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Aberrant IgA responses to the gut microbiota during infancy precedes asthma and allergy development

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Conflicts of interest: Maria Jenmalm and Thomas Abrahamsson have received honoraria for lectures and funding for a clinical trial from Biogaia AB, Sweden. The rest of the authors declare that they have no relevant conflicts of interest.
Abstract

Background: While a reduced gut microbiota diversity and low mucosal total IgA levels in infancy have been associated with allergy development, IgA responses to the gut microbiota have not yet been studied.

Objective: We sought to determine the proportions of IgA coating together with the characterization of the dominant bacteria, bound to IgA or not, in infant stool samples in relation to allergy development.

Methods: A combination of flow cytometry cell sorting and deep sequencing of the 16S rDNA gene was used to characterize the bacterial recognition patterns by IgA in stool samples collected at 1 and 12 month of age from children staying healthy or developing allergic symptoms up to seven years of age.

Results: The children developing allergic manifestations, particularly asthma, during childhood had a lower proportion of IgA bound to fecal bacteria at 12 months of age compared with healthy children. These alterations cannot be attributed to differences in IgA levels or bacterial load between the two groups. Moreover, the bacterial targets of early IgA responses (including the coating of Bacteroides genus) as well as the IgA recognition patterns differed between healthy children and children developing allergic manifestations. Altered IgA recognition patterns in children developing allergy were observed also already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breast fed children.

Conclusion: An aberrant IgA responsiveness to the gut microbiota during infancy precedes asthma and allergy development, possibly indicating an impaired mucosal barrier function in allergic children.
Key message: Aberrant and reduced IgA responses to the gut microbiota during infancy precede development of asthma and allergic disease during the first seven years of life.

Capsule summary: Early characterization of IgA coating patterns may represent a novel way to identify infants with increased risk to develop asthma and allergic disease. Moreover, interventions enhancing infant mucosal barrier function may represent efficacious preventive strategies required to combat the asthma and allergy epidemic.

Key words: Allergic disease; asthma; SIgA; IgA index; IgA recognition patterns; microbiome composition; gut microbiota, childhood.

Abbreviations used:
A: Allergic
ARC: Allergic rhinoconjunctivitis
H: Healthy
IgA: Immunoglobulin A
IgA+: IgA coated
IgA-: non IgA bound
PCA: Principal Component Analysis
RDP: Ribosomal Database Project
SIgA: Secretory immunoglobulin A
TLR: Toll Like Receptor
**Introduction**

Allergic diseases have become a major public health problem in affluent societies. Reduced microbial exposure, both pre- and postnatally, has been proposed to underlie the increase in allergy development. The gut microbiota, hosting a complex bacterial community, is quantitatively the most important source of microbial stimulation and may provide a primary signal for appropriate immune development. 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, although no specific microbes with consistently harmful or allergy protective roles have yet been identified. Also, we observed that the differences in the gut microbiota diversity during infancy between healthy children and children developing allergies were mainly related to asthma and not allergic rhinoconjunctivitis development. Early establishment of a diverse gut microbiota, with repeated exposure to new bacterial antigens, may be more important than the distribution of specific microbial species in shaping a normal immune mucosal and systemic maturation.

A reduced mucosal barrier function may increase the risk for allergy development and immunoglobulin A (IgA) is the primary mediator of humoral mucosal immunity. Immunoglobulin A is the most abundantly produced antibody in humans, with the highest amount of secretion in the intestinal tract. Secretory IgA (SIgA) has a crucial role in the gut through its binding to bacterial antigens, thus preventing their direct interaction with the host via immune exclusion and maintaining the mucosal homeostasis. SIgA may also limit overgrowth of select species, thus stimulating diversity. Therefore, this antibody represents a key host mechanism in regulation of the commensal community, and innate receptor
signaling in T-cells seems to decide the specificity of IgA to constrain the composition of the intestinal bacteria, ensuring a benign symbiotic relationship. However, in contrast to IgG and IgM levels, the generation of this anti-inflammatory antibody is limited during early infancy and delayed development of mucosal IgA production, for instance in the absence of breastfeeding, may lead to infectious disease in young infants. Studies and clinical reports suggest that SIgA that originates from the mothers’ breast milk is important for immune regulation and protection against bacterial, viral and parasitic infections in suckling infants.

Whereas total levels of SIgA in saliva and fecal samples have been investigated in children developing allergy before, little is known about the identities of the bacterial taxa targeted by IgA in the infant gut and what role mucosal immune responses to the gut microbiota plays in childhood allergy development. However, earlier studies have shown that low levels of salivary and intestinal SIgA are associated with an increased risk for allergic manifestations during early life. Recent advances in flow cytometry and next generation sequencing now allow studying the complex interactions between human antibodies and microbiota. In this study, we have used flow cytometry-based cell sorting and barcoded 16S rDNA 454-pyrosequencing to characterize the dominant gut bacteria, coated or non-coated with IgA, and determined total secretory IgA levels and bacterial load in stool samples collected during the first year of life in infants who either developed allergic manifestations or stayed healthy up to 7 years of age.
Methods

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

Study design

The infants included in this study were part of a larger randomized double-blind trial in South-Eastern Sweden between 2001 and 2003, evaluating the potential allergy prevention effect of probiotic *Lactobacillus reuteri* ATCC 55730, until 2(33) and 7 years of age.(34) The recruited children had a family history of allergic disease (1 or more family members with eczema, asthma, gastrointestinal allergy, allergic urticaria or allergic rhinoconjunctivitis), and more detailed inclusion and exclusion criteria are explained in the study of Abrahamsson et al.(33) Among the 188 infants completing the original study, infant stool samples collected at 1 and 12 months of life in 20 children developing allergy (Table EI in this article's Online Repository at www.jacionline.org) and 28 children staying healthy up to 7 years of age (Table EII), were randomly selected for this study (Fig. 1). Ten of the allergic children developed asthma. Other allergic diseases included eczema (n=9 at 7 years of age; n=17 at 2 years of age; no infants developed eczema before 1 month of age), allergic rhinoconjunctivitis (n=10) and allergic urticaria (n=1), with symptoms defined as described in detail previously.(33,34) The samples were immediately frozen at -20°C following collection and later stored at -70°C until use.

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 develop allergic
manifestations (Table I). All included infants were exclusively breast-fed for at least 1 month, and no infant received antibiotics before 1 month of age. The Regional Ethics Committee for Human Research at Linköping University approved the study. Informed consent was obtained from both parents before inclusion. The study is registered at ClinicalTrials.gov (ID NCT01285830).

Sample labeling and flow cytometry protocol

Stool samples were suspended in sterile saline solution (autoclaved H$_2$O; NaCl Sodium Chloride 99.5% PA-ACS-ISO, Panreac; Barcelona, Spain; Ref. 131689.1211) with 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA; Ref. A7030-100gr) in order to prevent non-specific antibody binding. The samples were stained with goat anti-mouse IgA labeled with FITC, used as an isotype control corresponding to unspecific binding, (Invitrogen, Frederick, MD, USA; Ref. M31001) or with goat anti-human IgA labeled with FITC (Invitrogen; Ref. H14001), according to the manufacturer instructions (Fig. E1 in this article’s Online Repository at www.jacionline.org). The sorting of the bacterial cells according to whether they were IgA coated (IgA+) or IgA non-coated (IgA-) was performed by MoFlo™ XDP Cell Sorter (Beckman Coulter, Inc; Brea, CA, USA), following Simon-Soro et al. 2015.

