have been demonstrated (55). Recently, a meconium microbiome type dominated by lactic acid bacteria with *Enterococcus, Lactococcus, Staphylococcus* and *Streptococcus* genera, was reported to be associated with infant mucus congestion (57)

suggesting that the pioneering gut microbiome may impact child health.

This study has several strengths such as the prospective design, repeated stool samplings for analysis with culture independent high-throughput 16S rRNA gene based molecular micobiology, repeated and careful characterization of the developing allergic phenotype. The main limitation is the relatively small sample size that may conceal potential differences. Confounding cannot be excluded as the small sample size also limits the possibility to control for a number of environmental exposures that impact intestinal colonization (56). Also, we did not correct for multiple testing due to the exploratory nature of the study and the small sample size. Collectively, this warrants conservative interpretation of the study findings that need validation in larger cohorts.

In summary, by using 454 pyrosequencing this study extends previous findings of the role of bacteria belonging to the Proteobacteria phylum in development of IgE-associated eczema (34) by indicating that early development of TLR4 responses are modulated by Gram-negative Proteobacteria, specifically the *Enterobacteriaceae* family. Our results also suggest that a low relative abundance of Gram-positive *Ruminococcaceae* is associated with exaggerated TLR-2 responses and increased risk of developing IgE-associated eczema. These results should be further explored in large-scale studies elucidating the role of the gut microbiome in development of innate immunity in allergic disease.

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Author contributions: CEW, SLP, LE and MKT designed the study. DL processed sequence data. CEW and PR analyzed data. CEW drafted the manuscript. All authors contributed to data interpretation and critically reviewed and approved the manuscript.

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Figure legends

Fig 1. Microbiota composition at the phylum level, α- and ß-diversity at 1 week, 1 month and 1 year in vaginally delivered infants (n=7, n=5, n=11) and their mothers (n=11), and caesarean delivered infants (n= 7, n=6, n=9) and their mothers (n=8) in pregnancy. **a)** Median relative abundances (%) of the most abundant phyla are shown as staggered bar charts. Note that since median values are displayed, the sum does not add up to 100%. At 1 week of age, infants born by caesarean section (CS) had greater relative abundance of Proteobacteria and lower relative abundance of Bacteroidetes (all p<0.05) compared with vaginally delivered infants. Bacteroidetes remained in lower abundance throughout the first year of life in infants born by caesarean delivery; statistically significantly so at 1 week (p=0.024) with the same trend at 1 month of age (p=0.092). **b)** Median Shannon diversity index of the total microbiota and at the phylum level are shown. Compared with vaginally delivered infants, the α-diversity of Bacteroidetes was lower in CS born infants at 1 week (p=0.031) and 1 month (p=0.015).

Fig S1. The Pielou's evenness index at 1 week, 1 month and 1 year in infants with IgE-associated eczema (n=6, n=4, n=10) and their mothers (n=9), and in controls (n= 8, n=7, n=10) and their mothers (n=10) in pregnancy. The evenness of Actinobacteria was lower in infants with IgE-associated eczema at 1 year of age. *<0.05

Fig S2. The Bray-Curtis index displayed as pairwise ß-diversity within groups at 1 week, 1 month and 1 year in infants with IgE-associated eczema (n=6, n=4, n=10) and their mothers (n=9), and in controls (n= 8, n=7, n=10) and their mothers (n=10) in pregnancy. No statistically significant differences emerged.

Fig S3. Principal component analysis revealed no separation in relation to development of IgE-associated eczema. Infants who developed IgE-associated eczema and their mothers are displayed in red, and the controls and their mothers in blue. Infants of age 1 week, 1 month and 1 year together with their mothers are marked with 1,2,3 and M, respectively.

Fig S4. The Pielou's evenness index at 1 week, 1 month and 1 year in vaginally delivered infants (n=7, n=5, n=11) and their mothers (n=11), and caesarean delivered infants (n= 7, n=6, n=9) and their mothers (n=8) in pregnancy.

