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Dzidic, M., Abrahamsson, T., Artacho, A., Collado, M. C., Mira, A., Jenmalm, M., (2018), Oral microbiota maturation during the first 7 years of life in relation to allergy development, *Allergy. European Journal of Allergy and Clinical Immunology*, 73(10), 2000-2011.
<https://doi.org/10.1111/all.13449>

Original publication available at:

<https://doi.org/10.1111/all.13449>

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Article type : Original Article: Experimental Allergy and Immunology

Oral microbiota maturation during the first 7 years of life in relation to allergy development

Short title: **Oral microbiota maturation and allergy development**

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.13449

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Funding: Alex Mira: Spanish Ministry of Economy and Competitiveness (grant no. BIO2015-68711-R). Maria C. Jenmalm: The Swedish Research Council (2016-01698); the Swedish Heart and Lung Foundation (20140321); the Medical Research Council of Southeast Sweden (FORSS-573471); the Cancer and Allergy Foundation. Maria Carmen Collado: European Research Council (ERC-starting grant 639226).

Author contributions: T.R.A. and M.C.J. were responsible for sample collection and clinical evaluation of the children. A.M., M.C.J., and M.C.C., designed the study. M.D. performed the sample preparations, experimental work and analyses/presentation of the data. A.A., M.D. performed statistical analysis in R. All authors interpreted and discussed the results. M.D. drafted the manuscript. All authors contributed to and approved the final draft for publication. The authors declare no conflict of interest.

ABSTRACT

Background: Allergic diseases have become a major public health problem in affluent societies. Microbial colonization early in life seems to be critical for instructing regulation on immune system maturation and allergy development in children. Even though the oral cavity is the first site of encounter between a majority of foreign antigens and the immune system, the influence of oral bacteria on allergy development has not yet been reported.

Objective: We sought to determine the bacterial composition in longitudinally collected saliva samples during childhood in relation to allergy development.

Methods: Illumina sequencing of the 16S rDNA gene was used to characterize the oral bacterial composition in saliva samples collected at 3, 6, 12, 24 months and 7 years of age from children developing allergic symptoms and sensitization (n=47) and children staying healthy (n=33) up to seven years of age.

Results: Children developing allergic disease, particularly asthma, had lower diversity of salivary bacteria together with highly divergent bacterial composition at 7 years of age, showing a clearly altered oral microbiota in these individuals, likely as a consequence of an impaired immune system during infancy. Moreover, the relative amounts of several bacterial species, including increased abundance of *Gemella haemolysans* in children developing allergies and *Lactobacillus gasseri* and *L. crispatus* in healthy children, was distinctive during early infancy, likely influencing early immune maturation.

Conclusion: Early changes in oral microbial composition seem to influence immune maturation and allergy development. Future experiments should test the probiotic potential of *L. gasseri* and *L. crispatus* isolates.

Keywords: Allergy development, infancy, *Gemella haemolysans*, *Lactobacillus*, oral microbiota.

INTRODUCTION

During the past decades, allergic diseases have become a major public health problem in affluent societies.(1) Microbial colonization occurring early in life seems to be critical for instructing regulation on the maturation of the immune system and allergy development in children.(2, 3) Approximately 700 common microbial species have been detected in the oral cavity.(4) Typically, the commensal microbiota here have a symbiotic relationship with the host, although, under certain circumstances, some microbes can overcome host defences and become pathogenic.(5) At the birth and following hours, the infant's oral cavity is exposed to a large amount of microorganisms encountered through the birth canal and during breastfeeding, in the contact with parents and medical staff and through breathing.(6) Moreover, it has been observed that maternal intrapartum antibiotic administration contributed to the shaping of the microbial colonization pattern in the neonatal oral cavity.(7)

During the initial period, the microbiota composition of different niches of the neonate's body highly resemble each other.(8) Niche specific establishment of a microbiota of increasing complexity then occurs, concurrent with immune system maturation.(9) However, relatively little is known about how the microbiome develops at extra-intestinal sites during infancy. As yet, there are no published longitudinal studies regarding oral microbiota development during early childhood with culture independent next generation sequencing methodology.

Accumulating evidence shows a close relationship between microbial dysbiosis during infancy and allergy development during childhood.(2, 3) Factors such as early life antimicrobial exposure(10), caesarean delivery(11), formula feeding(12) and maternal consumption of antimicrobials during pregnancy(13) have been identified to have capacity to influence microbial composition, thus potentially contributing to allergic disease development in childhood. Most of the studies present today are describing the microbial colonization in the gut(3, 9), yet there are also indications that microbial colonization of the skin(14, 15) and respiratory tract might be associated with allergies.(16) While some studies are demonstrating the bacterial dysbiosis and lower microbial diversity already before the onset of the allergic disease(14, 17–21), other are describing and comparing the differences in microbiota in children having allergies and being healthy.(15) Because the oral cavity is the first line of encounter between the immune system and the majority of foreign antigens, it is plausible to believe that the oral microbiota might have a crucial role in allergy development. While gut, skin and nasopharyngeal microbial dysbiosis during infancy has earlier been associated with the aberrant development of immune responses and allergy(14, 18, 19), the influence of oral bacteria on allergy development has not yet been studied in longitudinal cohorts and needs to be further addressed.