DNA-extraction

DNA from sorted fecal bacteria, IgA+ and IgA-, was isolated using the MasterPure™ complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), following the manufacturer’s instructions with a previous glass bead beating (0.17 mm diameter) and an additional enzymatic lysis step with lysozyme (20mg/ml,
37°C, 30 min; Thermomixer comfort, Eppendorf, Hamburg, Germany).

16S rDNA gene amplification and sequencing

DNA from 192 samples in total was used for PCR amplification and pyrosequencing in order to describe bacterial composition of the sorted populations. A region of approximately 650-bp of the 16S rDNA gene was amplified using universal bacterial degenerate primers(35), which encompass the hypervariable regions V3-V5 of the gene. A secondary amplification was performed by using the purified PCR product as a template.(36)

Sequence processing and taxonomic classification

The resulting 16S rDNA read ends were trimmed in 10 bp sliding windows, with average value ≥20, using the Galaxy tool(37) and only reads longer than 250 bp were considered. The sequences were assigned to each sample by the 8 bp barcode through the Ribosomal Database Project (RDP) pipeline(38) version 11.3, and chimeric sequences were filtered out using UCHIME. (39)

Taxonomic assignment was performed by the RDP-classifier(38) where the reads were assigned a phylum, class, family and genus and phylogenetic ranks were allocated when scores exceeded 0.8 confidence threshold. Shannon indices, based on randomly selected 700 reads per sample, was utilized to estimate the samples’ diversity on gene and phylum level.

For analyzing IgA coating patterns, the threshold used for including the genera was ≥1% relative abundance in either the IgA+ or IgA- fractions. The abundance proportions of a given genera was used to calculate the ratio between IgA+ and IgA-
fractions, giving the IgA index.(40) Thus, this score was based on proportional representation, for every given genus, within the IgA+ (positive IgA index values) and IgA- fractions (negative IgA index values), reflecting the degree of mucosal immune responsiveness to the microbiota. LDA Effect Size (LEfSe)(41) was then used for high-dimensional biomarker discovery comparing the IgA-indices between healthy infants and infants developing allergic manifestations. Furthermore, Principal ComponentAnalyses (PCA) was performed by R software ade4 package.(42)

**Bacterial load analysis with qPCR**

qPCR amplifications were performed in order to measure the bacterial load (number of bacterial cells normalized by the number of human cells) using primers targeting the single-copy housekeeping bacterial gene FusA and the human β-actin gene (Table EIII).

**Determination of secretory IgA concentrations in stool samples**

A commercially available ELISA kit was used for the determination of total secretory IgA concentrations in feces samples (ImmuChrom ELISA kit, ImmuChrom GmbH, Heppenheim, Germany) following the manufacturer’s instructions.

**Statistics**

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. For a more comprehensive description of the statistical methods, please see this article’s Online Repository at www.jacionline.org.
Results

Proportion of fecal bacteria bound to IgA in relation to allergy development

Infants developing allergic symptoms during the first 7 years of life had significantly lower proportions of IgA-coated fecal bacteria at 12 months of age than healthy children (Fig. 2A), while similar proportions were observed at 1 month of age. A low proportion of IgA-coated fecal bacteria at 12 months of age also preceded development of asthma (Fig. 2B), but not allergic rhinoconjunctivitis (Fig. E2 in this article’s Online Repository at www.jacionline.org). Moreover, independently of allergy development, an overall decreasing proportion of fecal bacteria bound to IgA from 1 to 12 months of age was observed, likely reflecting a change from predominantly maternally breast milk derived to child derived IgA antibodies.(22,43)

The influence of possible confounding factors was also evaluated. However, supplementation of the probiotic bacterium *L. reuteri*, delivery mode, antibiotic treatments and partial breastfeeding at 12 months of age did not affect the proportion of IgA-coated fecal bacteria (Fig. E3).
Bacterial load, but not total SIgA levels, differ in healthy children and children developing allergic manifestations

In order to better understand detected differences in IgA proportions between healthy children and children developing allergic manifestations, bacterial load and total SIgA levels in stool samples were measured. The bacterial load was higher at 12 months of age in children staying healthy than in those developing allergic manifestations (Fig. 3A), but not significantly so for asthma (p=0.11) and ARC (p=0.61). A similar bacterial load was observed at 1 month of age in children staying healthy and developing allergy (Fig. 3A).

Total fecal SIgA levels were similar in healthy children and children developing allergic manifestations (Fig. 3B), asthma (p=0.38 and p=0.71, for 1 and 12 months, respectively) and allergic rhinoconjunctivitis (p=0.77 and p=0.78, for 1 and 12 months, respectively). The fecal SIgA levels decreased significantly from 1 to 12 months of age in both groups (Fig. 3B).
Bacterial targets of IgA responses in children developing allergic manifestations and children staying healthy up to 7 years of age

Bacterial 16S rDNA gene sequencing of IgA+ and IgA- fractions was performed in order to assess early IgA responses in children staying healthy and children developing allergic manifestations during the first 7 years of age. After quality filtering and removal of chimeric sequences, 190 samples with 633,378 high-quality sequence reads remained, with an average of 3,316 reads per sample and a mean length of 515 bp.

While the analysis of the relative abundance of dominant bacterial families was generally similar between children developing allergy and staying healthy (Fig. E4 in this article’s Online Repository at www.jacionline.org), clear differences were observed upon analyzing the bacterial targets of IgA responses, represented as IgA index. IgA responses to the gut microbiota were demonstrated to differ between healthy children and children developing allergic manifestations (Fig. 4A, B) and asthmatic symptoms, particularly at 1 month but also 12 months of age (Fig. 4C, D).

At 1 month of age the genus *Faecalibacterium* was mainly IgA free (IgA-) in children developing allergic manifestations (including asthma but not allergic rhinoconjunctivitis; Fig. E5A). Moreover, the genera *Parabacteroides* and *Anaerococcus* were primarily not targeted by IgA in children developing allergic manifestations, when compared with healthy children.

At 12 months of age, the IgA responses of children developing allergic manifestations were mainly not targeting the *Bacteroides* genus (Fig. 4B), with similar findings observed for children developing allergic rhinoconjunctivitis (Fig. E5B). Regarding children developing asthmatic symptoms, *Escherichia/Shigella* was predominantly
IgA free (Fig. 4D), while *Lachnospiraceae incertae sedis*, a *Firmicutes* phylum member, was predominantly IgA bound in children developing allergic symptoms, when compared with healthy children (Fig. 4B). Furthermore, the genera *Roseburia* and *Erysipelotrichaceae incertae sedis* were generally IgA-targeted in healthy children, but not in children showing allergic manifestations during the first 7 years of life. In contrast, decreased IgA responses to the *Veillonella* genus was observed in healthy children.