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Table 1. Characteristics of the study population

Characteristics	**Controls (N=10)**	**IgE-associated eczema (N=10)**	***P* value**
Boys n (%)	4 (40)	3 (30)	1
Maternal allergy n (%)	10 (100)	10 (100)	1
Paternal allergy n (%)	5 (56)*	7 (70)	0.65
Maternal age (years [SE])	34.4 (1.0)	34.4 (1.0)	0.69
Birth weight (g [SE])	3184 (61)	3446 (152)	0.14
Birth length (cm [SE])	49.1 (0.5)	49.8 (0.8)	0.48
Head circumference (cm [SE])	34.2 (0.2)	35.0 (0.5)	0.19
Gestation (Wk [SE])	39.2 (0.3)	39.2 (0.5)	0.96
Cesarean section n (%)	4 (40)	5 (50)	1
Older siblings n (%)	5 (50)	4 (40)	1
Day care at 6 mo n (%)	2 (20)	0	0.47
Play group at 6 mo n (%)	5 (50)	7 (70)	0.65
Day care at 1 yr n (%)	4 (40)	3 (30)	1
Play group at 1 yr n (%)	3 (30)	6 (60)	0.37
Furred pets in home first year n (%)	5 (50)	7 (70)	0.65
Ever breastfed n (%)	9 (90)	10 (100)	1
Breastfed at 6 mo n (%)	6 (60)	8 (80)	0.63
Breastfed at 12 mo n (%)	2 (20)	5 (50)	0.35
Ever formula 0-6 mo n (%)	9 (90)	9 (90)	1

Probiotic group n (%)	5 (50)	6 (60)	1
Ever had yogurt in first yr n (%)	3 (30)	4 (40)	1
Eczema and SPT+ n (%)	0	10 (100)	**<0.001**
Food allergy (IgE-mediated) n (%)	0	5 (50)	**0.03**
Sensitized at any age n (%)	0	10 (100)	**<0.001**
Egg	0	9 (90)	<**0.001**
Milk	0	3 (30)	0.21
Peanut	0	4 (40)	0.09
House dust mite	0	6 (60)	**0.01**
Grass pollens	0	2 (20)	0.47
Cats	0	2 (20)	0.47

Differences between the groups were determined by Fisher's exact test for categorical data, and Student's t test for continuous data. Bold font indicates statistically significant difference p<0.05. *Allergy status of one father unknown. SPT-skin prick test

Table 2. Relative abundance >1% at any age of dominant phyla, classes, families and genera in stool samples at 1 week, 1 month and 12 months of age infants that remained non-allergic (controls) and in infants developing IgE-associated eczema and their mothers, median % (25th-75th percentile)