In this study, we aimed to evaluate the longitudinal development of oral microbiota during infancy and childhood in saliva samples from children developing allergies and children staying healthy up to 7 years of age by using culture-independent next generation sequencing methodologies.

METHODS

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

Sample collection and study design

The infants 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 prevention effect of probiotic *Lactobacillus reuteri* ATCC 55730 until 2 and 7 years of age was evaluated.(22, 23) Among the 188 infants completing the original study, longitudinal salivary samples, collected at 3, 6, 12 and 24 month and 7 years of age, in 47 children developing allergic disease and 33 children staying healthy up to 7 years of age were selected for this study. The selection was based on sample availability and a clear allergy diagnosis. Allergic disease included eczema, gastrointestinal allergy, asthma, allergic rhinoconjunctivitis (ARC) and allergic urticaria. All allergic children (having allergic symptoms) in the current study were also sensitized. Infants were regarded as sensitized if they had at least one positive skin prick test and/or detectable circulating allergen specific-IgE antibodies.(22, 23) Skin prick testing and circulating IgE antibodies analyses were performed for all the children of the cohort, including those that did not have any allergic symptoms, in order to determine their atopy status. Skin prick tests were performed on the volar aspects of the forearm with egg white, fresh skimmed cow milk, and standardized cat, birch, and timothy extracts at 6, 12, and 24 months, and 7 years of age (at this time point also peanut, house dust mite (Der p) and dog). Circulating IgE antibodies to egg white, cow's milk cod, wheat, peanut, and soybean, was analysed at 6, 12, and 24 months of age.(23) Asthma diagnosis required at least one of following two criteria: 1. Doctor diagnosis and asthma symptoms and/or medication during the last 12 months; 2. Wheeze or nocturnal cough and a positive reversibility test with spirometry. Please see the supplementary section for detailed information.

Possible confounders, such as mode of delivery, breastfeeding, probiotics supplementation, maternal allergy and antibiotics use during the first two years of age were obtained from medical records and questionnaires (Table 1). 90% and 77% of all the children included were exclusively breast-fed up to 1 and 3 months of age, respectively, and 96% were partially breastfed at 3 months of age. No infant received antibiotics before 1 month of age and one at 3 months of age.

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.

DNA extraction and 16S rDNA gene amplification and sequencing

DNA from 250 μ l of saliva samples was isolated with a MagNA Pure LC 2.0 equipment (1996-2016 Roche Diagnostics, Barcelona, Spain), using MagNA Pure LC DNA Isolation Kit III for Bacteria & Fungi (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions with additional enzymatic treatments. Extracted DNA was pre-amplified in order to increase total nucleic acid yield by using universal bacterial degenerate primers, encompassing the hypervariable regions V1-V5 of the gene. The DNA was sequenced on a MiSeq Sequencer according to manufacturer's instructions (Illumina).

Bacterial load and measurements by quantitative PCR

Total bacterial load (bacterial cells per ml of saliva) in saliva samples was measured by quantitative PCR using primers targeting 16S rDNA gene. Please see the Methods section in this article's supplementary information for more details.

Bioinformatics and statistics

Only overlapping paired-end reads were used for analysis. Sequences of <250 nucleotides in length were not considered; 5' 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 prior to taxonomical classification by RDP-classifier. Operational taxonomic units (OTUs) were generated by using CD-HIT OTU picking with 97% of similarity. The human oral microbiome database (HOMD) was used as a reference database for OTU assignment.

α – diversity analysis was utilized to estimate the samples' diversity and richness using the R-package Vegan. Constrained correspondence analysis (CCA) was used here to emphasize variation and bring out strong patterns in a dataset. This analysis was performed by R software ade4 package together with permutational multivariate analysis (Adonis) determining the differences in variance between groups.

Linear discriminant analysis effect size (LEfSe) was used to determine taxa, at both genus and species-level OTUs, that best characterize the populations of healthy children and children developing allergies. The plots obtained show the potential biomarkers in every group, ranked

according to their effect size. The bars represent the effect size of differences observed for a particular taxon between groups, where the length of the bar represents a log10 transformed Linear Discriminant Analysis (LDA) score.

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 are stated in figure legends. Sequences supporting the conclusions of this article are publicly available at the European Nucleotide Archive (ENA) database with the accession number PRJEB66628.

RESULTS

After quality filtering, 30,870,369 high-quality sequences were obtained, with an average of $92,700 \pm 3,652$ (SEM) reads per sample.

Bacterial diversity and density in saliva

An overall increase in microbial diversity and richness was observed through time, reaching over 450 species at 7 years of age. Children developing allergic diseases had significantly lower bacterial diversity at 7 years, when compared with children staying healthy (Fig. 1A, $p = 0.037$). Moreover, a similar trend was observed at 7 years of age in children developing asthma (Fig. 1B, $p = 0.044$). No significant differences were observed upon comparing species richness between children staying healthy and children developing allergic diseases during the first 7 years of age (Fig. 1C). However, children developing asthma tended to have higher bacterial richness at 12 and 24 months (Fig. 1D).

