

Allergy development is associated with consumption of breastmilk with a reduced microbial richness in the first month of life

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Abstract

Background: Early colonization with a diverse microbiota seems to play a crucial role for appropriate immune maturation during childhood. Breastmilk microbiota is one important source of microbes for the infant, transferred together with maternal IgA antibodies. We previously observed that allergy development during childhood was associated with aberrant IgA responses to the gut microbiota already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breastfed infants.

Objective: To determine the microbial composition and IgA-coated bacteria in breastmilk in relation to allergy development in children participating in an intervention trial with pre- and post-natal *Lactobacillus reuteri* supplementation.

Methods: A combination of flow cytometric cell sorting and 16S rRNA gene sequencing was used to characterize the bacterial recognition patterns by IgA in breastmilk samples collected one month *post-partum* from 40 mothers whose children did or did not develop allergic and asthmatic symptoms during the first 7 years of age.

Results: The milk fed to children developing allergic manifestations had significantly lower bacterial richness, when compared to the milk given to children that remained healthy. Probiotic treatment influenced the breastmilk microbiota composition. However, the proportions of IgA-coated bacteria, the total bacterial load and the patterns of IgA-coating were similar in breastmilk between mothers of healthy children and those developing allergies.

Conclusion: Consumption of breastmilk with a reduced microbial richness in the first month of life may play an important role in allergy development during childhood.

KEYWORDS

allergy, breastmilk, IgA, microbiota, mother-infant transfer

1 | INTRODUCTION

Human breastmilk is considered to be an optimal nutritional source for the immature immune system of the infant.¹ Among the bioactive factors, breastmilk contains immunoglobulins that can be transferred to the offspring through breastfeeding.² Although all immunoglobulin isotypes can be encountered in breastmilk, secretory IgA (SIgA) is the dominating isotype and considered most important due to its anti-inflammatory properties and important role in defending the mucous membranes, thus regulating the binding and invasion of commensals and pathogenic microorganisms.³ This passive immunization through breastfeeding is crucial as early production of secretory IgA in newborns is limited.³

The increasing prevalence of allergic diseases in affluent societies is hypothesized to be caused by reduced intensity and diversity of microbial stimulation.^{4,5} In support of this theory, the gut microbiota differs in composition and diversity during the first months of life in children who later do or do not develop allergic disease.⁶⁻⁸ Breastmilk hosts a diverse array of microbiota and potential probiotic bacteria, transferred together with maternal IgA antibodies,⁹ likely influencing the infant's developing mucosal immune system.

We have previously observed that allergy development during childhood was associated with divergent patterns of IgA-recognized bacteria in the gut already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breastfed children.¹⁰ However, the identities of the bacterial taxa targeted by IgA in the breastmilk and what role they may play in immune and allergy development are unknown. In this study, we aimed to characterize the composition and IgA-coating pattern of the breastmilk microbiota from mothers whose children developed allergic symptoms during early childhood or stayed healthy.

2 | METHODS

For detailed methods, experimental protocols and statistical analyses, see the Methods section in this article's Online Repository.

2.1 | Study design

The subjects included in this study were part of a larger randomized double-blind trial in Sweden, recruiting participants between 2001 and 2003, where the potential allergy-preventive effect of probiotic *Lactobacillus reuteri* ATCC 55730 in the infants with family history of allergic disease was evaluated.^{11,12} The mothers were supplemented with *L reuteri* during pregnancy from post-menstrual week 36 + 0 to delivery and the infants continued with the same treatment from days 1-2 of life until 12 months of age. Among the 184 mothers of children that completed the 7-year follow-up in the original study, breastmilk samples, at one month *post-partum*, from 24 mothers whose children did not and

Key Message

Our findings support the hypothesis that perinatal transmission of rich bacterial communities and immunomodulatory factors from mother to offspring may shape immune maturation during infancy and beyond. Therefore, modulating the interactions between breastmilk microbiota and IgA may be a novel strategy to establish a rich microbial communities and reduce the risk of allergies.

from 26 mothers whose children did develop allergic disease during early childhood, were randomly selected for flow cytometry-based sorting of IgA-coated bacteria in the current study. From these flow cytometry-sorted samples, subsequent 16S rRNA gene characterization was performed on the IgA-coated and IgA-free fractions of breastmilk bacteria from 20 mothers whose children did not and from 20 mothers whose children did develop allergic manifestations as well as from total, non-sorted breastmilk samples from the same mothers. Selection of the samples used for 16S rRNA sequencing in this study was based on the sample availability and required sample volume for Illumina sequencing, and a clear allergy diagnosis (based on proven symptoms to allergy provocation) of the child. Allergic disease included eczema ($n = 12$), gastrointestinal allergy ($n = 1$), asthma ($n = 10$), allergic rhinoconjunctivitis ($n = 14$) and allergic urticaria ($n = 3$). The criteria of these diagnoses are described in detail in Ref.^{11,12} and in the supplementary information of this manuscript. The majority of the children included in the current study were exclusively breastfed during the first month of life (93%).

There were no differences regarding potential confounders, such as sex, mode of delivery, birth order, maternal atopy, breastfeeding, antibiotics and probiotic supplementation, between the infants who did or did not have allergic manifestations (Table 1).

Total IgA levels were measured by ELISA in a study by Böttcher et al.¹³ The studies were approved by the Regional Ethics Committee for Human Research in Linköping, Sweden (Dnr 99323, M122-31 and M171-07, respectively).

2.2 | Sample preparation and flow cytometry-based sorting

The breastmilk samples were stained with goat anti-mouse IgA labelled with fluorescein isothiocyanate (FITC), used as an isotype control corresponding to unspecific binding (Sigma; reference SLBD9273), or with goat anti-human IgA labelled with FITC (Life Technologies; reference A18782), according to the manufacturer's instructions. The sorting of the bacterial cells according to whether they were IgA-coated (IgA+) or IgA-free (IgA-) was performed with the MoFlo XDP Cell Sorter (Beckman Coulter, Brea, Calif), according to the procedures of Simón-Soro et al.¹⁴

TABLE 1 Descriptive data of children compare in this study

Children	Healthy (% [no.]) n = 20	Developing allergic disease (% [no.]) n = 20	P value*	Developing asthma (% [no.]) n = 10	P value*
Girls	50 (10)	60 (12)	.53	60 (6)	.71
Older siblings	40 (8)	35 (7)	1.00	30 (3)	.70
Caesarean delivery	20 (4)	20 (4)	1.00	20 (2)	1.00
Furred pets	15 (3)	10 (2)	1.00	10 (1)	1.00
Breastfeeding					
1 mo exclusive	90 (18)	95 (19)	1.00	90 (9)	1.00
3 mo exclusive	85 (17)	70 (14)	.45	50 (5)	.08
12 mo partial	20 (4)	25 (5)	1.00	0 (0)	.27
Maternal atopy	85 (17)	80 (16)	1.00	70 (7)	.37
Antibiotic treatment (1-12 m)	35 (7)	30 (6)	1.00	50 (5)	.46
Day care (1-12 m)	10 (2)	5 (1)	1.00	10 (1)	1.00
Probiotics— <i>Lactobacillus reuteri</i>	45 (9)	55 (11)	.53	60 (6)	.70

*The x2 test was used to detect potential differences in frequencies between groups, except when the expected frequency for any cell was <5, in which case the Fisher exact test was used.

