Microbiota development and mucosal IgA responses during childhood in health and allergic disease

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Cover illustration “Baby surrounded by bacteria” by Iván Míllan Rodriguez.

To my family

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ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following papers (here included as the authors last version, with the permission from the publisher), which will be referred to in the text by their roman numerals.

I **Majda Dzidic**, Maria Carmen Collado, Thomas R. Abrahamsson, Alejandro Artacho, Malin Stensson, Maria C. Jenmalm, Alex Mira; Oral microbiome development during childhood: an ecological succession influenced by postnatal factors and associated with tooth decay; © *The ISME Journal*, Jun 2018, 12:2292–2306.

II **Majda Dzidic**, Thomas R. Abrahamsson, Alejandro Artacho, Bengt Björkstén, Maria Carmen Collado, Alex Mira, Maria C. Jenmalm; Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development; © *J Allergy Clin Immunol*; Mar 2017, 139:1017-1025.

III **Majda Dzidic**, Thomas R. Abrahamsson, Alejandro Artacho, Maria Carmen Collado, Alejandro Mira, Maria C Jenmalm; Oral microbiota maturation during the first 7 years of life in relation to allergy development; *Allergy* (© 2018 EAACI and John Wiley and Sons Ltd), Mar 2018; 73: 2000-2011.

IV **Majda Dzidic**, Alex Mira, Alejandro Artacho, Thomas R. Abrahamsson, Maria C. Jenmalm, Maria Carmen Collado; Relationship between breastmilk IgA-coated microbiota, probiotic supplementation and allergy development in childhood; *Submitted to Pediatric Allergy and Immunology*, Jun 2019.

Parts of the general introduction have been borrowed from the following reviews:

Majda Dzidic*, Alba Boix-Amorós*, Marta Selma-Royo*, Alejandro Mira, Maria Carmen Collado; Gut Microbiota and Mucosal Immunity in the Neonate; *Medical Sciences,* Jul 2018;6:56.

Christina E. West, **Majda Dzidic**, Susan L. Prescott, Maria C. Jenmalm; Bugging allergy; role of pre-, pro- and synbiotics in allergy prevention; Allergol Int, Oct 2017, 66:529-538.

SUMMARY

Background: It has been proposed that altered microbial colonization patterns during infancy may be partly responsible for the increase of allergic diseases in developed countries. 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. However, little is known about the significance of early mucosal immune responses to the gut microbiota in childhood allergy development, and the findings regarding the protective effect of breastmilk microbiota in the risk of allergy development have been inconclusive. Furthermore, 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 during childhood has not yet been reported.

Objectives: The general aim of this thesis was to assess the microbial composition and diversity of oral, fecal and breastmilk samples, together with its interaction with IgA, in order to study the role of microbial development during early childhood in health and allergic disease.

Subjects: The infants and mothers included in this study were part of a larger randomized double-blind trial in Sweden, between 2001 and 2003, where potential allergy preventive effects of *Lactobacillus reuteri* ATCC 55730 were evaluated until 2 and 7 years of age. In this thesis, we used longitudinally collected stool and oral samples from infants, obtained at 1 and 12 months and 3, 6, 12, 24 months and 7 years of age, respectively. Furthermore, we analyzed breastmilk samples, collected at one month *post partum*, from the corresponding mothers.

Methods: Next-generation sequencing technologies targeting the 16S rRNA gene, in combination with cell activated cell sorting, were used in order to address mucosal IgA responses towards gut and breastmilk bacteria. Furthermore, sequencing of the 16S rRNA gene was used in order to describe oral microbiota colonization, in longitudinally obtained saliva samples, from children developing allergy or staying healthy. Bacterial load levels in different microbial habitats were obtained by qPCR methodology and total IgA levels of stool samples were determined by ELISA immunoassays.

Results and conclusion: Colonization of the oral cavity during early childhood is transitional, increasing in complexity with time, and several external factors appear to greatly influence oral microbiota maturation, having either a short or a long-term impact. Early changes in oral microbial composition seem to influence immune maturation and allergy development in childhood, and the presence of specific bacterial species may be important for this progress. Furthermore, altered IgA responses towards the gut microbiota during infancy preceded asthma and allergy manifestations during the first 7 years of life, and consumption of breastmilk with a reduced microbial richness in the first month of life may increase the risk for allergy development during childhood. Findings observed here need to be confirmed in larger cohorts and the

importance of postnatal environmental factors for early microbiota development should be addressed further. Future research should go beyond characterization of bacterial community composition and investigate the functional mechanisms between early colonizing microorganisms, immune maturation and allergy and asthma development during childhood.

RESUMEN - *Castellano*

Antecedentes: Los patrones de colonización microbiana alterados durante la infancia pueden ser en parte responsables del aumento de enfermedades alérgicas en los países desarrollados. La microbiota intestinal difiere en composición y diversidad durante los primeros meses de vida en niños que luego desarrollan o no una enfermedad alérgica. Sin embargo, poco se sabe sobre la importancia de las respuestas inmunitarias tempranas de la mucosa a la microbiota intestinal en el desarrollo de alergias infantiles. Además, los estudios con respecto al efecto protector de la microbiota de la leche materna en el riesgo de desarrollar alergias no han sido concluyentes. Aunque la cavidad bucal es el primer lugar de encuentro entre la mayoría de los antígenos exógenos y el sistema inmunológico, no existen datos sobre la influencia de las bacterias orales en el desarrollo de alergias durante la infancia.

Objetivos: El objetivo general de esta tesis fue evaluar la composición y diversidad microbiana en muestras orales, intestinales y de leche materna, junto con su interacción con IgA, para estudiar el papel de la colonización microbiana durante edades tempranas de la vida en condiciones de salud y de enfermedad alérgica.

Sujetos: Los bebés y las madres incluidas en este estudio forman parte del ensayo aleatorio doble ciego más grande de Suecia, entre 2001 y 2003, donde se evaluaron los posibles efectos preventivos sobre la alergia de *Lactobacillus reuteri* ATCC 55730 hasta los 2 y 7 años. En esta tesis, utilizamos muestras de heces recogidas a los 1 y 12 meses, y muestras orales de bebés, obtenidas longitudinalmente a los 3, 6, 12, 24 meses y 7 años. Además, analizamos muestras de leche materna, recogidas a un mes después del parto de las madres correspondientes.

Métodos: Se utilizaron tecnologías de secuenciación de segunda generación dirigidas al gen 16S rARN, en combinación con citometría de células marcadas por fluorescencia, para abordar las respuestas de IgA de la mucosa hacia las bacterias intestinales y de la leche materna. Además, se utilizó la secuenciación del gen 16S para describir la colonización oral de la microbiota, en muestras de saliva, de niños que desarrollaron alergias o de aquellos que se mantuvieron sanos. Los niveles de carga bacteriana en diferentes hábitats microbianos se obtuvieron mediante la metodología de qPCR y los niveles totales de IgA de las muestras de heces se determinaron mediante inmuno-ensayo ELISA.

Resultados y conclusión: La colonización de la cavidad bucal durante la infancia temprana es progresiva, aumenta en complejidad con el tiempo, y varios factores externos parecen influir en gran medida en la maduración de la microbiota oral, ya sea con un impacto a corto o largo plazo. Los cambios tempranos en la composición microbiana oral parecen influir en la maduración inmune y el desarrollo de alergias en la infancia, y la presencia de especies bacterianas específicas puede ser importante para este proceso. Además, las respuestas de IgA alteradas hacia la

microbiota intestinal durante la infancia precedieron a las manifestaciones de asma y alergia durante los primeros 7 años de vida, y el consumo de leche materna con una riqueza microbiana reducida en el primer mes de vida puede aumentar el riesgo de desarrollar alergia durante la infancia. Los hallazgos observados en la presente tesis deben confirmarse en cohortes más grandes y la importancia de los factores ambientales postnatales para el desarrollo temprano de la microbiota debe abordarse más a fondo. Las investigaciones futuras deben ir más allá de la caracterización de la composición de la comunidad bacteriana e investigar los mecanismos funcionales entre los microorganismos colonizadores tempranos, la maduración inmunitaria y la alergia, así como el desarrollo del asma durante la infancia.

RESUMEN - *Valenciano*

Antecedents: S'ha proposat que els patrons de colonització microbiana alterats durant la infància podrien ser en part els responsables de l'augment de malalties al·lèrgiques als països desenvolupats. La microbiota intestinal difereix en composició i diversitat durant els primers mesos de vida en els nens que després van desenvolupar una malaltia al·lèrgica. No obstant això, poc es sap sobre la importància de les respostes immunes de la mucosa a la microbiota intestinal en el desenvolupament d'al·lèrgies infantils. A més, les investigacions amb relació a l'efecte protector de la microbiota de la llet materna en el risc de desenvolupar al·lèrgies no han sigut concloents. Encara que la cavitat bucal és el primer lloc de trobada entre la majoria dels gèneres externs i el sistema immunològic, encara no s'ha descobert la influència dels bacteris en el desenvolupament d'una al·lèrgia durant la infància.

Objectius: L'objectiu general d'aquesta tesi va ser avaluar la composició microbiana i la diversitat de mostres orals, fecals i llet materns, juntament amb la seva interacció amb IgA, per estudiar el paper del desenvolupament microbià durant el període de la infància primerenca a la salut i la malaltia al·lèrgica.

Subjectes: Les mares i xiquets inclosos en aquest estudi formen part d'un estudi aleatori doble-cec a Suècia, entre el 2001 i el 2003, on es van avaluar els possibles efectes preventius de la suplementació amb *Lactobacillus* ATCC 55730 fins als 2 i 7 anys. En aquesta tesi, s'utilitzaren mostres de bebès arreplegades longitudinalment, obtinguts a 1 i 12 mesos, 3, 6, 12, 24 mesos i 7 anys, respectivament. A més, s'analitzaren les mostres de llet materna, arreplegades a un mes postpart de les corresponents mares.

Mètodes: S'han utilitzat tecnologies de seqüenciació de nova generació dirigides al ARNr 16S, en combinació amb la classificació de les cèl·lules activades, per abordar les respostes de la mucosa cap als bacteris intestinals i de la llet materna. A més, s'utilitzà la seqüenciació d'Illumina MiSeq del gen 16S per descriure la colonització microbiana oral, i es van obtenir mostres longitudinals de saliva de menuts que varen desenvolupar al·lèrgies i d'alguns que es van mantenir saludables. Els nivells de càrrega bacteriana en diferents nínxols microbians s'han obtingut mitjançant la metodologia de qPCR i els nivells totals d'IgA de les mostres fecals es determinaren mitjançant l'immunoassaig ELISA.

Resultats i conclusions: La colonització de la cavitat bucal durant la primera infància és transitòria, augmenta la seva complexitat amb el temps, i diversos factors externs influeixen en gran mesura el procés de maduració de la microbiota oral, amb un impacte a curt i llarg termini. Els canvis primerencs en la composició microbiana oral pareixen influir en la maduració del sistema immunològic i el desenvolupament d'al·lèrgies a la infància, així com la presència d'espècies bacterianes específiques pot ser important per a aquest progrés. A més, les respostes

d'IgA alterades cap a la microbiota intestinal durant la infància precedeixen a les manifestacions relatives a la malaltia asmàtica i al·lèrgiques durant els primers 7 anys de vida. Per altra banda, el consum de llet materna amb una microbiota de riquesa reduïda al primer mes de vida podria augmentar el risc de desenvolupar al·lèrgia durant la infància. Els resultats observats en aquest estudi haurien de confirmar-se en cohorts humanes més grans i la importància dels factors ambientals post natals que influeixen en el desenvolupament de la microbiota primerenca han de ser més estudiats. Les investigacions futures deuen anar més enllà de la caracterització de la composició de la comunitat bacteriana i investigar els mecanismes funcionals entre els microorganismes colonitzadors primerencs, la maduració del sistema immunològic i el desenvolupament de l'al·lèrgia i l'asma durant la infància.

ABBREVIATIONS

A	Allergic
AB	Antibiotics taken
APRIL	A proliferation-inducing ligand
ARC	Allergic rhinoconjunctivitis
As	Asthmatic
BAFF	B cell-activating factor
BF	Breastfed
C-section	Caesarean section
CA	Caries active
CCA	Constrained correspondence analysis
CF	Caries free
CFU	Colony forming unit
DC	Dendritic cells
FE_{no}	Fractional exhaled nitric oxid
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
H	Healthy
HMO	Human milk oligosaccharides
HOMD	Human oral microbiome database
Ig	Immunoglobulin
IL	Interleukin
LDA	Logarithmic discriminant analysis
LEfSe	Linear discriminant analysis effect size
MAMPs	Microbe-associated molecular patterns
OUT	Operational taxonomic unit
PCA	Principal Component Analyses
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RDP	Ribosomal Database Project
rRNA	Ribosomal RNA
SCFA	Short chain fatty acid
SFB	Segmented filamentous bacteria
SIgA	Secretory IgA
SPT	Skin prick test
Th	T helper cell
TLR	Toll Like Receptor
Treg	Regulatory T Cells
VD	Vaginal delivery
WHO	World Health Organization

LITERATURE OVERVIEW

Human Microbiota

The microbial cells that colonize the human body are at least as abundant as our somatic cells, although they certainly possess far more genes, with higher genetic diversity, than the human genome.[1–4] Specifying the definition of the human microbiome has been challenging due to confusion about terminology where, for instance, "*microbiota*" (the microbial taxa, including bacteria, yeasts, filamentous fungi, archaea and viruses, associated with humans) and "*microbiome*" (the collection of these microbes and their genes) are often used interchangeably.[5] However, the term "*microbiota*" in the current thesis refers to the bacterial taxa described at different body habitats. Interestingly, although host-associated microbiota is likely acquired from the surrounding environment, the composition of mammalian microbial communities, particularly the gut, varies greatly from common free-living microorganisms. This indicates that long co-evolution between mammalian hosts and colonizing microbes has formed specialized microbial communities that thrive in habitats like the gut.[6] The resulting relationship between the human host and microbial symbionts benefits both parties; the microbial colonizers are provided with continuous source of nutrients while the host obtains metabolites from bacterial digestion, pathogen protection and immune system education, among other beneficial functions.[7–9]

One of the most ground-breaking progresses in the biological sciences in the past decades has been the understanding of the fact that microbial communities inhabit nearly every environment and have crucial roles in human health and disease. Numerous studies have focused on describing the complexity of microbial communities on particular body habitats, including gut [10–14], oral cavity [11, 12, 14, 15], skin [12, 14, 16, 17], milk [18–21], reproductive tract [22], and respiratory tract.[23] A common characteristic for each habitat is that the establishment of microbes is highly interpersonal and abundances, among different taxa, vary radically. Moreover, community composition is more similar within than between different environments while interpersonal dissimilarity within habitats is larger than intra-individual variability over time.[11] The complexity of the microbial community depends on the particular habitat and only selected microorganisms will be able to survive and colonize under the conditions characteristic for that body site. One of the principal objectives in human microbiota studies is to reveal how the composition, diversity and functions of the constituent microorganisms influence and regulate health and disease. By understanding this, we will be able to learn how to manipulate the

composition and metabolic activities of the microbiome, thus maximizing the health benefits to the host. Advances in this research area of biological science have been possible to achieve due to development of different culture-independent omics technologies based on massive sequencing in combination with innovative bioinformatics and system biology approaches.

Colonization with pioneer microbiota

Although some authors consider that the fetus resides in a sterile environment [24, 25], this idea has been challenged in other studies.[26–29] This is primarily due to discovery of the presence of complex bacterial community in the meconium [13, 30–32], and placenta [33, 34], reflecting a potential *in utero* microbial colonization, but also by the manifestation of bacteria and bacterial DNA at the maternal-fetal interphase. Several studies have examined the potential bacterial transmission through placental barrier, in healthy term pregnancies, and through the umbilical cord blood [35], fetal membranes [36, 37], and amniotic fluid [36, 38] from healthy neonates. However, the bacterial load levels in the fetal environment has been estimated to be extremely low, suggesting the possibility for (environmental) contamination upon sampling.[24]

Through the birth and following hours, the neonate encounters a wide range and large amount of microorganisms found in the mother´s vagina, gastrointestinal tract and skin, but also from microbes of other individuals present at the delivery and from the immediate environment.[7] This postnatal microbiota acquisition is of crucial role for the infants' future health, where by creating resilience against pathogens, it stimulates the immune system and initiates appropriate immune priming and education of immune cells. The timing of bacterial colonization influences the immune system development and, in turn, a life-long symbiotic relationship between the host and the microbiota.[7, 39, 40] Studies have concluded that during the early postnatal period there is a "*window of opportunity*" [39, 41], where the immune system interaction with specific microbe-associated molecular patterns (MAMPs) initiates a cascade of events that are crucial for a proper immune development of the infant.[39, 42] Therefore, particular microbial exposure here will determine if the specific immune events will be durably imprinted, or not. MAMPs are necessary for maintaining the dialogue between the host and the microbiota, thus promoting an adequate microbial colonization.[43] Likely, during this time-limited early period of colonization, the immune system of the infant appears to be more susceptible for microbial instructions.[39, 41] The microbial communities are highly dynamic during the first year of life [44], assembling an adult-like community structure possibly by preadolescence.[13, 44–46] Inadequate microbial acquisition during this critical time frame may shift the microbiome out of the healthy state, resulting in a less resilient microbial community, that in turn could favors the blooming of opportunistic pathogens and disturb microbial immune priming.[39, 40, 47] Succession and

assembly of the microbial community, of the respective human habitat may be affected by early exposure of the infant, *e.g.* the availability of each microbe in the local environment of the host and host selection, which depends on diet and genetics.[40, 47]

Microbial Niches

Oral microbiota development

The human oral microbiota is an ecological community consisting of symbionts and pathogenic microorganisms found in the oral cavity. The oral cavity consists of a wide range of niches, including the tongue, teeth and gingival crevice and the buccal mucosa, all with different characteristics and specific selective environment thus supporting diverse bacterial communities. A unique feature of the oral environment, compared to other body parts, is the presence of both non-shedding surfaces (teeth) and shedding (mucosal) surfaces, facilitating the microbial colonization with diverse characteristics.[7] Due to the high flow rate and generally low levels of nutrients that impede bacterial multiplication, the large numbers of bacteria (10^8 per ml) encountered in saliva are mainly representing the types of organisms found on the dorsal surface of the tongue.[7]

Unraveling how the oral microbiome is shaped and defined is important for the understanding of both oral and general systemic health. Research on development and maturation of the human microbiome during infancy has mainly been focused on the gut while knowledge of the establishment and structure of the neonatal oral microbiome colonization following delivery is still limited. *Streptococcus* and *Actinomyces*, belonging to Firmicutes and Acinetobacteria phyla respectively, are considered to be pioneer oral colonizers that are acquired at birth and following hours (Fig. 1).[48, 49] Gradually, by production and excretion of metabolic products from these colonizers, the growth of more strict anaerobes like *Veillonella* and *Fusobacteria,* is favored.[48, 49] As the infant grows, microbial communities become more complex and diverse, reaching the adult-like stability later in childhood.[48, 50, 51] The early microbial environment of the oral cavity is heavily shaped by microorganisms of maternal origin, by contact with the outside world through breathing and through contact with parents and medical staff.[48, 52, 53] This is how the process of permanent colonization of the oral cavity begins. Moreover, early oral microbiota development has been shown to be influenced by breastfeeding habits and delivery mode.[54, 55] To study the pattern of oral microbiota development is important because it has been shown that early colonization of certain pathogens may increase the rates of oral diseases such as dental caries.[56] In addition, the potential modulation of the immune system by microbes of the oral

mucosa and other tissues is relatively unknown in comparison with the large interest and resources devoted to understanding the role played by gut microorganisms.

Gut microbiota development

Gut bacteria is the key player of microbial host stimulation, providing specific signals for adequate immune stimulation and development.[57] Facultative bacteria such as *Lactobacillus* and *Staphylococcus* dominate the gut microbiota during the first days of life since the absence of oxygen prevents the colonization of obligate anaerobes. A few days later, *Enterococcus* and *Enterobacteriaceae* appear and, together with further expansion of facultative bacteria, create an anaerobic environment by consuming the oxygen. The following state will favour the colonization with the obligate anaerobes *Bifidobacterium*, *Bacteroides* and *Clostridium,* where *Bifidobacterium* is a dominant bacterial genus in the gut microbiota of breastfed infants.[58, 59] It is believed that the *pioneer microbiota,* consisting of Firmicutes, Bacteroidetes, Proteobacteria (*Enterobacteriaceae)*, *Veillonella* and especially *Bifidobacterium* (Actinobacteria phylum) [39, 60], is responsible for initial education of the evolving immune system as it provides the favorable environment for further microbial settlement, specific compounds and protection from the systemic immune system.[61] The early intestinal microbiota is characterized by low diversity and a relative dominance of the phyla Proteobacteria, Actinobacteria and Firmicutes (Fig. 1).[13] With introduction of solid foods, the early microbes with a genetic profile that facilitate lactate utilization are replaced by bacteria enriched in genes coding the digestion of different carbohydrates and genes involved in vitamin synthesis and degradation of xenobiotics.[61]

The settlement of bacteria on the gut surface can protect against pathogen penetration, a process known as 'colonization resistance', which is of great importance for the prevention of pathogen-induced gastrointestinal inflammation.[43] Commensal bacteria have been shown to control the potential pathogen-infection by competition for nutrients, adhesion sites, pH, receptors and production of specific metabolites and antimicrobial peptides, and by creating an environment hostile for pathogen survival end establishment (for instance, lactobacilli in vaginal environment that reduce local pH, and produce specific compounds that prevent expansion of other bacterial taxa).[43, 62, 63]

Human breastmilk microbiota

Breastmilk hosts a wide range of microbiota and potential probiotic bacteria (at concentrations of approximately 10^6 bacteria/ml sample [19]) that are believed to orchestrate the infant's developing mucosal immune system.[64, 65] Although information about the function of breastmilk bacteria is scarce, it has been proposed that they play a crucial role as seeding

colonizers of the infant microbiota, to facilitate infant digestion, to compete for nutrients and space with pathogens and to improve intestinal barrier functions by augmenting mucin production.[18, 66] Moreover, the breastmilk microbiota may promote an adequate initial intestinal immune homeostasis that encourages a shift from an intrauterine T helper cell 2 (Th2) predominant to a Th1/Th2 balanced response and to trigger regulatory T cell differentiation in infant.[40, 66] Culture-independent techniques, including 16S rDNA sequencing and metagenomics analysis, have demonstrated a complex and diverse group of bacteria in human milk, including the presence of *Staphylococcus, Lactobacillus, Pseudomonas, Streptococcus*, and *Acinetobacter,* with large degree of individual-specific profiles.[18, 19, 67, 68] *Lactobacillus* from breastmilk have been shown to prevent colonization and growth of gastrointestinal pathogens, including *Escherichia coli* and *Shigella* spp, and reduce the incidence of gastrointestinal, respiratory, and total infections.[69–71] *Streptococcus* and *Staphylococcus* species are the most commonly abundant bacterial families in human milk.[18, 21, 72, 73] The progressive establishment of the infant microbiota [18, 65, 72, 74], favored by breastfeeding, is crucial for instructing their immune system to tolerance [68] and many epidemiologic studies have documented differences in the microbial composition of gut in breastfed and formula-fed infants.[75–77]

Multiple studies have observed the sharing of several microbial strains, including *Bifidobacterium, Lactobacillus, Enterococcus*, and *Staphylococcus*, between breastmilk and infant stool.[78–80] Neonates who mainly breastfeed, during the first period of life, share 28% of their stool microbes with their mother's milk microbes – a percentage that increases in dose dependent manner.[72] The fact that the bacteria encountered in the infant gut mostly resemble the bacteria from their own mother [72], suggests the importance of breastfeeding for an initial colonization of the infant.

The origin of the bacteria in breastmilk is not yet clearly established and breast tissue itself contains a diverse community of bacteria, identified even in non-lactating woman.[81, 82] It is believed that maternal commensal skin flora, ingested by the infant during breastfeeding, may contribute to the bacterial communities both in baby's mouth and mother's milk duct colonization.[83, 84] However, this theory does not fully explain the presence of strict anaerobes, including *Bifidobacterium, Clostridium*, or *Bacteroides* species, in breastmilk. [68] Another theory is that bacteria from the mother's intestinal tract may translocate to mammary gland and reach the infant by breastfeeding.[18, 65, 85] The theory underlying the so named entero-mammary pathway, speculates that the bacteria from intestinal lumen invade the maternal gut, by internalization in dendritic cells (DC), and reach the mammary grand by the lymphatic system and blood circulation.[86] However, this hypothesis is still not fully explored and needs to be further investigated.

Maternal factors, including the mode of delivery, geographical locations (mothers from Europe, Africa and Asia express different milk microbiota profiles [20, 72, 87]), maternal health and antibiotics use (associated with decrease in milk microbiota diversity)[88], have all been shown to influence milk microbiota composition and diversity. While some studies have reported that lower bacterial diversity has been demonstrated in milk from mothers that deliver by caesarean section [89–91], other studies have not found any link between the mode of delivery and milk microbiota diversity.[20, 72] However, more studies are needed in order to understand the impact of different factors on milk microbiota and further mother-infant transfer.

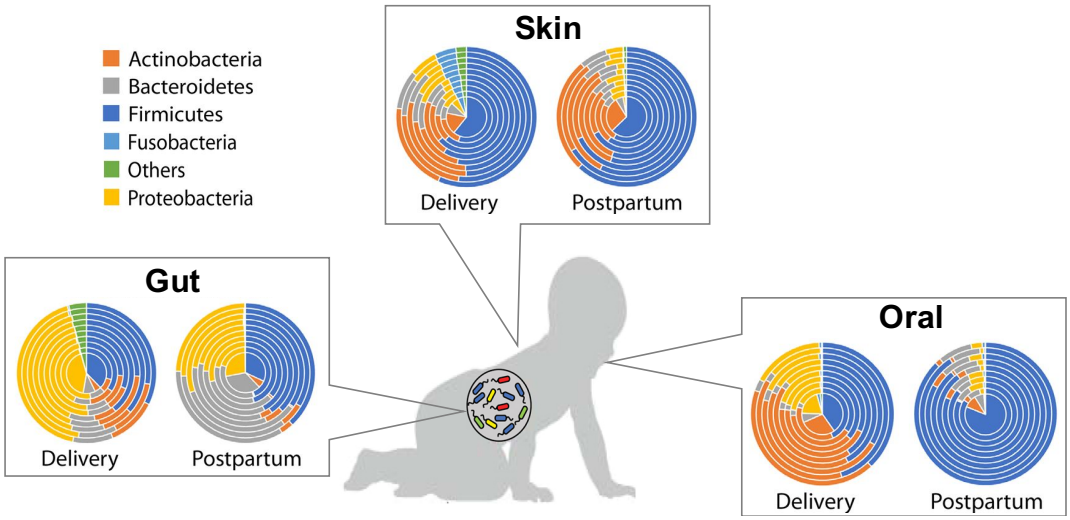

Fig. 1. A global overview of microbial composition across the different infant body niches. Key phyla of the infant microbiota composition are presented at different stage of early life. Concentric circular diagrams represent interindividual variability. Adapted from [92].