In subjects with allergies, the effect of asthma medication on microbiota diversity (using species-level OTUs), at 7 years of age was taken into account. When comparing healthy children and children developing allergies that were not taking asthma medication, a similar trend was observed (Shannon diversity index; Median_{Healthy} = 2.82, Median_{Allergic} = 2.26, $p = 0.066$). As only four asthmatic children were not taking asthma medication at 7 years, this could not be statistically evaluated.

In order to better understand the progress of bacterial density through the children's age, the bacterial load (bacterial cells/ml saliva) in saliva samples was measured. While there were no significant differences between children developing allergies/asthmatic symptoms and children

staying healthy (Fig. S2), an overall growth of bacterial density was observed from 3 months to 7 years, reaching levels of 10^8 bacterial cells/ml saliva. Also, children developing allergic disease tended to have higher levels of bacterial load at 7 years of age ($p=0.054$, Fig. S2A), when compared to children staying healthy. Individuals with low or high bacterial load through time did not correspond to any of the clinical variables measured, such as sex, antibiotic use or probiotic supplementation.

Microbial colonization patterns

Adonis testing supported the clustering of children's oral microbiota according to time of development, giving significant p-values (Fig. 2). Furthermore, canonical correspondence analyses demonstrated that, during the first 2 years of life, no clear separation of microbial patterns between children staying healthy and children developing allergies was detected (Fig. 2A). However, differences in microbiota patterns appeared at 7 years of age between healthy and allergic children, and a similar trend was observed when including children having asthmatic symptoms only (Fig. 2B).

The LEfSe algorithm was applied for biomarker discovery. Bacterial genera that were increased in abundance in healthy children, as compared with children developing allergies during the first 7 years of age, were *Eubacterium* and *Neisseria* at 3 months (Fig. 3A), *Lactobacillus*, *Alloprevotella*, *Corynebacterium*, *Selenomonas* and *Eubacterium* at 6 months (Fig. 3B) and *Lactobacillus*, *Selenomonas*, *Veillonella*, *Megasphaera*, *Fusobacterium* and *Lachnoanaerobaculum* at 7 years (Fig. 3C). Genera that were associated with allergy development were *Bacteroides* at 3 months and *Gemella* at 7 years (Fig. 3 A/C).

Bacterial genera that were increased in abundance in children developing asthmatic symptoms as compared with healthy children were *Alloprevotella* at 12 months of age (Fig. 4A) and *Staphylococcus* at 24 months of age (Fig. 4B). Upon checking the staphylococci species present (hits with >97% of identity over at least 350 bp alignment length), *S. capitis* (79% of the *Staphylococcus* sequences), *S. hominis* (15 %) and *S. warneri* (4%) were the most abundant. Bacterial genera that were increased in abundance in healthy children were *Lactobacillus* and *Atopobium* at 24 months of age (Fig. 4B) and *Fusobacterium*, *Capnocytophaga*, *Lactobacillus* and *Streptococcaceae* at 7 years of age (Fig. 4C).

Microbial species biomarkers

The differences in bacterial composition between children staying healthy and children developing allergies and/or asthma were further evaluated at species-level OTUs (>97% of nucleotide identity) by using LefSe for biomarker discovery. At 3 months of age (Fig. 5A), bacterial species that were significantly increased in abundance in children staying healthy until 7 years were, among others, *Prevotella* sp. and *Neisseria mucosa/sicca/flava*, while *Streptococcus parasanguinis* and *Gemella haemolysans* were more prevalent in children developing allergies. At 6 months (Fig. 5B), *Bacteroidales* [G-2] sp. and *Corynebacterium matruchotii* were some of the bacteria that were observed in increased abundance in children staying healthy, while *Streptococcus salivarius/cristatus/vestibularis* and *Selenomonas* sp. were associated with allergy development. At one and two years, *Veillonella dispar*, *Lactobacillus gasseri* and *Neisseria oralis/flava/mucosa* were among bacterial species that were increased in abundance in children staying healthy (Fig. 5C-D). At the same time point, OTUs belonging to the genera *Gemella* (including *G. sanguinis* and *G. haemolysans*) and *Streptococcus* (including *S. mitis/dentisani*, *S. lactarius* and *S. cristatus*), as well as *Alloprevotella* sp. were associated with allergy development (Fig. 5C-D). At 7 years of age (Fig. 5E), *Gemella haemolysans*, *Prevotella* sp. and *Streptococcus lactarius* were associated with allergy development, while the larger diversity detected in healthy children at this age was reflected in a larger list of over-represented species, including *Prevotella salivare*, *Veillonella rogosae* and *Lactobacillus gasseri*.

It was also of interest to compare microbial biomarkers between children staying healthy and children developing asthmatic symptoms (Fig. 6) showing that *Lactobacillus crispatus* and *L. gasseri* were found in increased abundance in children staying healthy at different time points during that 7-year period.

Influencing factors

The CCA statistic tool was used to examine the influence of confounding factors on microbial colonization patterns. No effect of delivery mode, antibiotic treatments, partial breastfeeding and maternal allergy on microbial composition in children developing allergies or asthmatic symptoms could be observed (data not shown). However, probiotic supplementation with *L. reuteri* ATCC 55730 during the first year of age appeared to influence the association of microbial composition with asthma, but not allergy development, at 12 months, 24 months and 7 years of age (Fig. S2;

p=0.0083, Adonis permutational analysis).