Possible differences in bacterial diversity in children developing allergy and staying healthy were also of interest. Thus, Shannon indices for IgA+ and IgA- fractions were calculated. No differences, neither at genus (Fig. E6) or at phylum level (Fig. E7A-B) were found in relation to allergy development, however, except that children developing asthma had increased diversity at 12 months among IgA coated *Bacteroidetes* and *Proteobacteria* (Fig. E7C) but not in the IgA-free fraction (Fig. E7D).
IgA recognition patterns of gut microbiota differ between healthy and allergic children

A principal components analysis (PCA), based on the calculated IgA indices, was used in order to evaluate the differences in IgA responses to the gut microbiota between healthy children and children developing allergic manifestations, including asthma. Interestingly, the IgA recognition patterns differed already at 1 month of age when comparing healthy children and children developing allergic (Fig. 5A) and asthmatic symptoms (Fig. 5C) but not ARC (Fig. E8A in this article’s Online Repository at www.jacionline.org). Clear separation was observed at 12 months of age for healthy children and children developing allergic disease (Fig. 5B), asthma (Fig. 5D) and ARC (Fig. E8B).

No effect of potential confounding factors (probiotic supplementation, the delivery mode, antibiotic treatment and partial breastfeeding at 12 months) on IgA recognition was observed (Fig. E9).
Discussion

The data presented in the current study demonstrate that the first year of life represents an early-life critical period in which aberrant gut microbiota IgA responses are linked to the risk of developing asthma and allergic disease. SIgA functions as a first line of defense by interfering with the microbiota and thus protect the intestinal tissue from invasion and destruction by pathogenic and commensal bacteria.(18,44)

Thus, intact production and function of IgA is a key mechanism to preserve intestinal health by directly influencing the properties of the microbiota and enhancing mucosal barrier function.(18) Furthermore, SIgA may also limit overgrowth of selected species, thus enabling an increased microbiota diversity.(18,21) This can be particularly crucial during early childhood, when the microbiota plays a central role in immune modulation and where microbial recognition by maternal and infant antibodies must be appropriately orchestrated for an optimal maturation of the immune system.(45-47) In line with this, low mucosal total IgA levels(28-30) a reduced gut microbiota diversity in infancy(8-16) and decreased seroreactivity to gut microbiota antigens(48) have been associated with allergy development. However, intestinal IgA responses to the infant gut microbiota have not previously been studied in relation to allergy development. To investigate this, we have used a combination of flow cytometry and high-throughput deep sequencing to characterize the patterns of bacterial recognition by IgA in stool samples collected at 1 and 12 month of age from children staying healthy or developing allergic symptoms up to seven years of age.

Interestingly, development of allergic disease, particularly asthma, during childhood was associated with a reduced proportion of IgA bound to fecal bacteria at 12 months
of age. To better understand these differences, we sought to investigate if the bacterial load and total fecal IgA levels might be of any influence. The results showed that the lower proportions of IgA coated fecal bacteria among children developing allergic manifestations were independent of total fecal SIgA levels that were relatively similar to healthy children, especially at 12 months of age. Moreover, the decreased proportions of IgA coated bacteria in allergic children are probably not due to lower IgA antibodies-to-bacteria ratios because bacterial densities were actually lower in children developing allergies. Thus, in addition to the lower bacterial diversity detected by other studies,(8-16) allergic children seem also to be exposed to lower microbial densities in the gut. These two factors could lead to decreased stimulation of the immune system via TLR:s,(19) affecting the production and microbial recognition patterns of IgA, thus leading to lower proportion of IgA coating in allergic than healthy children. It would be interesting to further investigate the role of factors influencing IgA production, such as vitamin A-derived retinoic acid, TGF-b, IL-10, BAFF and APRIL(49), in the aberrant IgA responses in children developing allergy in future studies. As we previously found that a low gut microbiota diversity in infancy was mainly related with asthma, but not allergic rhinoconjunctivitis, development at school age(16), we here aimed to determine the importance of IgA responses to the gut microbiota particularly for asthma development. Speculatively, the association with asthma could be due to the fact that viral lower respiratory tract infections have been linked to asthma development among atopic children.(1,50,51) Thus, low IgA responses to the microbiota may result in a reduced mucosal barrier function. This may cause an increased susceptibility to airway viral infections, leading to amplification of Th2 responses and subsequent asthma development.(1,50,51)
Previous studies in adults have determined that IgA might be more reactive against disease driving bacteria,(44,52-54) in line with the theory that the immune system can distinguish between pathogens and commensals through sensing pathogen-associated behaviors, including adherence to the intestinal epithelium and tissue invasion.(53,54) Also, SIgA may enable an increased microbiota diversity by limiting overgrowth of selected species.(18,21) In the current study, we observed that the IgA recognition patterns differed between healthy children and children developing allergic symptoms, including asthmatic disease and allergic rhinoconjunctivitis, with clearly divergent IgA index patterns already at 1 month of age. As the IgA antibodies at 1 month of age in exclusively breast fed infants are predominantly maternally derived,(22,43) the divergent responses observed at this time point suggest that the immunological interactions between mother and offspring influence allergy development, in line with previous studies.(45-47) For example, breast milk derived SIgA had a large impact on microbial colonization in neonatal mice and was crucial for healthy intestinal epithelial barrier function and immune homeostasis in the offspring.(25)

Interestingly, the gut commensals *Faecalibacterium* and *Bacteroides* were mainly IgA free at 1 and 12 months of age in children showing allergic manifestations but were predominantly IgA coated in healthy children, especially at 12 months. These two genera are important human gut symbionts, involved in production of butyrate, an end product of colonic fermentation that is important in maintaining a healthy gut.(55-57) Furthermore, decreased diversity of the *Bacteroidetes* phylum in infant stool samples have been linked to delivery by Cesarean section(4,58) and allergy development.(4,12) Other commensals that seem to be ignored by the IgA recognition
in children developing allergic symptoms were *Parabacteroides*, at 1 month of age, and *Roseburia*, at 12 months of age. The *Parabacteroides* species *distasonis* have been shown to reduce inflammatory responses in murine models with chronic colitis,(59) while *Roseburia* is another well-known butyrate-producer(60), which was reduced in patients suffering from ulcerative colitis.(61) In all, decreased coating of these commensals might reflect a lower stimulation of the mucosal immune system in the infants developing allergic diseases.