Microbiota and Immune System

Mucosal immunity in the gut

Despite the mostly symbiotic relationship of the host-microbial interactions, the close contact with microbial communities present at mucosal sites results in continuous immune challenge. It is still not fully understood how the neonatal gut tissue adapts to this constant microbial exposure, but features of the maternal breastmilk (discussed in detail below) are considered to direct some of these early responses to commensal microbes.[93] Moreover, properties of the neonatal immune system during the first period of life might also explain its ability to accept and

tolerate microbiota colonization. The early and developing immune system is characterized by dampened pro-inflammatory cytokine responses, resulting in a more regulatory profile [63], that favors the establishment of the microbiota.

The gut mucosal barrier provides a dynamic interface between the sterile milieu of surrounding tissues and the external environment with its enormous microbial load. This barrier consists of various layers, including the mucus layers, intestinal epithelium layer, layer with immune effector cells that are either associated with the epithelium or embedded in the *lamina propria* mucosae and gut-associated lymphoid tissue (GALT) layer, where adaptive immune responses can be initiated (Fig. 2).[94, 95] The epithelium, as a main component of mucosal barrier, blocks the invasion of pathogenic and commensal bacterial to the surrounding gut tissue.[96] This defense is achieved by two barrier subtypes, chemical and physical.[97] The chemical protection consists of antimicrobial peptides and lysozyme, among other bioactive factors, with the main function to separate the gut bacteria from the intestinal epithelial cells of the small intestine.[98, 99] Physical barriers inhibit microbial invasion by the mucus layer, the glycocalyx (a network of carbohydrate moites of glycoproteins/glycolipids) on the microvilli of absorptive inter-epithelial cells, and the cell tight junctions firmly uniting intestinal epithelial cells thus maintaining the integrity and the structure of the mucosal barrier.[97] Mucus, a viscous fluid consisting of mucin glycoproteins, is secreted by goblet cells that are enriched in the large intestine. The large intestinal epithelium, in contrast to the small intestine, are lined by a two-layered mucus layer: the outer loose and the inner firm mucus layer.[100] The outer mucus layer is inhabited by numerous bacteria, due to it loose physiology, while the inner mucus layer is denser and contains various antimicrobial molecules including immunoglobulin A (IgA), thus preventing the penetration of bacteria.[100] For bacteria overcoming both the mucus barrier, tight junctions connecting the epithelial cells is a final wall that hamper the invasion.[101]

The epithelial defense is exceptionally well-established and complex in the gut, where it contains large amounts of commensal microbes that are involved in digestion of polysaccharides, production of vitamins, maturation of the immune system and prevention of pathogenic bacterial colonization.[102] In return, the intestine provides symbionts with a proper milieu with abundant energy derived from ingested food.[97] The relationship between the host and prokaryotic cells requires a continuous interaction, where the signaling program sent by commensals present at the site is interpreted by diverse cell compartments, in order to educate the mucosal immune system and ensure hypo-responsiveness against commensals while being prepared for protection against pathogens.[95, 103, 104] The importance of host recognition of commensals has been confirmed in germ-free mice that demonstrate an altered immune system organization and reduced cellularity, when compared to mice harboring a complex microbiota.[94]

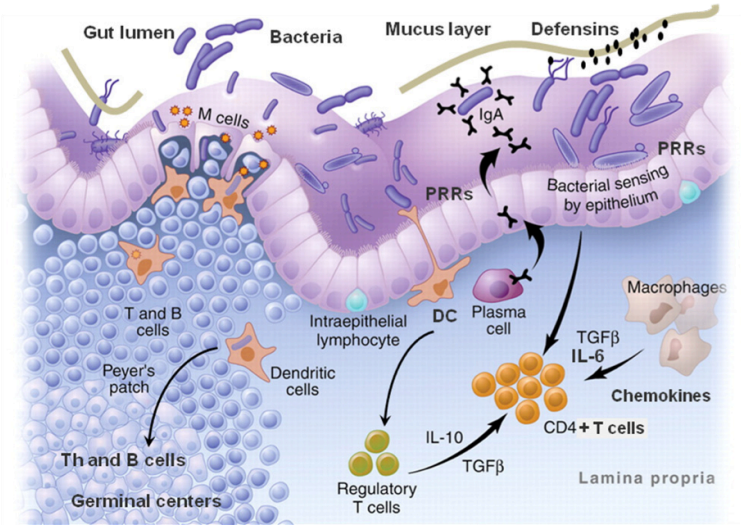

Fig. 2. Schematic view of the gut mucosa. The intestinal mucus barrier is composed of different compartments that are important for maintaining immune homeostasis and integrity. The thick layer of mucus facing the gut lumen contains secretory immunoglobulin IgA, antimicrobial peptides and commensals. The underlying intestinal epithelium is composed of enterocytes, mucus producing goblet cells, Paneth cells (that secrete antimicrobial peptides), intraepithelial lymphocytes and hormone-producing enteroendocrine cells. The majority of intraepithelial lymphocytes are memory CD8+ cells and they are responsible for maintaining the integrity of epithelium. Gut-associated lymphoid (GALT) consists of Peyer's Patches, clusters of lymphoid follicles several smaller lymphoid aggregates. Peyer's Patches consist of B cell follicles with naïve B cells and interfollicular T cell zones. In the core of each B cell follicle, there is a germinal centre where activated B cells expand, undergo class switching, somatic hypermutation and maturate into IgA producing plasma cells. The Peyer's Patches also contain microfold (M) cells with ability to take up the antigen from the gut and present it to the immature dendritic cells (DC) or macrophages, present in subepithelial dome region of the Peyer's Patches. Dendritic cells in lamina propria, supporting the epithelium, are also responsible for sampling gut bacteria. Upon invasive infections, Th17 cells are needed for the elimination of microbes trying to penetrate the surface epithelial cell layer. Foxp3+ regulatory T cells, also found here, are important for maintaining the intestinal immune homeostasis in the presence of a nonpathogenic commensal bacteria. Modified from TT. MacDonald and G. Monteleone. Immunity, Inflammation and Allergy in the Gut. Science 2005; 307:1920-1925.

Mucosal immune system control of microbiota

Although intestinal bacteria are mainly restricted to the luminal side of the gut, some microbes succeed to overcome the barrier protections and penetrate the intestinal epithelial cells. Commonly, these commensals get here phagocytosed and eliminated by macrophages present in lamina propria of the tissue.[105] Also, the mucosal immune system may use some of the invading bacteria in order to direct specific immune responses (Fig. 2), including the initiation of protective secretory IgA production by B cells through regulatory T cells (Tregs) activation.[106] The IgA antibodies are then distributed throughout all mucosal surfaces where it may exert directed immune responses, against commensals.[94]

The importance of the immune system for microbiota shaping is confirmed in immune deficient mice that, for instance, lack IgA antibodies. These mice then show priming of serum IgG responses against commensal bacteria, which is likely due to the fact that the commensals can overcome the mucosal barrier protection thus causing systemic immune responses.[94, 107] Moreover, since IgA antibodies have been shown to promote the adhesion of microbiota in the gut to the epithelial surface, thus selecting for the growth and colonization of specific bacterial species, the lack of this antibody may affect the microbial composition and establishment of non-beneficial species.[108] Similarly, disruptions of regulatory T cells networks (for instance due to Foxp3 deficiency) have been shown to induce chronic intestinal inflammation in the presence of a triggering intestinal microbiota and lack of tolerance within the intestine.[109, 110]

Findings here suggest that the host appears to have some control over the microbial consortia composition of the gut and that defects in the host immune system can cause a dysbiosis. However, it is still to be determined in detail to which extent, and how, the immune system influences the composition and location of commensals in other niches, including the oral cavity where this information is still very meagre.

The importance of microbiota in shaping the immune system

Microbial colonization of the neonatal gut might direct initial immune maturation, playing an essential role for a balanced postnatal innate and adaptive immune system. The microbial pleiotropic effect on the host includes its involvement in shaping the architecture of the immune system, in regulating the balance between immune cell types present and in influencing the development of immune cell populations.[93] The early studies comparing germ-free and colonized mice demonstrated the importance of microbial colonization for a proper formation of lymphoid tissue and further immune development.[111] Besides having reduced numbers of intestinal CD4+ cells, IgA producing cells and aberrant Peyer´s patches, germ-free mice are typically Th2 skewed [112], but a balance of Treg/Th2 responses can be achieved by introduction of symbiotic bacteria.[94, 113] However, this needs to occur during the critical developmental window, in the first 1,000 days of life of the neonate (and first postnatal week in mouse studies) when many biological systems are established.[42] Bacterial cell wall components, including LPS and flagellin [114, 115], and commensal microbiome metabolites display immune-regulatory properties within the gut mucosa that contribute to host-microbiota homeostasis.[94, 111]

Effects on T helper cell subsets. T cells are crucial for protection of the host from various pathogenic microorganisms. Based on their unique cytokine profile, effector T helper cells can be

classified into Th1, Th2, Th17 and Treg subpopulations (Fig.3).[116] Homeostasis in the gut mucosa is maintained by an interplay between potentially pro-inflammatory and anti-inflammatory cells, and the balance of effector lymphoid cells and Treg cells can have a great influence on the mucosal responses upon stress/tissue damage. It has been demonstrated that the microbiota composition is important for Th cell differentiation and also that the metabolites produced by different symbionts may be instrumental for the bacterium's success as a commensal.[94, 117, 118]

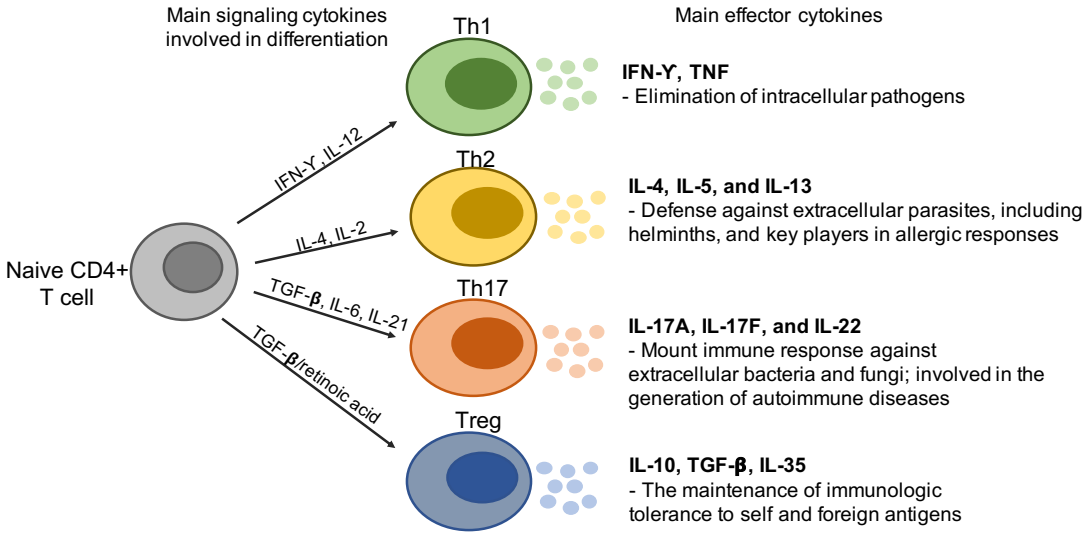

Fig. 3. Schematic view of T helper cell subsets. The graphic presents the differentiation of naïve CD4+ T cell, key regulators of the immune system, into different T helper cells (Th) and regulatory T cells (Treg) that possess distinctive biological functions. The CD4+ T cells´ ability to differentiate into distinct cell types is determined by the presence of key-signaling cytokines. Once programmed, activated cells secret effector cytokines that are important for the adaptive immunity.[119]

By producing their key cytokine IFNγ, Th1 cells stimulate immune responses against intracellular microbial infections, complement activating and opsonizing antibody production, while Th2 secrete IL-4, IL-5 and IL-13 to induce humoral immune responses against parasites and allergens.[116, 120] Th17 cells, involved in defenses against fungi and extracellular bacteria, produce the pro-inflammatory cytokine IL-17 that may also be involved in tissue damage and in different autoimmune diseases.[121] Even though the $CD4^+$ Th17 subset are considered to be pathogenic because of their abundant presence in a variety of inflammatory conditions, other studies have revealed that Th17 may also have a homeostatic role in the gut.[122]

Clostridial strains belonging mainly to cluster IV and XIVa, have been demonstrated to favor the anti-inflammatory responses by influencing the differentiation of Tregs, in mice.[123]. In germ-

free mice with a damaged gut epithelium, the colonization with the cluster IV and XIVa resulted in reduced disease symptoms.[123] In a mouse model, the recognition of flagellin of the *Lachnospiraceae* family of Clostridiales by Tregs provide tolerance to commensal bacteria by promoting the survival of antigen-specific IgA^+ B cells.[124] While the mechanisms in which the commensal-induced regulatory T cells perform systemic anti-inflammatory functions is not yet known, it has been observed that some Tregs in the gut possess T cell receptors with specificity for distinct commensal bacteria.[125] Other bacterial surface antigens, including Polysaccharide-A of *Bacteroides fragilis* provokes an IL-10 response by regulating $CD4^+$ T cell transformation toward Foxp3+ Treg, which in turn prevents epithelial barrier damage by inhibiting the differentiation of Th17 cells.[126] Moreover, in homeostatic conditions, *B. fragilis* elicits the induction of Th1 or Treg cells in the circulation, defending the host from pathogenic infections or by preventing immune activation by self-antigens.[117, 126] Together, these findings show that commensal bacteria have a general impact on immunity that reaches well beyond mucosal tissues.

Interactions between B-cells and microbiota. Through their production of antibodies that protect the host against pathogenic invasions, B cells are an important part of the adaptive immune system. These cells can limit aggressive immune reactivity, mainly by production of cytokine IL-10, and they can be activated in both T cell-dependent and independent manners.[127] In T cell-independent pathways, intestinal lamina propria DCs directly present antigen to B cells and activate them through B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL).[128, 129] IgA is a dominant antibody at mucosal surfaces where it plays a crucial role in maintaining gut homeostasis.[130] IgA secreting-B cells home to the intestinal lamina propria where IgA is produced in dimeric form (containing the joining (J) chain) and transported by the polymeric immunoglobulin receptor across the epithelium barrier and positioned on the apical surface.[118] Upon release into intestinal lumen, secretory IgA (SIgA) binds and prevents the uptake of microbial antigens [131], neutralizes pathogenic bacterial toxins [132] and disarm and clear potentially invasive microbes without starting any inflammatory responses or bacterial killing.[133] It is believed that the gut microbiota has an impact on B cells isotype switching due to the fact that microbiota alterations in mice, as a consequence of broad spectrum antibiotics or germ-free conditions, can lead to a switch to IgE, through enhanced Th2 responses [134], rather than IgA and subsequent activation of basophils, mast cells and inflammation.[135, 136] Moreover, IgA+ plasma cells in gut lymphoid tissues and lamina propria are greatly reduced in germ-free or antibiotics-treated mice.[137, 138]

It has been observed that IgA antibodies at mucosal surfaces may have polyreactive features and coat a broad, but defined, subset of microbiota.[136, 139] This suggests that the IgA, though originating from adaptive immunity, have innate-like recognition characteristics that may assist

the adaptation to a broad range of microbiota and their antigens encountered at mucosal surface of the gut.[136] It has also been reported in murine models that the majority of IgA responses here are T cell-dependent, highly antigen-specific and featured with high-affinity binding.[136, 140] SIgA has been shown to induce members of the gut microbiota, including some species of *Bacteroides,* to down-regulate their expression of pro-inflammatory surface antigens.[141] By coating the commensals, SIgA monitors bacterial entry into Peyer´s patches and germinal center in the lamina propria, thus establishing a positive antigen-specific IgA loop.[142] Selected commensals can induce systemic IgA-mediated immunity, thus constitutively protecting against bacterial sepsis in mouse model.[143] Overall, IgA might be important for diversity and balance of commensals through controlling their expansion in the gut, which is evident in their role in gene-expression regulation of commensals, thus selecting species that possess less inflammatory activity on the host's tissue.[141] IgA has also been shown to promote bacterial adhesion to the mucus layer of the intestinal epithelium, enriching for the growth and colonization of particular microbial strains, as observed *in vitro* and *in vivo*.[108, 144, 145] Furthermore, commensals, particularly *Bacteroides fragilis*, appear to be capable of modifying their surface architecture in order to attract IgA binding and allow robust mucosal colonization and single-strain stability in epithelial cell models.[145] Studies have shown that lack of intestinal stimulation by commensals results in lower levels of IgA+ plasma cells, and thus reduced abundance of IgA [146, 147], which could be due to aberrant development of lymphoid tissue that is important for IgA production.[148]

Besides SIgA, IgM and IgG antibody subclasses are also able to bind to microbiota and their responses are mainly induced through T-cell independent pathways.[149, 150] In contrast to mice where IgM responses are characterized by low affinity binding [149], human hosts have more abundant and specified IgM responses that help to maintain diversity of bacterial communities together with IgA. [151] Even considerable IgG amounts, with low affinity binding feature, have been detected in the secretory compartment of the gut as well, in homeostatic conditions.[139] However, lack of active translocation of secretory IgA and IgM to mucosal surfaces of the gut have been observed to lead to increased serum IgG antibodies against gut commensals and pathogens in mice, reflecting the importance of secretory anti-inflammatory antibodies in restricting systemic exposure to microbiota antigens.[152]

Short chain fatty acids and microbiota. The colonic microorganisms play a crucial role in metabolic activities associated with homeostasis in large intestine. Short chain fatty acids (SCFAs), including acetate, butyrate, and propionate [153], are principle metabolic outputs of the microbiota. Their quantity and relative amounts in adults have been closely linked to diet, where a high-fiber diet increases blood concentrations of SCFAs [154], as well as microbiota

composition, diversity, and activity.[155] The main producers of SCFAs are classified as *Ruminococcaceae* (cluster IV) and *Eubacterium* (cluster XIVa) in the order Clostridia, class Clostridia, and phylum Firmicutes.[153] SCFAs mainly bind to G-protein coupled receptors encountered on the cells of the intestinal tract, immune cells (including Tregs) and adipose tissue. Among other physiological functions, SCFAs, in concentration depended manners, regulate T cell polarization and accumulation [156], the chemotaxis and the viability of neutrophils [157] and immune regulation in monocytes and macrophages.[158] For instance, propionate may suppress the Th1-type immune response in stimulated human peripheral blood mononuclear cells [159], butyrate can inhibit the proliferation and apoptosis in activated T lymphocytes [153], and butyrate and propionate play a complex role in Treg differentiation and intestinal tract immune regulation.[153, 160]

Exclusive breastfeeding during infancy has been was associated with lower absolute concentrations of total SCFAs, including acetate, butyrate, propionate and valerate, and higher concentrations of lactate in stool samples.[161] Moreover, a higher relative proportion of acetate has also been related to exclusive breastfeeding.[161] This may in part be due to the breakdown of breastmilk-acquired human milk oligosaccharides by *Bifidobacterium* [162], in the gut of breastfed infants [163, 164], resulting in an increased production of acetate and lactate.[165] A positive correlation between bifidobacterial counts and fecal acetate has also been confirmed in a study Martin *et al.*[166] Although evidence is still limited, higher acetate in breastfed infants might provide protection against intestinal pathogens and allergic disease.[167, 168] Therefore, strategies to increase SCFA levels might be a preventive target for allergic diseases during childhood.

Factors Influencing Early Microbial Colonization

Early establishment of microbial communities and species interactions appear to be influenced by the order and timing of species immigration to the host tissue.[40] Factors including mode of delivery (vaginal or caesarean delivery), breastfeeding habits (duration of breastfeeding and formula feeding), gestational age, the number of older siblings, pet ownership and antibiotics use may all shape the patterns of early microbial colonization, thus favoring the expansion of specific microbial niches.[169, 170] These events may alter a priority effect of bacterial colonization and contribute to long-lasting consequences during early stages of gut microbiota development, although the importance for the host health remains unclear.[40]

Delivery mode

The initial neonatal colonization differs depending on delivery mode (Fig. 4). Infants born by caesarean section (C-section) are not exposed to their mother´s vaginal and fecal bacteria and their microbiota resembles the colonizers found on the skin, oral cavity and the surrounding environment [163], including *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*.[171] Moreover, these infants are less likely to be colonized by *Bifidobacterium* and *Bacteroides* at both three days and one month of age, while having increased presence of *C. difficile*.[172, 173] Furthermore, decreased levels of intestinal *Bacteroides* genus has also been detected in the microbiota of C-section infants at three-four months of age, together with higher abundance of the genera *Escherichia,* when compared to vaginally delivered babies.[77] This was further confirmed in another study where caesarean section delivered infants either lacked or showed a delayed colonization (by up one year for some infants) of the phylum Bacteroidetes.[174] This was accompanied with lower total microbiota diversity as well, which may be a consequence of the absence of this phylum.[174] Moreover, C-section delivered neonates have been observed to have lower leucocyte counts, lower haemoglobin and cytokine GM-CSF levels in cord blood [175], and reduced Th1 responses during the first two years of life.[174] Delivery by C-section has also been associated with an increased risk for immune disorders, including allergic rhinitis, asthma and intestinal bowel disease.[176, 177]

Breastfeeding

Breastfeeding plays an important role in shaping the microbiota and the immune system. The presence of myriad bioactive components in breastmilk with multifunctional and anti-inflammatory characteristics that protect against infections, together with a diverse array of microbiota, support the recommendation from World Health Organization (WHO) of exclusive maternal breastfeeding during the first 6 months of life for all healthy women and children. [64, 178–180] This first span of life with recommended exclusive breastfeeding coincides with a critical window for microbial imprinting, which in turn may be important for infant´s immune development. Moreover, protection provided by breastmilk may extend beyond the weaning period, thus having long-term benefits for the infant.[181–183]

However, there is increasing concern that the current recommendation of delaying solid foods complementation may be problematic for the risk of developing eczema and allergic disease. Conflicting guidelines, recommending the optimal time point for the introduction of complementary foods, have been presented [180, 181, 184–186], and some researchers support the hypothesis that later introduction of foods increases allergic responses.[187] The recommendations from WHO were not taking into account the association between early complementary foods introduction and allergy prevention, which must be further evaluated.

However, gut colonization through continued breastfeeding may promote tolerance and protection when complementary feeding is initiated.

Breastfed newborns have been demonstrated to have a more stable and uniform bacterial population, when compared to the formula fed babies.[188] While some studies have not observed differences in *Bifidobacterium* levels between breastfed and formula fed [189–191], Bezirtzoglou *et al.* detected that breastfed infants harbor more than double counts of *Bifidobacterium* cells when compared to formula fed.[188, 192] Within the genus *Bifidobacterium*, B. *breve*, *B. adolescentis*, *B. longum*, and *B. bifidum* have been isolated in both formula fed and breastfed infants, whereas *B. infantis* is a common commensal in breastfed neonates and *Bacteroides fragilis* in formula fed neonates.[172, 190] Another cohort-study demonstrated that exclusively breastfed, four-month-old infants had increased levels of bacterial taxa that are commonly used as probiotics, such as *Lactobacillus johnsonii/L.gasseri*, *L. paracasei/L. casei*.[163] However, differences between breastfed and formula fed are diminished between the first and second year of life.[59, 190]

Other human breastmilk components

HMOs. Among the bioactive factors, breastmilk contains various immunological substances, including immunoglobulins, cytokines, chemokines, human milk oligosaccharides (HMO) and growth factors that can be transferred to the offspring through breastfeeding.[193] Colostrum, the first form of milk produced by the mammary glands, contains especially high concentrations of HMOs, which are indigestible by human enzymes alone. HMO are responsible for selectively promoting the growth and colonization of beneficial commensal bacteria, including *Bifidobacterium* and *Lactobacillus*, where they serve as a substrate for fermentation thus selecting for growth of specific bacterial species.[194] Furthermore, the breakdown products of HMOs from commensals, lactate and SCFAs, suppress growth of pathogens like *Escherichia coli* and *Clostridium perfringens,* thus giving commensals an advantage over pathogens in the developing infant gut.[195]

Immunoglobulins. Breastmilk antibodies are believed to reflect the numerous pathogens which the mother has had contact with in the gut and airways, thus providing an important defense against the same pathogens likely encountered by her infant.[196, 197] Although all immunoglobulin isotypes can be encountered in breastmilk, SIgA is considered most important for their anti-inflammatory properties and defenses of the mucous membranes.[198] SIgA acts locally on the newborn's mucosal surfaces, thus regulating the binding and penetration of commensals and pathogenic microorganisms to epithelial cells.[196] While the concentrations of IgA in breastmilk are high during the first weeks *post partum*, the levels decrease as the infant's endogenous levels of this antibody augments.[199, 200] Interestingly, the quantities of SIgA in

milk from mothers delivering preterm are higher, which may reflect the importance of breastmilk protection of premature infants, whose immune system is more vulnerable to early life infections.[201]

Breastmilk components including SIgA and TGF-β are believed to protect the infant from developing allergies, whereas others might act in the opposite fashion (*e g* arachidonic acid and the cytokines IL-4, IL-5, IL-13 that are most intimately involved with IgE production and induction of eosinophils).[202] For instance, low levels of SIgA in breastmilk have been associated with increased risk of cow's milk allergy in infants in some studies.[203] Additionally, mothers having allergies were observed to have decreased SIgA responses to the antigen ovalbumin, compared to healthy mothers, but the presence of these antibodies was not predictive for allergy development in their infants.[204] However, it is still unclear how the presented immunomodulatory factors in breastmilk might affect and predict the development of allergic diseases in those children.

Antibiotic use

The use of antibiotics in early life may have an effect on microbial succession, diversity, and resistance to treatment that can last long past infancy. It has been observed that antibiotics may cause a shift in the composition of the gut microbiota towards a profile dominated by Proteobacteria and low abundance of Actinobacteria populations.[205, 206] Antibiotics have also been shown to decrease the overall bacterial diversity of the infant's microbiota, making the baby more susceptible to infections, and select for the growth of drug-resistant bacteria.[207, 208] In a study by Tanaka *et al.,* the fecal microbiota of newborn that were treated with broad-spectrum antibiotics during the first four days of life, showed less bacterial diversity and decreased colonization with *Bifidobacterium* and *Enteroccoccus*, during the first weeks of life.[205] At one month of age, overgrowth of *Enterobacteriaceae* and *Enterococcus* was observed in infants in the antibiotic-treated group. Interestingly, a similar trend was observed microbiota development of C-section delivered neonates whose mothers were administered with a closely related antibiotic.[205] Correspondingly, antibiotics treatment during early infancy appeared to influence the maturation of Th1 immune responses, the balance in activity and development of Th1/Th2/Th17 as well as regulatory T cell function in mice.[209] Some studies have also shown that prenatal exposure to antibiotics increases the risk of subsequent asthma and wheeze development [210–213], as well as eczema.[214] However, the biological mechanisms behind deserves to be evaluated further. Additionally, the use of antibiotics during infancy has been linked to increased risk in developing allergic diseases like asthma, food allergy, eczema and autoimmune diabetes (Fig. 4).[215, 216] Taken together, early exposure to antibiotics may shape

the microbiota ecology of an infant, by causing lasting perturbations on developing bacterial communities.