DISCUSSION

Given that the oral cavity is the first site of encounter between a majority of foreign antigens and the immune system, it is plausible that oral microbiome maturation might influence allergy development during childhood. In the present study, we use cultivation-independent techniques to characterise the development of microbial oral communities during the first 7 years of life in 33 healthy children and 47 children that developed allergic symptoms.

Development of allergic disease during childhood, and particularly asthma, was associated with a significantly lower bacterial diversity at 7 years of age. In comparison to gut microbiota studies where children developing allergic disease tend to have lower bacterial diversity than healthy children already during the first months of life(17, 20, 21), the association of disease with oral microbiota diversity appears to increase with age. Likely, the diverse intestinal microbiota might be of great importance for a primary establishment and for maturation of a balanced postnatal innate and adaptive immunity. However, this study suggests that infant oral microbiota composition, including the abundance of *G. haemolysans* in children developing allergic symptoms and *L. gasseri* and *L. crispatus* in children staying healthy, is more important than the oral microbiota diversity for later allergy development. Oral cavity development during infancy was accompanied by an overall steady increase in both diversity and richness of the oral microbiome, reaching 300 OTUs and bacterial densities of 10^6 cells/ml saliva already at three months of age. Further oral microbiota acquirement is probably facilitated by microbial colonization through diet and transmission from parents, caregivers and siblings.(6) Even though children developing allergies and asthmatic symptoms tended to have higher species richness and bacterial load, specifically after the first year of life, in comparison to children staying healthy, no statistically significant differences during the first 7 years of life could be observed. The deficient mucosal immune system of the oral cavity may favour an altered species colonization, even for those species appearing at low abundance.

Increased abundances of the genus *Bacteroides* at 3 months of age and *Gemella* at 7 years were associated with allergy development. The identification of disease-associated bacteria, especially at an early age, could provide potential biomarkers of allergy risk. *Bacteroides* species are among the earliest-colonizing and one of the most numerically dominant commensals of the gut microbiota.(2, 4) They provide many beneficial effects to the host, including breakdown of complex dietary

carbohydrates and modulation of mucosal glycosylation, and immune maturation.(24) However, *Bacteroides* is not a common oral inhabitant under healthy circumstances.(4) In addition, as these bacteria are potent stimulators of the immune system, the host immune responses can differ between the immunomodulatory molecules from different species of *Bacteroides*(25), possibly leading to aberrant immune development. *Gemella* is a predominant genus of the mucosal epithelium(4, 26) and in this study, it was related to allergy development in saliva samples particularly collected at 7 years of age, while at species level *G. haemolysans* was found to be associated with allergy development not only at 7 years of age, but also at 2 years, 1 year, and as early as 3 months of age. *G. haemolysans* has previously been shown to produce human IgA1 protease activity(27), a feature that is unique to this species within the genus *Gemella*, suggesting that its potential use as an early diagnostic marker in altered mucosal immunity deserve further investigation.

The primary colonizers of the oral microbiota, including both mucosal and tooth surfaces, are commonly streptococci, accounting for approximately 80% of early biofilms.(6) Most oral streptococci are commensal, frequently acquired during breastfeeding(28), although some are known to cause infective endocarditis when disseminated through the blood stream.(29) We observed that *S. parasanguinis*, a member of viridans streptococci(30), and *S. lactarius*, belonging to the *S. mitis* group(31), were here associated with allergy development in saliva samples from 3, 12, 24 months and 7 years of age. Both of these species have been described as primary colonizers, with *S. parasanguinis* frequently found in the tongue dorsum(26) and *S. lactarius* isolated from breast milk of healthy women.(32) Moreover, children developing asthmatic manifestations also had higher abundance of several *Streptococci*, including *S. sanguinis* (at 6 months), *S. salivarius/vestibularis* and *S. cristatus* (at 24 months) and *S. australis* and *S. mitis* at 7 years. Early asymptomatic colonization of the nasopharynx with *Streptococcus*, during infancy, has been proposed as a strong asthma predictor.(19) Because pioneer colonizers may facilitate the environment for later colonizers, the initial competition for bacterial colonization might have direct implications for the spatial and temporal composition of the developing oral microbiome, and therefore play a crucial role in immune modulation.

Neisseria sicca/mucosa/flava were increased in abundance in children staying healthy. The genus *Neisseria* is an abundant member of the oropharyngeal flora(33), tongue, oral mucosa and dental

plaque(26). Furthermore, higher abundance of *Corynebacterium* at 6 months of age and the species *C. matruchotii* at 7 years of age were observed in children staying healthy, as compared with children developing allergy. *Corynebacterium*, particularly *C. matruchotii*, with its highly interactive filamentous structure is considered instrumental in oral biofilm architecture.(34) Members of this genus have been shown to utilize carbohydrates and metabolize lactate and acetate, likely maintaining pH homeostasis in a healthy oral biofilm.(35)