	1 week				**1 month**				**12 months**				**Mothers***			
	Controls (N=8) VD (N=4) Probiotic (N=5)	N **	IgE-eczema (N=6) VD (N=3) Probiotic (N=3)	N	Controls (N=7) VD (N=4) Probiotic (N=5)	N	IgE-eczema (N=4) VD (N=1) Probiotic (N=3)	N	Controls (N=10) VD (N=6) Probiotic (N=5)	N	IgE-eczema (N=10) VD (N=5) Probiotic (N=6)	N	Controls (N=10) VD (N=6) Probiotic (N=5)	N	IgE-eczema (N=9) VD (N=5) Probiotic (N=5)	N
Actinobacteria	0.1 (0-30.3)	7	0.6 (0.1-21.3)	6	65.5 (7.0-72.1)	7	51.9 (17.6-81.1)	3	31.9 (8.7-60.6)	10	29.1 (10.3-43.1)	10	6.3 (4.1-15.3)	10	9.9 (3.3-15.8	9
Bifidobacteriaceae	0.1 (0-28.7)	6	0.2 (0-19.7)	6	62.2 (6.9-70.8)	7	50.7 (15.7-81.1)	3	28.4 (4.8-56.7)	10	28.5 (9.5-39.3)	10	6.2 (2.0-11.6)	10	5.5 (0.0-9.1)	9
Bifidobacterium	0 (0-28.7)	6	0.2 (0-19.6)	6	62.2 (6.9-70.8)	7	50.6 (15.6-81.1)	3	28.4 (4.8-56.7)	10	28.5 (9.5-39.3)	10	6.2 (1.9-11.6)	10	5.5 (0.0-9.1)	9
Coriobacteriaceae	0.0 (0.0-0.0)	1	0.0 (0.0-0.0)	1	0.0 (0.0-0.7)	4	0.0 (0.0-0.6)	2	2.3 (0.5-3.7)	10	0.7 (0-1.0)	7	1.1 (0.3-3.7)	9	1.2 (0.9-4.4)	8
Bacteroidetes	0.0 (0.0-0.1)	4	0.0 (0.0-7.1)	2	0.0 (0.0-0.4)	4	0.4 (0.1-14.3)	3	0.3 (0.2-0.7)	9	0.2 (0.1-0.6)	9	3.3 (1.1-32.0)	10	3.1 (0.1-3.7)	7
Bacteroidaceae	0.1 (0.0-0.1)	4	0.0 (0.0-6.2)	2	0.0 (0.0-0.4)	4	0.4 (0-11.6)	3	0.3 (0.2-0.7)	9	0.2 (0.1-0.6)	9	2.5 (0.8-28.2)	10	0.6 (0-1.7)	6
Bacteroides	0.0 (0.0-0.1)	4	0.0 (0.0-6.2)	2	0.0 (0.0-0.4)	4	0.4 (0-11.6)	3	0.3 (0.2-0.7)	9	0.2 (0.1-0.6)	9	2.5 (0.8-28.2)	10	0.6 (0-1.7)	6
Proteobacteria	30.6 (12.3-79.1)	7	0.2 (0.0-13.4)	5	7.8 (4.2-8.5)	7	3.8 (1.4-7.1)	3	0.1 (0.0-0.2)	9	0.2 (0.1-3.4)	8	0.5 (0.2-0.9)	10	0.2 (0.2-0.6)	8
Enterobacteriaceae	20.3 (8.7-78.9)	6	0.1 (0.0-13.4)	4	7.7 (4.1-8.5)	7	3.7 (1.3-7.0)	3	0.1 (0.0-0.2)	7	0.2 (0.1-3.4)	8	0.2 (0.1-0.6)	8	0.1 (0.0-0.6)	6
Escherichia-Shigella	5.8 (0-16.3)	4	0.0 (0.0-0.0)	2	0.6 (0.0-1.2)	5	0.0 (0.0-1.5)	2	0.0 (0.0-0.1)	4	0.1 (0.0-0.6)	7	0.2 (0.1-0.5)	8	0.0 (0.0-0.1)	6
Firmicutes	20.0 (18.1-32.6)	8	59.1 (15.9-77.2)	6	26.7 (16.7-60.8)	7	16.6 (11.8-39.4)	4	52.7 (32.5-61.7)	10	58.7 (43.5-82.9)	10	73.1 (41.9-83.0)	10	81.2 (71.8-94.3)	9
Bacilli class	19.2 (12.9-20.1)	8	39.5 (11.1-71.9)	6	18.7 (11.8-27.5)	7	14.0 (8.0-38.6)	4	1.9 (1.3-16.0)	10	13.6 (5.4-41.5)	10	**0.9 (0.5-1.5)**	10	**4.7 (3.7-14.9)**	9
Streptococcaceae	11.6 (4.7-17.6)	8	5.0 (2.1-61.0)	6	12.0 (6.3-14.3)	7	5.1 (3.3-18.6)	4	1.7 (1.2-12.9)	10	8.5 (1.3-25.6)	9	**0.8 (0.4-1.5)**	10	**4.6 (1.2-14.8)**	9
Streptococcus	11.6	8	4.5	6	12.0	7	5.1	4	1.7	10	8.5	9	**0.8**	10	**4.6**	9