2.3 | DNA extraction

DNA from sorted breastmilk bacteria, both IgA-coated and IgA-free, as well as the total milk sample, was isolated by using the MasterPure complete DNA and RNA Purification Kit (Epicentre Biotechnologies), according to the manufacturer's instructions.

2.4 | 16S rRNA gene amplification and sequencing

DNA from sorted bacterial fractions (in total 80) together with total non-sorted breastmilk samples (in total 40) was used for PCR amplification and Illumina sequencing to describe the bacterial composition of breastmilk. Sequences supporting the conclusions of this article are publicly available at European Nucleotide Archive database (ENA) with accession number PRJEB30065.

2.5 | Sequence analysis

16S rRNA gene reads from the total milk samples were used in order to perform an accurate filtering of the flow cytometry IgA-sorted fractions that, due to low bacterial yield, were more susceptible to sequencing contaminations. This was done by eliminating OTUs in IgA-sorted fractions that were absent in corresponding total non-sorted milk samples.

For analysing IgA-coating patterns, the IgA index score (calculated according to the formula $\log(\text{IgA-coated}(\text{IgA+})/\text{IgA-free}(\text{IgA-}))$) was used to describe the degree of mucosal immune responsiveness to the microbiota.

Statistical analyses were performed in R version 3.2.2 and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA, Version 6.1f), where $P < .05$ was considered significant.

3 | RESULTS

3.1 | IgA proportions in breastmilk

On average, approximately 40% of bacteria in breastmilk appeared to be IgA-coated. Proportions of IgA-coated bacteria were similar in breastmilk samples of mothers whose children did or did not develop allergic (Figure 1A, $P = .567$) and asthmatic symptoms (Figure 1B) during the first 7 years of life. Allergic disease included the development of eczema, gastrointestinal allergy, asthma, allergic rhinoconjunctivitis or allergic urticaria during the first 7 years of life. Moreover, no differences in proportions of breastmilk IgA-coated bacteria in relation to allergy development (most commonly eczema), during the first 2 years of age, were observed (data not shown). IgA proportions observed did not seem to be influenced by the total IgA levels in breastmilk samples of these mothers ($n = 29$; Spearman correlation test $r = .32$, $P = .095$). The proportions of IgA-coated bacteria were lower in breastmilk from mothers supplemented with probiotics during the last month of pregnancy, as compared with placebo ($P = .04$; Figure S1A in this article's Online Repository), and particularly in breastmilk of probiotic supplemented mothers whose children stayed healthy ($P = .02$; Figure S1B).

3.2 | Bacterial diversity, richness and density in total non-sorted milk samples and IgA-coated fractions

The overall species richness (as determined by Chao1 index) in total non-sorted breastmilk samples was significantly higher ($P = .02$, Figure 2A) in mothers with healthy children, although

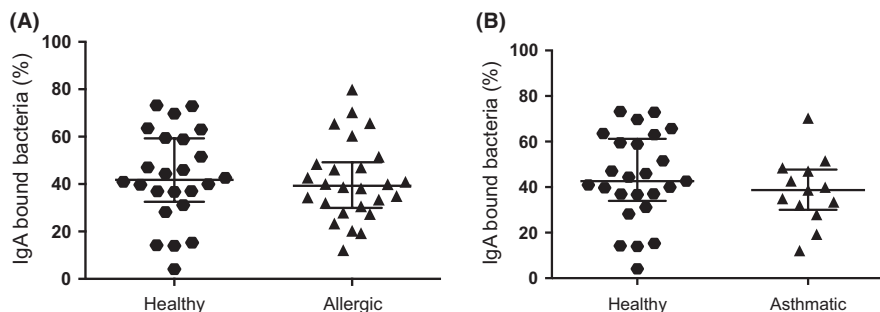


FIGURE 1 Proportions of IgA-coated breastmilk bacteria collected one month *post-partum*. A, Proportion of breastmilk IgA-coated bacteria in mothers whose children stayed healthy ($n = 24$, circles) or developed allergic symptoms ($n = 26$, triangles) during the first 7 y of life, as determined by flow cytometry-based cell sorting ($P = .567$). B, Proportion of breastmilk bacteria coated to IgA at one month of age in children staying healthy ($n = 24$) or developing asthmatic symptoms ($n = 13$) during the first 7 y of life ($P = .308$). Medians and interquartile ranges are indicated

the bacterial load (Figure S2) and species diversity (Shannon index, Figure 2B) were similar in breastmilk samples of mothers whose children did/did not develop allergic manifestations. The total species richness also tended to be higher in breastmilk from mothers whose children stayed healthy than from mothers whose children developed asthmatic symptoms ($P = .066$, Figure 2A). However, no significant differences between mothers of healthy and allergic subjects were observed upon comparing the richness and diversity of the IgA-coated fractions in breastmilk (Table S1Table S1). Additionally, no differences in bacterial load, species diversity or richness were observed between the total non-sorted breastmilk from the mothers treated with probiotics and placebo (data not shown).

3.3 | Bacterial composition in allergy development and probiotic supplementation

Bacterial 16S rRNA gene sequencing, of the total non-sorted milk and IgA-coated/free fractions, was performed in order to determine the milk microbial composition and to assess IgA responses towards specific bacteria. After quality filtering and removal of chimeric sequences, 40 total non-sorted breastmilk samples and 80 IgA

separated fractions remained with 2 000 107 and 1 525 770 high-quality reads, respectively. Sequencing of total non-sorted breastmilk samples resulted in an average of $48\,987 \pm 2725$ (SEM) reads per sample, while the IgA-separated breastmilk fractions had an average of $20\,322 \pm 1660$ (SEM) sequence reads per sample.