Children staying healthy up to 7 years had higher abundance of *Lactobacillus* both at genus and species level at 3, 6 and 24 months and 7 years when compared to children developing asthmatic symptoms, or to children developing allergies (at 24 months and 7 years). *Lactobacilli* colonize the gastrointestinal tract, including the oral cavity(36), and vagina(37), and may promote health by their influence on biofilm microbial composition, or by stimulating the host immune responses.(3) *L. crispatus* and *L. gasseri* were both associated with reduced allergy development. *Lactobacillus gasseri* is an important health-promoting immunomodulator of innate and systemic immune responses with an antimicrobial activity(38), and it has also been evaluated as a possible treatment of allergic rhinitis(39), demonstrating that supplementation with *L. gasseri* may be beneficial because of its effect on nasal blockage(39), and decreased nasal clinical symptoms scores in children suffering from allergic rhinitis.(40) As *L. crispatus* and *L. gasseri* were also observed to suppress allergic responses(41) and reduce mite-induced airway inflammation and hyperresponsiveness in mice models(42), these species may have a protective role in asthma development and deserve to be further investigated.

The use of antibiotics during the first years of life, birth mode, feeding habits and urban versus farm living have all been shown to affect microbiota composition(43), and several studies have found associations between these factors and allergy development.(3) To understand how these early-life risk factors may be related to allergy development during childhood, it is of great importance to consider how they affect the microbiome development in early infancy. Delivery mode, breastfeeding duration, antibiotics intake and maternal allergy seem not to have influence on the microbiota in relation to allergy and asthma development in our study population because the discovered differences are driven by health status (*e.g.* if children developed allergies or stayed healthy). However, the majority of the infants were exclusively breastfed until 3 months of age (Table 1), upon the first collection of the saliva samples, making it difficult to demonstrate the

possible differences between breastfed and not breastfed infants due to low statistical power. Supplementation with *L. reuteri* during the first year of life seemed to influence the association between oral microbiota composition and asthma development (Fig. S2). This was reflected in distinctive microbiota clustering at 12 and 24 months and 7 years of age between children taking probiotics and not developing asthma and children that did not take probiotics and developed asthma. However, the probiotic intervention in this study did not directly reduce asthma development in the cohort. Reduced allergen responsiveness have previously been observed in *L. reuteri* supplemented infants, suggesting enhanced capacity for immunoregulation during infancy(44), associated with reduced incidence of IgE-associated eczema in infancy.(22) *L. reuteri* colonizes in a close contact with intestinal mucosa, priming dendritic cells to produce increased levels of anti-inflammatory IL-10 and inhibit the proliferation of bystander effector T-cells.(45) This species may have a similar mode of action in the oral mucosa, beside its symbiotic relationship with the immune system, and may support the co-colonization of other oral microbes beneficial for immune system development. However, larger studies, including the replication of our findings in other geographic origins, are required to further investigate and confirm the role of *L. reuteri* in allergy development. Beside saliva samples, it would be interesting to address other oral habitats, including tongue dorsum and buccal mucosa, in order to obtain the overall picture of oral microbiota maturation. Even though the time between 24 months and 7 years of age might be important to address in this type of longitudinal studies, the majority of the studies published today are describing that the first year of life is primarily significant for the immune system development.

CONCLUSION

By 7 years of age, allergic children appear to have a higher density and lower diversity of salivary bacteria, as well as a highly divergent bacterial composition, showing a clearly altered oral microbiota in these individuals, likely a consequence of an impaired immune system. Several individual bacterial species during infancy were associated with allergy development. The bacterial species detected in the current study as clearly associated to allergic conditions even years before the appearance of allergic manifestations, could potentially be used as early biomarkers capable to predict the risk of allergy and asthma. In addition, the possibility that some of these early changes in microbiota composition could impact immune modulation, inflammation and allergy development should be considered. Thus, the potential immunomodulatory effect of oral microorganisms deserves further attention and future investigation in longitudinal and animal-model studies.

Table 1. Descriptive data of children included in the study.

*The χ^2 test was used to detect potential differences in frequencies between children developing allergic/asthmatic symptoms and children staying healthy, except when the expected frequency for any cell was < 5 , in which case the Fisher exact test was used. $N_{\text{Healthy}} = 33$, $N_{\text{Allergic}} = 47$, $N_{\text{Asthmatic}} = 20$

Fig. 1. Species richness and diversity of the total microbiota in infant saliva samples of children developing allergies and children staying healthy up to 7 years of age. Bacterial richness and diversity (here presented by Chao1 and Shannon estimate indices at OTU level), obtained at different time points until 7 years of age, were determined by 16s rDNA Illumina sequencing. (A) and (C) are describing bacterial diversity and richness, respectively, during the first 7 years of life in children staying healthy and children developing allergies. (B) and (D) are presenting species diversity and richness in children developing asthma and children staying healthy up to 7 years of age. Data are presented with mean and standard error. (* $p < 0.05$; Mann-Whitney U-test). 3 months ($N_{\text{Healthy}} = 28$; $N_{\text{Allergic}} = 36$; $N_{\text{Asthmatic}} = 20$), 6 months ($N_{\text{Healthy}} = 31$; $N_{\text{Allergic}} = 45$; $N_{\text{Asthmatic}} = 20$), 12 months ($N_{\text{Healthy}} = 27$; $N_{\text{Allergic}} = 35$; $N_{\text{Asthmatic}} = 16$), 24 months ($N_{\text{Healthy}} = 25$; $N_{\text{Allergic}} = 34$; $N_{\text{Asthmatic}} = 15$) and 7 years of age ($N_{\text{Healthy}} = 32$; $N_{\text{Allergic}} = 40$; $N_{\text{Asthmatic}} = 15$).