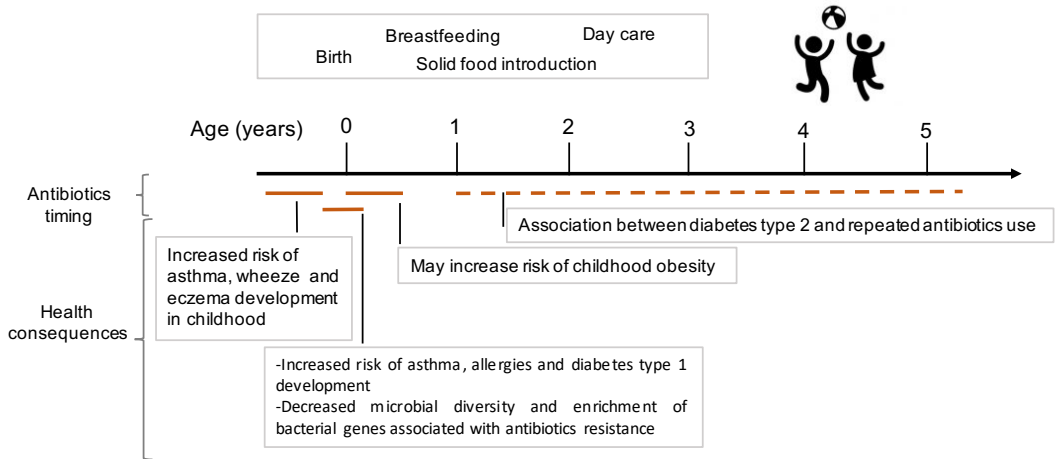

Fig. 4. Schematic overview describing the possible consequences of antibiotics use in early childhood. Adapted from: The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation, Langdon, Amy; Genome Medicine 2016.

Allergy and Hypersensitivity

Allergy is characterized by inappropriate hypersensitive immune reactions to innocuous antigens, developing due to interactions between environmental and genetic factors. The term *allergy* is today most commonly used to define the Type 1 (of four different types) hypersensitivity reaction, which is mediated by immunoglobulin E (IgE) antibodies (atopic allergy).[217, 218] However, allergy or allergic hypersensitivity also includes non-IgE mediated allergies and hypersensitivities, characterized by nonspecific immune interactions instead (Fig. 5).[217, 218] An allergic reaction occurs when the immune system is triggered by allergens to which the affected individual is sensitized, activating an antibody response that in turn cause a cascade of immune events.[134, 218] Type 1 is an immediate reaction that can either cause anaphylaxis (to food allergens, drugs and insect venoms), asthmatic and rhinoconjunctivitis reactions (due to inhalant allergens such as pollen, cat and dog dander and house dust mites) and skin and gastrointestinal reactions caused by food allergens such as peanuts, eggs and milk.[217–219] Allergic asthma, a heterogeneous disease influenced both by genetics and environmental factors, has become the most prevalent chronic childhood disease in recent decades.[220]

Atopy is a personal and/or familiar genetic predisposition to become sensitized and produce IgE antibodies upon ordinary exposure to allergens.[217] Atopic allergic diseases are characterized by

excessive Th2-like immunity to allergens, with elevated production of IgE inducing IL-4 and IL-13 and eosinophilia promoting IL-5.[218, 221] In the early phase, allergen cross-linking of IgE antibodies bound to mast cells and basophils triggers the release of inflammatory mediators, including histamine, prostaglandins, cytokines and chemokines. Later, in the late-phase reaction (after 6-8 hours), cytotoxic mediators from eosinophils are released, leading to chronic inflammation, which includes epithelial damage and increased mucus production. Moreover, the inflammatory mediators IL-4, IL-13, CXCL8 and GM-CSF that are released from infiltrating leucocytes, sustain the activation, proliferation and further recruitment of inflammatory cells.[218, 221] However, in non-allergic individuals, regulatory T cells and Th1 cells normally suppress these responses.[222] Children suffering from atopic dermatitis and/or food allergy early in life are more likely to develop inhalant allergen sensitization together with asthmatic and allergic rhinoconjunctivitis symptoms later in childhood, a process recognized as the allergic march.[223]

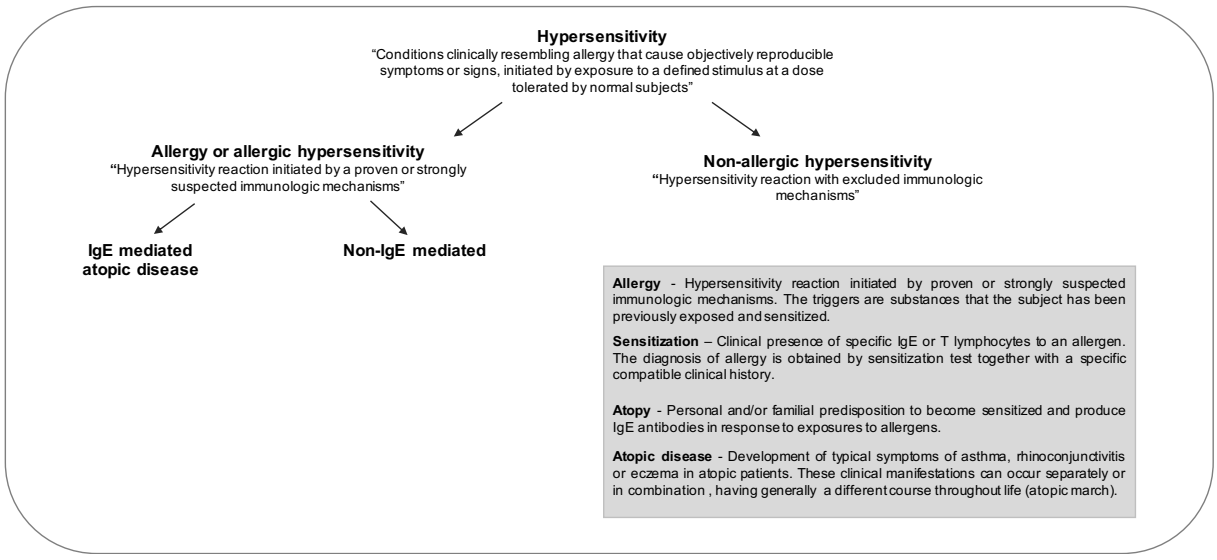

Fig. 5. Definitions of hypersensitivity and allergic disease. Adapted from [217].

Immune Development in Healthy and Atopic Children

It is now becoming clear that the early life period represents a window of both vulnerability and opportunity that subsequently impacts the immune development and tissue homeostasis. Maturation of the human fetal innate immunity is already initiated during the first trimester of pregnancy where relevant precursors population are detectable, in the thymus and bone marrow, between weeks 8 to 12.[224] It is evident that the possibly "harmful" responses, such as the production of Th1 key cytokine IFN-γ, are selectively restricted during intrauterine development but also during the early infancy.[27, 225, 226] A combination of epigenetic mechanism and

additional cellular processes contribute to overall weaken capacity of T cell activation, thus shifting the responses towards a more Th2 immuno-profile.[227–229] This pre-determined Th2 propensity from fetal stages may be responsible for the limited inflammatory functions of infantile leukocytes, leading to immunotolerance and low control over infections.[230] The Th2 bias of the newborn is also involved in maturation of Th1-like responses [231, 232] and appropriate development of regulatory T cell responses [233, 234], which are both important features for the mature immune phenotype development during childhood. A failure of silencing Th2 responses during maturation of the immune system may underlie development of Th2-mediated allergic disease [235] and various studies have reported that these early life neonatal Th2-biased responses are even more manifested, and difficult to attenuate during the immune maturation, in infants who later develop allergies.[231, 233, 236, 237]

Slow postnatal maturation of immunocompetence, including the ability to produce IL-12, IL-10 and IFN-γ, together with reduced circulating numbers and function of DCs and regulatory T cell, has been reported as a risk factor for allergic/asthmatic disease development in infants/children at high genetic risk susceptibility for these diseases.[238–242] This means that the children with family history of atopy have a more delayed development of immune function maturation, thus taking longer time to reach adult-like levels of immune competence.[224] The mechanisms underlying these immune deficiencies, that are evident already at birth [240, 243], are not well-understood but interestingly, susceptibility to severe lower tract respiratory infections (recognized as a strong risk in atopic asthma development) have been associated with poor production of the above mentioned cytokines and dendritic cells.[239, 241, 244] Moreover, establishment of an adequate mucosal barrier function, for instance by increasing the production of SIgA during infancy, seems important to counteract allergic responses.[41, 245, 246]

A longitudinal study of healthy infant peripheral blood observed that neonatal T cells have an increased propensity to differentiate into Treg cells in the first 12 months of life but that they also have the capacity to become Th17 cells already at age of 3 months.[247] This suggests that while the proportions of both resting and activated Tregs increase from birth to 6 months of age [248], in order to establish a regulatory immune profile and support the development of acquired immunity, the production of adaptive immunity Th17 -supporting cytokines, including IL-6 and IL-23, is greatly active in combating the pathogens and keeping colonizing microbes in check.[249, 250] Interestingly, high proportions of regulatory T cells in neonates, at birth and early infancy, have been positively associated with allergic sensitization at 18 or 36 months of life.[251] This implies that high proportions of regulatory T cells this early in life may counteract the important activation and maturation of the immune system, thus triggering the immune mechanisms that may be involved in sensitization later in life. Alternatively, the increased

numbers of regulatory T cells may also be masking their impaired function, as observed in a study by Hrdý *et al.* They detected that an impaired function of regulatory T cells present in the cord blood of children of allergic mothers, characterized by decreased expression of regulatory cytokine IL-10 and TFG-beta, which was compensated by an increased number of Treg in these neonates, in comparison with the healthy group.[252] Speculatively, this insufficient function of Treg early in life may be involved in compromised tolerance development in infancy and subsequent allergen sensitization in high-risk individuals during the childhood.

Innate immunity, including the surface expression of toll-like receptors and the production of their effector molecules, decreased level of phagocytosis and poorly developed complement system, are also compromised during the early neonatal period.[230, 253] For instance, the expression of TLRs involved in recognition of surface molecules of pathogens is either absent or expressed at very low levels in newborn mice.[254] Human studies are obviously harder to perform but nasal mucosal explants from young children, exposed to TLR stimulation, showed increased allergen-induced T cell reactivity and expansion, augmented production of Th1 cytokines, IL-10 secretion, and TLR4 expression, as compared with explants from adults.[255] Perinatal TLR responses are increased in cord blood cells of newborns, particularly in subjects who subsequently develop allergic disease. Babies from allergic mothers, or those infants that subsequently developed allergies, had increased production of TNF and IL-6, following stimulation of TLR 3/4/5 and TLR 4/5. In contrast, T cell-derived IFNγ responses were decreased when compared to non-allergic children.[256] In another longitudinal study of TLR responses in children developing allergies and children staying healthy, non-allergic children displayed increased production of IL-10, TNF and IL-6 in response to TLR stimulation over the first 5 years of life. On the contrary, allergic children show greater cytokine production at birth but the levels decreased over time, thus being lower by age of 5 in comparison to similar aged non-allergic children.[257] In another study, exposure to common environmental allergens, such as cockroach, or dust mite, was associated with increased cytokines responses at age 3 years, including production of IFN-α and IL-10. Moreover, reduced LPS-induced IL-10 responses as birth were related with recurrent wheeze by age of 3.[258]

Taken together, the innate immunity recognition receptor responses, the cytokine balance, transient Th2 activation and the function of regulatory T cells during the early period of life appears to be important for subsequent allergy development in childhood. The mechanisms that direct the immune maturation process are still not completely understood, but it is becoming clear that a wide range of environmental factors during pregnancy and early neonatal life can have profound effects and long-lasting consequences on the immune status of the infant and their further susceptibility to inflammatory diseases.[224] Additionally, it has now been

recognized that a major driver of a normal postnatal maturation is the microbiota inhabiting the gastrointestinal tract within a crucial developmental time window, as described elsewhere in this literature overview. It has also to be kept in mind that the potential role of the oral microbiota in that immune maturation has not been addressed.

Allergy and Microbial Dysbiosis

The prevalence of allergic diseases has increased drastically during the last decades and it is believed that factors associated with Western lifestyle, including the improved living standard, cleaner environments, the eradication of certain pathogens, antibiotics, increased time spent indoors, and smaller family size may be of great importance. These factors are assumed to reduce exposure to infectious and commensal microbes, a phenomenon known as "hygiene hypothesis", in early life which may contribute to a lack of shifting of allergen-specific Th2 responses to the Th1 phenotype.[61, 259] Moreover, development of allergic disease in the first year of life has been associated with reduced responses of Tregs to microbial stimuli, likely reflecting failure of immune tolerance development in infancy.[260] A reduced regulatory T cell function may also be reflected in Th1-mediated autoimmune-mediated diseases such as multiple sclerosis, type I diabetes and Crohn's disease also have increased in prevalence in affluent countries.[234, 261–264]

The gut microbiota differs in children who later do or do not develop allergic disease, particularly during the first months of life.[61] Moreover, the diversity of the microbiota plays also an important role in regulating allergy and mucosal immunity development during infancy.[59, 265–269] Several prospective studies have observed decreased microbial diversity to precede the development of eczema [265, 268–270], atopic sensitization and allergic rhinitis [267], and asthma.[266] Intestinal bacterial colonization during infancy is closely related to the development of the immune system compartments and the gut microbial stimulation may provide a primary signal for the maturation of a balanced postnatal innate and adaptive immune system.[61, 169, 220, 271, 272] Germ-free mice have poorly developed gut and mesenteric lymphoid tissue together with limited production of IgA, stronger Th2 responses and augmented allergic phenotype.[224] This suggests that perturbations to the colonization and intestinal bacterial acquirement (dysbiosis), may disrupt the natural immune cell-microbe interactions, resulting in biased infant immune system towards a more hypersensitive (allergic) state.[273] This is in line with the results from various cohort studies where they observed that alterations in gut microbiota in early infancy and childhood, are associated with allergic disease development later in life.[168, 265, 268, 274–278] For instance, lower relative abundance of intestinal bacterial genera *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* were significantly decreased in children at risk of developing asthma. This was further confirmed in a mouse model of allergic asthma

where the colonization with mentioned genera reduced airway inflammation.[168] Therefore, microbial exposure is crucial for mounting balanced and tolerant immune responses, thus preventing subsequent allergy development. However, the exposure needs to occur during the important "*window of opportunity*" in early life, in parallel with the maturation of the neonatal immune system. Postnatal factors like pet ownership, family size, day care attendance, animal exposure and the history of certain infectious diseases, during this early period of life, were found to influence the risk of developing allergic diseases.[279, 280]

A lack of diverse microbial exposure might result in the development of atopic diseases in early life.[281] For instance, development of IgE-associated eczema during the first two years of life was associated with a reduced diversity of the gut microbiota at 1 month of age.[265] Toddlers with eczema harboured significantly lower abundance of *Bifidobacterium* and *Bacteroides* and higher prevalence of *Enterobacteriaceae*.[275, 282] Allergic symptoms among Swedish children have been associated with low levels of house dust endotoxin when compared to Estonian households where endotoxin levels are higher (and allergy prevalence lower).[283] Moreover, children growing up on farms in Central Europe are exposed to a wider range or microbial diversity, which is related to protection from development of asthma.[284] Following the presented hypothesis, the prevalence of allergic diseases was found to be significantly higher in urban children when compared with rural children, likely due to higher level of bacterial endotoxin, and other microbial products, in the environment where the rural children were living.[285, 286] For instance the lipopolysaccharide from *Acinetobacter lwoffi*, isolated from a farming environment, was observed to have potential allergy-protection abilities by inducing Th1 polarization of dendritic cell *in vitro*.[287] One central mode of action through which microbes trigger immune responses is through recognition of microbial motifs by pattern recognition receptors, including TLRs.[224] Local mucosal administration of natural/synthetic TLR agonists has been shown to result in protection against experimental asthma in several murine models.[224] In line with this, transmaternal asthma-protective effects of *Acinetobacter lwoffii* was observed to depend on intact toll-like receptor signaling the mothers as well as TLR expression patterns in the placenta.[288]

Noteworthy, ethnicity, genetics, diets, living environments, or other factors, together with study methodologies, sampling and analytical tools can influence the investigated microbial composition.

Allergy and Probiotic Interventions

The endorsed definition of probiotics is "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"*.[289] They have wide range of effect where the main aim is to restore or improve a dysbiotic microbiota.[290–292] Species within the genus *Lactobacillus* has been commonly used as probiotics,[293] with *L. reuteri* being effective against antibiotic-

associated diarrhea [294] and *L. salivarius* preventive against *Listeria* infections.[295] Moreover, *Bifidobacterium* species have also been widely acknowledged to have immunomodulatory properties and health-promoting probiotics potential.[296] Most studies used single strains of *Lactobacillus* and *Bifidobacteium*, or their combination [263] in both humans and mouse models in order to determine the potential probiotic effects of these bacteria to treat various infections, inflammatory conditions and allergies.[297–300] Generally, probiotics are mainly used to treat gastrointestinal disorders, including infectious diarrhea caused by rotavirus.[301] Even though the results have not been always conclusive, the studies are suggesting that the beneficial effect of mentioned potential probiotics depends on the pathogens present, particular patient groups, disease condition and host immunity.[296]

The timing of probiotic intervention is critical, as early life events occurring during the "*window of opportunity*", where both the immune system and initial bacterial colonization are vulnerable, can have long-term impact on children's health. Therefore, it is of great interest to understand if microbiome-host response interactions can be modified at early life or even during pregnancy. Speculatively, starting probiotic supplementation already from the second trimester of pregnancy, when circulating fetal T cells have developed [302], may have a significant effect on fetal immune physiology and perhaps even affect microbial colonization of the infant postnatally. Hence, supplementation with probiotics have been performed in pregnant and breastfeeding mothers in order to potentially reduce the immune-related disease risk in children. For instance, probiotics administration to pregnant woman (2-4 weeks before delivery) and to their infants, six months postnatally, have been shown to significantly decrease the risk of development of atopic eczema during the first 7 years of life.[303, 304] Also, an effect on gut microbiota composition in the neonate has been reported in some studies of pre- and postnatal probiotic supplementation [305, 306], while solely maternal probiotic administration was not able to influence the infant microbiota diversity postnatally.[307] The probiotic effect on microbial composition and immune system modulation depends possibly on treatment duration as well as probiotic strain selection, as discussed by Forsberg *et al.*[308], where certain strains are more efficient colonizers than others. Also, since bacterial isolates are unique organisms with strain-specific properties and different mechanisms of action, a combination of different probiotic strains may increase the beneficial health effect.[309]

Possible mechanisms of probiotic interventions through mothers may be linked to an increase of immunomodulatory factors, including cytokines and growth factors, in breastmilk.[303] Beside probiotics, prebiotics, substrates that are selectively utilized by host microorganisms conferring a health benefit [289, 310], and synbiotics, the combination of probiotics and prebiotics, have also been used to favorably modulate gut microbiota.[263] An example of prebiotics are complex,

non-digestible carbohydrates that benefit the host by selectively favoring the growth or activity of specific bacteria in the colon, thus positively affecting gut function. Human milk oligosaccharides, for instance, are assumed to be naturally functional prebiotics with potential health benefits in breastfeeding infants.[311] Previously, human milk has been the only source for significant levels of HMOs, while these components have been mostly absent from infant formula. However, many infant formula on today's market contain mixtures of industrial-made SCFAs (including galactooligosaccharides - GOS, fructooligosaccharides - FOS and inulin) that mimic the prebiotic effects of human milk and promote a microbial colonization that resembles that of breastfed infants.[311] A potential allergy protective effect of these probiotics, during infancy, has also been evaluated.[263] A systemic review for allergy prevention reported a preventive effect on eczema in studies with combined pre- and postnatal supplementation, while results in asthma and recurrent wheeze have been inconclusive.[312, 313] The protective effects of synbiotics for eczema development have also been reported in two randomized, double-blind, placebo-controlled trial.[314, 315] However, these prebiotic components are structurally very different from the oligosaccharides occurring naturally in human milk, which might also imply that they have distinctive effects than natural HMOs.

In conclusion, and as previously addressed [308, 316–318], probiotics administered prenatally and postnatally may provide microbial stimuli that support the early maturation of both innate and adaptive immune responses, while limiting inappropriate inflammatory responses to different allergens. Moreover, the potential role of prebiotics and synbiotics in improving health and reducing risk of allergic diseases mediated by microbial aberrations should not be excluded. This field would benefit from research focusing on specific mechanisms of action, addressing host response processes and understanding how different probiotics strains and prebiotic structures associates to the function and health outputs. Therefore, more research is required in the selection of probiotics strains, prebiotic substances, treatment combinations, timing and duration of administration.

Methodologies for Characterizing the Microbiota

The classic microbiology methods for culturing bacteria from human subjects exclude a numerous uncultured or non-identified bacterial taxa while, at the same time, they are not providing a correct abundance of the taxa present. During the last decade, microbial analyses have shifted from culture-dependent techniques to culture-independent genetic sequencing, providing a fundamental breakthrough in research areas concerning microbial communities.

Quantitative PCR in real time (qPCR) and fingerprinting methodologies, including temperature gradient gel electrophoresis and Denaturing Gradient Gel Electrophoresis, have been widely used for quantification and assessment of the structure of microbial communities within a sample.[319] However, these traditional molecular techniques are rapidly being replaced by high-throughput next generation sequencing techniques, that together with advanced bioinformatics tools, have facilitated the profiling and functionality of human microbiome and improved the identification of bacterial taxa associated with certain diseases.[320]

454 Pyrosequencing

In 454 pyrosequencing (Roche Applied Science, Basel, Switzerland) of 16S rRNA genes, barcoded primers for amplifying 16S rRNA genes are used to examine and compare both dominant and low-abundance microbial communities from multiple samples. 16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify, study and compare complex bacterial consortia within a given sample.[319] Here, sequences from different samples can be identified in the same run by using a barcoded multiplex approach, which means that a unique sequence (adaptor) is incorporated into the primer and barcode amplicons are generated. Furthermore, emulsion PRC is carried out for clonal amplification by using capture beads complementary to the sequences on the adaptors, thus allowing DNA fragments to attach to the beads. The DNA fragments are then separated into single-stranded DNA and copied numerous times on each bead, creating millions of identical copies of DNA sequence. Next, beads carrying single-stranded DNA, together with other reagents (including DNA polymerase, ATP sulfurylase, luciferase, apyrase) are transferred to the wells proceeding with the pyrosequencing reaction (Fig. 6). This method relies on light generation detection upon nucleotide incorporation into a growing chain of DNA, creating sequence read length of approximately 400-500 base pairs, extended to 800 bp with the Titanium chemistry used in the current thesis.[321, 322] However, this platform has been phased out by the manufacturer and was only used in Chapter I.

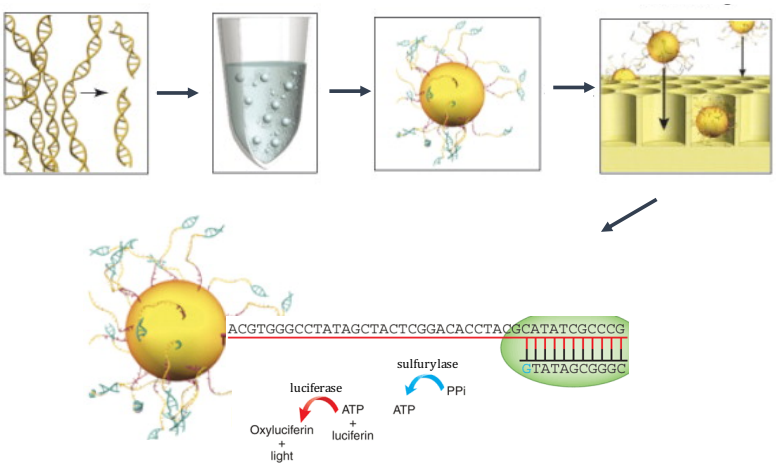

Fig. 6. 454 pyrosequencing overview. Isolated genomic DNA is fragmented, separated to single strands and ligated to adapters. Fragments are then bound to beads (one fragment per bead) and the beads are sorted in the droplets of oil emulsion where PCR amplification occurs. The emulsion is then broken, and beads carrying single-stranded DNA templates are enriched and deposited into wells for a further pyrophosphate sequencing reaction. Complementary four nucleotides are then incorporated into the template strand (red) by the annealed primer and the DNA polymerase. Incorporation of the base generates inorganic pyrophosphate (PPi), which is converted to ATP by the sulfurylase. Luciferase uses the ATP to convert luciferin to oxyluciferin, producing light.[323]

Illumina sequencing

Currently, the Illumina MiSeq platform is dominantly being used for 16S rRNA-mediated documentation of diverse bacterial samples. Extracted and purified DNA is immobilized on a flow cell surface and prepared for Illumina sequencing by isothermal PCR that amplifies single DNA molecules. The DNA sequencing is then performed by repeated cycles of single-base extensions using four fluorescent reversible dye terminators (one for each nucleotide, Fig. 7).[321, 324]

Fluorescence is identified after base incorporation by a four-channel fluorescent scanner. Using Illumina, shorter sequence lengths than those from the 454 platforms are generated. This may create difficulties in identifying taxonomic affiliation down to genus or species level, and therefore, selection of primers targeting specific variable regions of 16S rRNA gene sequence is critical here. However, Illumina MiSeq platform supports sequencing of templates from both ends of a DNA fragment, known as paired-end sequencing, providing a wider sequence of the region of interest.[321, 324]

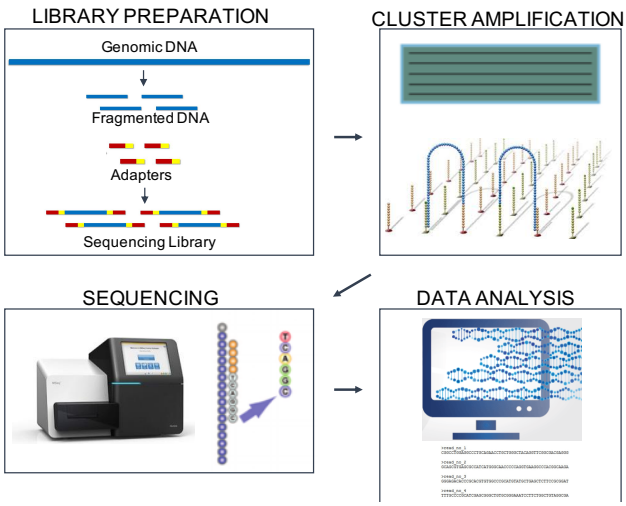

Fig. 7. Illumina sequencing overview. Illumina next generation sequencing includes four steps: 1. Library preparation - Random fragmentation of a genomic DNA (around 250-300 bases long fragments) is followed by 5´ to 3´ ligation of specific custom adapters to both fragment ends. 2. Cluster amplification – Sequencing library is loaded into a flowcell with the fragments attaching to the adapters in a random pattern. Each fragment is then amplified into a clonal cluster through bridge amplification. 3. Sequencing - Fluorescent labeled nucleotides (dNTPs), together with other sequencing reagents, are added to growing DNA copy strands. dNTPs are modified containing a terminator which blocks further polymerization. Moreover, only a single fluorescent color is used and therefore, each of the four bases must be added in a separate cycle of DNA synthesis and imaging. The emission wavelength and intensity from fluorescent labeled nucleotides is then used to identify the base incorporated. This process is repeated in order to create a read length of desired bases. 4. Data analysis - Reads are aligned to a reference sequence using bioinformatics software.[325]

Flow cytometry and fluorescence activated-bacterial sorting

Flow cytometry is a technology used for detection and identification of physical and chemical characteristics of cells or particles in a fluid as it passed through an interrogation point. Sheath fluid directs the cell suspension, causing cells to pass through a laser beam one cell at a time. The light that the particles absorb from a laser beam, scatters or emits light to all the angles due to its intrinsic and extrinsic physical properties. A detector, located in front of the laser beam, measures a forward scatter indicating the size of a particle. The light scattered at wide angles, known as side scatter (which indicates cellular granularity), is collected and directed to several mirrors and filters that enable the reflection of wanted light to a specific pass filter (red, orange, yellow, green light) and to finally be detected by a wavelength-specific photomultiplier tube sensor. The signal of the photons is then amplified, converted to voltage pulses, which are called events. The side scatter is proportional to the particle´s complexity and roughness (granularity).[326]

Fluorescence activated cell sorting (FACS) is a unique tool for separating the subpopulations of interest, by detecting their certain, targeted characteristics. Cell sorter functions basically as a traditional flow cytometer that has the option of separating cells. Briefly, when the cell sorter

detects the cell of interest, for instance bacteria coated with an antibody that have been marked with a fluorescent dye, an electric charge is given to the droplet of sheath fluid containing the specific cell. As the droplet passes through the electrostatic field, charged droplets are deflected appropriately and collected into a tube. Uncharged droplets, containing cells that do not possess the feature of interest (for instance bacteria that are not coated with an antibody), are collected into a separate tube or discarded into the waste tank (Fig. 8). This technique is extremely useful for the separation of subpopulations, for subsequent molecular analyses, including high throughput DNA sequencing.[326] The combination of 16S rDNA sequencing and flow cytometry-based sorting has enabled the identification of IgA-coated and non-coated fractions of the microbiota described in this thesis.