The relative abundance of genera in total breastmilk, that is non-sorted samples, is presented in Figure 3A,B. Infants developing allergies during the first 7 years of life tended to have higher abundance of the genera *Enterococcus* ($P = .01$; adj. P -value = 0.18; Figure S3A) and *Pseudomonas* ($P = .01$; adj. P -value = .18; Figure S3B). The genus *Enterococcus* was found in significantly higher abundance ($P = .001$; adj. P -value = 0.02) in breastmilk given to children that developed asthmatic symptoms, when compared to breastmilk given to the children that remained healthy (Figure 3B, Figure S3A). No effect of the confounding factors such as probiotic supplementation during pregnancy, maternal atopy, sex and the delivery mode influenced the microbial abundance between the groups, as determined with the MaAsLin multivariate statistical algorithm.

Microbiota composition patterns of total non-sorted breastmilk were significantly different ($P = .04$ Adonis testing) between mothers that received, or not, probiotic supplementation (Figure 4A) with significantly increased relative abundance ($P = .03$, Wilcoxon rank-sum test adjusted P -value) of the genus *Rothia* in mothers treated

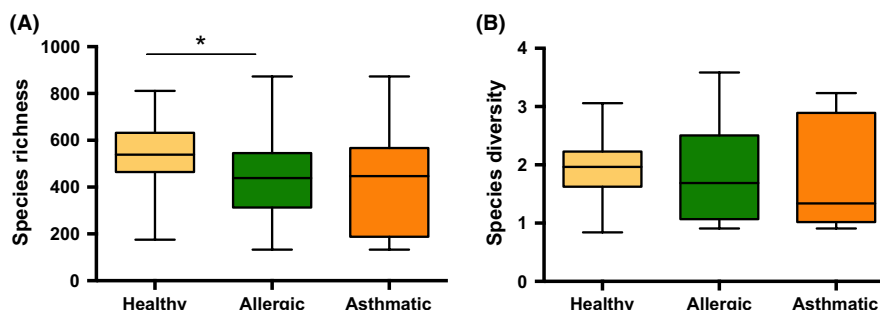


FIGURE 2 Bacterial richness (A) and diversity (B) at 16S rRNA OTU's species level, as described with Chao1 and Shannon indices, respectively, of the total non-sorted breastmilk samples from mothers whose children stayed healthy up to 7 y of age ($n = 20$), mothers whose children developed allergic manifestations ($n = 20$) and/or asthmatic symptoms ($n = 10$). Medians and interquartile ranges are indicated. * P -value < .05, Mann-Whitney U test

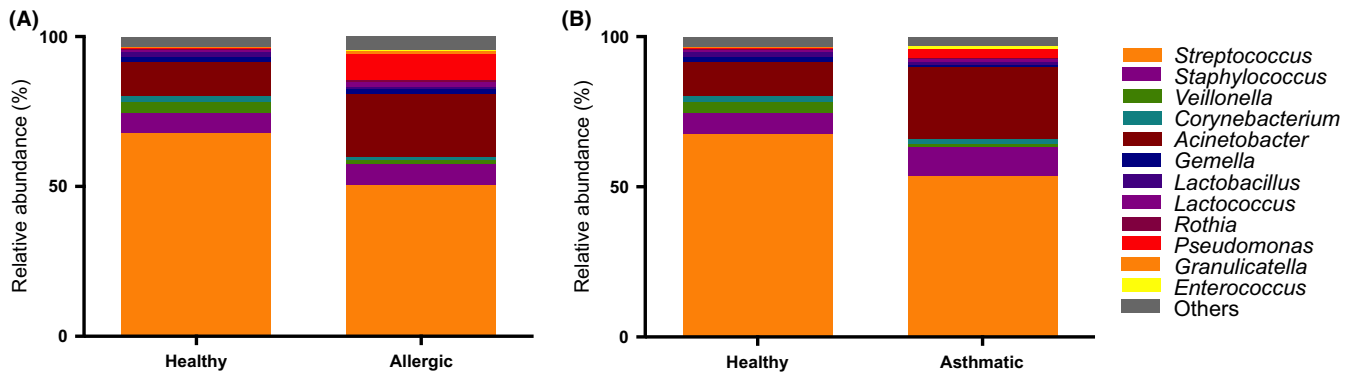


FIGURE 3 Microbiota composition of most dominant bacterial genera in total non-sorted milk samples. A, The relative abundance (>0.5% of the total) of dominant bacterial genera in 20 mothers whose children stayed healthy and in 20 mothers whose children developed allergic manifestation during their first 7 years of life. B, The relative abundance of dominant bacterial genera in breastmilk samples in 20 mothers whose children stayed healthy and 10 mothers whose children developed asthmatic manifestation

with placebo. However, upon comparing IgA pattern recognitions of bacteria in mothers who were treated, or not, with probiotics supplementation during the last month of the pregnancy, no statistically significantly different IgA responses could be observed for bacterial genera presented (Figure 4B).

3.4 | IgA responses towards milk microbiota in allergy development

Although the analysis of the relative abundance of dominant bacterial genera in breastmilk, and the composition patterns of

sorted IgA fractions (Figure S4) were generally similar between the IgA-coated and IgA-free fractions, upon considering the health status of the children, some differences at genus level were observed when analysing the bacterial targets of IgA responses, represented as the IgA index here. The value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA-positive fraction, to negative values (genera found dominantly in the IgA negative fraction). However, no statistically significant differences could be observed in IgA-coating patterns of breastmilk bacteria given to children that did and did not develop allergic and/or asthmatic symptoms (Figure 5A,B).