	(3.9-17.6)		(1.9-61.0)		(6.3-14.3)		(3.3-18.6)		(1.2-12.9)		(1.3-25.6)		**(0.4-1.5)**		**(1.2-14.8)**	
Staphylococcaceae	3.7 (0.8-6.2)	8	3.3 (1.0-5.0)	5	1.3 (0.0-1.4)	7	0.1 (0.0-0.2)	3	0.0 (0.0-0.0)	1	0.0 (0.0-0.0)	2	0.0 (0.0-0.0)	4	0.0 (0.0-0.0	2
Staphylococcus	3.7 (0.8-6.2)	8	3.3 (1.0-5.0)	5	1.3 (0.0-1.4)	7	0.0 (0.0-0.2)	3	0.0 (0.0-0.0)	1	0.0 (0.0-0.0)	2	0.0 (0.0-0.0)	4	0.0 (0.0-0.1)	2
Enterococcaceae	0.2 (0.0-1.2)	5	0.1 (0.0-13.3)	4	2.9 (0.0-8.7)	5	0.3 (0.2-10.2)	3	0.0 (0.0-0.2)	5	2.5 (0.1-7.0)	9	0.0 (0.0-0.0)	1	0 (0-0.1)	3
Enterococcus	0.2 (0.0-1.2)	5	0.1 (0.0-13.3)	4	2.9 (0.0-8.7)	5	0.3 (0.2-10.2)	3	0.0 (0.0-0.2)	5	2.5 (0.1-7.0)	9	0.0 (0.0-0.0)	1	0 (0-0.1)	3
Lactobacillaceae	0.0 (0.0-0.0)	4	0.0 (0.0-0.0)	2	1.3 (0.5-3.2)	6	4.3 (0.3-9.3)	3	0.0 (0.0-0.0)	2	0.0 (0-0.4)	6	0.0 (0.0-0.0)	2	0.1 (0-0.2)	5
Lactobacillus	0.0 (0.0-0.0)	4	0.0 (0.0-0.0)	2	1.3 (0.5-3.2)	6	4.3 (0.3-9.3)	3	0.0 (0.0-0.0)	2	0.0 (0-0.4)	6	0.0 (0.0-0.0)	2	0.1 (0-0.2)	5
Clostridia class	0.8 (0.3-9.4)	8	0.0 (0.0-10.2)	4	6.6 (3.4-8.4)	6	0.2 (0.2-0.5)	3	35.4 (11.3-50.6)	10	32.0 (24.2-48.5)	10	66.0 (38.6-74.0)	10	62.7 (48.4-77.0)	9
Clostridiaceae	0.0 (0.0-6.2)	4	0.0 (0.0-2.2)	2	0.5 (0.0-1.5)	4	ND	0	1.4 (0.3-3.3)	9	0.5 (0.1-1.5)	8	1.3 (0.3-4.4)	10	0.6 (0.2-1.9)	8
Clostridium	0.0 (0.0-6.2)	4	0.0 (0.0-2.2)	2	0.5 (0-1.5)	4	ND	0	1.4 (0.3-3.3)	9	0.5 (0.1-1.5)	8	1.3 (0.3-4.4)	10	0.6 (0.2-1.9)	8
Ruminococcaceae	**0.1 (0.0-0.1)**	7	**ND**	0	ND	0	ND	0	0.5 (0.1-2.7)	8	0.3 (0.2-2.1)	8	30.5 (11.4-36.9)	10	7.5 (4.5-16.5)	9
Ruminococcus	0.0 (0.0-0.1)	4	ND	0	ND	0	ND	0	0.0 (0.0-0.1)	4	0.0 (0.0-0.0)	3	7.3 (0.8-14.1)	7	1.1 (0.0-1.6)	9
Subdoligranulum	ND	0	ND	0	ND	0	ND	0	0.0 (0.0-0.0)	4	0.0 (0.0-0.3)	7	7.2 (5.4-11.9)	10	3.8 (2.3-9.9)	9
Lachnospiraceae	0.0 (0.0-0.0)	6	0.0 (0.0-0.0)	4	0.0 (0.0-2.7)	3	0.0 (0.0-0.2)	1	29.9 (22.7-38.2)	10	17.6 (4.9-48.1)	10	20.8 (15.1-38.2)	10	33.1 (31.6-47.3)	9
Anaerostipes	0.0 (0.0-0.0)	2	ND	0	ND	0	ND	0	0.2 (0.0-2.9)	7	0.5 (0.0-2.4)	8	1.0 (0.5-1.1)	10	1.9 (0.01-4.8)	8
Blautia	0.0 (0.0-0.0)	4	0.0 (0.0-0.0)	3	0.0 (0.0-2.7)	3	0.0 (0.0-0.2)	1	19.2 (14.5-32.0)	10	9.9 (2.6-23.6)	8	7.8 (4.6-22.0)	10	20.8 (15.1-27.2)	9
Coprococcus	0.0 (0.0-0.0)	2	0.0 (0.0-0.0)	1	ND	0	ND	0	0.1 (0.0-0.2)	6	0.0 (0.0-0.2)	6	1.2 (0.6-2.8)	10	1.6 (1.2-2.3)	9
Dorea	ND	0	ND	0	ND	0	ND	0	0.1 (0.0-0.3)	5	0.1 (0.0-1.2)		1.6 (0.9-2.8)	10	2.6 (1.0-5.2)	9
Verrucomicrobia	ND	0	ND	0	ND	0	ND	0	0.0 (0.0-9.0)	6	0.0 (0.0-0.0)	3	1.5 (0.0-5.1)	7	0.6 (0.0-1.2)	7
Akkermansia	ND	0	ND	0	ND	0	ND	0	0.0 (0.0-9.0)	6	0.0 (0.0-0.0)	3	1.5 (0.0-5.1)	7	0.6 (0.0-1.2)	7