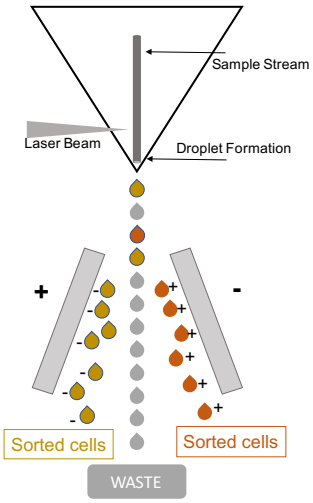

Fig. 8. Fluorescence activated cell sorting. A fluorescent-marked antibody specific for a particular cell surface protein is added to a mixture of cells prior to passing through a laser beam, where the complex is monitored. Droplets, containing single cells (events), are given a positive or a negative charge, depending on whether the cell is coated with fluorescently-tagged antibody or not. Droplets are then directed into collection tubes according to their charge.

Original study design and study subjects included in the papers of this thesis

The results of all the papers included in this thesis are based on a prospective, randomized, multi-center trial conducted at the Department of Pediatrics in university hospitals in South Eastern Sweden, between 2001 and 2005. 232 families with allergic disease (*i.e.* one or more family members with eczema, asthma, gastrointestinal allergy, allergic urticaria, or allergic rhinoconjunctivitis) were recruited to the study, where the potential allergy protective effect of the probiotic *Lactobacillus reuteri* ATCC 55730 was evaluated in infants until 2 and 7 years of age.[327, 328] The mothers started taking *L. reuteri,* or placebo, four weeks before term and continued daily until delivery. After birth, within 1-3 days, the infant continued with the same study product as the corresponding mother, daily up to 12 months of age. The mothers were encouraged to breastfeed. The families were advised to not use any other probiotic available on the market, during the study period. The infants were clinically followed by research nurses at 1, 3, 6, 12 and 24 months of age and telephone interviews with parents at 2, 4, 5, 8, 10 and 18 months. A final follow up was done by a pediatrician at two and seven years of age (Fig. 9). In total 188 infants completed the study until 2 years of age and 184 until 7 years of age.

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.[329] Among the 188 infants completing the original study, longitudinal salivary samples from 90 children were included in chapter I. In chapter III, 47 children that developed allergic symptoms and sensitization and 33 children that stayed healthy were included.

Stool samples were collected from the infants at age 5-7 days and at one month and 12 months of age. The samples were immediately frozen at -20°C following collection and later stored at -70°C. In the study presented in chapter II of this thesis, 28 healthy children and 20 children that developed allergic symptoms were included.

Breastmilk samples (approximately 10 mL) were collected within the first 3 days after delivery and at 1 month, in sterile plastic containers by the mother at home, immediately placed in the freezer, and brought to the hospital and stored at −70°C within 3 days.[330] In chapter IV of this thesis, we used breastmilk samples collected one-month *post partum* from 40 mothers whose children did or did not develop allergic and asthmatic symptoms during the first 7 years of age.

Lactobacillus reuteri preparation

The strain *L. reuteri* ATCC 55730, which was employed in the original study, was originally isolated from the breastmilk of a Peruvian mother (strain American Type Culture Collection 55730, BioGaia AB, Stockholm, Sweden). Safety and tolerance have been evaluated in healthy individuals (with up to 10^{11} bacteria/dose) [331] and in clinical trials with children.[332–336] Clinical studies have confirmed beneficial effects in acute diarrhea in children [333, 335], reduction of infections in a day-care setting [334], improved feeding tolerance in formula fed premature neonates [336], and or the management of infantile colic.[337]

The probiotic preparation consisted of freeze-dried *L. reuteri*, suspended in refined coconut oil and refined peanut oil, containing cryo-protective components. The daily intake, five oil droplets, corresponded to 10^8 colony forming units (CFU). The placebo consisted of the same oil without any bacteria and was not possible to differentiate from the active product by smell, taste or visual appearance.[327]

Diagnostic criteria of allergic diseases and asthma

Infants were regarded as sensitized if they had at least one positive skin prick test and/or detectable circulating IgE antibodies to allergens.[327, 328] 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 (Soluprick®, ALK, Hørsholm, Denmark) at 6, 12 and 24 months of and 7 years of age (here also mite (Der p)).[328] Histamine hydrochloride (10 mg/ml) was used as positive and albumin diluents as negative control. The test was regarded as positive if the mean diameter of the wheal was >3mm. Circulating IgE antibodies to egg white and cow's milk were analyzed at 6, 12, and 24 months of age in venous blood (UniCap® Pharmacia CAP SystemTM, Pharmacia Diagnostics, Uppsala, Sweden). The cut off level was 0.35 kU/L, according to the protocol of the manufacturer.

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. The diagnosis atopic eczema was defined upon visible pruritic, chronic or chronically relapsing non-infectious dermatitis symptoms with typical features and distribution. Also, it required that the infant was also sensitized.[265] Moreover, symptoms related to allergic disease, physical examination, spirometry and measurement of fractional exhaled nitric oxid (FE_{NO}) were observed. 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.[266, 327] All asthmatic children were also included in the allergic group analyses. For more details, please see [265, 266, 327–330].

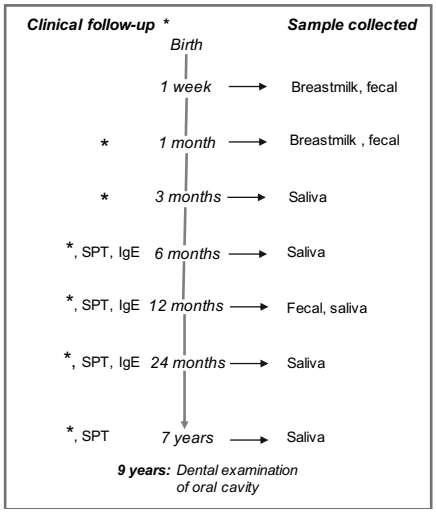

Fig. 9. Schematic overview of the original study. SPT: skin prick test; IgE: Measurements of circulating IgE antibodies, to various allergens, in venous blood.

RATIONALE OF THIS THESIS

In conclusion, the advent of new, advanced high-throughput sequencing technologies, together with its combination with flow cytometry techniques, now allow the study of the complex interactions between human antibodies and microbiota. IgA antibody is the primary mediator of humoral mucosal immunity and low secretory IgA levels in saliva and stool samples have been associated with increased risk for allergic manifestations during early life. However, 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 play in childhood allergy development. In the current thesis, we have studied the degree of microbial coating with IgA and patterns of IgA targeting of gut microbiota in children staying healthy or developing allergic and asthmatic disease. In addition to stool samples, we have also studied the much less explored oral microbiota development in the same cohort, identifying the patterns of bacterial succession associated with health and allergy, as well as the different postnatal factors influencing bacterial development. As an unhealthy oral microbiome can have important effects beyond the oral cavity, microbiological studies in longitudinal samples through childhood are essential in providing information about colonization patterns of oral commensals and their potential benign effect in preventing oral and systemic diseases. Breastfeeding, representing a passive immunization from mother to child, is an important factor in shaping microbial colonization of the infant. Therefore, IgA-coating towards breastmilk microbiota was also examined, with the hope that the integration of data from these different studies and sample types would shed light on the role of microbial exposure and initial colonization in preventing or promoting allergic disease development during childhood.

AIMS OF THE THESIS

The general aim of this thesis was to assess the microbial composition of oral, fecal and breastmilk samples, together with its interaction with IgA, in order to study the role of microbial development during early childhood in health and allergic disease.

The specific aims of each individual chapters were:

I To study the temporal evolution and maturation of the oral microbial ecosystem during infancy and childhood and its relation to delivery mode, breastfeeding habits, antibiotic use and dental caries status, in longitudinally collected oral samples in 90 children followed from birth to 7 years of age.

II 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 during childhood.

III 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.

IV To characterize the IgA-bound/non-bound breastmilk microbiota from mothers whose children develop, or not, allergic symptoms during early childhood.

Chapter I - ORAL MICROBIOME DEVELOPMENT DURING CHILDHOOD: AN ECOLOGICAL SUCCESSION INFLUENCED BY POSTNATAL FACTORS AND ASSOCIATED WITH TOOTH DECAY

- Majda Dzidic, Maria Carmen Collado, Thomas R. Abrahamsson, Alejandro Artacho, Malin Stensson, Maria C. Jenmalm, Alex Mira; Oral microbiome development during childhood: an ecological succession influenced by postnatal factors and associated with tooth decay; © *The ISME Journal*, Jun 2018, 12:2292–2306.

Abstract

Information on how the oral microbiome develops during early childhood and how external factors influence this ecological process is scarce. We used high-throughput sequencing to characterize bacterial composition in saliva samples collected at 3, 6, 12, 24 months and 7 years of age in 90 longitudinally followed children, for whom clinical, dietary and health data were collected. Bacterial composition patterns changed through time, starting with "early colonizers", including *Streptococcus* and *Veillonella*; other bacterial genera such as *Neisseria* settled after 1 or 2 years of age. Dental caries development was associated with diverging microbial composition through time. *Streptococcus cristatus* appeared to be associated with increased risk of developing tooth decay and its role as potential biomarker of the disease should be studied with species-specific probes. Infants born by C-section had initially skewed bacterial content compared with vaginally delivered infants, but this was recovered with age. Shorter breastfeeding habits and antibiotic treatment during the first 2 years of age were associated with a distinct bacterial composition at later age. The findings presented describe oral microbiota development as an ecological succession where altered colonization pattern during the first year of life may have long-term consequences for child´s oral and systemic health.

Introduction

The development and structure of the neonatal microbiome have been partially elucidated, with a main focus on the microbial population inhabiting the lower intestinal tract, while information about the oral cavity colonization following delivery is still limited.[48] As yet, no published longitudinal studies have characterized oral microbiota development during infancy and childhood with culture independent next generation sequencing methodologies, particularly in association with tooth decay. It is believed that by production and excretion of metabolic products of pioneer colonizers (including facultative anaerobes *Streptococcus* and *Actinomyces*), acquired at birth and the following hours, the environment can be altered, thus benefiting and selecting the growth of other species (including more strictly anaerobic genera like *Veillonella* and *Fusobacteria*).[48, 49] As the baby grows, microbial communities evolve and increase in microbial diversity.[50, 51] During this period the oral microbiota is characterized by high variability and current knowledge indicates that it reaches adult-like stability around two years of age.[48]

Most evidence available today shows that the early oral environment is strongly shaped by the mother [49, 338, 339] and maternal oral microbiota has been proposed to colonize the placenta [33] where it could influence fetal immune tolerance towards the mother´s microbiome.[340] Further transition into a more mature and complex microbial ecosystem is mainly influenced by the external environment as well as vertical transmission from the parents.[49, 339, 341, 342] An essential question is to identify which factors and at what time point they can influence the progression of microbial colonization. Previous studies of the lower gastrointestinal tract microbiota have reported that the gut microbiota of infants delivered by caesarean section (C-section) was mainly colonized by skin bacteria, had lower numbers of *Bifidobacterium* and *Bacteroides* species and were more often colonized with *Clostridium difficile* in comparison to vaginally born infants.[172, 343] However, research regarding the influence of delivery mode on the early oral microbiota development, by using next generation sequencing on longitudinal samples, has not yet been reported.

Breastmilk has long been considered a superior food for infants, increasing resistance to infections, providing nutrition and being a source of bacteria (10^6 bacterial cells/ml), who serve as inoculum for the newborn.[18, 19, 73, 85] The genus *Streptococcus* is one of the dominant bacterial groups found in human milk [19, 73] and various species, including *Streptococcus salivarius,* are frequently found in the infant oral cavity.[344] The metabolic products derived from *Streptococcus* species from the dietary oligosaccharides in breastmilk might pave the way for the establishment of other microorganisms in the oral cavity, thus influencing attachment and

growth of selected bacteria.[48, 49, 345–348] However, the longitudinal impact of these initial colonizers on the oral ecosystem and the influence of breastfeeding habits on children's oral and systemic health are widely unknown and deserve to be investigated.

Knowledge about the effect of other external factors like antibiotic use, especially at an early age, on subsequent microbiome development is also scarce. In children, long-term alterations of the gut microbiome as a consequence of early antibiotic administration have been described and proposed to have negative effects for systemic health, including obesity and allergy.[349, 350] However, the long-term effect of antibiotic use for children's oral microbiota is currently unknown.

An important consequence of oral microbiome development for health is the protection against tooth decay (dental caries), considered among the most prevalent diseases worldwide.[351] Tooth decay is caused by an interaction between acidogenic bacteria, a carbohydrate substrate and host susceptibility, leading to bacterial dysbiosis and demineralization of tooth tissue.[51, 352] The acid-tolerant bacterial species *Streptococcus mutans* is recognized to be an important pathogen in dental caries, [353, 354] and its early presence in edentulous children (from 3 months of age), is suggesting that the soft tissue may play a role as a reservoir for oral pathogenic microorganisms.[50, 355] Given that early colonization with cariogenic microorganisms has been associated with higher caries incidence [353], microbiological studies in longitudinal samples through early childhood may reveal those bacteria increasing caries risk that could be used as early diagnostic biomarkers. This could also provide important information for active and passive immunization strategies against oral diseases.[356] Moreover, an unhealthy oral microbiome can have important effects beyond the oral cavity, including elevated cardiovascular risk.[357, 358] For instance, *in vitro* studies have demonstrated the ability of periodontal bacteria to increase the probability of thrombus formation, which could lead to ischemic cardiovascular events.[359, 360] Therefore, it is of interest to understand the colonization patterns of oral commensals during childhood and the potential benign effect of oral bacteria in preventing oral and systemic diseases, including microorganisms which have been associated with health conditions.[361, 362]

A more detailed understanding of oral microbial communities development in health and disease fundamental and the use of high-throughput sequencing techniques now allow exploring microbial composition and diversity in low volume oral samples to an unprecedented level of detail [363], in comparison with culturing or early molecular methodologies. In this study, we aimed to address the temporal evolution and maturation of the oral microbial ecosystem during infancy and childhood and its relation to delivery mode, breastfeeding habits, antibiotic use and

dental caries status, in longitudinally collected oral samples in 90 children followed from birth to seven years of age.

Methods

Sample collection and study design

The infants included in the study were part of a larger randomized double-blind trial in Sweden between 2001 and 2003 evaluating the potential allergy prevention effect of probiotic *Lactobacillus reuteri* ATCC 55730 until 2 and 7 years of age.[327, 328] Among the 188 infants completing the original study, longitudinal salivary samples were collected in 90 children. The participants were instructed not to eat or drink for two hours preceding the sampling. Non-stimulated saliva samples at 3, 6, 12 and 24 months of age 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 −80°C. At 7 years of age, paraffin-stimulated whole saliva was collected (≈3 ml) in a sterile test tube and immediately frozen at −80°C. By 9 years of age, the children were examined at public dental clinics at which the children received their regular dental care [329], and the caries status was evaluated. The oral examination included radiographs and the registration of manifest and initial caries lesions in the primary dentition according to Koch *et al.* and Alm *et al.*[364, 365]

Possible confounders, such as mode of delivery, breastfeeding habits (exclusive or partial breast feeding), infant health and antibiotics use during the first two years of age were obtained from medical records and semi-structured questionnaires (see I-Table 1).[329] 91% and 80% of all children included were exclusively breast-fed up to 1 and 3 months of age, respectively, while 97% were partially breastfed at 3 months of age. No infant received antibiotics before 1 month of age while 2% took antibiotics during the first 3 months of life. 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. Written informed consent was also given by the parents or guardians before the dental examination.

DNA extraction

250 ul of each saliva sample were centrifuged at 15000 g for 30 min and the pellet, together with 50 ul of the supernatant, was used for further analysis. DNA was isolated by MagNA Pure LC 2.0 Instrument (1996-2016 Roche Diagnostics, Barcelona, Spain), using MagNA Pure LC DNA Isolation Kit III for Bacteria and 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 rRNA gene amplification and sequencing

Prior to sequencing of 16S rRNA gene, extracted DNA was pre-amplified 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.[366] 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 rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part #15044223 Rev. A). The gene-specific primer sequences used in this protocol were selected from Klindworth *et al.*[367], with forward 5' primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse 5´primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC, targeting the 16S rRNA 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 rRNA gene amplification, the DNA was sequenced on a MiSeq Sequencer according to manufacturer's instructions (Illumina) using the 2x300 bp paired-end protocol. Sequences supporting the conclusions of this article are publicly available at the European Nucleotide Archive (ENA) database with the accession number PRJEB66628.

Bacterial load and *Streptococcus dentisani* measurements with quantitative PCR

Total bacterial load (number of bacterial cells per ml of saliva) and the presence of *Streptococcus dentisani* in saliva samples were 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 and 65°C for total bacterial load and *S. dentisani*, respectively. Each reaction mixture of 10 mL was composed of SYBR Green PCR Master Mix (Roche), 0.5 mL of the specific primer (concentration 10 mmol/L), and 2 mL of DNA template. For *S. dentisani* the forward primer was 5´GTA ACC AAC CGC CCA GAA GG 3´ and the reverse primer 5´CCG CTT TCG GAC TCG ATC A 3´ (Integrated DNA Technologies (IDT); San Diego, California,

USA) targeting the carbamate kinase gene, and for total bacterial density measurements the universal forward and reverse primers were 5´GTG CCA GCM GCC GCG GTA A 3´ and 5´GCG TGG ACT ACC AGG GTA TCT 3´ (IDT), respectively, targeting the bacterial 16S rRNA gene. The obtained Ct values were transformed in bacterial cell numbers by a standard curve calibrated by flow cytometry.[19]

Bioinformatics and statistics

Only overlapping paired end reads were used for analysis. A sequence quality assessment was carried out using the PRINSEQ program.[368] Sequences of <250 nucleotides in length were not considered; sequence end-trimming was performed by cutting out nucleotides with a mean quality of <30 in 20-bp windows. Chimeric 16S sequences were filtered out using USEARCH program.[369]

Obtained sequences were taxonomically classified by the RDP-classifier [370] where reads were assigned a phylum, class, family and genus and phylogenetic ranks were allocated when scores exceeded an 0.8 confidence threshold. Operational taxonomic units (OTUs) were generated by using CD-HIT OTU picking with 97% of similarity.[371] Human oral microbiome database (HOMID) was used as a reference database for OTU assignment.[372] For the *Streptococci*-species analyses, sequences were clustered into operational taxonomic units (OTUs) at 100% similarity by BLAST analysis [373] and >350 bp alignment length, against the RDP database.[374] A few species appeared to be identical in the sequenced region, namely *Streptococcus infantis*, *S. mitis* and *S. dentisani*, and could not be distinguished from each other.

α–diversity analyses (presented here as Shannon and Chao1 indices), were utilized to estimate samples' diversity and richness at the 97% OTU level using the R-package Vegan.[375] 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 [376] using the function *CCA*, which is based on Chi-squared distances. Adonis tests were done with the R library 'vegan'.[375] It performs a permutational multivariate analysis of variance using distance matrices and fitting linear models to them. The test allows modelling the whole compositional variability at once by taking into account different sources of variation as well as interactions between them as it is defined in a linear model.

Linear discriminant analysis effect size (LEfSe), a method for biomarker discovery on the online interface Galaxy (http://huttenhower.sph.harvard.edu) [377], was used to detect the taxa, at

both genus and OTU level, characterizing the populations of caries-free and caries active children.

Statistical analyses were performed in R version 3.2.2 and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA, Version 6.1f), where $p<0.05$ was considered significant. Specific statistical tests (including Mann-Whitney U-test for nonparametric comparisons) are stated in figure legends. When comparing the frequencies of different bacterial taxa between groups (e.g. caries-free and caries-experienced children), the balanced proportions of confounding factors, including breastfeeding length, mode of delivery and antibiotic intake, were checked by Chi-square test, and non-significant differences between the groups were found.

Table I-1. The characteristics of children included in this study.

Children	Prevalence (%)
Sex (% Girls)	43/90 (48 %)
Delivery mode (% caesarean section)	13/90 (14 %)
Breastfeeding (% breastfed, not exclusively)	
3 months	87/90 (97 %)
6 months	74/90 (82 %)
12 months	23/90 (26 %)
Antibiotics use (%)	
First year	27/90 (30 %)
Second year	40/90 (44 %)
Probiotics use (% *L.reuteri*)	40/90 (44 %)
Allergic manifestations at 7 yrs (%)	42/80 (52 %)
Caries status at 9 yrs (% Caries)	33/79 (42%)

Findings and Discussion

After quality filtering, 34,794,056 sequences were obtained, with an average of 93,532 ± 3,480 (SEM) sequences per sample.

Bacterial load, richness and diversity through time

Bacterial diversity and richness increased through time, reaching nearly 550 OTUs at 7 years of age with a Shannon diversity index of approximately 2.4 (Fig. I-1). The delivery mode and partial breastfeeding habits until 12 months of age did not have an impact on species richness (Fig. I-1A-B). However, bacterial diversity appeared to be higher in C-section delivered infants at 12 months of age (Fig. I-1A) and at two years of age in children not being breastfed through 12 months of age (Fig. I-1B).

Oral development, including the emergence of teeth, was accompanied by a steady increase in diversity and richness of the oral microbiome in this study, especially between 1 and 2 years of age. Interestingly, bacterial diversity at 2 years of age (Fig. I-1B), appears to be higher in children which abandoned breastfeeding before 12 months of age. Although this has not been studied before in oral microbiota, a similar trend was observed in gut microbiota analyses where children not being breastfed had higher microbial diversity [77, 163, 378], probably due to earlier introduction of solid food. Our results agree with a scenario in which following delivery, the oral cavity gets exposed to the environment, triggering the initiation of microbial colonization through diet, vertical transmission from parents and horizontal transmission from caregivers and siblings, thus increasing the bacterial diversity.[52, 379]

In order to determine the development of bacterial density through infancy, we measured total bacterial load (Fig. I-S1) in saliva samples. Although there were no differences regarding delivery mode (Fig. I-S1A) and breastfeeding habits (Fig. I-S1B), the density of bacteria increased significantly with age, probably reflecting the influence of environmental interactions and the emergence of teeth. Interestingly, bacterial density at each time point appeared to fall within two groups (low or high), and this bimodal distribution was maintained through time for each individual. This pattern could not be attributed to caries status, allergy development, mode of delivery, feeding habits, antibiotics intake or probiotic administration (data not shown). In the future, it would be interesting to determine whether the physicochemical properties of saliva may influence cell density.

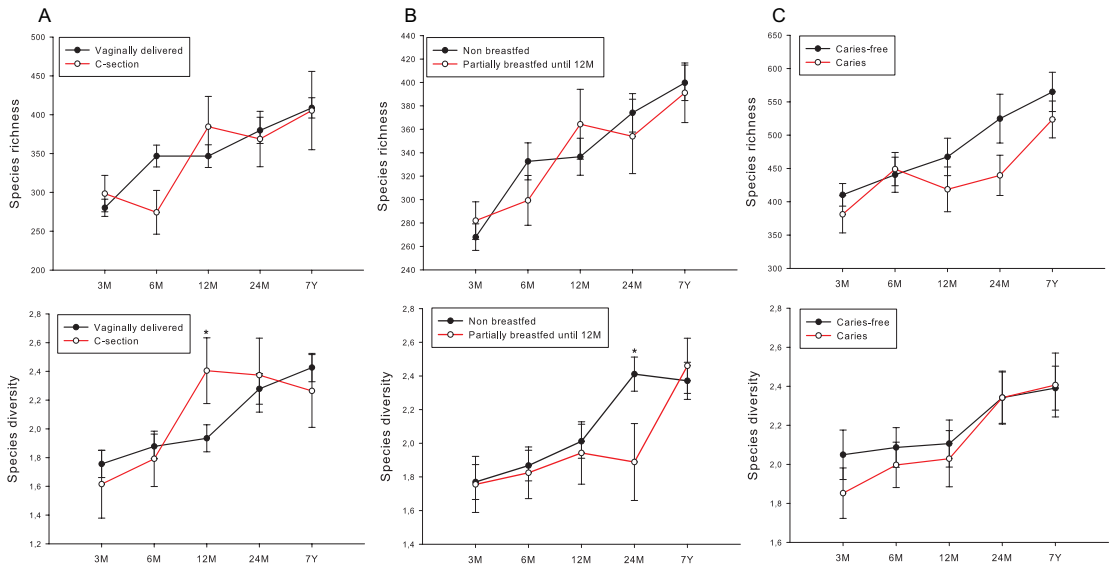

Fig. I-1. Species richness and diversity of total microbiota in infant saliva samples. **(A)** shows species richness and diversity in infants delivered vaginally (VD) or by caesarean section (C-section) at 3 months (N_{VD}=62; N_{CS}=11), 6 months (N_{VD}=72; N_{CS}=11), 12 months (N_{VD}=59; N_{CS}=10), 24 months (N_{VD}=56; N_{CS} =10) and 7 years of age (N_{VD}=68; N_{CS}=12). **(B)** represents species diversity and richness in infants breastfed for 12 months (BF) and in infants breastfed for less than 6 months of age (nBF). Analyzed samples were collected at 12 months (N_{nBF}=52; N_{BF}=17), 24 months (N_{nBF}=50; N_{BF}=16), and 7 years of age (N_{nBF}=59; N_{BF}=21). **(C)** shows species richness and diversity in children developing caries (CA) and children staying caries-free (CF) during the first 9 years of life. Saliva samples were collected at 3 months (N_{CF}=40; N_{CA}=26), 6 months (N_{CF}=43; N_{CA}=31), 12 months (N_{CF}=37; NCA=26), 24 months (N_{CF} = 35; N_{CA} = 24) and 7 years of age (N_{CF}= 45; N_{CA}=30). Data are presented with means and standard errors. *p < 0.05; Mann–Whitney U-test.