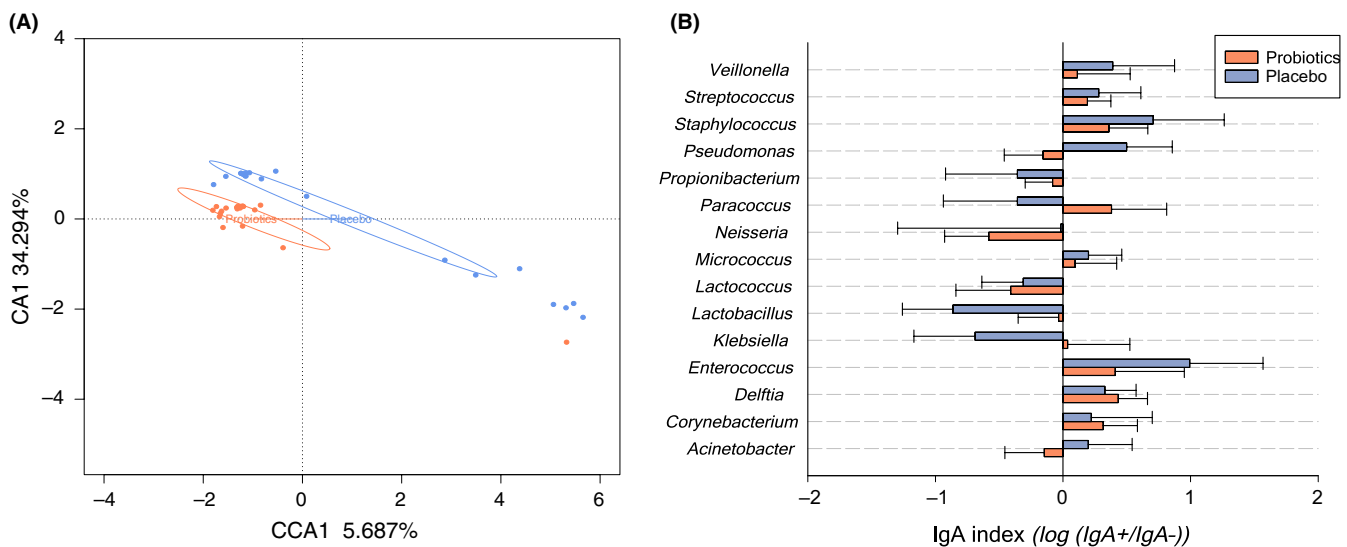


FIGURE 4 Breastmilk microbiota composition and IgA-coating patterns of the dominant genera (>0.5% of total), in probiotic supplementation. A, Constrained correspondence analyses (CCA) based on microbiota composition patterns (determined by 16S rRNA sequencing) in breastmilk of mothers treated with probiotics *Lactobacillus reuteri* or placebo. The percentage of variation explained by constrained correspondence components is indicated on the axes. P-Value for CCA plots was determined by Adonis ($P = .04$) and indicates if the factor provided (in this case *probiotics*) can significantly explain data variability. B, Plots represent IgA-coating patterns (defined by IgA index, reflecting the ratio in IgA-coated and IgA-free breastmilk microbiota) to dominant genera in breastmilk samples collected at one month *post-partum* from mothers who were treated with probiotic *L. reuteri* or placebo. For given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA-coated fraction, to negative values (genera found dominantly in the IgA-free fraction). $n_{\text{Probiotics}} = 20$, $n_{\text{Placebo}} = 20$

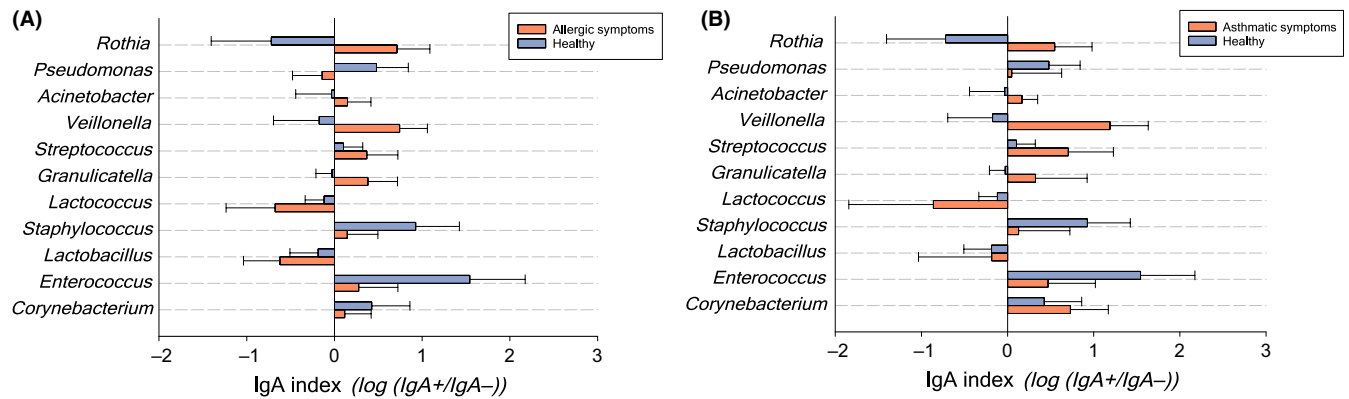


FIGURE 5 IgA responses towards breastmilk microbiota. Plots represent IgA-coating patterns to dominant genera (>0.5% of total) from mothers whose children stay healthy ($n = 20$) compared to children that develop allergic (A, $n = 20$) or asthmatic (B, $n = 10$) symptoms. For given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA-coated fraction, to negative values (genera found dominantly in the IgA-free fraction). Means with SEs are indicated. Wilcoxon rank-sum test with FDR correction was performed, and no statistically significant differences were observed

4 | DISCUSSION

The data presented in the current study demonstrate that total non-sorted breastmilk from mothers whose children developed allergic symptoms during early childhood had lower bacterial richness, when compared to milk fed to children staying healthy. Moreover, probiotic supplementation during pregnancy modifies the breastmilk microbiota composition and the proportion of IgA-coated bacteria.

The influence of breastmilk composition on later allergy development appears to be linked to higher richness of bacterial species and not to the relative abundance of specific bacteria. Previously, low total diversity of the gut and oral microbiota have been associated with atopy and asthma development during early childhood.^{6,8,15,16} Moreover, aberrant IgA immune responses towards gut microbiota, but not the proportions of IgA-coating of bacteria, in relation to subsequent allergy development during childhood were observed as early as 1 month *post-partum*, in the same cohort of children.¹⁰ At this time point, the IgA antibodies are predominantly maternally derived in exclusively breastfed children, as the levels of endogenously produced IgA in the baby during this period is limited.¹⁷ Therefore, any divergent responses observed at this time suggest that immunological interactions between mother and infant may play an important role in subsequent immune development. However, the data in the current study suggest that bacterial IgA responses in breastmilk do not correlate with that observed in the gut of the breastfed infants. Likely, not only the proportions of IgA-coated bacteria but also the composition and timing of bacterial colonization (including the establishment of specific bacterial species, recognized by IgA or not) during early infancy will have an effect on the proper immune system education. Thus, further research is needed in order to understand the exact function of vertical transmission of IgA-coated bacteria from mother to breastfeeding child.