Differences between the groups were determined with the Wilcoxon signed rank test. Bold font indicates statistically significant difference $p<0.05$. *Atopic mothers whose infants developed IgE-associated eczema or remained non-allergic **Number of stool samples where the specific taxon was detected. VD- vaginally delivered; ND- not detected

Table 3. Shannon diversity index of the overall microbiome and dominant phyla in stool samples of infants that remained non-allergic (controls) and in infants developing IgE-associated eczema, and their mothers, presented as median (25-75th percentile)

	Controls	**IgE-associated eczema**	***P* value**
1 week	N=8	N=6	
All	1.32 (0.94-1.58)	0.53 (0.36-1.34)	0.23
Actinobacteria	0.51 (0.34-0.65)	0.28 (0.05-0.37)	0.11
Bacteroidetes	0 (0-0.17)	0 (0-0.14)	0.81
Proteobacteria	0.04 (0.02-0.05)	0.01 (0-0.83)	0.60
Firmicutes	1.21 (1.04-1.61)	0.91 (0.40-1.46)	0.28
1 month	N=7	N=4	
All	1.45 (1.43-1.67)	1.18 (0.76-1.70)	0.79
Actinobacteria	0.34 (0.24-0.57)	0.36 (0.03-0.77)	0.79
Bacteroidetes	0 (0-0.58)	0 (0-0.23)	0.74
Proteobacteria	0.19 (0.12-0.59)	0.27 (0.10-0.42)	0.53
Firmicutes	1.59 (1.12-1.71)	1.45 (1.31-1-58)	0.93
1 year	N=10	N=10	
All	1.86 (1.78-2.18)	1.71 (1.48-1.80)	0.16
Actinobacteria	0.94 (0.92-1.08)	0.57 (0.26-0.77)	**0.002**
Bacteroidetes	1.17 (0.98-1.65)	0.89 (0.50-1.45)	0.31
Proteobacteria	0.12 (0-0.66)	0.06 (0-0.64)	0.70
Firmicutes	1.63 (1.20-1.84)	1.28 (1.20-1.49)	0.45
*Mothers**	N=10	N=9	
All	2.79 (2.56-2.99)	2.73 (1.80-2.99)	0.72
Actinobacteria	1.13 (0.93-1.38)	0.95 (0.61-1.63)	0.91
Bacteroidetes	1.92 (1.72-2.25)	1.22 (0.64-1.67)	**0.04**
Proteobacteria	0.55 (0.10-0.92)	0.95 (0.62-0.98)	0.33
Firmicutes	2.70 (2.36-2.81)	2.54 (1.66-2.72)	0.36

Differences between the two groups were assessed with the Wilcoxon signed rank test. Bold font indicates statistically significant difference $p<0.05$.
*All mothers were atopic, but this refers to mothers whose children developed IgE-associated eczema or remained non-allergic.

Supplementary information 1.

Let $n_1, \ldots, n_m$ denote the number of reads for the observed and taxonomic classified OTUs in one s

Shannon diversity index was obtained as

$$H = -\sum_{j=1}^{m} p_j \ln(p_j),$$

where the proportion of the OTUs were estimated as

$$p_j = \frac{n_j}{\sum_{j=1}^{m} n_j},$$

$j=1,\ldots,m$. Pielou's evenness index was obtained as

$$E = \frac{H}{\ln(m)}.$$

Bray-Curtis dissimilarity between samples j and k was obtained as

$$d_{jk} = \frac{\sum_{i=1}^{m} |x_{ij} - x_{ik}|}{\sum_{i=1}^{m} (x_{ij} + x_{ik})}$$

Where m is the amount of unique OTUs and x_{ij} is the number of reads of OTU i in sample j, x_{ik}

of reads of OTU i in sample k.