Fig. 2. Salivary microbiota colonization patterns in children developing allergies and children staying healthy up to 7 years of age. Constrained correspondence analyses (CCA), here used to emphasize variations in microbiota species-level patterns, show compositional characteristics of total microbiota at different time points. The percentage of variation explained by constrained correspondence components is indicated on the axes. (A) Microbial composition differences in saliva of infants staying healthy and infants developing allergies during the first 7 years of age ($p = 0.001$). (B) Microbial composition patterns of salivary samples in children developing asthmatic symptoms and children staying healthy up to 7 years of age ($p = 0.004$). Different colours represent different time points (M=Months, Y=Years). p values for CCA plots were determined by Adonis and indicate if the factor provided (in this case *time*) can significantly explain data variability. Sample sizes were: 3 months ($N_{\text{Healthy}} = 28$; $N_{\text{Allergic}} = 36$; $N_{\text{Asthmatic}} = 20$), 6 months ($N_{\text{Healthy}} = 31$; $N_{\text{Allergic}} = 45$; $N_{\text{Asthmatic}} = 20$), 12 months ($N_{\text{Healthy}} = 27$; $N_{\text{Allergic}} = 35$; $N_{\text{Asthmatic}} = 16$), 24 months ($N_{\text{Healthy}} = 25$; $N_{\text{Allergic}} = 34$; $N_{\text{Asthmatic}} = 15$); 7 years of age ($N_{\text{Healthy}} = 32$; $N_{\text{Allergic}} = 40$; $N_{\text{Asthmatic}} = 15$).

Fig. 3. Salivary bacterial genera associated with allergy development during the first 7 years of age. The plots show statistically significant genera associated with allergy development at (A) 3 months, (B) 6 months and 7 years of age (C). The LEfSe algorithm was used for biomarker discovery and the threshold for logarithmic discriminant analysis (LDA) score was 2. Sample sizes: 3 months ($N_{\text{Healthy}} = 28$; $N_{\text{Allergic}} = 36$), 6 months ($N_{\text{Healthy}} = 31$; $N_{\text{Allergic}} = 45$), and 7 years of age ($N_{\text{Healthy}} = 32$; $N_{\text{Allergic}} = 40$).

Fig. 4. Salivary bacterial genera associated with asthma development during the first 7 years of life. (A) At 12 months of age only the genus *Alloprevotella* was increased in abundance in children developing allergies. (B)

Genera associated with children developing allergies (grey) and children staying healthy (orange) at 24 months of age. (C) At 7 years of age, all significant differences corresponded to bacteria associated with healthy children. The LEfSe algorithm was used for biomarker discovery and the threshold for logarithmic discriminant analysis (LDA) score was 2. Sample sizes were: 12 months ($N_{\text{Healthy}}=27$; $N_{\text{Asthmatic}}=16$), 24 months ($N_{\text{Healthy}}=25$; $N_{\text{Asthmatic}}=15$) and 7 years of age ($N_{\text{Healthy}}=32$; $N_{\text{Asthmatic}}=15$).

Fig. 5. Salivary bacterial OTUs associated with allergy development during the first 7 years of life. Bars represent bacterial species at 3 (A), 6 (B), 12 (C) and 24 (D) months, and at 7 years (E) of age increased in abundance in children developing allergies (grey) and children staying healthy (orange). The LEfSe algorithm was used for biomarker discovery and the threshold for logarithmic discriminant analysis (LDA) score was 2. Sample sizes were: 3 months ($N_{\text{Healthy}}=28$; $N_{\text{Allergic}}=36$), 6 months ($N_{\text{Healthy}}=31$; $N_{\text{Allergic}}=45$), 12 months ($N_{\text{Healthy}}=27$; $N_{\text{Allergic}}=35$), 24 months ($N_{\text{Healthy}}=25$; $N_{\text{Allergic}}=34$) and 7 years of age ($N_{\text{Healthy}}=32$; $N_{\text{Allergic}}=40$).

Fig. 6. Salivary bacterial OTUs associated with asthma development during the first 7 years of life. Bars show bacterial species at 3 (A), 6 (B), 12 (C) and 24 (D) months and 7 years (E) of age increased in abundance in children developing asthmatic symptoms (grey) and children staying healthy (orange). The LEfSe algorithm was used for biomarker discovery and the threshold for logarithmic discriminant analysis (LDA) score was 2. Sample sizes were: 3 months ($N_{\text{Healthy}}=28$; $N_{\text{Asthmatic}}=20$), 6 months ($N_{\text{Healthy}}=31$; $N_{\text{Asthmatic}}=20$), 12 months ($N_{\text{Healthy}}=27$; $N_{\text{Asthmatic}}=16$), 24 months ($N_{\text{Healthy}}=25$; $N_{\text{Asthmatic}}=15$) and 7 years of age ($N_{\text{Healthy}}=32$; $N_{\text{Asthmatic}}=15$).