Streptococcus, *Acinetobacter*, *Staphylococcus* and *Veillonella* were the most commonly found bacterial genera in milk samples used in this study. Furthermore, lactic acid bacteria *Lactococcus*,

Lactobacillus and *Enterococcus* as well as oral inhabitant *Gemella* were also detected, in agreement with previous reports.¹⁸ Various studies have demonstrated that there is a mother-to-infant transfer of bacterial genera including *Lactobacillus*, *Staphylococcus*, *Enterococcus* and *Bifidobacterium* through breastfeeding.^{19–22} The constant intake during lactation of breastmilk bacteria leads to the establishment of an intestinal microbiota that deeply impacts on the newborn's immune maturation.²³ An interesting finding is the significantly higher levels of the lactic acid bacteria *Enterococcus* in breastmilk fed to children developing asthma. *Enterococcus* is a common inhabitant of breastmilk microbiota and one of the first microbes to colonize the infant gut after birth,^{8,24} being more abundant in the gut of atopic infants, from the same cohort, at 12 months of age.⁸ However, whether our finding here reflects an overgrowth of *Enterococcus* due to the absence of competing species, or whether *Enterococcus* is suppressing the growth of allergy protecting bacteria remains to be addressed.

An important function of SIgA is immune exclusion, a mechanism where this antibody binds to commensals, through its recognition of multiple antigenic epitopes on the surface, encountered in gut lumen and prevents their attachment to mucosal barrier.²⁵ Moreover, this antibody can also promote bacterial adhesion to the mucosa, as shown *in vitro* and *in vivo*,^{26,27} thus enriching for the growth of particular microbial strains.²⁸ In this study, we were not able to observe significant differences in the patterns of IgA-binding in breastmilk fed to children staying healthy and children developing allergies but the possibility that the true differences are at the lower bacterial resolution (for instance species and even different strains) should not be excluded. The fact that the majority of the mothers included in this study, and also in the original study of this cohort, suffered from allergic disease, should be considered as it also may affect both the IgA-coating patterns and the microbiota transferred to the child. Moreover, due to sample availability, in this study we have examined solely a part of the total original cohort which might limit the findings presented here.

IgA-coating proportions of breastmilk bacteria were higher in mothers treated with placebo, compared to those treated with probiotic *L reuteri*. As the mothers were treated with probiotics until delivery, likely the changes in the microbial composition were more significant in colostrum than in breastmilk samples extracted at one month *post-partum*, as in the present study. We have previously shown that *Lactobacilli* colonization was significantly increased in colostrum of the mothers treated with *L reuteri*, but not in samples obtained one month *post-partum*.²⁹ This could suggest that a part of the mother's IgA responses, while treated with probiotics, were directed towards *L reuteri*, but that the ending of supplementation was reflected in diminished targeting of this species and perhaps decreased proportions of IgA-coated microbiota. Additionally, microbiota patterns of total non-sorted milk were also significantly different between mothers treated with probiotics and those with placebo, with the genus *Rothia* (a common oral inhabitant associated with good oral health³⁰) significantly increased in the placebo group. However, the importance of these results and the detailed mechanisms behind the relationship of probiotic supplement and the development of allergic diseases during childhood needs to be further investigated, preferably in larger study cohorts including mothers not suffering from allergic disease.

Together with the maternal intestinal and vaginal microorganisms that are ingested by the neonate during the passage through the birth canal, breastmilk microbiota appears to contribute to the initial microbial colonization in infants, thus having a pivotal role in modulating and influencing the newborns' immune system. The bacterial species that initially colonize the mucosal surfaces likely define the ecosystem conditions, which, in turn, will affect the establishment of further co-colonization patterns. Also, milk microbes are transferred together with maternal IgA antibodies that may enable maintenance of a mutually beneficial relationship with a diverse set of commensals, while protecting against pathogens.

In conclusion, consumption of breastmilk with a reduced microbial richness in the first month of life correlates with an increased risk to develop allergy during childhood. In addition, our data show that probiotic supplementation during pregnancy alters the breastmilk microbiota composition and the proportion of IgA-coated bacteria. These findings open the possibility of modulating breastmilk microbiota and its interaction with antibodies as a strategy to promote a healthy microbial colonization with the purpose of reducing allergy risk.

CONFLICT OF INTEREST

Maria C. Jenmalm has received funding for a clinical trial and honoraria for lectures from BioGaia AB, as well as consultant fees and travel support from Nutricia/Danone.

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SUPPORTING INFORMATION

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

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SUPPLEMENTARY INFORMATION for - *Allergy development is associated with consumption of breastmilk with a reduced microbial richness in the first month of life*

METHODS

Study design

The subjects included in this study were part of a larger randomized double-blind trial in South-eastern Sweden, recruiting participants between 2001 and 2003, where the potential allergy preventive effect of probiotic *Lactobacillus reuteri* ATCC 55730 in the infants with family history of allergic disease was evaluated.^{1,2} The mothers were supplemented with *L. reuteri* during pregnancy from postmenstrual week 36+0 to delivery and the infants from day 1-2 of life until 12 months of age. Among the 184 mothers that completed the 7-year follow up in the original study, breastmilk samples from 51 mothers were used for flow cytometry based-sorting of IgA-coated bacteria in the current study. When comparing the IgA proportions in breastmilk from mothers that were treated with probiotics or placebo, 27 and 24 samples were used for these analyses. However, for comparisons of the breastmilk fed to children developing allergies or staying healthy, we excluded one of the samples as this child had allergic symptoms, but not sensitization, in order to get more accurate analyses.