The *Erysipelotrichaceaee* family is considered to be highly immunogenic and seems important in inflammation related disorders of the gastrointestinal tract as they are enriched in colorectal cancer.(62,63) Palm and colleagues found that a member of *Erysipelotrichaceous* was highly coated by IgA in specific pathogen free mice, relative to other members of the gut microbiota, proposing its role as a colitogenic bacteria.(54) Furthermore, deregulation of T-cells in mice affecting the selection of IgA plasma cells caused gut dysbiosis, including increased abundance of *Erysipelotrichaceous* that are known to induce immune hyperactivation.(64) *Escherichia* and *Shigella* genera are Proteobacteria proposed to express highly proinflammatory hexa-acylated endotoxin production and were enriched in adult asthmatic patients triggering airway inflammation.(65) Moreover, high abundance of fecal *Escherichia coli* was associated with development of IgE-associated eczema within the first year of life.(66) As allergy and asthma development were associated with reduced IgA responses to the *Erysipelotrichaceous* and *Escherichia/Shigella* genus respectively, at 12 months of age, this may suggest an impaired mucosal immune exclusion of this genus in children developing allergic disease, possibly leading to proinflammatory responses enhancing disease susceptibility.
In contrast, allergy development was associated with increased IgA responses at 12 months of age to Lachnospiraceae, a Gram positive barrier associated microbes that are colonizing the inner mucus layer, staying in close contact with host mucosa. (67,68) Furthermore, excessive growth of species belonging to Lachnospiraceae in mice with impaired IgA responses reduces Firmicutes diversity. (21) The increased IgA coating of these bacteria in children developing allergies might thus be an indication of an altered mucosal barrier function.

Factors that might influence the development of the intestinal microbiota and the mucosal immune system include the mode of delivery, exposure to antibiotics, partial breastfeeding at 12 months of age and probiotic supplementation. (14,23,58,69,70) These confounding factors seem not to have influence in our study population, since the discovered differences are driven by health status. However, larger studies are required to further investigate and confirm the role of these factors. Also, it needs to be determined whether our findings can be replicated in cohorts of other geographic origins and with different family history of allergic disease.

In conclusion, our work suggests that studies of IgA responses to gut microbiota during infancy could be used to determine the normal development of mucosal immunity and establishment of a healthy symbiosis with gut microbes, and how maternal immunity affects these processes. Early characterization of IgA coating patterns may represent a novel way to identify infants with increased risk to develop asthma and allergic disease, although this needs to be confirmed in larger cohorts. Furthermore, interventions enhancing infant mucosal barrier function may represent efficacious preventive strategies required to combat the asthma and allergy epidemic.
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Fig. 1. Schematic workflow of performed experiments. The proportion of the gut microbiota bound to IgA (IgA+) or not (IgA-), in infants, was analyzed by flow cytometry-based sorting of fecal samples prior to 16S rDNA 454-pyrosequencing. In addition, total secretory IgA levels were estimated by ELISA tests, and the bacterial density measured using universal primers targeting the single-copy bacterial gene FusA.

Fig. 2. Proportions of IgA-coated fecal bacteria in early infancy. A) The proportion of fecal bacteria bound to IgA at 1 month and 12 months of age in children staying healthy (n=28, circles) or developing allergic symptoms (n=20, triangles) during the first 7 years of life. B) The proportion of fecal bacteria bound to IgA at 1 month and 12 months of age in children staying healthy (n=28, circles) or developing asthma (n=10, triangles) during the first 7 years of life. Median and interquartile ranges are indicated. *p < 0.05; **p < 0.01 (Mann–Whitney U-test).

Fig. 3. Bacterial load and total fecal secretory IgA levels in healthy infants and infants developing allergic manifestations. A) The quantification of bacterial numbers was obtained by qPCR- detection with universal primers targeting the gene FusA (present in single-copy in bacterial cells) and normalized by the number of human cells, determined by qPCR- detection with primers for the human β-actin gene. n_{healthy}=28; n_{Allergic}=20. B) Total secretory IgA levels in stool samples were measured using ELISA immunoassay. 1 month of age: n_{healthy}=25; n_{Allergic}=19; 12 months of age: n_{healthy}=27; n_{Allergic}=19. Means with standard errors are indicated. * p<0.05; ** p<0.01; *** p<0.001 (Mann Whitney U-test and Wilcoxon matched pairs test for unpaired and paired comparisons, respectively).

Fig. 4. IgA responses to the gut microbiota, at 1 and 12 months of age. Plots are depicting IgA responses (defined by IgA index, reflecting the ratio in IgA+ and IgA-) to dominant genera (>1% of total) of the gut microbiota at 1 month (n_{Healthy}=27; n_{Allergic}=19; n_{Asthma}=10) and 12 months (n_{Healthy}=28; n_{Allergic}=20; n_{Asthma}=10) of age when comparing healthy children and children developing allergic (A, B) and asthmatic symptoms (C, D). For a given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA+ fraction, to negative values (genera found dominantly in the IgA- fraction), as a measure of the degree of mucosal immune responsiveness to the microbiota. LEfSe (Linear discriminant analysis Effect Size) algorithm, emphasizing both statistical and biological relevance, was used for biomarker discovery. Threshold for the logarithmic discriminant analysis (LDA) score was 2. Means with standard errors are indicated. * p<0.05; ** p<0.01.
Fig. 5. IgA recognition patterns of the gut microbiota at 1 (left panels) and 12 months (right panels). Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota at 1 month ($n_{\text{Healthy}}=27; n_{\text{Allergic}}=19; n_{\text{Asthma}}=9; \text{A, C})$ and 12 months ($n_{\text{Healthy}}=27; n_{\text{Allergic}}=19; n_{\text{Asthma}}=10; \text{B, D})$ of age when comparing healthy children with children developing allergic manifestations (A, B) or with children developing asthmatic symptoms (C, D).
Table I. Descriptive data of children included in this study.

<table>
<thead>
<tr>
<th>Children</th>
<th>Healthy % (no)</th>
<th>Developing allergy % (no)</th>
<th>P value*</th>
<th>Developing asthma % (no)</th>
<th>P value*</th>
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</thead>
<tbody>
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<td>Girls</td>
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<td>40 (8)</td>
<td>0.66</td>
<td>40 (4)</td>
<td>1.00</td>
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<td>Older siblings</td>
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<td>50 (10)</td>
<td>0.62</td>
<td>50 (5)</td>
<td>0.70</td>
</tr>
<tr>
<td>Caesarean delivery</td>
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<td>20 (4)</td>
<td>0.70</td>
<td>20 (2)</td>
<td>0.64</td>
</tr>
<tr>
<td>Furred pets</td>
<td>4 (1)</td>
<td>15 (3)</td>
<td>0.29</td>
<td>10 (1)</td>
<td>0.46</td>
</tr>
<tr>
<td>Maternal atopy</td>
<td>82 (23)</td>
<td>80 (16)</td>
<td>1.00</td>
<td>80 (8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Breast-feeding (1 to 12 mo)</td>
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<td>20 (4)</td>
<td>0.74</td>
<td>10 (1)</td>
<td>0.40</td>
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<td>Antibiotic treatment (1-12 mo)</td>
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<td>30 (6)</td>
<td>0.68</td>
<td>50 (5)</td>
<td>0.43</td>
</tr>
<tr>
<td>Day care (1-12 mo)</td>
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<td>10 (2)</td>
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<tr>
<td>Probiotic group</td>
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<td>60 (12)</td>
<td>0.66</td>
<td>60 (6)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*The $x^2$ test was used to detect potential differences in frequencies between groups, except when the expected frequency for any cell was less than 5, when the Fisher exact test was used.
References


(44) Mantis NJ, Rol N, Cortesey B. Secretory IgA’s complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 2011 Nov;4:603-611.