Bacterial composition during infancy

When bacterial composition was analyzed for all samples through child development, clear changes emerged through time (Fig. I-S2). Streptococci dominated salivary samples at all times. They were particularly high in proportion during the first months of age, and their decrease was accompanied by a rise in other genera. These general patterns were influenced by several perinatal and postnatal factors.

The influence of delivery mode and breastfeeding durations

Bacterial species composition development was influenced by delivery mode and breastfeeding habits (Fig. I-2A, B), but not by *L. reuteri* supplementation during the first year of age (data not shown). The impact of delivery mode was reflected in differences in bacterial composition at 3 and 6 months of age (Fig. I-2A, p=0.001, CCA analysis), followed by convergent microbial patterns at later age. Only the genus *Haemophilus* was found to be significantly more abundant (p=0.047) at 7 years of age in children delivered by C-section (Fig. I-S3). Thus, with the exception of this genus, no further colonizers were found to be significantly different between vaginally delivered and C-section delivered infants (Fig. I-S3). This could be due to infant

delivery mode affecting the direct transmission of initial bacteria from mother to newborn, having a short-term effect. This finding is in line with previous studies [55], where the Human Oral Microbe Identification Microarray was used, showing that microbial oral colonization in three-month-old infants delivered vaginally and those delivered by C-section was different. Similar findings of an early impact, but also more long-term effects [343, 380], have been reported for the microbiota of the lower gastrointestinal tract.[171, 172] When a multivariate analysis was performed including time, breastfeeding length and caries status as confounding factors, the effect of delivery mode on microbiota composition was no longer significant. Given that a significant breastfeeding length-delivery mode interaction was detected (p=0.026), part of the observed differences between children born by vaginal delivery and C-section can be due to the effect of breastfeeding.

The influence of partial compared to no breastfeeding until 12 months of age did appear to have a long-term effect, as evidenced by a divergent oral bacterial composition at 24 months and 7 years of age (Fig. I-2B, p=0.002) while bacterial colonization at early age appeared to be similar. This could be due to the fact that the majority of the infants in this cohort were breastfed during their first months of life (see Table I-1). A multivariate analysis revealed that the significant effect of breastfeeding on microbiota composition was maintained even after removing the effect of caries status, time and mode of delivery as confounding factors (p=0.036). Further work should therefore address the impact of formula feeding on microbiome development as findings presented here suggest that variations in the initial oral microbial communities may result in differences in the bacterial succession patterns that persist over time, analogous to the impact of early disturbance in ecological successions.[381]

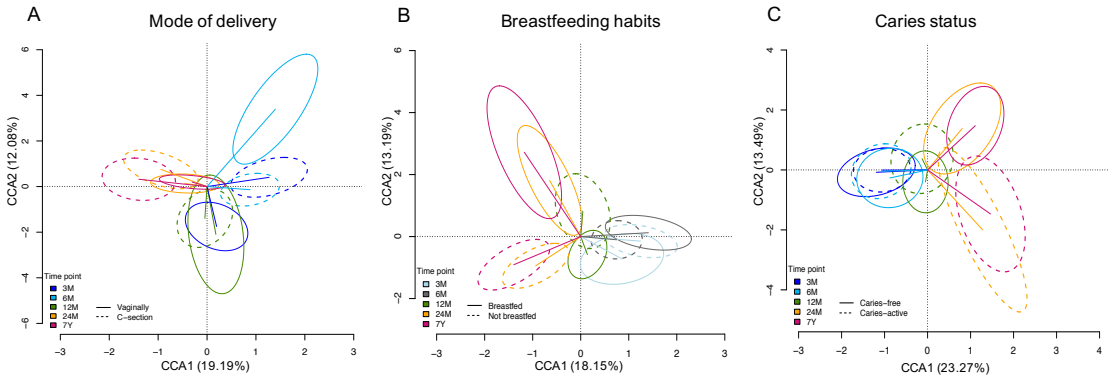

Fig. I-2. Salivary microbiota patterns through children's development. **(A)** Microbial pattern differences in saliva from infants delivered vaginally (VD) or by caesarean section (CS), p = 0.0016, at 3 months (N_{VD}=62; N_{CS}=11), 6 months (N_{VD}=72; N_{CS} =11), 12 months (N_{VD} =59; N_{CS}=10), 24 months (N_{VD}=56; N_{CS}=10) and 7 years of age (N_{VD} =68; N_{CS}=12). **(B)** Showing microbial composition pattern differences in infants who were breastfed for 12 months (BF) and in infants breastfed (nBF) for less than 6 months (p = 0.0017). The numbers of children were: 12 months (N_{nBF}= 52; N_{BF} = 17), 24 months (N_{nBF} = 50; N_{BF} = 16)

and 7 years of age (N_{nBF} =59; N_{BF}=21). **(C)** Microbial composition patterns in children developing caries (CA) and children staying caries-free (CF) during the first 9 years of life (p = 0.0018). Saliva samples were collected at 3 months (N_{CF}=40; N_{CA}=26), 6 months (N_{CF}=43; N_{CA}=31), 12 months (N_{CF}=37; N_{CA}=26), 24 months (N_{CF}=35; N_{CA}=24) and 7 years of age (N_{CF}=45; N_{CA}=30). Numbers accompanying the variables (delivery mode, breastfeeding and caries onset) are representing the time points plotted. p-Values for CCA plots were determined by Adonis analysis (a nonparametric statistical method, R package Vegan) and significant values indicate that the factor provided can explain part of the total variability.

Microbial colonization patterns

Dominant bacterial genera (present at >1%) which inhabited the oral cavity during the first 3-6 months, here called "Early colonizers", included *Streptococcus*, *Veillonella* and *Lactobacillus spp.* (Fig. I-3A). The most frequent bacterium of the oral cavity in the current study was *Streptococcus,* and children being breastfed until 12 months of age appeared to have higher abundance of this genus at one year of age (p=0.005). This finding is consistent with other reports [50, 382] and *Streptococcus* has been found to be one of the dominant bacterial groups in human breastmilk.[19, 85] Aging of the children was associated with lower levels of *Streptococcus*, although the decrease tended to be more notable in children abandoning breastfeeding before 12 months of age. This indicated that settlement of this genus is favored by breastmilk, either by direct transmission or by an appropriate nutrient supply.[19, 21] Moreover, this pioneer is often found in the oral cavity of the neonate because of its ability to adhere to and colonize the mucosal surface lining.[49] The metabolic products (such as lactic acid) derived from *Streptococcus* species from the dietary oligosaccharides in breastmilk might pave the way for the establishment of other microorganisms in the oral cavity, including bacterial genera like *Veillonella*.[48, 347] *Veillonella*, here ranging between 2 to 8% of total abundance with significantly higher levels at 7 years in children keeping breastfeeding until 12 months of age (p=0.037), is another bacterial genus commonly encountered in breastmilk [74, 91]. This genus requires organic acids as carbon source and therefore its presence is likely favored by the high levels of lactate derived from lactose fermentation, which this genus will transform to propionate and acetate.[383] An important lactose fermenter is obviously *Lactobacillus*, which in the oral cavity might be acquired by the neonate during vaginal delivery, as this genus is highly abundant in vaginal microbiota, [384] but also through breastfeeding since breastmilk has been proposed to favor the growth of vaginally acquired bacteria.[88, 171, 383] In the current study, no differences in *Lactobacillus* abundance were found between children being breastfed up to one year of age or not (Fig. I-3A) and neither between vaginally delivered and C-section infants (Fig. I-S3). Among the components of human breastmilk, oligosaccharides are thought to directly influence the gut microbial composition and to enrich bacterial functions associated to carbohydrate consumption and biosynthesis of amino acids and vitamins [163, 385] and a similar process may be taking place in the oral cavity. Early commensals of the oral cavity are likely having an ecological advantage over those arriving later

and may promote the change of the environment through the production and excretion of products of their metabolism, thus benefitting the growth of further oral bacterial communities. This process of microbial succession and increasing diversity, promoted by breastfeeding, could lead to subsequent formation of complex and steadier microbial communities, as proposed for gut microbiota.[40]

Bacterial genera *Gemella*, *Granulicatella, Haemophilus* and *Rothia*, here defined as "constant colonizers" (Fig. I-3B), were present already at 3 and 6 months of age with >1% of abundance, and their abundance increased with time. *Gemella* and *Granulicatella* are considered as common dental plaque inhabitants [386] and were found to increase in abundance through age, ranging from 5-10% and 2-8%, respectively. It is likely that the initiation of teeth eruption, starting around 6-8 months postnatally, creates new ecological niches in the oral cavity, giving rise to new adhesion surfaces, thus favoring their further colonization.

A third set of microorganisms were "late colonizers" and included *Actinomyces*, *Porphyromonas*, *Abiotrophia* and *Neisseria*, which became dominant in the oral cavity at a later stage, approximately after the first year of life (Fig. I-3C). Thus, the data suggest that the acquisition or dominance of each bacteria may occur optimally only at certain ages. Breastfeeding until 12 months of age was associated with significantly lower levels of *Actinomyces* ($p=0.044$) at 7 years of age and *Porphyromonas* ($p=0.049$) and *Neisseria* ($p=0.028$) at 12 months and 24 months of age, respectively. *Porphyromonas*, more specifically *Porphyromonas gingivalis,* is a gram-negative oral anaerobe involved in the pathogenesis of periodontitis, an inflammatory disease that destroys the tooth tissue and may lead to tooth loss.[387] The results are indicating that children being breastfed by 12 months of age, as compared with children no longer breastfed, have significantly lower abundance of this genus at one year of age. However, species-level taxonomic analysis revealed that 100% of *Porphyromonas* sequences correspond to *Porphyromonas catoniae* during the first 12 months of age. At 2 years, *P. gingivalis* appeared at 9% of the total, whereas *P. catoniae* accounted for 91% of the sequence reads. At 7 years of age, the proportions were 86.5% for *P. catoniae* and 13.4% *P. gingivalis*. Thus, an association between reduced breastfeeding length and risk of gum disease is uncertain. *Neisseria*, a common bacterial community member of the healthy human mouth [388], was found to be more abundant in children not being breastfed until 12 months of age, in line with previous research where species belonging to this genus were found more frequently in children being formula fed.[54] Thus, breastmilk had a long-term effect on oral microbiota composition, but this altered microbiota could not always be linked to healthy or disease-associated communities, and further work should study the long-term consequences for the child's oral and systemic health. Beside the potential health effect, the results presented here are suggesting that the transmission of bacteria from breastmilk and the nutrients supplied by it at

this critical time point in infant´s development, could affect the colonization window of specific bacterial genera, and depending on delivery mode and breastfeeding duration, this may lead to disturbances in the oral microbial succession patterns that persist over time.

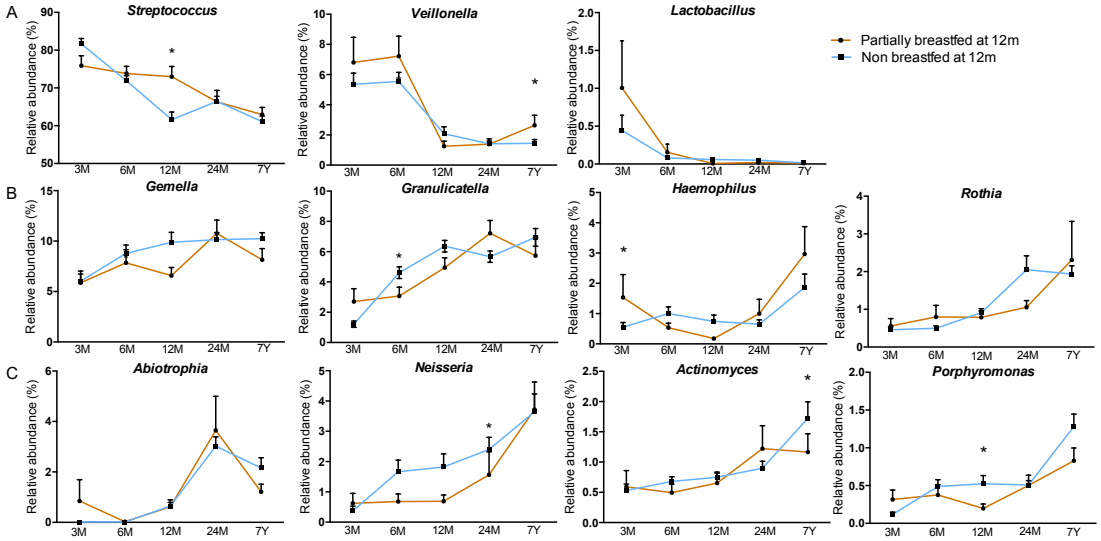

Fig. I-3. Microbiota composition of dominant bacterial genera in children being or not being breastfed until 12 months of age. **(A)** Genera classified as early colonizers. **(B)** Genera considered to "constantly increase" are already present at 3–6 months of age at >1% frequency, and are increasing in relative proportion with time. **(C)** Bacterial genera considered as "Late colonizers", are defined as those present at 3–6 months of age below 1% relative abundance, which undergo significant increase after 12 months of age. Plots are showing the relative abundance of dominant bacterial genera, as determined by Illumina sequencing of 16S rRNA gene, in saliva samples collected at 3 months, 6 months, 12 months (N_{nBF}=52; N_{BF} =17), 24 months (N_{nBF} =50; N_{BF}=16) and 7 years (N_{nBF}=59; N_{BF}=21) of age. *p< 0.05 by both Mann–Whitney U-test and Wilcoxon analysis.

The effect of antibiotics intake on microbiota development

The clinical data of this cohort allowed us to assess the influence of antibiotics intake in early life (first and second year) on developing microbiota. The antibiotics courses given were mainly due to early otitis media (in 89% of cases) and included Amoxicillin (34 % of cases) and Phenoxymethylpenicillin (42 % of cases) (Table I-SI). Upon comparing the microbial succession in children who did or did not take antibiotics during the first two years of life, significantly divergent colonization was observed at 24 months and 7 years of age, whereas bacterial composition at earlier time points were overlapping in children treated with antibiotics (Fig. I-4A). Multivariate analyses were also performed, considering the effect of time and different confounding factors on microbiota composition. Antibiotic use had a significant effect on microbiota composition once the effects of caries status and time were removed (p=0.05) and a significant antibiotic by time interaction was found (p=0.008). There was a lower effect of antibiotics on microbiota composition (p=0.067) once breastfeeding length was included in the

analysis, suggesting that part of the significance is due to the strong effect of breastfeeding on microbiota composition.

By comparing the most dominant genera (>1% of total microbiota) present in these two groups, the genus *Granulicatella* was higher in abundance at 24 months of age (p=0.003) in children not taking antibiotics while *Prevotella* (p=0.020) was more prevalent at 7 years of age in children treated with antibiotics in early life. The data suggest that the abundance of commensal genera such as *Granulicatella* [386] may be disturbed by antibiotics use while the presence of other genera, like *Prevotella*, which has been associated with several oral diseases [389], may be favored.

In order to obtain deeper insight of microbiota alterations upon antibiotics intake, the microbial composition was assessed at species-level OTUs (Fig. I-4B). The analysis revealed a high number of bacteria uniquely present in children that were treated with antibiotics more than once during the first two years of life including several *Actinomyces* species at 2 and 7 years of age. Moreover, the presence of species belonging to *Fusobacterium*, *Veillonella* and *Lactobacillus* was also associated with antibiotics intake during the first two years of life in our cohort. The fact that *Veillonella* spp use organic acids as their only carbon source strongly suggests that the oral microbiota of those children is more acidogenic. On the contrary, *Neisseria* and *Streptococcus mitis/dentisani*, were present in our samples at significantly higher levels in 7-year old children that did not take antibiotics. Thus, although a divergent microbiota does not necessarily imply a negative effect for health, most significant changes in microbial composition detected in the current study as a consequence of antibiotic administration, have previously been associated with oral diseases [361, 363, 390–394] and future studies will need to specifically address whether antibiotic use during infanthood has an effect on oral health.

It is of course possible that the divergent microbial succession patterns observed at 7 years of age might be affected by further antibiotics courses and other influencing factors, occurring during the remaining five years. However, given that the first years of age appear to represent a crucial period of microbiota development and immune system modulation and that early changes in ecological successions are those with the largest impact on community development [381], it is important to consider that early antibiotic treatment can have long-term consequences for microbiota development. It has to be emphasized that in adults, the original salivary microbial composition appears to be restored after antibiotic use [395], suggesting resilience of the oral microbiome; in children, long-term alterations of the gut microbiome, as a consequence of early antibiotic administration, have been proposed to have negative effects for systemic health, including obesity and allergy.[349, 350] Thus, the impact of early intake of antibiotics for human health deserves consideration.

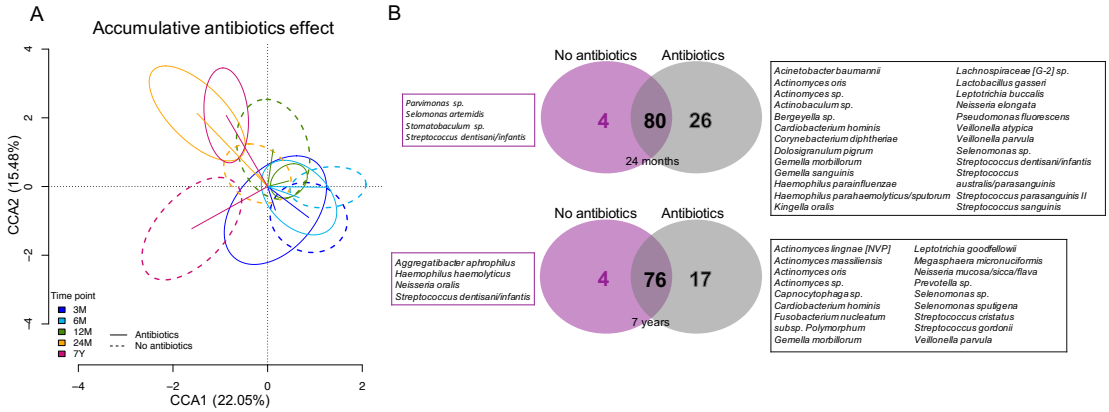

Fig. I-4. Accumulative antibiotics effect on salivary microbiota development. **(A)** shows salivary microbiota patterns in children treated with antibiotics during the first 2 years of life and children not taking any antibiotics during the first 2 years of life; p = 0.017. p values for CCA plots were determined by Adonis analysis (a nonparametric statistical method, R package Vegan) and indicate that the factor provided can explain part of the total variability. **(B)** represents the influence of antibiotics intake on bacterial species distribution at 24 months and 7 years of age, here represented with a Venn's diagram. The numbers indicate unique species found in children not treated with antibiotics (purple) and children treated with antibiotics (grey) and OTUs differentially distributed are stated in the tables. OTUs presented were filtered according to sequence length (>300 bp) and identity (>97%nucleotide similarity). Analyzed sample sizes were: 3 months (N_{NO}=28; N_{YES}=11), 6 months (N_{NO}=33; N_{YES}=13), 12 months (N_{NO}=29; N_{YES}=11), 24 months (N_{NO}=26; N_{YES}=10) and 7 years (N_{NO} =35; N_{YES}=11); NO = no antibiotics intake for the first 2 years of age; YES = antibiotic consumption during the first 2 years of age.

Oral microbiota in health and disease

Caries development did not appear to be related with bacterial diversity (Fig. I-1C) or bacterial load (Fig. I-S1C) during the first 7 years of life. Although there were no differences between children staying caries-free and children developing caries at age 9, the density of bacteria was increasing significantly with age, probably reflecting the influence of environmental interactions and the emergence of teeth. The overall species richness was higher in children that remained caries-free by 9 years of age, but the difference was not significant (Fig. I-1C). However, the potential association of lower bacterial diversity to caries risk should be further studied, as a lower bacterial diversity has been associated to caries in cross sectional studies.[396] A factor reducing the possible association of caries status to diversity could be the use of saliva samples, which provide a good representation of overall oral microbial diversity but may not fully correlate with bacterial composition at the tooth biofilm, where the disease takes place.[397]

Caries development at 9 years of age was preceded by divergent bacterial composition at 24 months of age, reaching the maximum at 7 years. (Fig. I-2C). At early age, no differences between caries-experienced and caries-free children were detected, suggesting that the

colonization patterns and ecological factors favoring caries development are associated with later age. A critical period may include the eruption of primary incisors, primary molars and permanent first molars, where cariogenic bacteria like *Mutans streptococci* can adhere through glucan binding proteins.[398] Although these caries-linked species are considered associated to hard-tissues, there are studies suggesting that they can be acquired at any time from under 6 months (prior to first tooth eruption) to over 3 years of age.[399, 400] Taken together, the data here suggest that different bacterial colonization patterns were present between caries-free children and children that developed caries, however they were significant only after the second year of age.

Bacterial composition and caries development

Since no significant differences were observed between caries-free and caries-active children at the genus taxonomic level (Fig. I-S4) and given that the genus *Streptococcus* was highly abundant in the infants' oral cavity, it was of great interest to investigate if there were any specific Streptococci species associated with caries development in the cohort. The genus *Streptococcus* comprises a large number of species that can have positive effects on human health and some of them have started to be used as probiotics in oral diseases.[401] The OTUs found corresponded to *S. mitis/infantis/dentisani* (identical in the sequenced 16S rRNA region), *S. salivarius*, *S. sanguinis*, *S. lactarius*, *S. cristatus* and *S. mutans* (Fig. I-5). *S. mitis/infantis/dentisani* were the most prevalent OTUs (ranging from 75-85%) and no difference was found between the children who did or did not develop caries at 9 years of age. *S. infantis* belongs to the *Streptococcus mitis* group [402] and has been associated with oral health as it significantly decreases during caries progression in the young permanent dentition.[403] *S. dentisani* is a bacterial species associated with good oral health and it has been isolated from caries-free individuals.[361] Because of the high sequence similarity within the *Streptococcus* genus PCR-amplified region used for Illumina sequencing, we could not distinguish which 16S rRNA reads belonged to *S. mitis, S. infantis* or *S. dentisani*. To clarify this, qPCR amplification with *S. dentisani*-specific primers was performed in order to determine the acquisition of this species through age. The quantities of *S. dentisani* were undetectable by qPCR during the first year of age, suggesting that the colonization of this species might be dependent of teeth eruption. This is in agreement with its normal association with dental plaque.[361] The levels of *S. dentisani* were higher in children remaining caries-free at 9 years of age in comparison with caries-experienced children, but the difference was not significant (Fig. I-S5).

Streptococcus salivarius was another commonly found species in children's saliva (Fig. I-5). Its abundance was highest at 3 months of age, ranging between 10-15% of the total streptococcal species, and decreasing steadily through time, likely opposing teeth eruption. This pioneer colonizer and a prominent member of the oral microbiota of the healthy mouth has been

detected hours after birth because of its unique ability to adhere and colonize tongue and cheek mucosa.[52] Although *S. salivarius* has been intended for use as a probiotic targeting the oral cavity [404], no differences in abundance levels of this species through age were discovered between children who did or did not develop caries at 9 years of age, perhaps due to its absence from dental plaque.[361] *Streptococcus lactarius* was another species encountered in infant´s saliva, predominantly at 3 and 6 months of age, to later decrease and even disappear. This species was isolated from breastmilk of healthy mothers [405], explaining its high abundance in early age when the majority of the children in this cohort were breastfed. Given the long-term impact of breastfeeding for microbiota development (Fig. I-2B), it is plausible that early colonization with *S. lactarius*, acquired from mother´s breastmilk, could benefit later colonization by other beneficial microbial species. However, the potential role of *S. lactarius* in health and disease has not been evaluated to date.

Colonization of *Streptococcus sanguinis* started between 6 and 12 months of age and followed a similar pattern of development between children who did and did not develop caries. This species is believed to play a benign role in the oral cavity and it has been described to colonize in association to tooth emergence, at a median age of 9 months.[406] Moreover, *S. sanguinis* is recognized for its antagonistic role in dental caries since it may compete with cariogenic mutans streptococci for colonization sites on tooth surfaces.[406] Interestingly, although at very low levels, the cariogenic *S. mutans* was detected in the oral cavity of the infants already at an early age, possibly acquired through their mothers as shown before [398], with a trend of significantly higher levels at 7 years ($p=0.06$) in children developing caries. This is in line with previous studies where proportions of *S. mutans* in saliva were higher in children with caries when compared to those who stayed caries-free.[51] Thus, although this species is considered mainly an inhabitant of hard tissues, our data show that it can be detected before tooth eruption and therefore the oral health of mothers and caretakers during infancy may play an important role in the transmission of this pathogen. However, *S. mutans* has also been detected in caries-free populations and not in all cases of childhood caries, suggesting that other species may be cariogenic pathogens.[389, 398] In this study, children developing caries had significantly higher abundance of *Streptococcus cristatus* already at 3 months ($p=0.026$) and 24 months of age ($p=0.033$), compared to the children that stayed caries-free until 9 years of age. Given that *S. cristatus,* among other species*,* has been associated with severe early childhood caries [354], even in the absence of *Streptococcus mutans*, its role as an important cariogenic species and potential caries risk biomarker should be further studied. Nevertheless, it must be emphasized that streptococci are extremely similar in their 16S rRNA gene sequence, particularly at the V3-V4 region analysed in the current work, and therefore the suggested association of *S. cristatus* with caries development should be confirmed by

species-specific methodology with higher discriminatory power, like qPCR with specific primers and probes.[407]

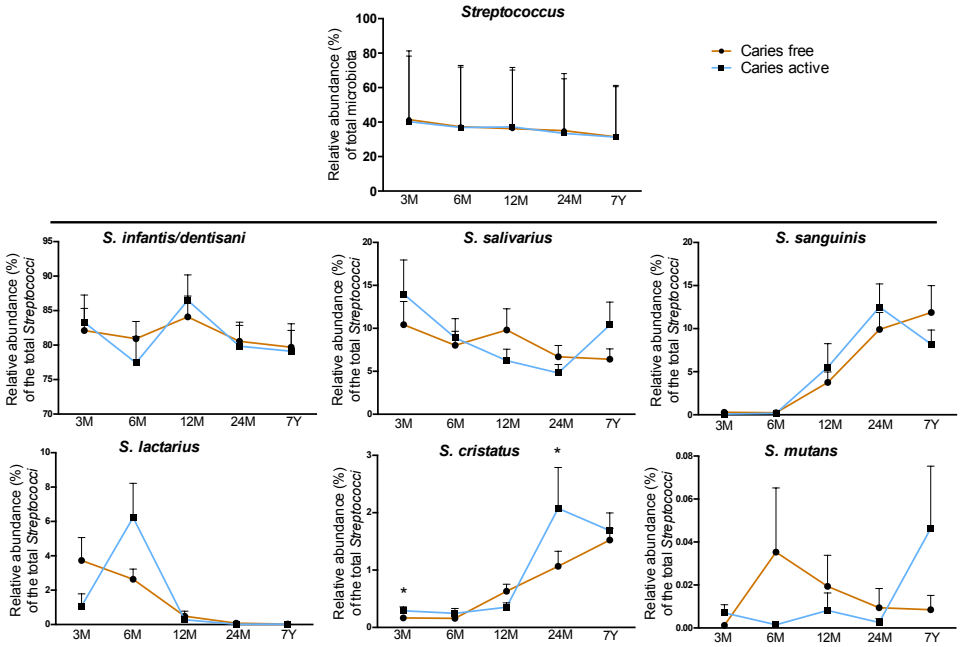

Fig. I-5. Relative abundance of most prevalent *Streptococci* species found in saliva samples of children staying caries-free and children developing caries during the first 9 years of age. Plots represent average relative abundance of *Streptococci* through time. Taxonomy assignments were performed with RDP classifier at 100% nucleotide identity. All data are presented as means with standard errors. *p < 0.05 by both Mann–Whitney U-test and Wilcoxon analysis.