Fig 1. a)

Relative abundance (%)

0
20
40
60
80
100

Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Other

1 Week
1 Month
1 Year
Mother
1 Week
1 Month
1 Year
Mother

Vaginal delivery
Caesarean delivery

b)

Shannon index

0
2
4
6
8

Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
All

1 Week
1 Month
1 Year
Mother
1 Week
1 Month
1 Year
Mother

Vaginal delivery
Caesarean delivery

Fig. S1)

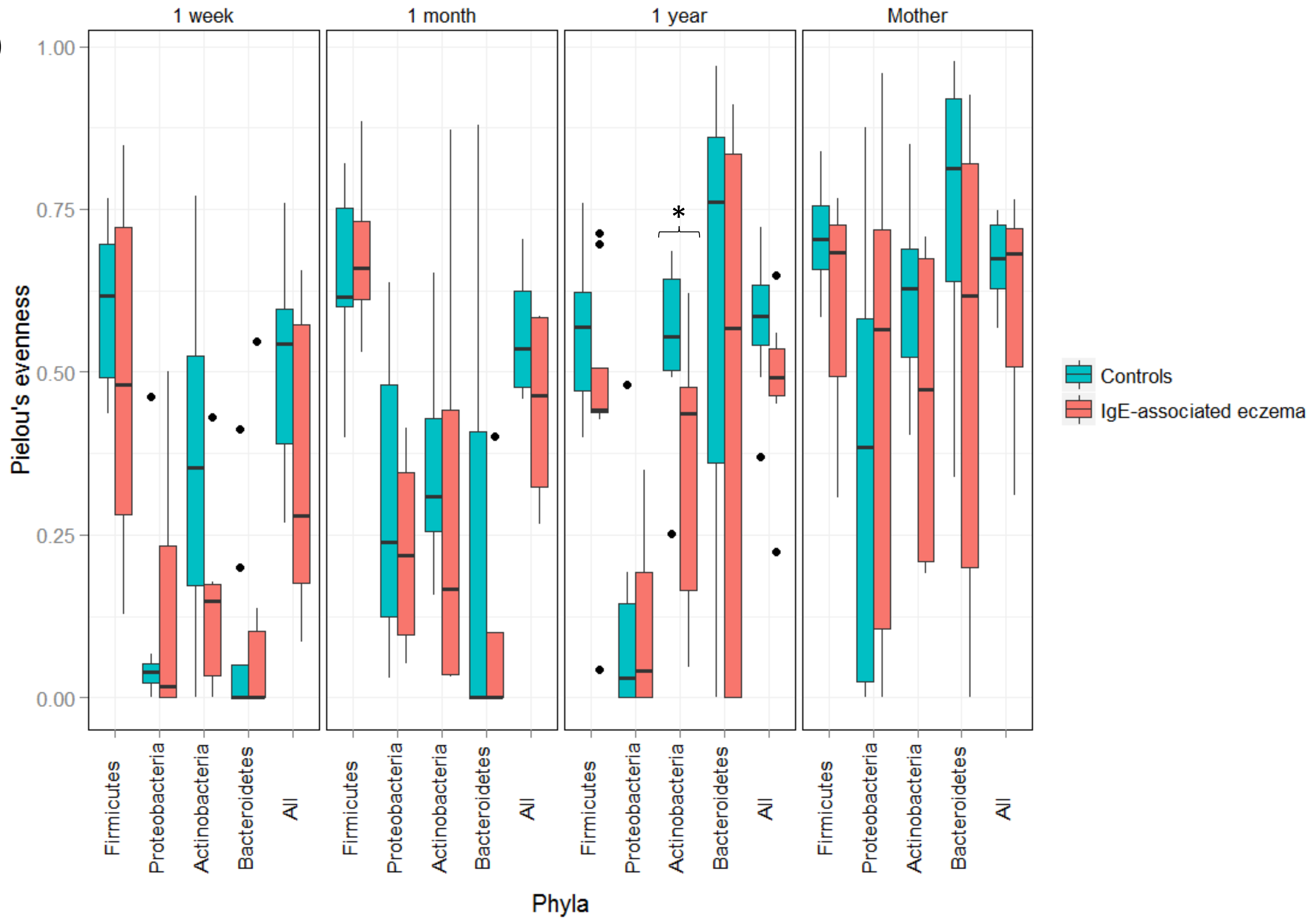

Fig. S2)