Fig. 1. Schematic workflow of performed experiments. The proportion of the gut microbiota bound to IgA (IgA+) or not (IgA-), in infants, was analyzed by flow cytometry-based sorting of fecal samples prior to 16S rDNA 454-pyrosequencing. In addition, total secretory IgA levels were estimated by ELISA tests, and the bacterial density measured using universal primers targeting the single-copy bacterial gene FusA.
Fig. 2. Proportions of IgA-coated fecal bacteria in early infancy. A) The proportion of fecal bacteria bound to IgA at 1 month and 12 months of age in children staying healthy (n=28, circles) or developing allergic symptoms (n=20, triangles) during the first 7 years of life. B) The proportion of fecal bacteria bound to IgA at 1 month and 12 months of age in children staying healthy (n=28, circles) or developing asthma (n=10, triangles) during the first 7 years of life. Median and interquartile ranges are indicated. *p < 0.05; **p < 0.01 (Mann–Whitney U-test).
Fig. 3. Bacterial load and total fecal secretory IgA levels in healthy infants and infants developing allergic manifestations. A) The quantification of bacterial numbers was obtained by qPCR- detection with universal primers targeting the gene FusA (present in single-copy in bacterial cells) and normalized by the number of human cells, determined by qPCR- detection with primers for the human β-actin gene. \( n_{\text{Healthy}}=28; \ n_{\text{Allergic}}=20 \). B) Total secretory IgA levels in stool samples were measured using ELISA immunoassay. 1 month of age: \( n_{\text{Healthy}}=25; \ n_{\text{Allergic}}=19 \); 12 months of age: \( n_{\text{Healthy}}=27; \ n_{\text{Allergic}}=19 \). Means with standard errors are indicated. * \( p<0.05 \); ** \( p<0.01 \); *** \( p<0.001 \) (Mann Whitney U-test and Wilcoxon matched pairs test for unpaired and paired comparisons, respectively).
Fig. 4. IgA responses to the gut microbiota, at 1 and 12 months of age. Plots are depicting IgA responses (defined by IgA index, reflecting the ratio in IgA+ and IgA-) to
dominant genera (>1% of total) of the gut microbiota at 1 month (n_{Healthy}=27; n_{Allergic}=19; n_{Asthma}=10) and 12 months (n_{Healthy}=28; n_{Allergic}=20; n_{Asthma}=10) of age when comparing healthy children and children developing allergic (A, B) and asthmatic symptoms (C, D). For a given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA+ fraction, to negative values (genera found dominantly in the IgA- fraction), as a measure of the degree of mucosal immune responsiveness to the microbiota. LEfSe (Linear discriminant analysis Effect Size) algorithm, emphasizing both statistical and biological relevance, was used for biomarker discovery. Threshold for the logarithmic discriminant analysis (LDA) score was 2. Means with standard errors are indicated. * p<0.05; ** p<0.01.
Fig. 5. IgA recognition patterns of the gut microbiota at 1 (left panels) and 12 months (right panels). Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota at 1 month (n_{Healthy}=27; n_{Allergic}=19; n_{Asthma}=9; A, C) and 12 months (n_{Healthy}=27; n_{Allergic}=19; n_{Asthma}=10; B, D) of age when comparing healthy children with children developing allergic manifestations (A, B) or with children developing asthmatic symptoms (C, D).
SUPPLEMENTARY MATERIALS

Aberrant IgA responses to the gut microbiota during infancy precedes asthma and allergy development.
Authors: M. Dzidic, T. R. Abrahamsson, A. Artacho, B. Björkstén, M. C. Collado, A. Mira, M. C. Jenmalm

Study design

The infants included in this study were part of a larger randomized double-blind trial in South-Eastern Sweden between 2001 and 2005, evaluating the potential allergy prevention effect of probiotic *Lactobacillus reuteri* ATCC 55730, until 2(1) and 7 years of age.(2) Among the 188 infants completing the original study, infant stool samples collected at 1 and 12 months of life in 20 children developing allergy (Table EI) and 28 children staying healthy up to 7 years of age (Table EII), were randomly selected for this study. Ten of the allergic children developed asthma. Other allergic diseases included eczema (n=9 at 7 years of age; n=17 at 2 years of age; no infants developed eczema before 1 month of age), allergic rhinoconjunctivitis (n=10) and allergic urticaria (n=1), with symptoms defined as described in detail previously.(1,2) The samples were immediately frozen at -20°C following collection and later stored at -70°C until use.

There were no differences regarding potential confounders, such as sex, mode of delivery, birth order, maternal atopy, breast-feeding, antibiotics, and probiotic supplementation, between the infants who did or did not develop allergic manifestations. All included infants were exclusively breast-fed for at least 1 month, and no infant received antibiotics before 1 month of age.(3) Informed consent was obtained from both parents before inclusion.
Sample labeling and flow cytometry protocol

Stool samples were suspended in sterile saline solution (autoclaved H₂O; NaCl Sodium Chloride 99.5% PA-ACS-ISO, Panreac; Barcelona, Spain; Ref. 131689.1211) with 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA; Ref. A7030-100gr) in order to prevent non-specific antibody binding. After short sonication, the samples were stained with goat anti-mouse IgA labeled with FITC (Invitrogen, Frederick, MD, USA; Ref. M31001) or with goat anti-human IgA labeled with FITC (Invitrogen; Ref. H14001), according to the manufacturer instructions. Anti-mouse IgA was used as an isotype control, determining the fluorescence intensity corresponding to unspecific binding (Fig. E1).

The sorting of the bacterial cells according to whether they were IgA coated (IgA+) or IgA non-coated (IgA-) was performed by MoFlo™ XDP Cell Sorter (Beckman Coulter, Inc; Brea, CA, USA) using blue laser (488 nm; 200 mW power) and red diode laser (635 nm; 25 mW power) as a light source. The lasers were aligned using Flow-Check™ (10 µm) and Flow-Set™ (3µm) spheres. Bacterial cell separation was performed according to their fluorescence and forward scatter, reflecting the IgA coating and cell size, respectively, following the protocol by Simon-Soro et al.(4)

DNA-extraction

DNA from sorted fecal bacteria, IgA+ and IgA-, was isolated using the MasterPure™ complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), following the manufacturer’s instructions with a previous glass bead beating (0.17 mm diameter) and an additional enzymatic lysis step with lysozyme (20mg/ml, 37°C, 30 min; Thermomixer comfort, Eppendorf, Hamburg, Germany). DNA was
finally purified by isopropanol/ethanol extractions as previously reported(5) and the concentration and purity were measured by calculating A260/280 ratios in Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