Subsequently, 16S rRNA gene characterization was performed on the IgA-coated and IgA-free fractions of breastmilk bacteria from 20 mothers whose children did not and from 20 mothers whose children did develop allergic manifestations as well as from total, non-sorted breastmilk samples from the same mothers. The selection of the samples used for 16S rRNA sequencing in this study, was based on the sample availability and a clear allergy diagnosis (based on proven symptoms to allergy provocation) of the child. There were no differences regarding potential confounders, such as sex, mode of delivery, birth order, maternal atopy, breastfeeding, antibiotics, and probiotic supplementation, between the infants who did or did

not have allergic manifestations. Allergic disease included eczema (n=12), gastrointestinal allergy (n=1), asthma (n=10), allergic rhinoconjunctivitis (n=14) and allergic urticaria (n=3). The criteria of these diagnoses are described in detail in^{1,2}. All children with allergic disease in the current study were also sensitized (*i.e.* they had at least one positive skin prick test (evaluated at 6, 12 and 24 months and 7 years of age) and/or detectable circulating allergen specific-IgE antibodies (assessed at 6, 12 and 24 months), while the healthy children were non-sensitized. Skin prick tests were performed on the forearm with egg white, fresh skimmed cow milk and standardized cat, dog, birch, peanut and timothy extracts at 6, 12, 24 months and 7 years of age (here even mite (Der p)).² Moreover, symptoms related to allergic disease, physical examination, spirometry and measurement of fractional exhaled nitric oxide (FE_{NO}) were observed. Children were diagnosed with allergy if they have had symptoms of and/or have been treated for the actual allergic disease during the last twelve months. A diagnosis of gastrointestinal allergy required vomiting, diarrhea, or systemic reaction after ingestion of a potentially allergenic food and a confirmation by challenge, unless there was a clear history of a severe systemic reaction.^{1,3} Asthma diagnosis was based on at least one of following two criteria: 1. Doctor diagnosis and asthma symptoms and/or medication during the last twelve months; 2. Wheeze or nocturnal cough and a positive reversibility test and/or pathological FE_{NO} value.^{1,3} All asthmatic children were also included in the allergic group. For further details, please see the publications from Abrahamsson T. *et al.*¹⁻³

Breastmilk samples were collected one-month *post partum* by the mother at home. They were immediately placed in the freezer and brought to the hospital and stored at -70°C within 3 days. All the children included in the current study were exclusively breastfed during the first month of life. Total IgA levels were measured by ELISA in a study by Böttcher *et al.*⁴

The studies were approved by the Regional Ethics Committee for Human Research in Linköping, Sweden (Dnr 99323, M122-31 and M171-07, respectively). An informed consent was obtained from both parents before inclusion in the study.

Sample preparation and flow cytometry-based sorting

After thawing, the breastmilk fatty layer and whey were removed by centrifugations. The resulting sample fraction was further suspended in sterile saline solution (autoclaved H₂O; NaCl Sodium Chloride 99.5% PA-ACS-ISO; Panreac, Barcelona, Spain; reference 131689.1211) with 5% BSA (Sigma-Aldrich, St Louis, Mo; reference A7030-100gr) to prevent nonspecific antibody binding. The samples were stained with goat anti-mouse IgA labelled with fluorescein isothiocyanate (FITC), used as an isotype control corresponding to unspecific binding (Sigma; reference SLBD9273), or with goat anti-human IgA labelled with FITC (Life Technologies; reference A18782), according to the manufacturer's instructions. The sorting of the bacterial cells according to whether they were IgA-coated or IgA-free was performed with the MoFlo XDP Cell Sorter (Beckman Coulter, Brea, Calif), according to the procedures of Simon- Soro et al.⁵

DNA Extraction

DNA from sorted breastmilk bacteria, both IgA⁺ and IgA⁻, as well as the total milk sample (2 ml) was isolated by using the MasterPure complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, Wis), according to the manufacturer's instructions, with a previous glass bead beating (0.17 mm in diameter) and an additional enzymatic lysis step with lysozyme (20 mg/mL, 37°C, 30 minutes; Thermo-mixer comfort, Eppendorf, Hamburg, Germany), lysostaphin (2000 units/mg protein, 37 °C, 60 min; Sigma-Aldrich, Madrid, Spain) and mutanolysin (4000 units/mg protein, 37 °C, 60 min; Sigma-Aldrich).

16S rDNA gene amplification and sequencing

DNA from sorted bacterial fractions (in total 80) together with total non-sorted breastmilk samples (in total 40) was used for PCR amplification and Illumina sequencing to describe the bacterial composition of breastmilk. Universal bacterial degenerate primers 8F—5'-AGAGTTTGATCMTG GCTCAG-3' and 926R—5'-CCGTCAATTCMTTTRAGT- 3', which encompass the hypervariable regions V1–V5 of 16S ribosomal RNA (rRNA) gene were used for an initial amplification in order to increase the bacterial yield. Purification of PCR products was completed using Nucleofast 96 PCR filter plates (Macherey-Nagel, Düren, Germany).

An Illumina amplicon library was performed following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part #15044223 Rev. A). The gene-specific primer sequences used in this protocol were selected from Klindworth et al. ⁶, and target the 16S rRNA gene V3 and V4 regions, resulting in a single amplicon of approximately 460 bp. After 16S rRNA gene amplification, the DNA was sequenced on a MiSeq Sequencer according to manufacturer's instructions (Illumina) using the 2 × 300 bp paired-end protocol. Sequences supporting the conclusions of this article are publicly available at European Nucleotide Archive database (ENA) with accession number PRJEB30065.

Total bacterial load

Total bacterial load (number of bacterial cells per ml breastmilk) was measured by quantitative PCR. Amplifications were performed in duplicates on a LightCycler 480 Real-Time PCR System (Roche Technologies) by using annealing temperatures of 60 °C. Each reaction mixture of 10mL was composed of SYBR Green PCR Master Mix (Roche), 0.5 mL of the specific primer (concentration 10 mmol/L), and 2 mL of DNA template. The universal forward and reverse primers were 5'-CGTGCCAGCAGCCGCGG-3' and 5'-

TGGACTACCAGGGTATCTAATCCTG-3', targeting a 293bp long region of the bacterial 16S rRNA gene. The obtained Ct values were transformed in bacterial cell numbers by a calibrated standard curve.⁷

Bioinformatics

The PRINSEQ program was used for a sequence quality assessment.⁸ Sequences of <250 nucleotides in length were discarded; sequence end-trimming was performed by cutting out nucleotides with a mean quality of <30 in 20-bp windows. Chimeric 16S sequences were filtered out using USEARCH program.⁹ OTUs were built at 97% of identity by using vsearch program and Qiime modules (version qiime2-2017.12) were used for taxonomic annotation.¹⁰ In order to assign taxonomy up to species level to each OTU's centroid, we classified them using a naïve bayes classifier model previously fitted against the Green Genes database version 13.5.

16S rRNA gene reads from the total milk samples were used in order to perform an accurate filtering of the flow cytometry IgA-sorted fractions that, due to low bacterial yield, were more susceptible to sequencing contaminations. This was done by eliminating OTUs in IgA sorted fractions that were absent in corresponding total non-sorted milk samples. Moreover, OTUs were also removed in cases where either IgA positive or IgA negative fractions presented an abundance of less than five reads, compared to its corresponding OTU in non-sorted milk sample. In addition, we filtered out low signal OTUs that were presented in less than five sequences through the total set of samples.