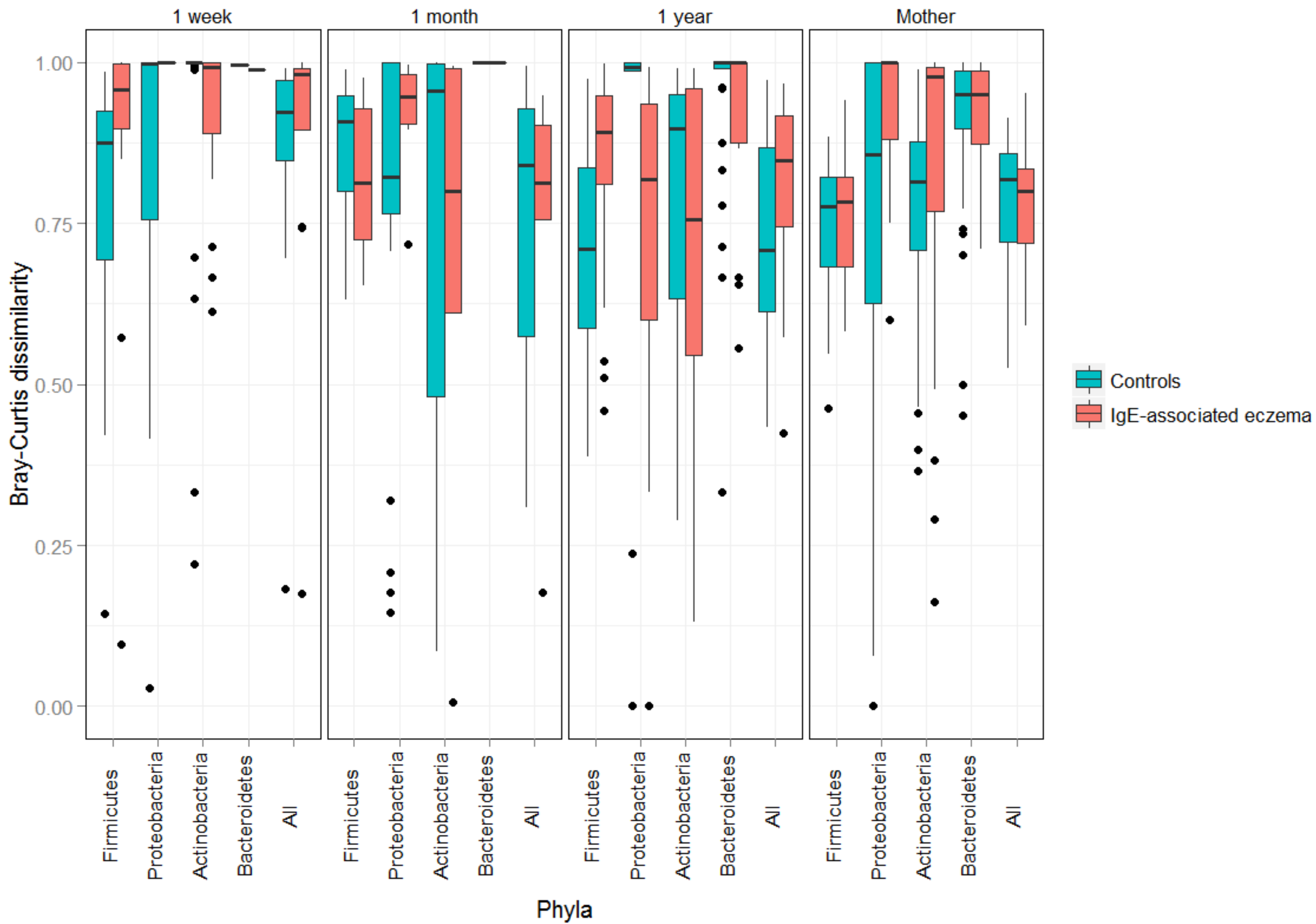

Fig. S3)