16S rDNA gene amplification and sequencing
DNA from 192 samples in total was used for PCR amplification and pyrosequencing in order to describe bacterial composition of the sorted populations. A region of approximately 650 bp of the 16S rDNA gene was amplified using universal bacterial degenerate primers 357F-CCTACGGGAGGCAGCAG and 926R-CCGCTCAATTCCMTTTRAGT(6), which encompass the hypervariable regions V3-V5 of the gene. This was performed using the high-fidelity AB-Gene DNA polymerase (Thermo Scientific, Waltham, Mass., USA) with an annealing temperature of 52°C and 20 cycles, in order to minimize amplification biases.(7) A secondary amplification (25 cycles, annealing temperature of 52°C) was performed by using the purified PCR product as a template.(8) The primers in this step were modified to contain the pyrosequencing adaptors A and B linked to an 8-bp barcode, specific to each sample. Barcodes were different in at least 3 nucleotides, from each other, to avoid errors in sample assignments. The purification of PCR products was performed using Nucleofast 96 PCR filter plates (Macherey-Nagel, Düren, Germany) and the final concentration of the DNA per sample was measured by PicoGreen fluorescence (454 Life Science, Roche, Brandford, USA) in a QuantiFlour fluorometer from Promega Biosystems (model nr E6090; Sunnyvale, CA, USA). The PCR products were mixed in equimolar amounts and pyrosequencing was performed from the forward primer end using 454
GS-FLX pyrosequencer with Titanium chemistry (Roche, Basel, Switzerland) at the Center for Public Health Research (CSISP) in Valencia, Spain. One eighth of a plate was used for each pool of 20-25 samples.

**Sequence processing and taxonomic classification**

The resulting 16S rDNA read ends were trimmed in 10 bp sliding windows, with average value $\geq 20$, using the Galaxy tool(9) as quality has been shown to dramatically decrease towards the end of the sequences.(10) Only reads longer than 250 bp were considered, as well as those without mismatches in the primer region. The sequences were assigned to each sample by the 8 bp barcode through the Ribosomal Database Project (RDP) pipeline(11) version 11.3, and chimeric sequences were filtered out using UCHIME.(12)

Taxonomic assignment was performed by the RDP-classifier(11) where the reads were assigned a phylum, class, family and genus and phylogenetic ranks were allocated when scores exceeded 0.8 confidence threshold. $\alpha$ – diversity analysis (Shannon indices), based on randomly selected 700 reads per sample, was utilized to estimate the samples’ diversity on gene and phylum level. Here, the sequences over 97% identity, with 90% sequence alignment length, were considered to correspond to the same operational taxonomic unit (OTUs), thus representing a group of reads presumably belonging to the same species.(13)

For analyzing IgA coating patterns, the threshold used for including the genera was $\geq 1\%$ relative abundance in either the IgA+ or IgA- fractions. Further, a pseudocount that was equal to 0.001, was added to every genera dedicated in both the IgA+ and IgA-
fractions, thus evading the fractions with a value of zero. The abundance proportions of a given genera was log-transformed before calculating the ratio between IgA+ and IgA- fractions, giving the IgA index. Thus, this score was based on proportional representation, for every given genus, within the IgA+ (positive IgA index values) and IgA- fractions (negative IgA index values), reflecting the degree of mucosal immune responsiveness to the microbiota. LDA Effect Size (LEfSe) was then used for high-dimensional biomarker discovery comparing the IgA-indices between healthy infants and infants developing allergic manifestations. The used algorithm controls for the large number of statistical comparisons.

Principal Component Analyses (PCA) is a statistic tool used to emphasize variation and bring out strong patterns in a dataset. This analysis was performed by R software ade4 package which is using a Principal Component Analysis constrained to Euclidean metric for multivariate data analysis and graphical display, where samples with similar IgA index values appear closer to each other in the two dimensional space.

**Bacterial load analysis with qPCR**

qPCR amplification and detection were performed in order to measure the bacterial load per human cell using primers targeting the single-copy housekeeping gene FusA, that is present in bacterial cells. Single-copy cellular sequence β-actin was used as a quality control for isolated genomic DNA (Table EIII). The amplifications were performed in duplicates, on a LightCycler 480 Real-Time PCR System (Roche Technologies), using an annealing temperature of 62°C. Each reaction mixture of 10 µl was composed of SYBR Green PCR Master Mix (Roche), 0.5 µl of the specific primer
(concentration 10 µM) and 2 µl of DNA template. The bacterial cell numbers, in each sample, was calculated by comparing the Ct values obtained from those in standard curves. These were generated using serial tenfold dilutions of DNA extracted from 10 million bacteria from infant stool samples and from 5 million human umbilical vein endothelial cells (HUVEC, Advancell, Barcelona, Spain)(18) quantified and sorted by flow cytometry.

**Determination of secretory IgA concentrations in stool samples**

A commercially available ELISA kit was used for the determination of total secretory IgA concentrations in feces samples (ImmuChrom ELISA kit, ImmuChrom GmbH, Heppenheim, Germany). 1 ml of a fecal suspension (100 mg of stool sample in 5 ml wash buffer) was centrifuged for 10 min at 10000xg, and the resulting supernatant (diluted 1:250 with wash buffer) was used in the assay. Samples were additionally diluted 1:10 and 1:5 for samples collected at 1 and 12 months of age respectively, due to high antibody concentrations. The manufacturer's guidelines were followed. The plates were read at 450 nm with 620 nm as the reference wavelength on Infinite F200 plate reader (Tecan Trading AG, Männedorf, Switzerland). All samples were analyzed in duplicate and the detection limit was 3.1 ng/ml.

**Statistics**

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. The chi-squared test was used to detect the potential influence of confounding factors between the groups. Fisher’s exact test was performed when
expected frequency for any cell was less than five. \(\alpha\) – diversity analysis were performed using the statistical software R with the \textit{vegan} package.\(^{(19)}\) Bar plots are presented as mean values with error bars representing the SEM while the scatter plots are demonstrating median with interquartile ranges. Specific statistical test are noted in figure legends. For the PCA-analysis, \textit{DMwR} package of R was used for removing two outliers, sample code 5 (Table E1) and 20 (Table EII). This was done according to \textit{Local Outlier Factor} (LOF) – algorithm that identifies density-based outliers by comparing the LOF-values.\(^{(20)}\)
Fig. E1. Scatter plots presenting how the gating strategy was performed. A) A blank control (unlabelled bacterial cells) was used to determine the threshold for autofluorescence. B) Anti-mouse IgA antibodies were used as an isotype control, determining the fluorescence intensity which was corresponding to unspecific binding (indicated as area R2 in the plot). Green fluorescence above the isotype control threshold was considered as indicative of true IgA-coating (indicated as area R3 in the plot). C) An example of a scatter plot from an anti-human IgA sample that was gated according to the controls above. In this particular patient sample, collected at 12 months of age, most bacteria appear as IgA-coated cells.

Fig. E2. Proportion of fecal bacteria bound to IgA in children developing allergic rhinoconjunctivitis (n=10, triangles) and children staying healthy (n=28, circles) up to 7 years of age.