α -diversity analyses (presented here as Shannon and Chao1 indices), were utilized to estimate the samples' diversity and richness at the 97% OTU level using the R-package Vegan.¹¹ Constrained correspondence analysis (CCA) was used here to emphasize variation and bring out strong patterns in the dataset. This analysis was performed by R software ade4 package

together with permutational multivariate analysis (Adonis) determining the differences in variance between groups.

For analyzing IgA-coating patterns, the threshold used for including the genera was 0.5 % or greater in relative abundance in either the IgA positive or IgA negative fractions. A pseudocount that was equal to 0.001 was added to every genus dedicated in both the IgA positive or IgA negative fractions, thus avoiding the fractions with a value of zero. The abundance proportions of a given genera were log-transformed before calculating the ratio between the IgA positive or IgA negative fractions, resulting in the IgA index.^{12,13} The IgA index (calculated according the formula $\log(IgA+/IgA-)$) score reflects the degree of mucosal immune responsiveness to the microbiota, where the positive values represent the genera predominantly found IgA-coated while the negative values the bacterial genera predominantly found IgA uncoated.

The MaAslin multivariate statistical framework was used in this study in order to evaluate if the confounding factors, including probiotics supplementation during pregnancy and maternal atopy, could influence microbial community abundance.¹⁴ Statistical analyses were performed in R version 3.2.2 and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA, Version 6.1f), where $p < 0.05$ was considered significant. Specific statistical tests (including Mann–Whitney U-test/ Wilcoxon rank-sum test for nonparametric comparisons together with false discovery rate control giving the adjusted p-value) are stated in figure legends.

RESULTS

Table S1. Chao1 and Shannon indices describing the species richness and diversity, respectively, in total milk samples, in the IgA bound and IgA free fractions. Mean and standard error of the mean (SEM) are indicated.

HEALTHY non-sorted (n=20)		ALLERGIC non-sorted (n=20)		ASTHMA non-sorted (n=10)		p-value
Chao1	SEM	Chao1	SEM	Chao1	SEM	H vs Allergic / H vs Asthmatic
542.26	32.96	430.30	40.74	424.88	71.38	0.018*/ 0.065
HEALTHY IgA-bound (n=40)		ALLERGIC IgA-bound (n=40)		ASTHMA IgA-bound (n=20)		p-value
Chao1	SEM	Chao1	SEM	Chao1	SEM	H vs Allergic / H vs Asthmatic
62.67	6.26	65.88	9.39	70.33	17.05	0.733/0.820
HEALTHY IgA-free (n=40)		ALLERGIC IgA-free (n=40)		ASTHMA IgA-free (n=20)		p-value
Chao1	SEM	Chao1	SEM	Chao1	SEM	H vs Allergic / H vs Asthmatic
57.09	5.45	58.14	6.75	58.42	11.78	0.898/ 0.820

HEALTHY non-sorted (n=20)		ALLERGIC non-sorted (n=20)		ASTHMA non-sorted (n=10)		p-value
Shannon	SEM	Shannon	SEM	Shannon	SEM	H vs Allergic / H vs Asthmatic
1.96	0.11	1.87	0.19	1.82	0.30	0.351/0.411
HEALTHY IgA-bound (n=40)		ALLERGIC IgA-bound (n=40)		ASTHMA IgA-bound (n=20)		p-value
Shannon	SEM	Shannon	SEM	Shannon	SEM	H vs Allergic / H vs Asthmatic
2.45	0.10	2.38	0.11	2.42	0.18	0.490/0.893
HEALTHY IgA-free (n=40)		ALLERGIC IgA-free (n=40)		ASTHMA IgA-free (n=20)		p-value
Shannon	SEM	Shannon	SEM	Shannon	SEM	H vs Allergic / H vs Asthmatic
2.40	0.09	2.32	0.15	2.18	0.28	0.875/0.859

* Mann-Whitney U test.

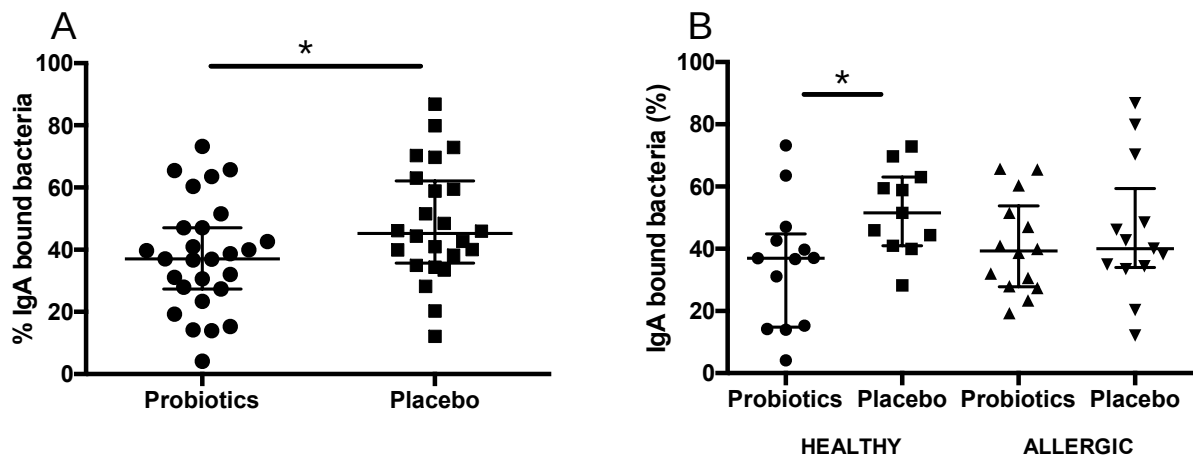


Fig. S1. IgA-coating levels in the breastmilk of mothers treated, or not, with *L. reuteri*, as determined with flow cytometry based – cell sorting. (A) Proportions of IgA bound bacteria in breastmilk of mothers treated with *L. reuteri* probiotics and mothers treated with placebo. $p = 0.04$; $n_{\text{Placebo}} = 27$, $n_{\text{Probiotics}} = 24$ (B) Proportions of IgA

bound bacteria in breastmilk of mothers, treated with probiotics or not, whose children stayed healthy ($p=0.02$) or developed allergic diseases during the first 7 years of age. Healthy= $n_{\text{placebo}}=11$, $n_{\text{probiotics}}=13$; Allergic: $n_{\text{placebo}}=13$, $n_{\text{probiotics}}=14$. Media with interquartile ranges are indicated; * Mann-Whitney U test.