If the association between *S. cristatus* and dental caries is confirmed, it must be born in mind that this species has been found to interrupt the formation of *P. gingivalis* biofilms by repressing the production of several virulence factors in this major periodontal pathogen.[408] In our dataset, a scatterplot of the relative frequencies of *Porphyromonas* and *S. cristatus* shows an L-shape (correlation p-value for the hyperbolic regression was p=0.057), suggesting potential antagonistic behaviour (Fig. I-S6), a feature that has been demonstrated in subgingival plaque samples from adults.[409] Given that most *Porphyromonas* sequences in our samples corresponded to *P. catoniae* (*P. gingivalis* accounted only for 13.4% of total *Porphyromonas* reads by 7 years of age), the potential antagonism between *S. cristatus* and *P. gingivalis* may not be apparent until a later age.

In addition, LefSe analyses were performed in order to examine potential biomarkers for early caries diagnosis. No specific group of species/genera at early age could be associated with caries development at 9 years of age (data not shown), suggesting that other ecological determinants including host interactions with microbiota, play a crucial role and should be integrated in caries

risk assessment models.[410, 411] Interestingly, even though the supplementation with *L. reuteri* during the first year of life has been associated with reduced caries prevalence at 9 years of age [329], no differences in caries development related to this Lactobacilli could be detected in the present study. Given that some of the infants included in the study developed allergies during their early childhood (see Table I-I), the groups were balanced according to allergy status and no relationship was found between allergies and caries onset. Even though mode of delivery and breastfeeding until 12 months of age have been shown to impact oral microbiota development in this study, no correlation between delivery mode or breastfeeding duration with dental caries could be detected. However, this could be due to low statistical power of the groups compared. Although microbiota composition clearly differed at 7 years of age between caries-free and caries-experienced children (Fig. I-2C), the absence of robust individual biomarkers of caries risk at an earlier age underlines that microbial-based early diagnostic tests should not be based on single species, and new potential bacterial risk indicators should be identified [412], including *S. cristatus* as proposed above. Given the enormous inter- and intra-individual variability in bacterial composition at caries lesions [413], and the multi-factorial nature of oral diseases where microbial, environmental and host-associated variables are involved, a holistic, ecological approach to caries risk assessment where information about the host, the habits (including the diet and oral hygiene) and the microbes are integrated will likely provide a better estimate of caries prediction.[410, 411, 414]

CONCLUSIONS

Only limited information is available on oral microbiome development in infants, and most studies have focused on taxonomic analysis. Thus, functional, metagenomic analyses are pending to fully understand the microbial contribution to oral health and disease.[415] Previous studies addressing oral microbiota development in early life have been hampered by retrospective approaches, small sample sizes, lack of deep sequence coverage, limited period of follow-up and analyses at single time points. The current study demonstrates that the infant's oral cavity gets colonized by microorganisms in a timely manner, increasing in complexity with time. In general, the data presented in the current manuscript is consistent with a model where microbiota development follows an ecological succession.[416]

In this scenario, several early colonizing species pave the way for the settlement of other microorganisms, which further expand microbial diversity towards a mature community which is more robust and resilient to change, partly because of the developed immune tolerance.[340] The presence of several species (particularly *S. cristatus*) at an early age was associated in this study to a higher frequency of dental caries at 9 years of age. Therefore, these findings open the possibility to use this species, together with others identified in other studies, as potential biomarkers of

caries risk. The oral cavity is a complex and heterogenous ecosystem with many variables influencing microbial composition and function. Several external factors appear to strongly influence microbiota development, including mode of delivery, which had a short-term effect, and others like breastfeeding length or antibiotic treatment, which appeared to have a long-term impact. It is interesting to note that, on the contrary, the oral microbiome composition in adults appears to be extremely resilient to antibiotic treatment.[395] This highlights that developmental milestones that are critical for oral microbiota succession occur in particular during infancy, and that an appropriate microbial colonization pattern can be instrumental for future health. Thus, microbial exposure, feeding habits and medical interventions during those initial and fragile stages may have a lifelong impact on general microbiome composition, and their potential consequences for human health should be carefully studied.

Chapter I – Supplementary Information

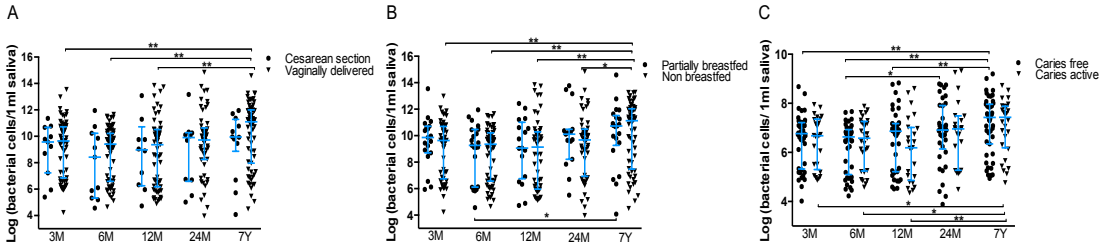

Fig. I-S1. Bacterial load of the total microbiota in infant saliva samples. Bacterial density using salivary samples that were obtained at different time points until 7 years of age, was determined by qPCR using universal primers targeting 16s rDNA bacterial gene. Figures are showing bacterial density depending on the mode of delivery **(A)**, partial breastfeeding until 12 months of age **(B)** and dental caries status **(C)**. Data are presented with median with interquartile ranges. Delivery mode: 3 months (N_{VD}=62; N_{CS}=11), 6 months (N_{VD}=72; N_{CS}=11), 12 months (N_{VD}=59; N_{CS}=10), 24 months (N_{VD}=56; N_{CS}=10) and 7 years of age (N_{VD}=68; N_{CS}=12); Partial breastfeeding at 12 months: 12 months (N_{nBF}=52; N_{BF}=17), 24 months (N_{nBF}=50; N_{BF}=16) and 7 years of age (N_{nBF}=59; N_{BF}=21); Caries status: 3 months (N_{CF}=40; N_{CA}=26), 6 months (N_{CF}=43; N_{CA}=31), 12 months (N_{CF}=37; N_{CA}=26), 24 months (N_{CF}=35; N_{CA}=24) and 7 years of age (N_{CF}=45; N_{CA}=30). M=months; Y=years. (*p <0.05,**p<0.01; Mann-Whitney U-test).

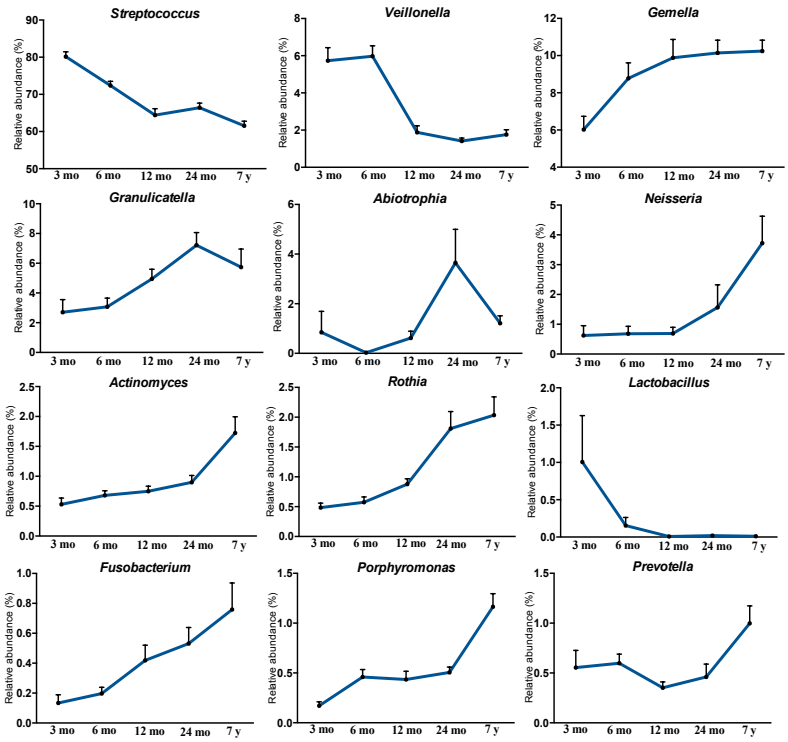

Fig. I-S2. Oral microbiota development through time. Plots show the relative abundance of dominant bacterial genera at 3, 6, 12, 24 months and 7 years of age.

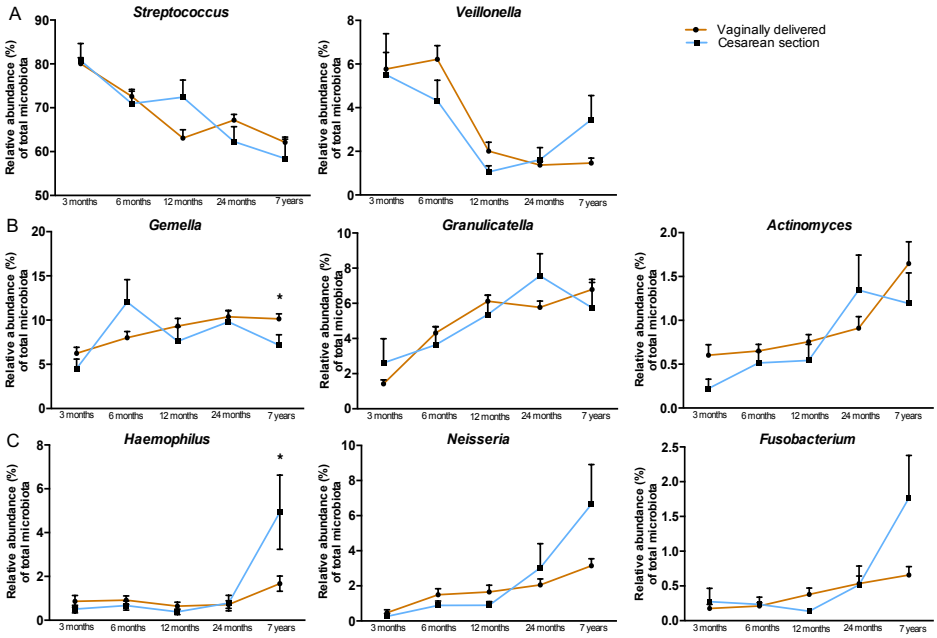

Fig. I-S3. Microbiota composition of the most dominant bacterial genera in children delivered vaginally or through caesarean section. **(A)** Genera considered as early colonizers. **(B)** Genera considered to "Constantly increase" are already present at 3-6 months of age, at >1%, and they are increasing with time. **(C)** Bacterial genera considered as "Late colonizers", present at 3-6 months of age but below 1% of abundance and significant increase after 12 months of age. Plots are showing the relative abundance of dominant bacterial genera, as determined by Illumina sequencing of 16S rDNA gene, in saliva samples collected at 3 months (N_{VD}=62; N_{CS}=11), 6 months (N_{VD}=72; N_{CS}=11), 12 months (N_{VD}=59; N_{CS}=10), 24 months (N_{VD}=56; N_{CS}=10) and 7 years of age (N_{VD}=68; N_{CS}=12); M=months; Y=years. (*p <0.05; Mann-Whitney U-test).

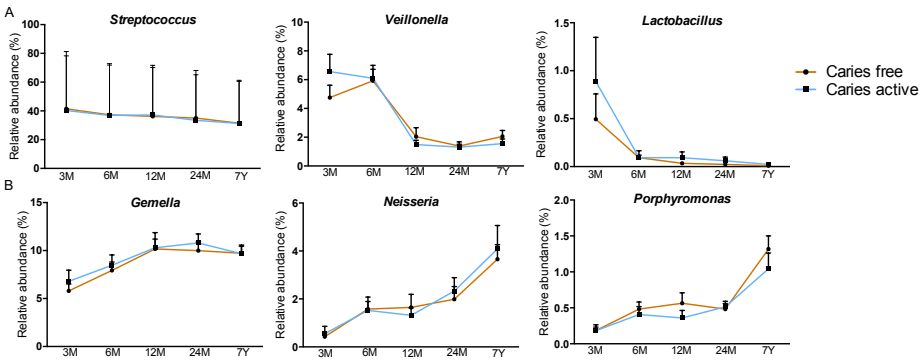

Fig. I-S4. Microbiota composition of the most dominant bacterial genera in children developing dental caries and children staying caries-free until 9 years of age. **(A)** Genera considered as early colonizers. **(B)** Genera that are constantly increasing through the age. Plots are showing the relative abundance of dominant bacterial genera, as determined by Illumina sequencing of 16S rDNA gene, in saliva samples collected at 3 months (N_{CF}=40; N_{CA}=26), 6 months (N_{CF}=43; N_{CA}=31), 12 months (N_{CF}=37; N_{CA}=26), 24 months (N_{CF}=35; N_{CA}=24) and 7 years of age (N_{CF}=45; N_{CA}=30). CF: caries free; CA: caries active; M=months; Y=years. (*p <0.05; Mann-Whitney U-test).

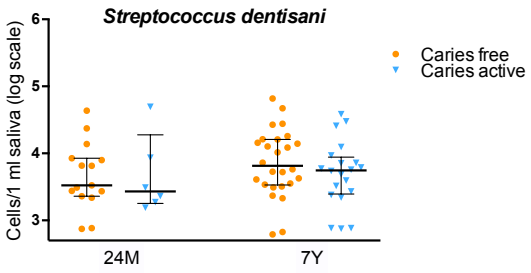

Fig. I-S5. *Streptococcus dentisani* levels in infant saliva samples determined by qPCR quantification. Detection limit was established >2*10^2 cells/ml saliva. Data are presented with median and interquartile ranges. 24 months (24M): N_{CF}=15; N_{CA}=6; 7 years (7Y): N_{CF}=26; N_{CA}=19.

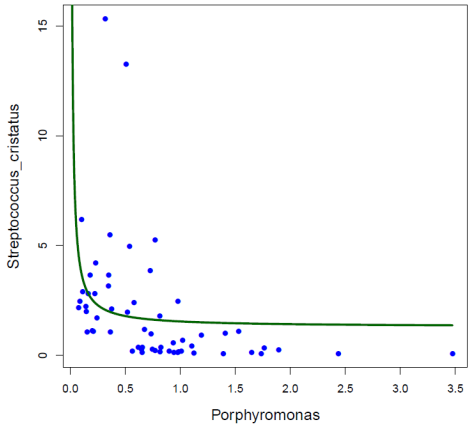

Fig. I-S6. Correlation between *Streptococcus cristatus* and *Porphyromonas spp.* in children's salivary samples. Scatterplot shows the relative proportions of the two bacteria, which approximate a hyperbolic regression (p=0.057), suggesting potential antagonism between the two taxa. Samples with 0% proportion were removed from the analysis.

Table I-S1. Accumulative antibiotics intake during the first two years of life.

Patient code	Antibiotics courses *(age in months)*
1	Phenoxymethylpenicillin *(9)*, Phenoxymethylpenicillin *(10)*, Amoxicillin *(11)*, Phenoxymethylpenicillin *(22)*
2	Phenoxymethylpenicillin *(7)*, Phenoxymethylpenicillin *(18)*
3	Erythromycin *(9)*, Phenoxymethylpenicillin *(13)*, Loracarbef *(17)*
4	Phenoxymethylpenicillin *(9)*, Ampicillin *(10)*, Phenoxymethylpenicillin *(20)*, Amoxicillin *(21)*
5	Phenoxymethylpenicillin *(3)*, Amoxicillin *(3)*, Amoxicillin *(10)*, Amoxicillin *(13)*, Flucloxacillin *(15)*, Amoxicillin *(15)*, Amoxicillin *(19)*, Amoxicillin *(24)*
6	Phenoxymethylpenicillin *(10)*, Phenoxymethylpenicillin *(12)*, Phenoxymethylpenicillin *(23)*
7	Phenoxymethylpenicillin *(21)*, Benzyl-penicillin and Phenoxymethylpenicillin *(23)*
8	Phenoxymethylpenicillin *(8)*, Amoxicillin *(9)* Phenoxymethylpenicillin *(16)*
9	Phenoxymethylpenicillin *(6)*, Amoxicillin *(7)*, Amoxicillin *(10)* Amoxicillin *(11)*, Phenoxymethylpenicillin *(19)*, Amoxicillin *(21)*, Amoxicillin *(22)*
10	Bactrim *(6)*, Furadantin *(6-11, as prophylaxis)*, Phenoxymethylpenicillin *(20)*
11	Phenoxymethylpenicillin *(2)*, Amoxicillin *(3)*, Phenoxymethylpenicillin *(13)*, Amoxicillin *(13)*
12	Ampicillin *(11)*, Erythromycin *(11)*, Amoxicillin *(14)*, Erythromycin *(17)*, Erythromycin *(19)*

Chapter II – ABERRANT IgA RESPONSES TO THE GUT MICROBIOTA DURING INFANCY PRECEDE ASTHMA AND ALLERGY DEVELOPMENT

- Majda Dzidic, Thomas R. Abrahamsson, Alejandro Artacho, Bengt Björkstén, Maria Carmen Collado, Alex Mira, Maria C. Jenmalm; Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development; © *J Allergy Clin Immunol*; Mar 2017, 139:1017-1025.

Abstract

Although 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. Here 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. A combination of flow cytometric 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 months of age from children staying healthy or having allergic symptoms up to 7 years of age. The children with allergic manifestations, particularly asthma, during childhood had a lower proportion of IgA bound to fecal bacteria at 12months of age compared with healthy children. These alterations cannot be attributed to differences in IgA levels or bacterial load between the 2 groups. Moreover, the bacterial targets of early IgA responses (including coating of the *Bacteroides* genus), as well as IgA recognition patterns, differed between healthy children and children with allergic manifestations. Altered IgA recognition patterns in children with allergy were observed already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breast-fed children. 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.

Introduction

Allergic diseases have become a major public health problem in affluent societies.[417] Reduced microbial exposure, both pre- and postnatally, has been proposed to underlie the increase in allergy development.[42, 57, 418, 419] 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.[57] 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,[42, 168, 420–422, 265–270, 275, 278], 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.[266] 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.[57]

A reduced mucosal barrier function may increase the risk for allergy development[417] and immunoglobulin A (IgA) is the primary mediator of humoral mucosal immunity.[423] Immunoglobulin A is the most abundantly produced antibody in humans, with the highest amount of secretion in the intestinal tract.[423, 424] 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.[423, 425] SIgA may also limit overgrowth of select species, thus stimulating diversity.[110, 423] 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.[424] 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.[58, 426] Studies and clinical reports suggest that SIgA that origins from the mothers' breastmilk is important for immune regulation and protection against bacterial, viral and parasitic infections in suckling infants.[58, 427–430]

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.[245, 246, 431] Recent advances in flow cytometry [432] and next generation sequencing [413] 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 rRNA 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

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 [327] and 7 years of age.[328] 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.*[327] 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 II-SI) and 28 children staying healthy up to 7 years of age (Table II-SII), were randomly selected for this study (Fig. II-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.[327, 328] 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 II-I). All included infants were exclusively breast-fed for at least 1 month, and no infant received antibiotics before 1 month of age.[265] 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_2O; 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. II-S1).

The sorting of the 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. 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.*[413]

Table II-I. Descriptive data of children included in this study.

Children	Healthy % (no)	Developing allergy % (no)	P value*	Developing asthma % (no)	P value*
Girls	46 (13)	40 (8)	0.66	40 (4)	1.00
Older siblings	57 (16)	50 (10)	0.62	50 (5)	0.70
Caesarean delivery	14 (4)	20 (4)	0.70	20 (2)	0.64
Furred pets	4 (1)	15 (3)	0.29	10 (1)	0.46
Maternal atopy	82 (23)	80 (16)	1.00	80 (8)	1.00
Breast-feeding (1 to 12 mo)	29 (8)	20 (4)	0.74	10 (1)	0.40
Antibiotic treatment (1-12 mo)	36 (10)	30 (6)	0.68	50 (5)	0.43
Day care (1-12 mo)	14 (4)	10 (2)	1.00	(0)	0.56
Probiotic group	54 (15)	60 (12)	0.66	60 (6)	1.00

*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.

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 [433] and the concentration and purity were measured by calculating A260/280 ratios in Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

16S rRNA 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 rRNA gene was amplified using universal bacterial degenerate primers 357F-CCTACGGGAGGCAGCAG and 926R- CCGTCAATTCMTTTRAGT [434], 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.[366] A secondary amplification (25 cycles, annealing temperature of 52°C) was performed by using the purified PCR product as a template.[435] 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 rRNA read ends were trimmed in 10 bp sliding windows, with average value ≥20, using the Galaxy tool [436] as quality has been shown to dramatically decrease towards the end of the sequences.[437] 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 [374] version 11.3, and chimeric sequences were filtered out using UCHIME.[438]

Taxonomic assignment was performed by the RDP-classifier [374] where the reads were assigned a phylum, class, family and genus and phylogenetic ranks were allocated when scores exceeded 0.8 confidence threshold. α – 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.[439]

For analyzing IgA coating patterns, the threshold used for including the genera was ≥ 1% relative abundance in either the IgA+ or IgA- fractions. Further, a pseudocount that was equal to 0.001,

was added to every genus 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.[140] 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) [377] was then used for high-dimensional biomarker discovery comparing the IgA-indices between healthy infants and infants developing allergic manifestations.

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 [376] 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.[440] Single-copy cellular sequence β-actin was used as a quality control for isolated genomic DNA (Table II-SIII). 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) [441] 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. α – diversity analysis were performed using the statistical software R with the vegan package.[442] 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 tests are noted in figure legends. For the PCA-analysis, DMwR package of R was used for removing two outliers, sample code 5 (Table II-SI) and 20 (Table II-SII). This was done according to *Local Outlier Factor* (LOF) – algorithm that identifies density-based outliers by comparing the LOF-values.[443]

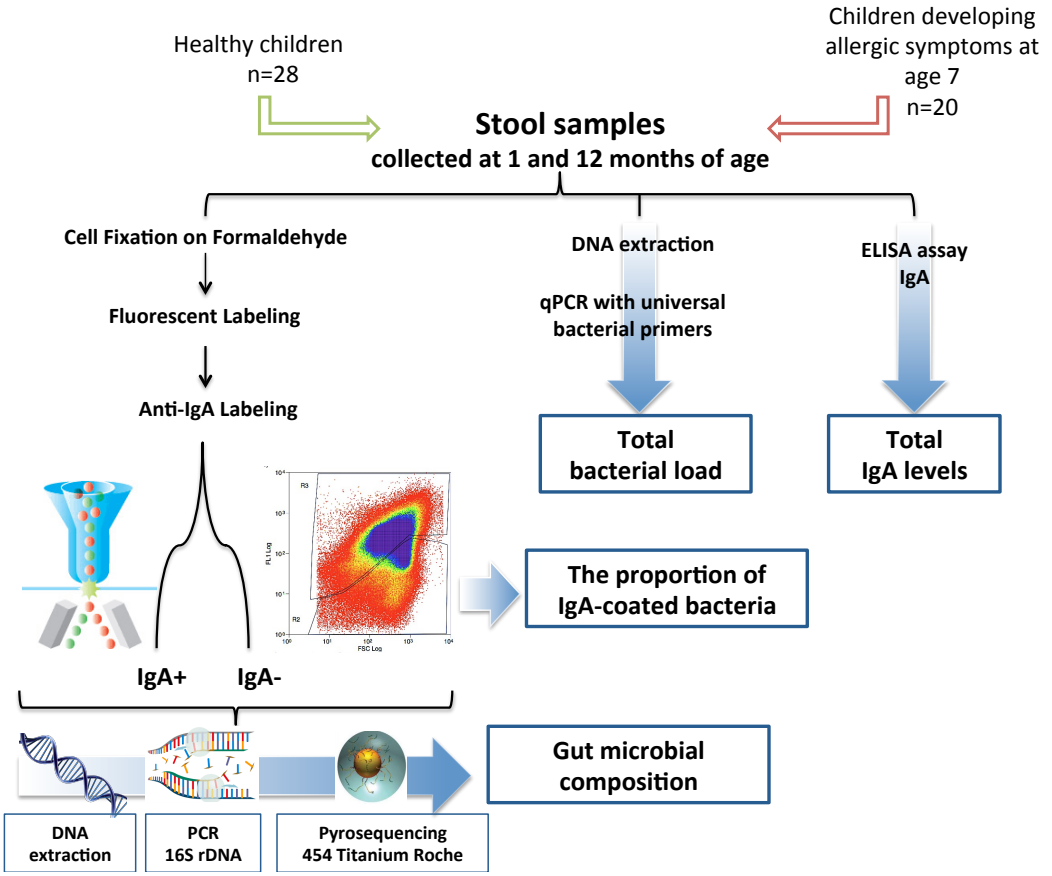

Fig. II-1. Schematic workflow of performed experiments. The proportion of the gut microbiota bound to IgA (IgA+) or not (IgA-), in infants, was analyzed by flow cytometry-based sorting of fecal samples prior to 16S rRNA 454-pyrosequencing. In addition, total secretory IgA levels were estimated by ELISA tests, and the bacterial density measured using universal primers targeting the single-copy bacterial gene FusA.

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. II-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. II-2B), but not allergic rhinoconjunctivitis (Fig. II-S2). 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 breastmilk derived to child derived IgA antibodies.[426, 444]

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. II-S3).