Fig. E3. Proportion of fecal bacteria bound to IgA in relation to probiotic supplementation, mode of delivery, antibiotic treatment and breastfeeding. A) The proportion of faecal bacteria bound to IgA at 1 month and 12 months of age in children treated with L. reuteri (n=27, circles) or placebo (n=21, triangles). B) The proportion of faecal bacteria bound to IgA at 1 month and 12 months of age in children delivered vaginally (n=40, circles) or by Caesarean section (n=8, triangles). C) The proportion of faecal bacteria bound to IgA at 12 months of age in children untreated (n=31, circles) and treated (n=17, triangles) with antibiotics during their first year of life. D) The proportion of faecal bacteria bound to IgA at 12 months of age in children who were not breastfed (n=36, circles) or who were still partially breastfed (n=12, triangles) at 12 months of age. Median and interquartile ranges are indicated.

Fig. E4. Microbiota composition of the most dominant bacterial families as determined by pyrosequencing of 16S rDNA gene. Plots are showing the relative abundance (>1% of the total) of dominant bacterial families, coated (A, C) or not (B, D) with IgA, in stool samples collected at 1 month (A, B) and 12 months (C, D) of age in 20 infants showing allergic manifestations and 28 infants staying healthy up to 7 years of age. A, allergic; H, healthy; IgA+, IgA-coated bacterial fraction; IgA-, IgA free bacterial fraction. * p < 0.05 (Mann-Whitney test).

n1 month: H IgA+ =27; A IgA+ =19; H IgA- =28; A IgA- =20;
n12 month: H =28; A =20.
**Fig. E5.** IgA responses to the gut microbiota in healthy children and children developing allergic rhinoconjunctivitis during the first 7 years of life. Plots are depicting IgA responses (defined by IgA index, reflecting the differences in IgA+ and IgA-) to dominant genera (>1% of total) of the gut microbiota at A) 1 month (n_{Healthy}=27; n_{ARC}=9) and B) 12 months (n_{Healthy}=28; n_{ARC}=10) of age when comparing healthy children and children developing allergic rhinoconjunctivitis (ARC) during the first 7 years of age. For a given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA+ fraction, to negative values (genera found dominantly in the IgA- fraction). LEfSe (Linear discriminant analysis Effect Size) algorithm was used to detect bacteria with statistically different IgA index values, that could represent potential biomarkers for disease. Means with standard errors are indicated. * p<0.05.

**Fig. E6.** Diversity of the total microbiota in stool samples. Shannon index, based on randomly selected 700 reads per sample (samples with lower amount of reads were excluded), was used to estimate the samples’ diversity of fractions coated or non-coated with IgA, obtained at 1 and 12 months of age from infants who did (A) or did not (H) develop allergic manifestations during the first 7 years of life. Shannon indices were calculated after clustering of sequences in **Operational Taxonomic Units** – (OTUs) at 97% nucleotide identity. Data are shown as median and interquartile ranges. A, allergic; H, healthy; IgA+, IgA-coated bacterial fraction; IgA-, IgA free bacterial fraction.

n\textsubscript{1 month}: H IgA+ =22; A IgA+ =19; H IgA- =24; A IgA- =14;
n\textsubscript{12 month}: H IgA+ =28; A IgA+ =19; H IgA- =27; A IgA- =20.

**Fig. E7.** Bacterial phyla diversity of IgA-coated and non-coated bacteria in stool samples of healthy children and children developing allergic/asthmatic symptoms. Shannon index, based on randomly selected 700 reads per sample, was used to estimate the samples’ diversity of the most dominant phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Samples with lower amount of reads were excluded from the analysis. A) and B) are showing the phylum diversity of IgA-coated (IgA+) and IgA-non coated (IgA-) fractions respectively, at different time points, from infants who did (A) or did not (H) develop allergic manifestations during the first 7 years of life. C) and D) are illustrating Shannon
diversity index for the most dominant phyla in IgA+ and IgA- fractions respectively, from infants who did (As) or did not (H) develop asthmatic manifestations during the first 7 years of life. Data are presented as median and interquartile ranges. * p<0.05; ** p<0.01; *** p<0.001 (Mann-Whitney U test).

A, allergic; As, asthmatic disease; H, healthy; IgA+, IgA-coated bacterial fraction; IgA-, IgA non-coated bacterial fraction.

n1 month: H IgA+ =22; A IgA+ =19; H IgA- =24; A IgA- =14;
n12 month: H IgA+ =28; A IgA+ =19; H IgA- =27; A IgA- =20.

Fig. E8. IgA recognition patterns of the gut microbiota in children with/without allergic rhinoconjunctivitis. Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota at A) 1 month (nHealthy=27; nARC=9) and B) 12 months (nHealthy=27; nARC=10) of age when comparing healthy children and children developing allergic rhinoconjunctivitis during the first 7 years of age.

Fig. E9. IgA recognition patterns of the gut microbiota in relation to probiotic supplementation, mode of delivery, antibiotics treatment and breastfeeding. Plots are showing Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota. IgA recognition patterns in children treated with L. reuteri or placebo at A) 1 month of age (nL.reuteri=20; nplacebo=26) and B) 12 months of age (nL.reuteri=21; nplacebo=27). IgA recognition patterns in children that were vaginally delivered or by Caesarian section (C-section) at C) 1 month of age (nVaginally=38; nC-section=8) and D) 12 months of age (nVaginally=40; nC-section=8). E) The patterns of IgA recognition for the children, at 12 months of age, who were untreated (n=31) and treated (n=17) with antibiotics during their first year of life. F) IgA recognition patterns towards microbiota, in children who were not breastfed (n=36) or who were still partially breastfed (n=12) at 12 months of age.
Table EI. Allergic manifestations and sensitization in the 20 children with allergic symptoms. The children were followed prospectively during the first 7 years of life.

<table>
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<th>Children</th>
<th>Allergy symptoms at 2 years</th>
<th>Allergy symptoms at 7 years</th>
<th>Breastfed at 12 months</th>
<th>Mode of delivery</th>
<th>Antibiotic treatment first year</th>
<th>Probiotics/Placebo treatment</th>
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<td>SPT+, AD</td>
<td>SPT+, ARC, AD</td>
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<td>Probiotics</td>
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AB, asthma bronchiale; AD, atopic dermatitis; ARC, allergic rhinoconjunctivitis; CS, caesarean delivery; SPT, skin prick test; U, allergic urticarial; VD, vaginal delivery. *No information about skin prick test reactivity was available at 7 years of age in this child.
Table EII. 28 children without allergic symptoms and sensitization. The children were followed prospectively for the first 7 years of life.

<table>
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<th>Mode of delivery</th>
<th>Antibiotic treatment first year</th>
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CS, caesarean delivery; VD, vaginal delivery.
Table EIII. Primers used for qPCR-amplification. For the analysis of bacterial load in stool samples, FusA single copy gene (present in bacterial cells) and $\beta$-actin gene (present in human cells) were targeted with universal primers presented below.