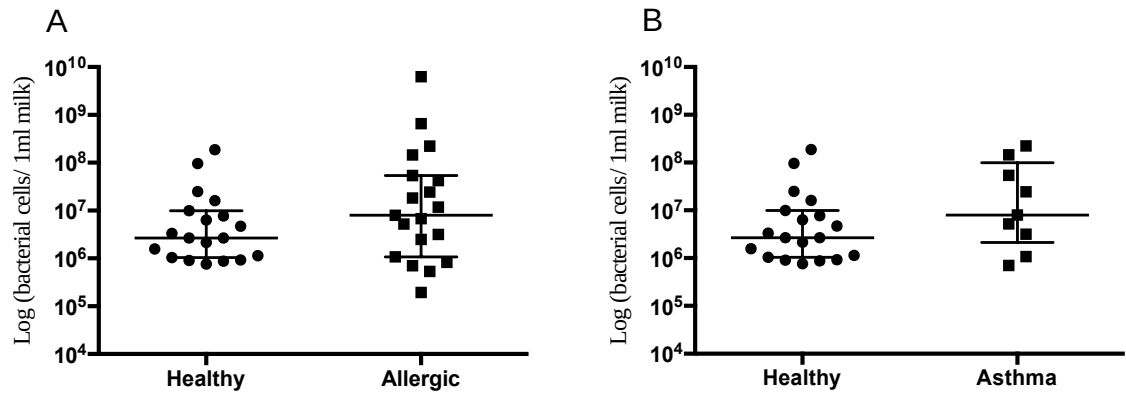


Fig. S2. Bacterial load in breastmilk collected at one month *post partum*. Quantification of bacterial numbers was obtained by using qPCR detection with universal primers targeting the 16S rRNA bacterial gene. (A) Bacterial load in breastmilk samples fed to children developing allergies or staying healthy. (B) Bacterial load in breastmilk samples fed to children developing asthmatic disease or staying healthy. $n_{\text{Healthy}}=19$; and $n_{\text{Allergic}}=19$; $n_{\text{Asthmatic}}=9$. Media with interquartile ranges are indicated and no statistically significant differences were observed.

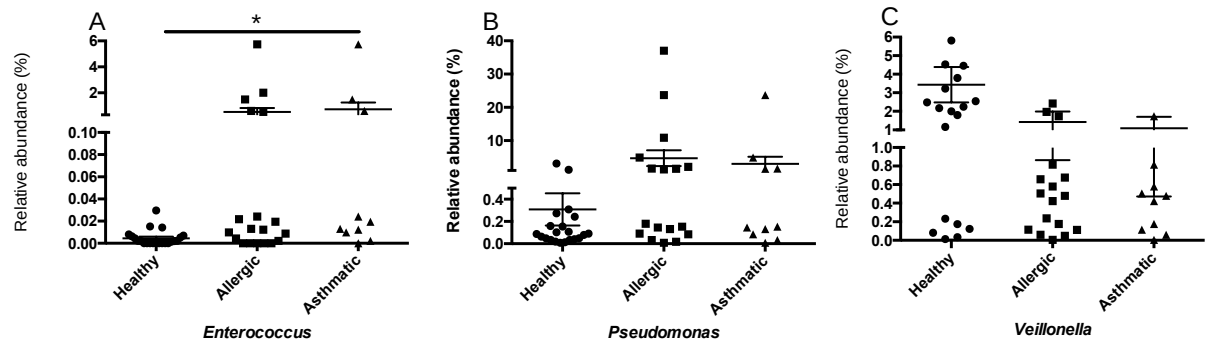


Fig. S3. Relative abundance of selected bacterial genera found in non-sorted breastmilk samples of mothers whose children stay healthy or develop allergic and/or asthmatic manifestations. Mean with SEM are presented. * $p < 0.05$, Mann-Whitney U test, FDR adjusted p-value.

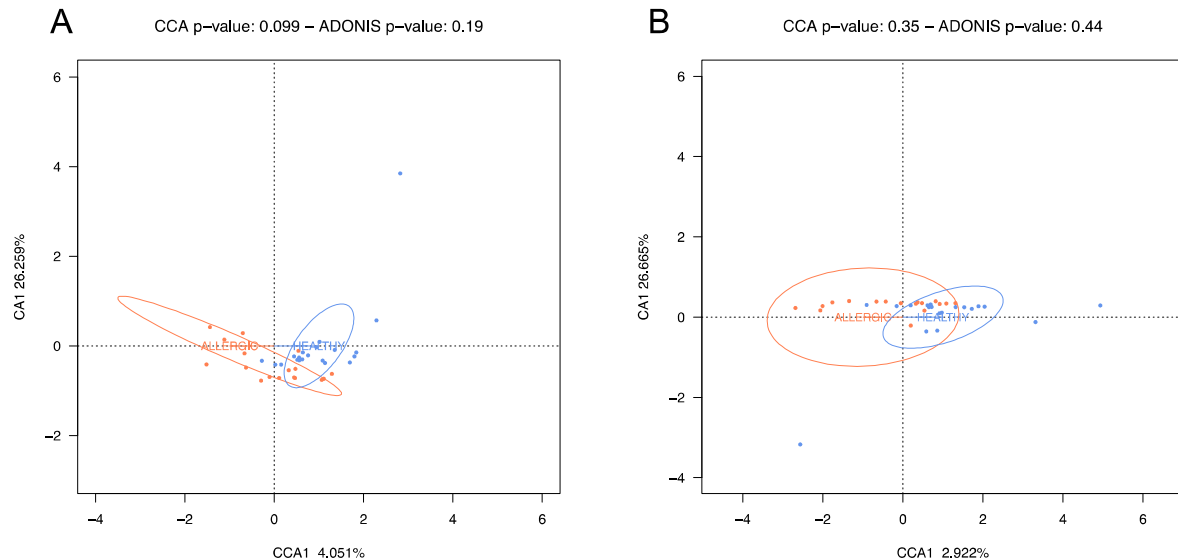


Fig. S4. Breastmilk microbiota composition patterns of sorted IgA fractions, IgA-coated and IgA-free, in allergy development. Constrained correspondence analyses (CCA) based on breastmilk microbiota patterns of the dominant bacterial genera (>0.5% of total) coated with IgA (A) or not coated with IgA (B), from mothers whose children did (n=20) or did not (n=20) develop allergic diseases (p=0.19 and p=0.44 respectively).

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