Acknowledgements

We would like to acknowledge the technical assistance performed by Ann-Marie Fornander and Camilla Janefjord.

Conflicts 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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Table 1. Descriptive data of children included in the study

Children	Healthy (% [no.])	Developing allergy (% [no.])	P value*	Developing asthma (% [no.])	P value*
Girls	57 (19)	51 (24)	0.57	70 (14)	0.40
Caesarean delivery	15 (5)	13 (6)	0.75	20 (4)	0.72
Breastfeeding					
1 month exclusive	93 (31)	87 (41)	0.46	90(18)	0.63
3 months exclusive	76 (25)	79 (37)	0.91	70 (14)	0.53
3 months partially	100 (33)	94 (44)	0.26	95 (19)	0.38
12 months partially	21 (7)	21 (10)	0.99	10 (2)	0.46
Antibiotic treatment					
first year	30 (10)	30 (14)	0.96	40 (8)	0.47
second year	48 (16)	43 (20)	0.60	65 (13)	0.25
Day care					
first year	12 (4)	4 (2)	0.22	5 (1)	0.64
second year	76 (25)	72 (34)	0.73	90 (18)	0.29
Probiotic group	55 (17)	45 (21)	0.55	50 (10)	0.91

*The χ^2 test was used to detect potential differences in frequencies between children developing allergic/asthmatic symptoms and children staying healthy, except when the expected frequency for any cell was < 5 , in which case the Fisher exact test was used. $N_{\text{Healthy}} = 33$, $N_{\text{Allergic}} = 47$, $N_{\text{Asthmatic}} = 20$

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Oral microbiota maturation during childhood

SUPPLEMENTARY MATERIALS

Oral microbiota maturation during the first 7 years of life in relation to allergy development

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METHODS

Sample collection and study design

The infants 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 prevention effect of probiotic *Lactobacillus reuteri* ATCC 55730 until 2 and 7 years of age was evaluated.(1, 2) Among the 188 infants completing the original study, longitudinal salivary samples were collected at 3, 6, 12 and 24 month and 7 years of age in 47 children developing allergic symptoms and 33 children staying healthy up to 7 years of age. The selection of saliva samples for this study was based on sample availability. The included children were not statistically different from the children included in the main trial with regards to the variables presented in Table 1. The participants were instructed not to eat or drink for two hours preceding the sampling. Non-stimulated saliva samples were collected from the buccal cavity, using a hand pump (Nalgene #6131, ThermoFisher, Stockholm, Sweden) connected to a thin plastic tube, and immediately frozen and kept at -20°C . In connection with the oral examination and saliva sampling, clinical data about symptoms of allergic disease, adverse events, infections, use of antibiotics, and possible confounding factors were registered.(1, 2) Infants were regarded as sensitized if they had at least one positive skin prick test and/or detectable circulating allergen specific-IgE antibodies.(1, 2) Skin prick tests were performed on the forearm with egg white, fresh skimmed cow milk and standardized cat, dog, birch, peanut, mite (Der p) and timothy extracts. (2) Moreover, symptoms related to allergic disease, physical examination, spirometry and measurement of fractional exhaled nitric oxid (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. 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)

Possible confounders, such as mode of delivery, breastfeeding, probiotics supplementation, maternal allergy and antibiotics use during the first two years of age were obtained from medical records and questionnaires (see Table I). 90% and 77% of all the children included were exclusively breast-fed up to 1 and 3 months of age, respectively, and 96% were partially breastfed at 3 months of age. No infant received antibiotics before 1 month of age and one at 3 months of age.

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.

DNA extraction

250 ul of saliva sample was centrifuged at 15000 g for 30 min and the pellet, together with 50 ul of the supernatant, were used for further analysis. DNA was isolated with a MagNA Pure LC 2.0 equipment (1996-2016 Roche Diagnostics, Barcelona, Spain), using MagNA Pure LC DNA Isolation Kit III for Bacteria & Fungi (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions with an additional enzymatic lysis step with lysozyme (20 mg/ml, 37°C, 60 min; Thermomixer 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). DNA was resuspended in 100 ul of elution buffer and frozen at -20°C until further analysis.

16S rDNA gene amplification and sequencing

Prior to sequencing of the 16S rDNA gene, extracted DNA was pre-amplified in order to increase total nucleic acid yield by using universal bacterial degenerate primers 8F–AGAGTTTGATCMTGGCTCAG and 926R–CCGTCAATTCMTTTRAGT, which encompass the hypervariable regions V1-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 10 cycles, in order to minimize amplification biases.(4) The 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 rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part #15044223 Rev. A). The gene-specific primer sequences used in this protocol were selected from the Klindworth et al. publication and target the 16S rDNA gene V3 and V4 regions, resulting in a single amplicon of approximately 460 bp. Overhang adapter sequences were used together with the primer pair sequences for compatibility with Illumina index and sequencing adapters. After 16S rDNA gene amplification, the DNA was sequenced on a MiSeq Sequencer according to manufacturer's instructions (Illumina) using the 2x300 bp paired-end protocol.