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Actin: Isogen Life Science, Barcelona, Spain; FusA: Biomedal, Sevilla, Spain.
References


Online Repository - Figures
Aberrant IgA responses to the gut microbiota during infancy precedes asthma and allergy development.
Authors: M. Dzidic, T. R. Abrahamsson, A. Artacho, B. Björkstén, M. C. Collado, A. Mira, M. C. Jenmalm

Methods

A

Fig. E1. Scatter plots presenting how the gating strategy was performed. A) A blank control (unlabelled bacterial cells) was used to determine the threshold for autofluorescence. B) Anti-mouse
IgA antibodies were used as an isotype control, determining the fluorescence intensity which was corresponding to unspecific binding (indicated as area R2 in the plot). Green fluorescence above the isotype control threshold was considered as indicative of true IgA-coating (indicated as area R3 in the plot). C) An example of a scatter plot from an anti-human IgA sample that was gated according to the controls above. In this particular patient sample, collected at 12 months of age, most bacteria appear as IgA-coated cells.
Results

Fig. E2. Proportion of fecal bacteria bound to IgA in children developing allergic rhinoconjunctivitis (n=10, triangles) and children staying healthy (n=28, circles) up to 7 years of age.
Fig. E3. Proportion of fecal bacteria bound to IgA in relation to probiotic supplementation, mode of delivery, antibiotic treatment and breastfeeding. A) The proportion of faecal bacteria bound to IgA at 1 month and 12 months of age in children treated with *L. reuteri* (n=27, circles) or placebo (n=21, triangles). B) The proportion of faecal bacteria bound to IgA at 1 month and 12 months of age in children delivered vaginally (n=40, circles) or by Caesarean section (n=8, triangles). C) The proportion of faecal bacteria bound to IgA at 12 months of age in children untreated (n=31, circles) and treated (n=17, triangles) with antibiotics during their first year of life. D) The proportion of faecal bacteria bound to IgA at 12 months of age in children who were not breastfed (n=36, circles) or who were still partially breastfed (n=12, triangles) at 12 months of age. Median and interquartile ranges are indicated.
Fig. E4. Microbiota composition of the most dominant bacterial families as determined by pyrosequencing of 16S rDNA gene. Plots are showing the relative abundance (>1% of the total) of dominant bacterial families, coated (A, C) or not (B, D) with IgA, in stool samples collected at 1 month (A, B) and 12 months (C, D) of age in 20 infants showing allergic manifestations and 28 infants staying healthy up to 7 years of age. A, allergic; H, healthy; IgA+, IgA-coated bacterial fraction; IgA-, IgA free bacterial fraction. * $p < 0.05$ (Mann-Whitney test). $n_{1\text{ month}}$: H IgA+ = 27; A IgA+ = 19; H IgA- = 28; A IgA- = 20; $n_{12\text{ month}}$: H = 28; A = 20.
Fig. E5. IgA responses to the gut microbiota in healthy children and children developing allergic rhinoconjunctivitis during the first 7 years of life. Plots are depicting IgA responses (defined by IgA index, reflecting the differences in IgA+ and IgA-) to dominant genera (>1% of total) of the gut microbiota at A) 1 month \((n_{\text{Healthy}}=27; \ n_{\text{ARC}}=9)\) and B) 12 months \((n_{\text{Healthy}}=28; \ n_{\text{ARC}}=10)\) of age when comparing healthy children and children developing allergic rhinoconjunctivitis (ARC) during the first 7 years of age. For a given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA+ fraction, to negative values (genera found dominantly in the IgA- fraction). LEfSe (Linear discriminant analysis Effect Size) algorithm was used to detect bacteria with statistically different IgA index values, that could represent potential biomarkers for disease. Means with standard errors are indicated. * \(p<0.05\).
Fig. E6. Diversity of the total microbiota in stool samples. Shannon index, based on randomly selected 700 reads per sample (samples with lower amount of reads were excluded), was used to estimate the samples’ diversity of fractions coated or non-coated with IgA, obtained at 1 and 12 months of age from infants who did (A) or did not (H) develop allergic manifestations during the first 7 years of life. Shannon indices were calculated after clustering of sequences in Operational Taxonomic Units – (OTUs) at 97% nucleotide identity. Data are shown as median and interquartile ranges. A, allergic; H, healthy; IgA+, IgA-coated bacterial fraction; IgA-, IgA free bacterial fraction.

n_{1\, month}: \ H \ IgA^+ =22; \ A \ IgA^+ =19; \ H \ IgA^- =24; \ A \ IgA^- =14;

n_{12\, month}: \ H \ IgA^+ =28; \ A \ IgA^+ =19; \ H \ IgA^- =27; \ A \ IgA^- =20.
Fig. E7. Bacterial phyla diversity of IgA-coated and non-coated bacteria in stool samples of healthy children and children developing allergic/asthmatic symptoms. Shannon index, based on randomly selected 700 reads per sample, was used to estimate the samples’ diversity of the most dominant phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Samples with lower amount of reads were excluded from the analysis. A) and B) are showing the phylum diversity of IgA-coated (IgA+) and IgA-non coated (IgA-) fractions respectively, at different time points, from infants who did (A) or did not (H) develop allergic manifestations during the first 7 years of life. C) and D) are illustrating Shannon diversity index for the most dominant phyla in IgA+ and IgA- fractions respectively, from infants who did (As) or did not (H) develop asthmatic manifestations during the first 7 years of life. Data are presented as median and interquartile ranges. * p<0.05; ** p<0.01; *** p<0.001 (Mann-Whitney U test).

A, allergic; As, asthmatic disease; H, healthy; IgA+, IgA-coated bacterial fraction; IgA-, IgA non-coated bacterial fraction.

n_{1 month}: H IgA+ =22; A IgA+ =19; H IgA- =24; A IgA- =14;
n_{12 month}: H IgA+ =28; A IgA+ =19; H IgA- =27; A IgA- =20.
Fig. E8. IgA recognition patterns of the gut microbiota in children with/without allergic rhinoconjunctivitis. Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota at A) 1 month (n_{Healthy}=27; n_{ARC}=9) and B) 12 months (n_{Healthy}=27; n_{ARC}=10) of age when comparing healthy children and children developing allergic rhinoconjunctivitis during the first 7 years of age.
Fig. E9. IgA recognition patterns of the gut microbiota in relation to probiotic supplementation, mode of delivery, antibiotics treatment and breastfeeding. Plots are showing Principal component
analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota. IgA recognition patterns in children treated with *L. reuteri* or placebo at A) 1 month of age (*n*$_{L\text{-}reuteri}$=20; *n*$_{\text{placebo}}$=26) and B) 12 months of age (*n*$_{L\text{-}reuteri}$=21; *n*$_{\text{placebo}}$=27). IgA recognition patterns in children that were vaginally delivered or by Caesarian section (C-section) at C) 1 month of age (*n*$_{\text{Vaginally}}$=38; *n*$_{\text{C\text{-}section}}$=8) and D) 12 months of age (*n*$_{\text{Vaginally}}$=40; *n*$_{\text{C\text{-}section}}$=8). E) The patterns of IgA recognition for the children, at 12 months of age, who were untreated (*n*=31) and treated (*n*=17) with antibiotics during their first year of life. F) IgA recognition patterns towards microbiota, in children who were not breastfed (*n*=36) or who were still partially breastfed (*n*=12) at 12 months of age.