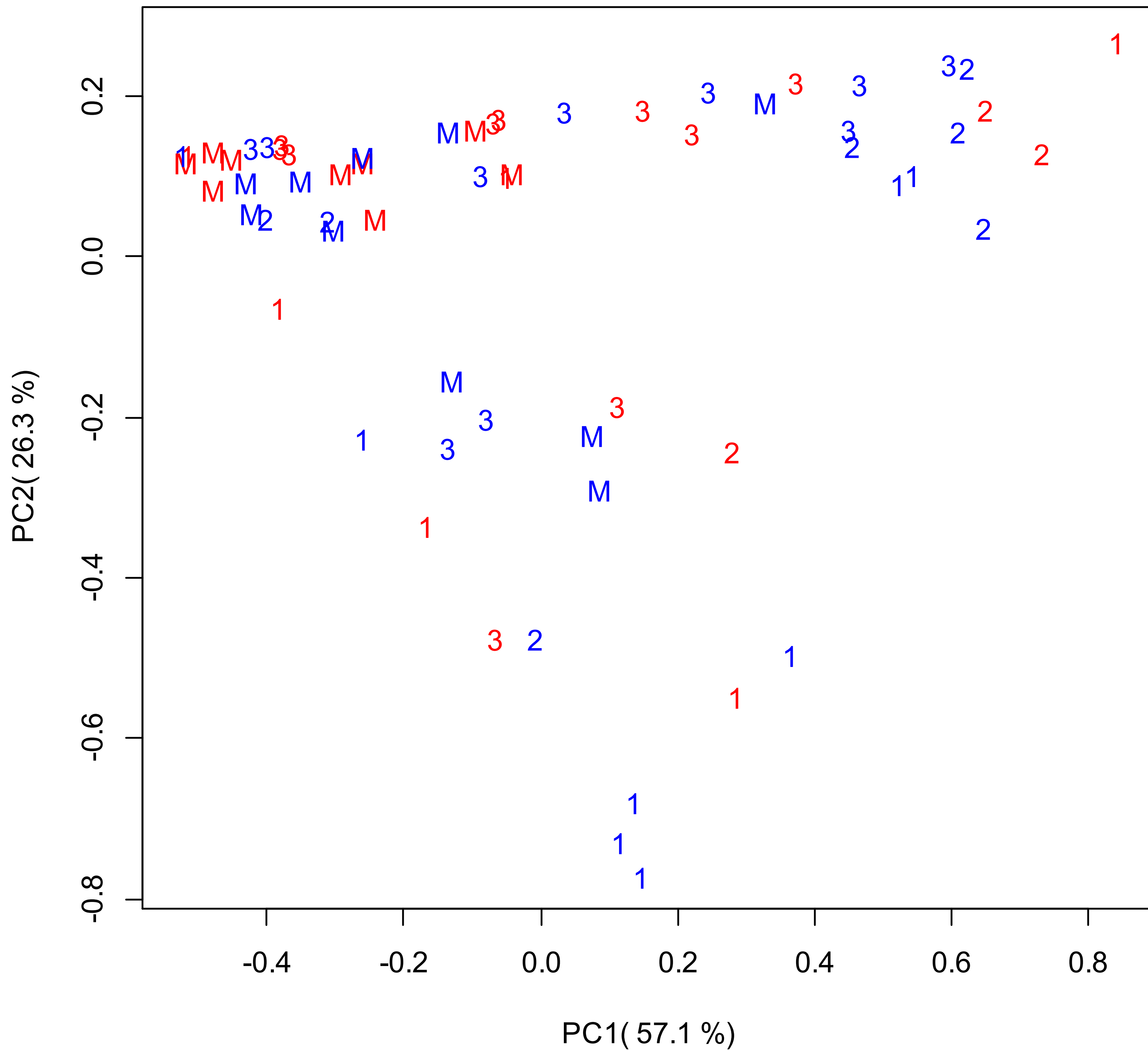

Fig. S4)