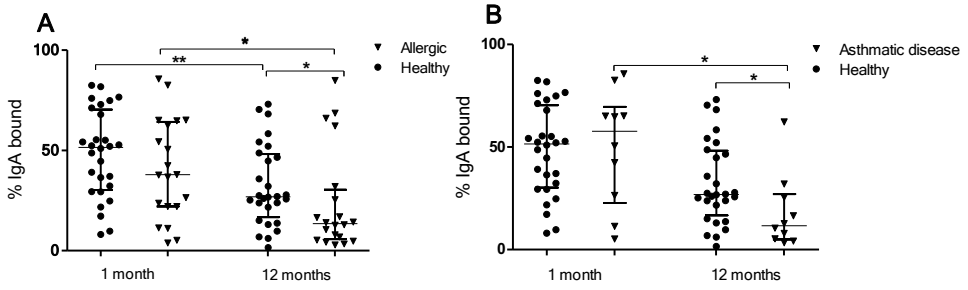

Fig. II-2. Proportions of IgA-coated fecal bacteria in early infancy. **(A)** The proportion of fecal bacteria bound to IgA at 1 month and 12 months of age in children staying healthy (N=28) or developing allergic symptoms (N=20) during the first 7 years of life. **(B)** The proportion of fecal bacteria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

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. II-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. II-3A).

Total fecal SIgA levels were similar in healthy children and children developing allergic manifestations (Fig. II-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. II-3B).

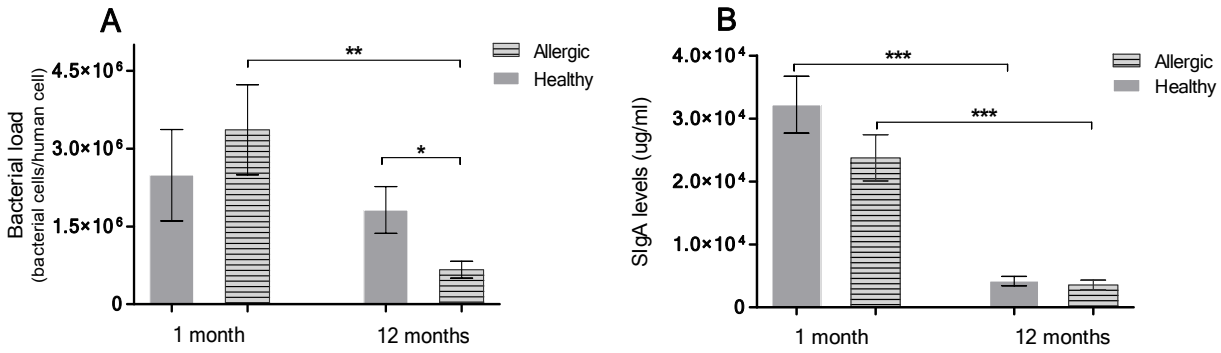

Fig. II-3. Bacterial load and total fecal secretory IgA levels in healthy infants and infants developing allergic manifestations. **(A)** The quantification of bacterial numbers was obtained by qPCR- detection with universal primers targeting the gene FusA (present in single-copy in bacterial cells) 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).

Bacterial targets of IgA responses in children developing allergic manifestations and children staying healthy up to 7 years of age

Bacterial 16S rRNA 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. II-S4), 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. II-4A, B) and asthmatic symptoms, particularly at 1 month but also 12 months of age (Fig. II-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. II-S5A). 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. II-4B), with similar findings observed for children developing allergic rhinoconjunctivitis (Fig. II-S5B). Regarding children developing asthmatic symptoms, *Escherichia/Shigella* was predominantly IgA free (Fig. II-4D), while *Lachnospiraceae incertae sedis*, a *Firmicutes* phylum member, was predominantly IgA bound in children developing allergic symptoms, when compared with healthy children (Fig. II-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. II-S6) or at phylum level (Fig. II-S7A, 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. II-S7C) but not in the IgA-free fraction (Fig. II-S7D).

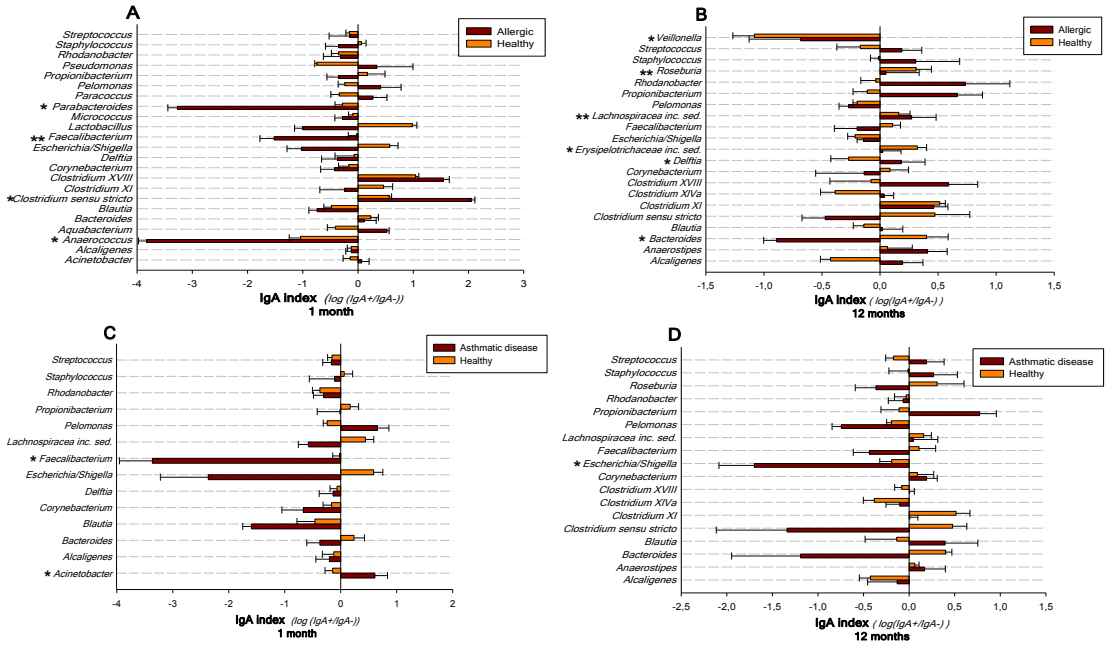

Fig. II-4. IgA responses to the gut microbiota, at 1 and 12 months of age. Plots are depicting IgA responses (defined by IgA index, reflecting the ratio in IgA+ and IgA-) to dominant genera (>1% of total) of the gut microbiota at 1 month ($n_{Healthy}$=27; $n_{Allergic}$=19; n_{Asthma}=10) and 12 months ($n_{Healthy}$=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. * $p<0.05$; ** $p<0.01$.

IgA recognition patterns of gut microbiota differ between healthy and allergic children

A *principal component 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. II. 5A) and asthmatic symptoms (Fig. II-5C) but not ARC (Fig. II-S8A). Clear separation was observed at 12 months of age for healthy children and children developing allergic disease (Fig. II-5B), asthma (Fig. II-5D) and ARC (Fig. II-S8B).

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. II-S9).

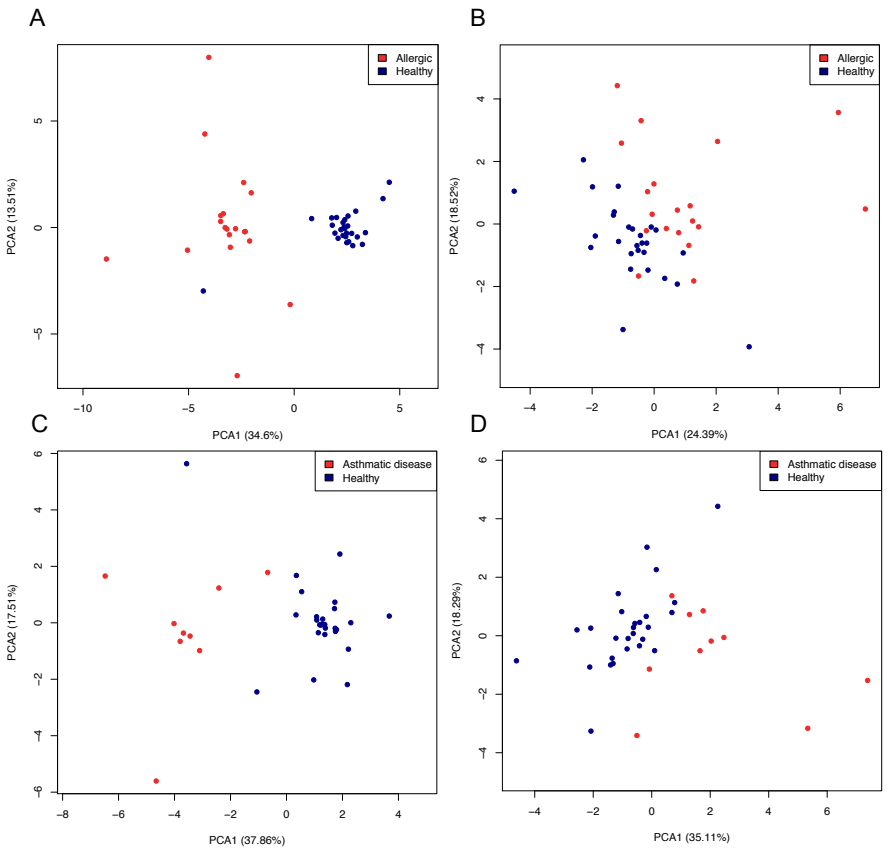

Fig. II-5. IgA recognition patterns of the gut microbiota at 1 (left panels) and 12 months (right panels). Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota 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)**.

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.[130, 423] 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.[423] Furthermore, SIgA may also limit overgrowth of selected species, thus enabling an increased microbiota diversity.[110, 423] 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.[235, 445, 446] In line with this, low mucosal total IgA levels [245, 246, 431] a reduced gut microbiota diversity in infancy [265–270, 420–422] and decreased seroreactivity to gut microbiota antigens [447] 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 months 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, [265–270, 420–422] 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 [424], 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 [448], 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 [266], 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.[417, 449, 450] 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.[417, 449, 450]

Previous studies in adults have determined that IgA might be more reactive against disease driving bacteria [130, 141, 451, 452], 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.[141, 452] Also, SIgA may enable an increased microbiota diversity by limiting overgrowth of selected species.[110, 423] 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,[426, 444] 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.[235, 445, 446] For example, breastmilk 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.[428]

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.[453–455] Furthermore, decreased diversity of the *Bacteroidetes* phylum in infant stool samples have been linked to delivery by Cesarean section [57, 174] and allergy development.[57, 265] 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,[456] while *Roseburia* is another well-known butyrate-producer [457], which was reduced in patients suffering from ulcerative colitis.[458] In

all, decreased coating of these commensals might reflect a lower stimulation of the mucosal immune system in the infants developing allergic diseases.

The *Erysipelotrichaceae 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.[459, 460] Palm and colleagues found that a member of *Erysipelotrichaceae* 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.[452] *F*urthermore, deregulation of T-cells in mice affecting the selection of IgA plasma cells caused gut dysbiosis, including increased abundance of *Erysipelotrichaceae* that are known to induce immune hyperactivation.[461] *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.[462] Moreover, high abundance of fecal *Escherichia coli* was associated with development of IgE-associated eczema within the first year of life.[463] As allergy and asthma development were associated with reduced IgA responses to the *Erysipelotrichaceae* 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.[464, 465] Furthermore, excessive growth of species belonging to *Lachnospiracea* in mice with impaired IgA responses reduces Firmicutes diversity.[110] 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.[58, 163, 343, 422, 466] 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.

CONCLUSIONS

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.

Chapter II- Supplementary Information

Table II-SI. Allergic manifestations and sensitization in the 20 children with allergic symptoms. The children were followed prospectively during the first 7 years of life.

Children	Allergy symptoms at 2 years	Allergy symptoms at 7 years	Breastfed at 12 months	Mode of delivery	Antibiotic treatment first year	Probiotics/ Placebo treatment
1	SPT+, AD	SPT+, ARC, AD	Yes	VD	No	Probiotics
2	AD	SPT+, AB, ARC, AD	No	VD	No	Probiotics
3	SPT+, AD, AB	SPT+, AB, ARC, AD	Yes	VD	Yes	Placebo
4	SPT+, AD	SPT+, AB, AD	No	CS	Yes	Probiotics
5	SPT+, AD	SPT+, AD	No	CS	No	Placebo
6	SPT+, AD	ARC	No	CS	No	Probiotics
7	SPT+, AD	AD	Yes	VD	No	Placebo
8	SPT+, AD	AB, *	No	VD	No	Placebo
9	SPT+, AD	SPT+, ARC	No	VD	Yes	Probiotics
10	SPT+	SPT+, AB, ARC	No	VD	Yes	Placebo
11	SPT+, AD	SPT+ AB, AD	No	CS	No	Probiotics
12	SPT+, AD	SPT+, AB, AD	No	VD	Yes	Probiotics
13	SPT+, AD	SPT+, ARC	Yes	VD	No	Placebo
14	SPT+, AD	-	No	VD	No	Placebo
15	SPT+, AD	-	No	VD	No	Probiotics
16	SPT+, AD	SPT+, ARC, AD	No	VD	No	Probiotics
17	SPT+, AB	SPT+, AB	No	VD	Yes	Placebo
18	SPT+, AD	SPT+, AB, ARC, AD	No	VD	No	Probiotics
19	SPT+, AD	-	No	VD	No	Probiotics
20	SPT+, AB	SPT+, AB, ARC, U	No	VD	Yes	Probiotics

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 II-SII. 28 children without allergic symptoms and sensitization. The children were followed prospectively for the first 7 years of life.

Children	Breastfed at 12 months	Mode of delivery	Antibiotic treatment first year	Probiotics/ Placebo treatment
1	No	VD	Yes	Placebo
2	No	VD	Yes	Probiotics
3	No	VD	No	Probiotics
4	No	VD	No	Placebo
5	No	VD	Yes	Probiotics
6	No	VD	Yes	Placebo
7	No	VD	Yes	Placebo
8	No	VD	No	Placebo
9	No	VD	Yes	Probiotics
10	No	VD	No	Probiotics
11	No	VD	No	Placebo
12	Yes	VD	Yes	Probiotics
13	Yes	VD	Yes	Probiotics
14	No	CS	Yes	Probiotics
15	Yes	VD	Yes	Placebo
16	Yes	VD	No	Probiotics
17	Yes	VD	Yes	Placebo
18	Yes	CS	No	Probiotics
19	Yes	CS	No	Placebo
20	Yes	VD	No	Probiotics
21	No	VD	No	Placebo
22	No	VD	No	Placebo
23	No	VD	No	Placebo
24	No	VD	No	Probiotics
25	No	VD	No	Placebo
26	No	VD	No	Probiotics
27	No	VD	No	Probiotics
28	No	VD	No	Probiotics

CS, caesarean delivery; VD, vaginal delivery.

Table II-SIII. Primers used for qPCR-amplification. For the analysis of bacterial load in stool samples, FusA single copy gene (present in bacterial cells) and β-actin gene (present in human cells) were targeted with universal primers presented below.

		5´ to 3´ direction	PCR-product lenght (bp)
β-actin	F	TTGTTACAGGAAGTCCCTTGCC	101
	R	ATGCTATCACCTCCCCTGTGTG	
FusA	F	TTGTTACAGGAAGTC	155
	R	ATGCTATCACCTCCC	

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

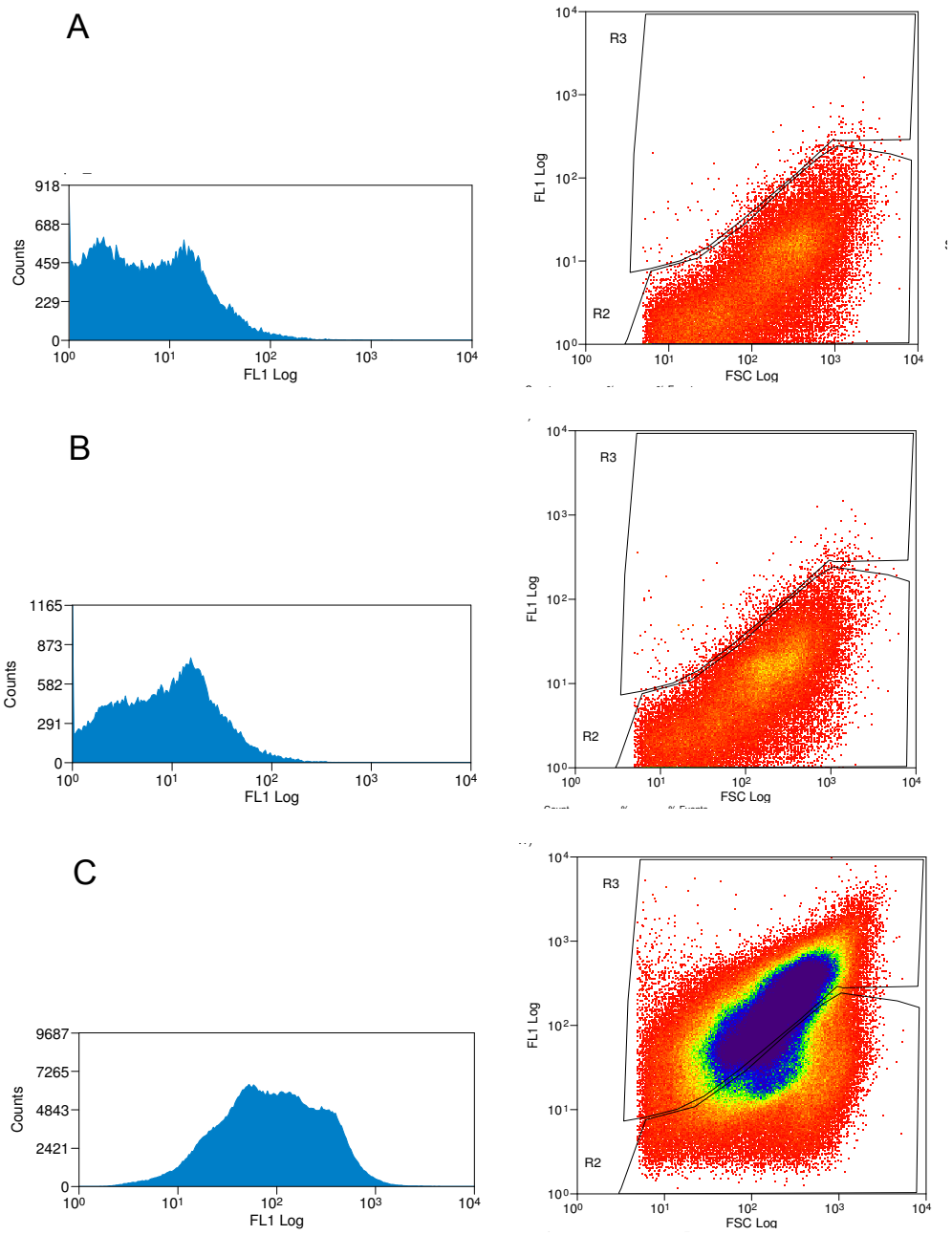

Fig. II-S1. Scatter plots presenting how the gating strategy was performed. **(A)** A blank control (unlabelled bacterial cells) was used to determine the threshold for autofluorescence. **(B)** Anti-mouse IgA antibodies were used as an isotype control, determining the fluorescence intensity which was 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.

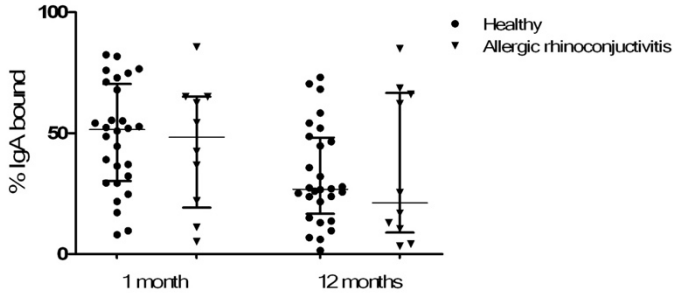

Fig. II-S2. Proportion of fecal bacteria bound to IgA in children developing allergic rhinoconjunctivitis (N=10, triangles) and children staying healthy (N=28, circles) up to 7 years of age.

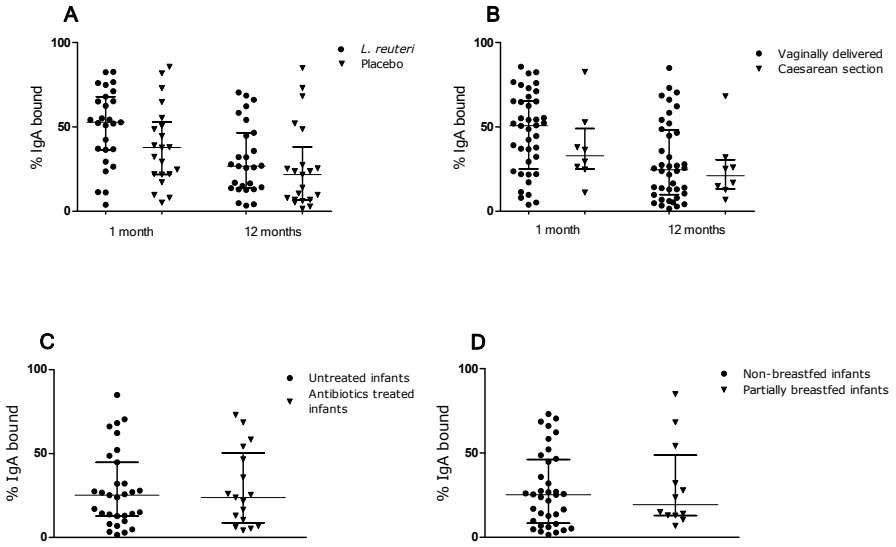

Fig. III-S3. Proportion of fecal bacteria bound to IgA in relation to probiotic supplementation, mode of delivery, antibiotic treatment and breastfeeding. **(A)** The proportion of faecal bacteria bound to IgA at 1 month and 12 months of age in children treated with *L. reuteri* (N=27, circles) or placebo (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.

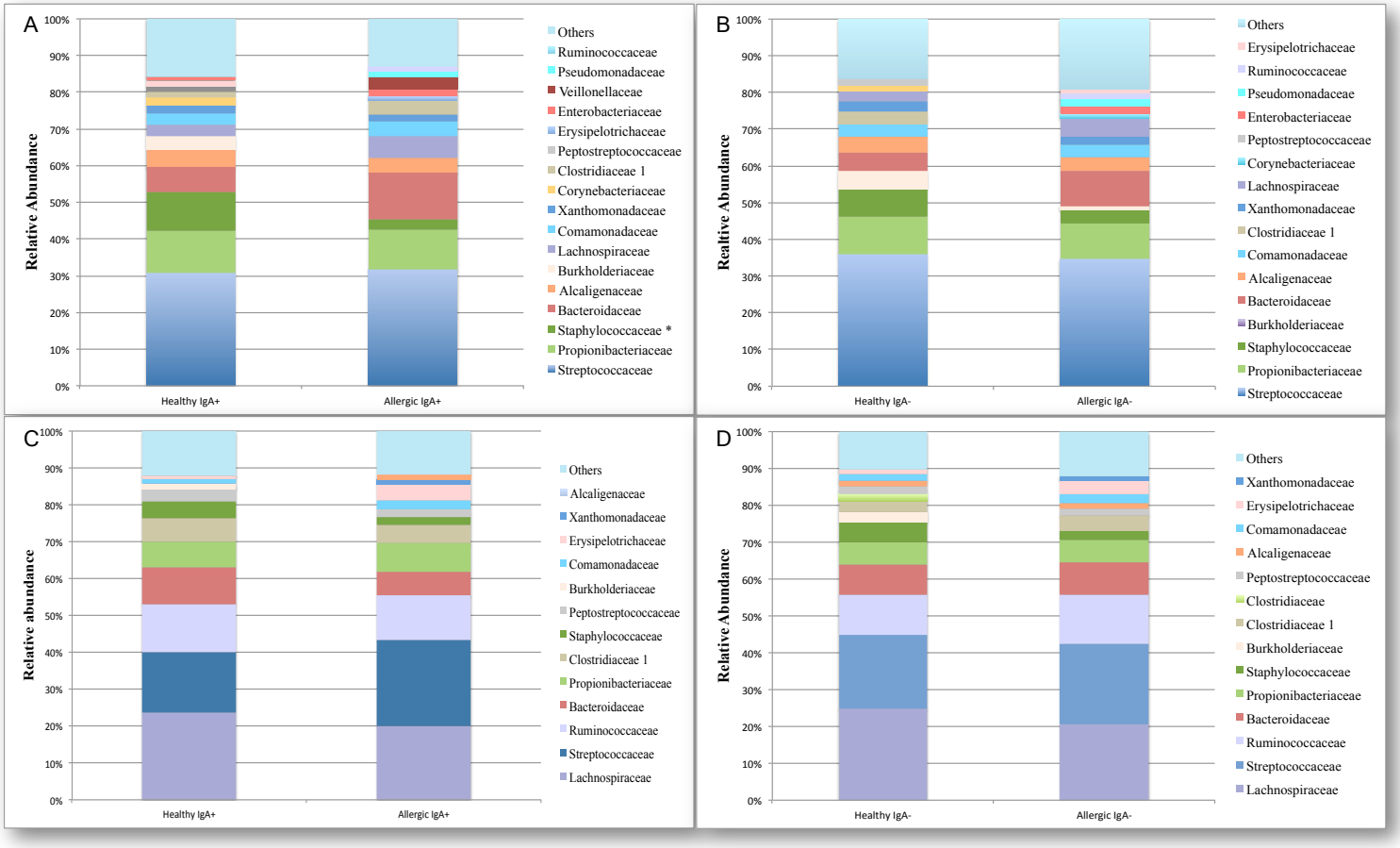

Fig. II-S4. Microbiota composition of the most dominant bacterial families as determined by pyrosequencing of 16S rDNA gene. Plots are showing the relative abundance (>1% of the total) of dominant bacterial families, coated **(A, C)** or not **(B, D)** with IgA, in stool samples collected at 1 month **(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). $N_{1\ month}$: H IgA+ =27; A IgA+ =19; H IgA- =28; A IgA- =20; $N_{12\ month}$: H =28; A =20.

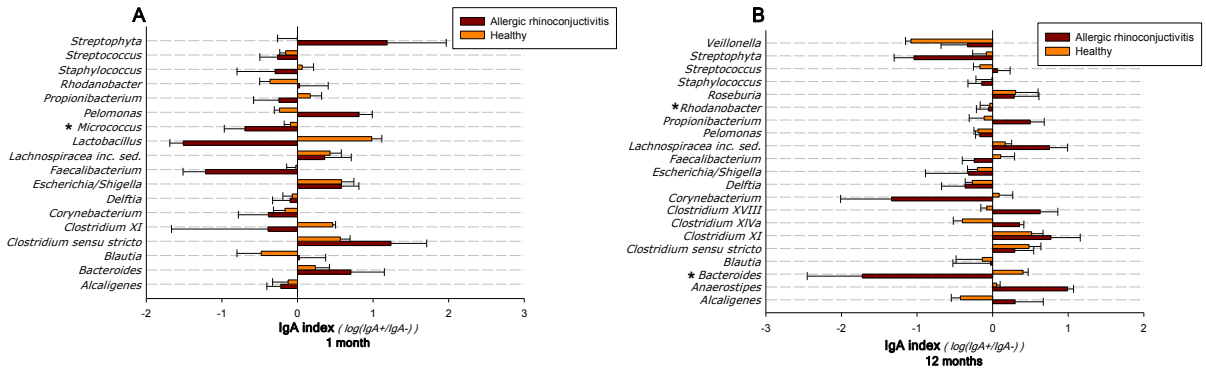

Fig. II-S5. IgA responses to the gut microbiota in healthy children and children developing allergic rhinoconjunctivitis during the first 7 years of life. Plots are depicting IgA responses (defined by IgA index, reflecting the differences in IgA+ and IgA-) to dominant genera (>1% of total) of the gut 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. * $p<0.05$.