Bacterial load and measurements with quantitative PCR

Total bacterial load (bacterial cells per ml of saliva) in saliva samples 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 10 mL was composed of SYBR Green PCR Master Mix (Roche), 0.5 mL of the bacterial universal primer (concentration 10 mmol/L), and 2 mL of DNA template. The forward primer was 5'GTG CCA GCM GCC GCG GTA A 3' and the reverse primer 5'GCG TGG ACT ACC AGG GTA TCT 3' (Integrated DNA Technologies (IDT); San Diego, California, USA), targeting the 16S rDNA gene.(5) The obtained Ct values were transformed to bacterial cell numbers by a standard curve calibrated by flow cytometry.(6)

Bioinformatics and statistics

Only overlapping paired end reads were used for analysis. A sequence quality assessment was carried out using the PRINSEQ program. Sequences of <250 nucleotides in length were not considered; 5' 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.(7)

Obtained sequences were taxonomically classified by the RDP-classifier (8) where the reads were assigned a phylum, class, family and genus and phylogenetic ranks were allocated when scores exceeded 0.8 confidence threshold. Operational taxonomic units (OTUs) were generated by using CD-HIT OTU picking with 97% of similarity.(9) Normalized OTU tables were used for downstream analysis. The human oral microbiome database (HOMD) was used as a reference database for OTU assignment.(10)

Microbiota diversity metrics were determined from normalised OTU tables. α – diversity analysis (presented here as Shannon and Chao1 indices), were utilized to estimate the

samples' diversity and richness using the R-package Vegan.(11) Constrained correspondence analysis (CCA, a.k.a. canonical correspondence analysis) is a statistic tool used to emphasize variation, taking advantage of the fact that the factor provided can explain part of the total variability, and bring out strong patterns in a dataset. This analysis was performed by R software ade4 package (12), using the function *CCA* (related to correspondence analysis) and based on Chi-squared distances. Furthermore, the Adonis statistic for permutational multivariate analysis was used to measure differences in variance between groups.

Linear discriminant analysis effect size (LEfSe), a method for biomarker discovery on the online interface Galaxy (<http://huttenhower.sph.harvard.edu>) (13), was used to determine taxa that best characterize the populations of healthy children and children developing allergies. LEfSe scores measure the consistency of differences in relative abundance between taxa in the groups analysed, where a higher score indicates higher consistency. In this study, LEfSe was used for biomarker discovery at both genus and species-level OTUs. The threshold for the logarithmic LDA score was set at 2.0. The influence of confounding factors was determined by the CCA statistic tool and Adonis permutational multivariate analysis.

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. Comparison between categories was performed by Mann-Whitney U test. Specific statistical tests are stated in figure legends.

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RESULTS

Figure S1. Bacterial load in infant saliva samples. Bacterial density in salivary samples, obtained at different time points until 7 years of age, was determined by qPCR using universal primers targeting the 16S bacterial gene. Figures show number of bacterial cells/ml in allergic (A) and asthmatic children (B) when compared to children staying healthy up to 7 years of age. Children developing allergies tended to have higher levels of bacterial load at 7 years of age ($p=0.054$), when compared to children staying healthy (A). Data are presented with median with interquartile ranges. Mann-Whitney U-test was used for statistical comparisons. 3 months ($N_{\text{Healthy}}=28$; $N_{\text{Allergic}}=36$; $N_{\text{Asthmatic}}=20$), 6 months ($N_{\text{Healthy}}=31$; $N_{\text{Allergic}}=45$; $N_{\text{Asthmatic}}=20$), 12 months ($N_{\text{Healthy}}=27$; $N_{\text{Allergic}}=35$; $N_{\text{Asthmatic}}=16$), 24 months ($N_{\text{Healthy}}=25$; $N_{\text{Allergic}}=34$; $N_{\text{Asthmatic}}=15$) and 7 years of age ($N_{\text{Healthy}}=32$; $N_{\text{Allergic}}=40$; $N_{\text{Asthmatic}}=15$).

Figure S2. The effect of probiotic supplementation with *Lactobacillus reuteri* on the oral microbiota composition in relation to asthma development. Constrained correspondence analyses (CCA), here used to emphasize variations in microbiota species-level patterns, show compositional characteristics of total microbiota at different time points. The percentage of variation explained by constrained correspondence components is indicated on the axes. The plot is showing the impact of probiotic supplementation, during early infancy, on the oral microbiota composition in saliva samples collected at 3, 6, 12, 24 months and 7 years of age in children staying healthy and children developing asthmatic manifestations ($p=0.0083$). Different colours present different time points (M=Months, Y=Years) and probiotic supplementation in children and asthma development are marked with either 0 or 1 describing the absence or presence, respectively. p values for CCA plots were determined by Adonis and indicate if the factor provided (in this case time) can significantly explain data variability. Sample sizes were: 3 months ($N_{\text{Probiotics}}=14$; $N_{\text{Placebo}}=9$), 6 months ($N_{\text{Probiotics}}=16$; $N_{\text{Placebo}}=12$), 12 months ($N_{\text{Probiotics}}=13$; $N_{\text{Placebo}}=7$), 24 months ($N_{\text{Probiotics}}=10$; $N_{\text{Placebo}}=8$); 7 years of age ($N_{\text{Probiotics}}=15$; $N_{\text{Placebo}}=10$).