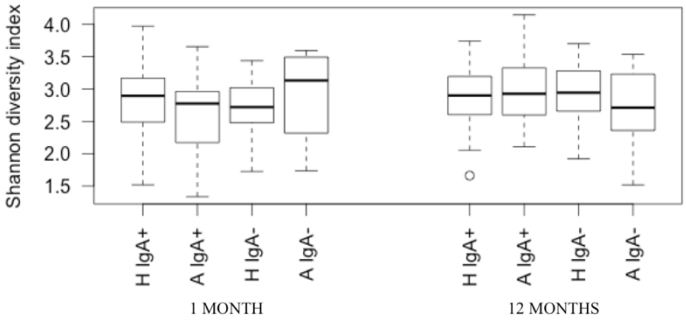

Fig. II-S6. Diversity of the total microbiota in stool samples. Shannon index, based on randomly selected 700 reads per sample (samples with lower amount of reads were excluded), was used to estimate the samples' diversity of fractions coated or non-coated with IgA, obtained at 1 and 12 months of age 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.

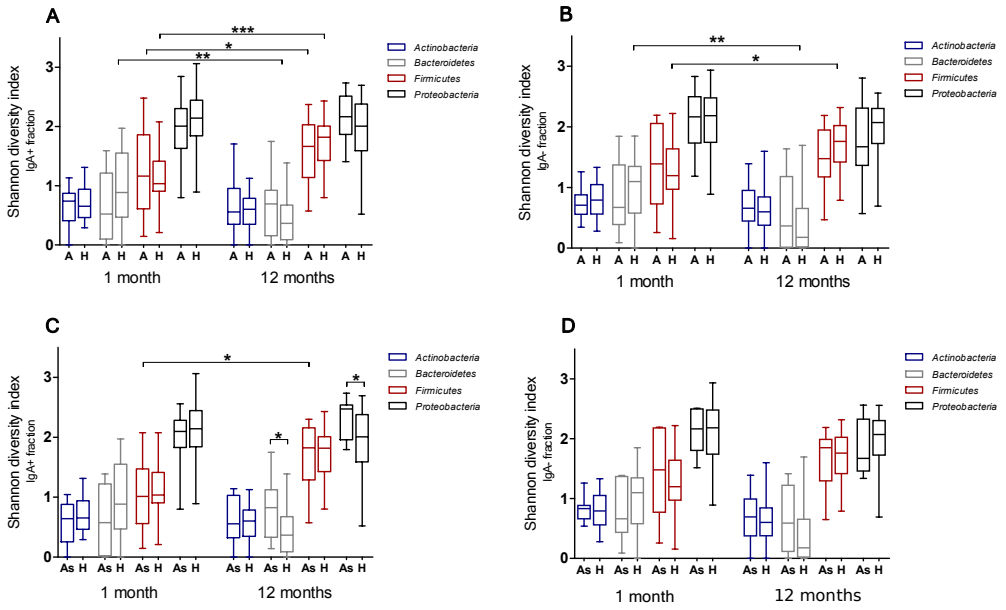

Fig. II-S7. Bacterial phyla diversity of IgA-coated and non-coated bacteria in stool samples of healthy children and children developing allergic/asthmatic symptoms. Shannon index, based on randomly selected 700 reads per sample, was used to estimate the samples' diversity of the most dominant phyla: 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.

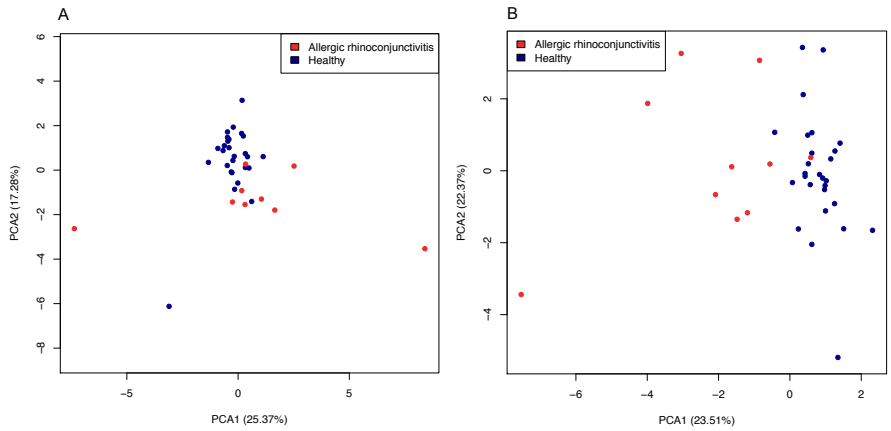

Fig. II-S8. IgA recognition patterns of the gut microbiota in children with/without allergic rhinoconjunctivitis. Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ and IgA-) of the dominant genera (>1% of total) of the gut microbiota 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.

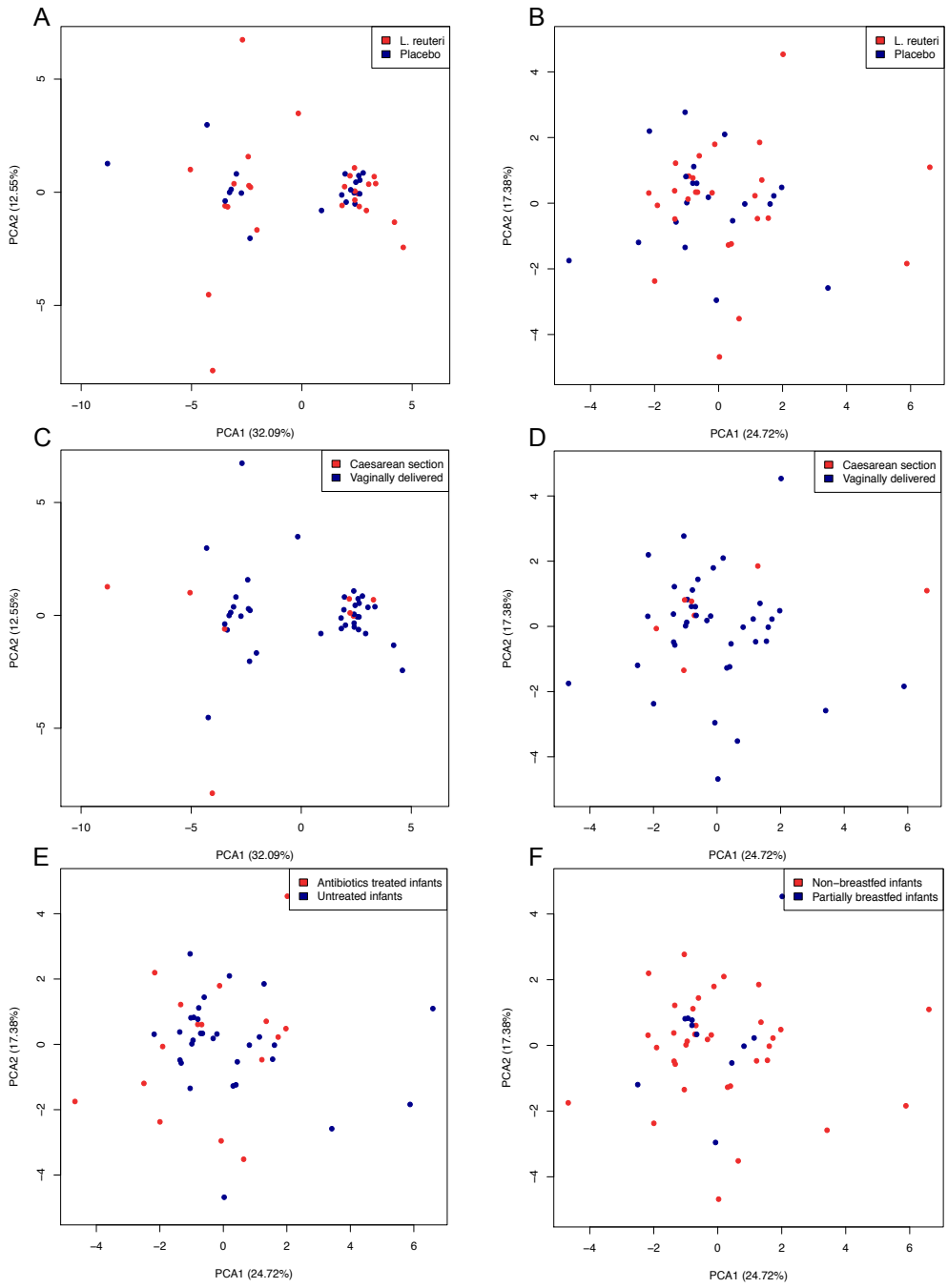

Fig. II-S9. IgA recognition patterns of the gut microbiota in relation to probiotic supplementation, mode of delivery, antibiotics treatment and breastfeeding. Plots are showing Principal component analysis (PCA) based on the IgA index (reflecting the differences in IgA status, e.g. the ratio of IgA+ 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 ($N_{L.reuteri}$=20; $N_{placebo}$=26) and **(B)** 12 months of age ($N_{L.reuteri}$=21; $N_{placebo}$=27). IgA recognition patterns in children that were vaginally delivered or by Caesarian section (C-section) at **(C)** 1 month of age ($N_{Vaginally}$=38; $N_{C\text{-}section}$=8) and **(D)** 12 months of age ($N_{Vaginally}$=40; $N_{C\text{-}section}$=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.

Chapter III – ORAL MICROBIOTA MATURATION DURING THE FIRST 7 YEARS OF LIFE IN RELATION TO ALLERGY DEVELOPMENT

- Majda Dzidic, Thomas R. Abrahamsson, Alejandro Artacho, Maria Carmen Collado, Alejandro Mira, Maria C Jenmalm; Oral microbiota maturation during the first 7 years of life in relation to allergy development; *Allergy (© 2018 EAACI and John Wiley and Sons Ltd.),* Mar 2018; 73:2000-2011.

Abstract

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. In this study, we aimed to determine the bacterial composition in longitudinally collected saliva samples during childhood in relation to allergy development. 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 7 years of age. 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, were distinctive during early infancy, likely influencing early immune maturation. 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.

Introduction

During the past decades, allergic diseases have become a major public health problem in affluent societies.[467] 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.[263, 264] Approximately 700 common microbial species have been detected in the oral cavity.[11] Typically, the commensal microbiota here have a symbiotic relationship with the host, although, under certain circumstances, some microbes can overcome host defenses and become pathogenic.[468] 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.[48] Moreover, it has been observed that maternal intrapartum antibiotic administration contributed to the shaping of the microbial colonization pattern in the neonatal oral cavity.[469]

During the initial period, the microbiota composition of different niches of the neonate's body highly resemble each other.[171] Niche specific establishment of a microbiota of increasing complexity then occurs, concurrent with immune system maturation.[39] 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.[263, 264] Factors such as early life antimicrobial exposure[470], caesarean delivery[343], formula feeding[471] and maternal consumption of antimicrobials during pregnancy[213] 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 [39, 263], yet there are also indications that microbial colonization of the skin[17, 472] and respiratory tract might be associated with allergies.[473] While some studies are demonstrating the bacterial dysbiosis and lower microbial diversity already before the onset of the allergic disease[168, 265, 266, 472, 474, 475], other are describing and comparing the differences in microbiota in children having allergies and being healthy.[17] 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[472, 474, 475], 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

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.[327, 328] 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.[327, 328] Infants were regarded as sensitized if they had at least one positive skin prick test and/or detectable circulating allergen specific-IgE antibodies.[327, 328] Skin prick tests were performed on the forearm with egg white, fresh skimmed cow milk and standardized cat, dog, birch, peanut and timothy extracts at 6, 12, 24 months and 7 years of age (here also mite (Der p)).[328] 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.[266, 327] All asthmatic children were also included in the allergic group. For further details, please see the publications from Abrahamsson *et al.*[266, 327, 328]

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.

Table III-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 x^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. $N_{Healthy}$= 33, $N_{Allergic}$=47, $N_{Asthmatic}$=20

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.[366] 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. Overhand 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.[476] The obtained Ct values were transformed to bacterial cell numbers by a standard curve calibrated by flow cytometry.[19]

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.[477]

Obtained sequences were taxonomically classified by the RDP-classifier [374] 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.[371] Normalized OTU tables were used for downstream analysis. The human oral microbiome database (HOMD) was used as a reference database for OTU assignment.[372]

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.[442] 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 [376], 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) [377], 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. 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 ± 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. III-1A, p=0.037). Moreover, a similar trend was observed at 7 years of age in children developing asthma (Fig. III-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. III-1C). However, children developing asthma tended to have higher bacterial richness at 12 and 24 months (Fig. III-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.

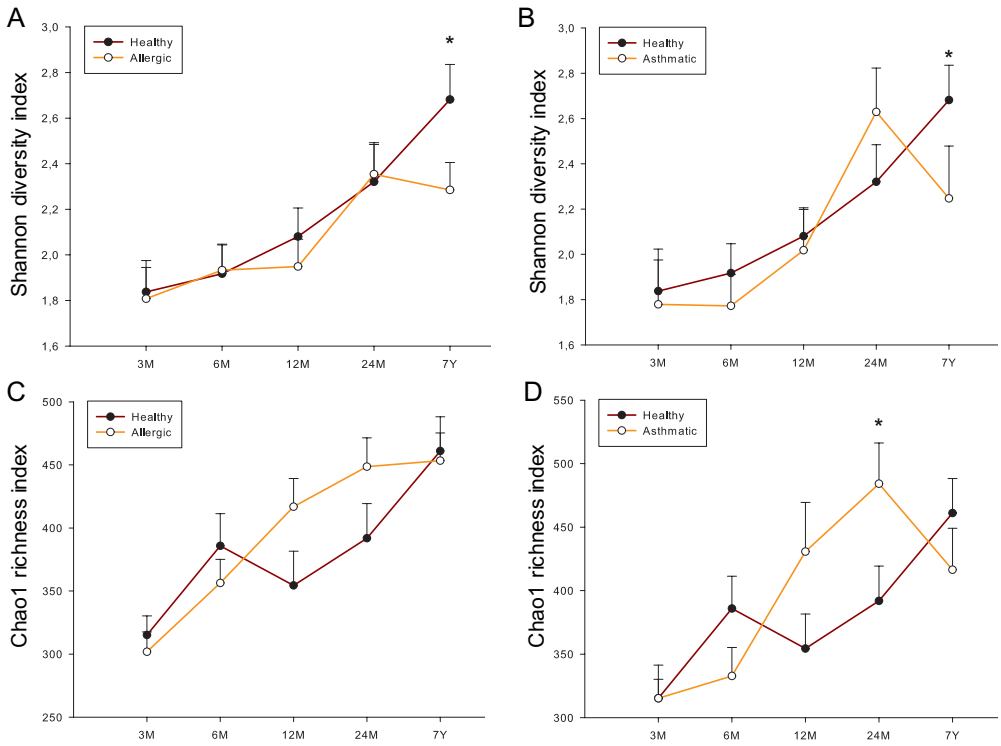

Fig. III-1. Species richness and diversity of the total microbiota in infant saliva samples of children developing allergies and children staying healthy up to 7 years of age. Bacterial richness and diversity (here presented by Chao1 and Shannon estimate indices at OTU level), obtained at different time 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 ($N_{Healthy}$=28; $N_{Allergic}$=36; $N_{Asthmatic}$=20), 6 months ($N_{Healthy}$=31; $N_{Allergic}$=45; $N_{Asthmatic}$=20), 12 months ($N_{Healthy}$=27; $N_{Allergic}$=35; $N_{Asthmatic}$=16), 24 months ($N_{Healthy}$=25; $N_{Allergic}$=34; $N_{Asthmatic}$=15) and 7 years of age ($N_{Healthy}$=32; $N_{Allergic}$=40; $N_{Asthmatic}$=15).

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. III-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. III-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. III-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. III-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. III-2B).

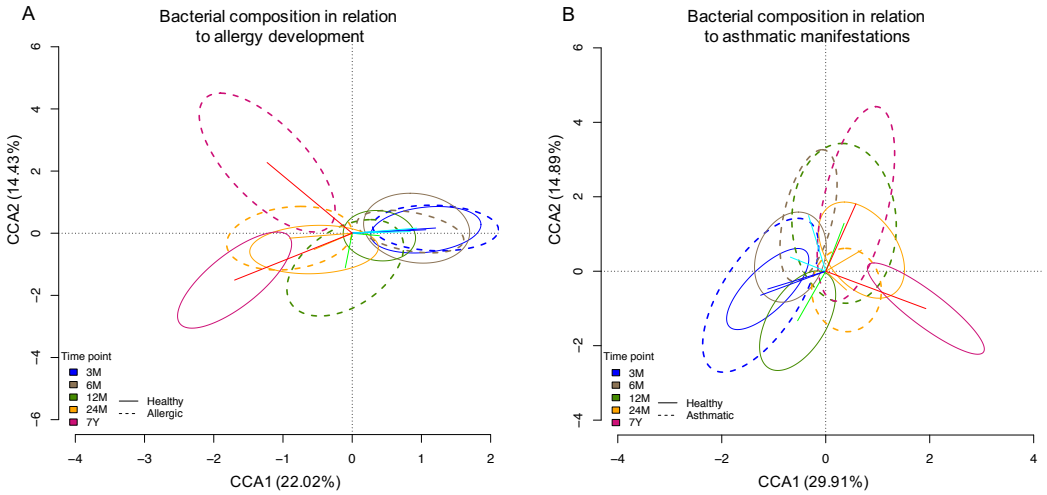

Fig. III-2. Salivary microbiota colonization patterns in children developing allergies and children staying healthy up to 7 years of age. Constrained correspondence analyses (CCA), here used to emphasize variations in microbiota species-level patterns, show compositional characteristics of total microbiota 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 colors 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 ($N_{Healthy}$=28; $N_{Allergic}$=36; $N_{Asthmatic}$=20), 6 months ($N_{Healthy}$=31; $N_{Allergic}$=45; $N_{Asthmatic}$=20), 12 months ($N_{Healthy}$=27; $N_{Allergic}$=35; $N_{Asthmatic}$=16), 24 months ($N_{Healthy}$=25; $N_{Allergic}$=34; $N_{Asthmatic}$=15); 7 years of age ($N_{Healthy}$=32; $N_{Allergic}$=40; $N_{Asthmatic}$=15).

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. III-3A), *Lactobacillus*, *Alloprevotella*, *Corynebacterium*, *Selenomonas* and *Eubacterium* at 6 months (Fig. III-3B) and *Lactobacillus*, *Selenomonas*, *Veillonella*, *Megasphera*, *Fusobacterium* and *Lachnoanaerobaculum* at 7 years (Fig. III-3C). Genera that were associated with allergy development were *Bacteroides* at 3 months and *Gemella* at 7 years (Fig. III-3A, 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. III-4A) and *Staphylococcus* at 24 months of age (Fig. III-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. III-4B) and *Fusobacterium*, *Capnocytophaga*, *Lactobacillus* and *Streptococcaceae* at 7 years of age (Fig. III-4C).

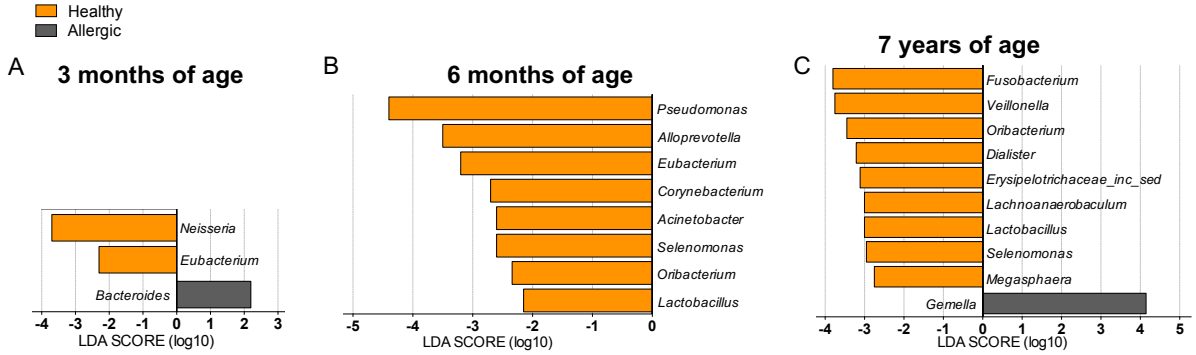

Fig. III-3. Salivary bacterial genera associated with allergy development during the first 7 years of age. The plots show statistically significant genera associated with allergy development at **(A)** 3 months, **(B)** 6 months and 7 years of age **(C)**. The LEfSe algorithm was used for biomarker discovery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

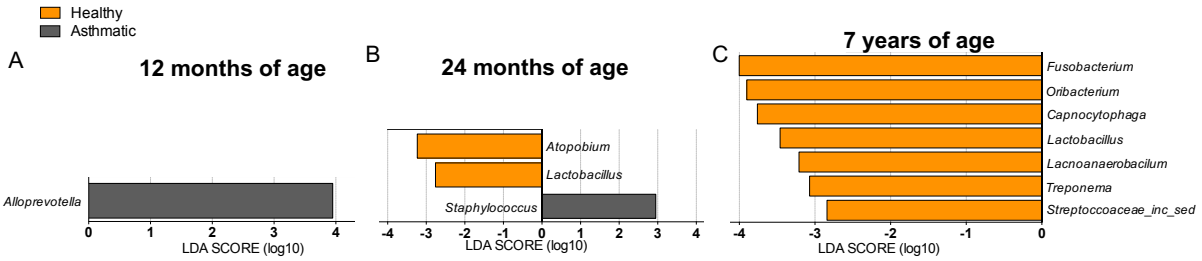

Fig. III-4. Salivary bacterial genera associated with asthma development during the first 7 years of life. **(A)** At 12 months of age only the genus *Alloprevotella* was increased in abundance in children developing allergies. **(B)** Genera associated with children developing allergies (grey) and children 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 ($N_{Healthy}$=27; $N_{Asthmatic}$=16), 24 months ($N_{Healthy}$=25; $N_{Asthmatic}$=15) and 7 years of age ($N_{Healthy}$=32; $N_{Asthmatic}$=15).

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. III-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. III-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. III-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. III-5C, D). At 7 years of age (Fig. III-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.*

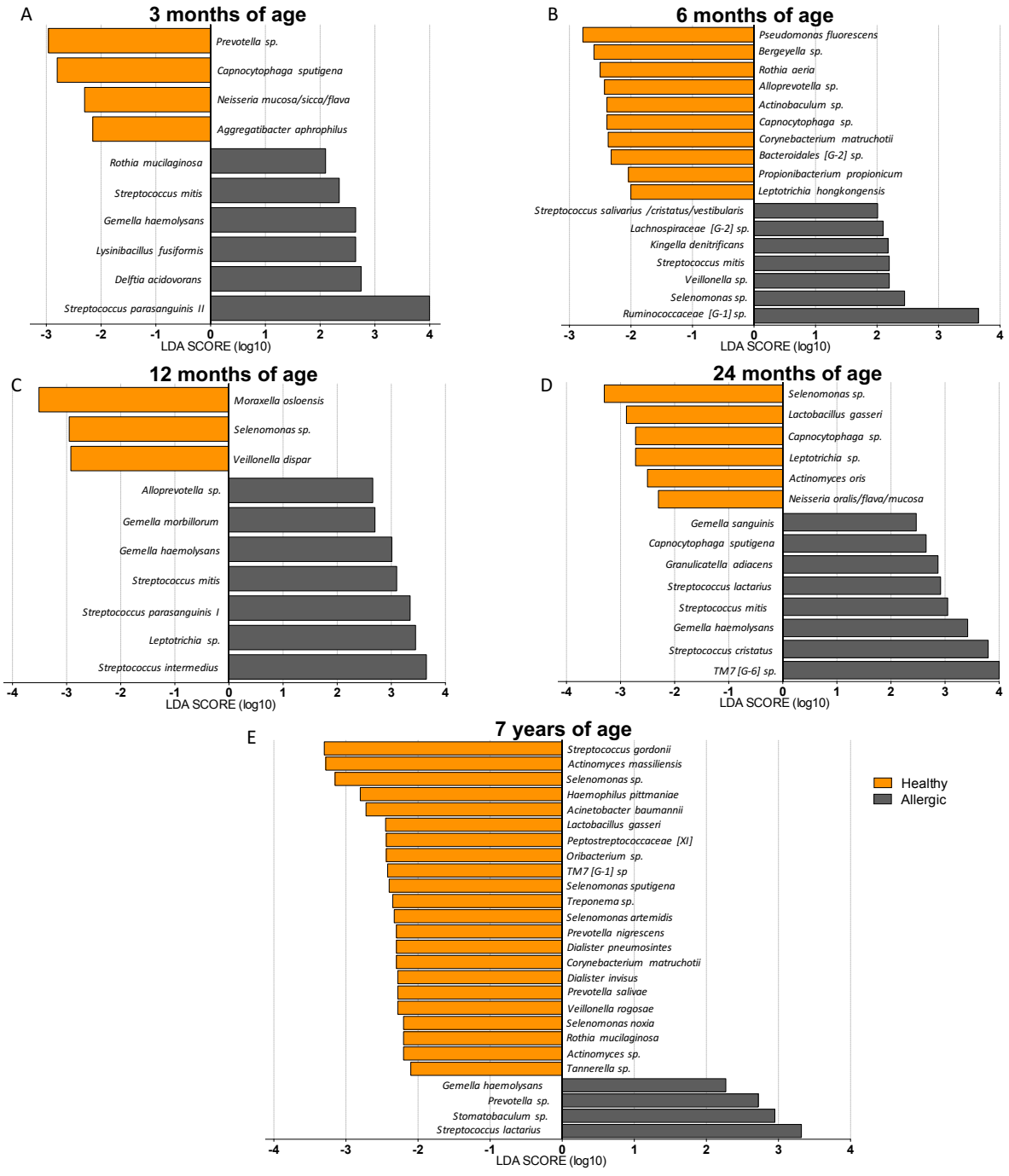

Fig. III-5. Salivary bacterial OTUs associated with allergy development during the first 7 years of life. Bars represent bacterial species at 3 **(A)**, 6 **(B)**, 12 **(C)** and 24 **(D)** months, and at 7 years **(E)** of age increased in abundance in children developing allergies (grey) and children 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 ($N_{Healthy}$=28; $N_{Allergic}$=36), 6 months ($N_{Healthy}$=31; $N_{Allergic}$=45), 12 months ($N_{Healthy}$=27; $N_{Allergic}$=35), 24 months ($N_{Healthy}$=25; $N_{Allergic}$=34) and 7 years of age ($N_{Healthy}$=32; $N_{Allergic}$=40).

It was also of interest to compare microbial biomarkers between children staying healthy and children developing asthmatic symptoms (Fig. III-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.

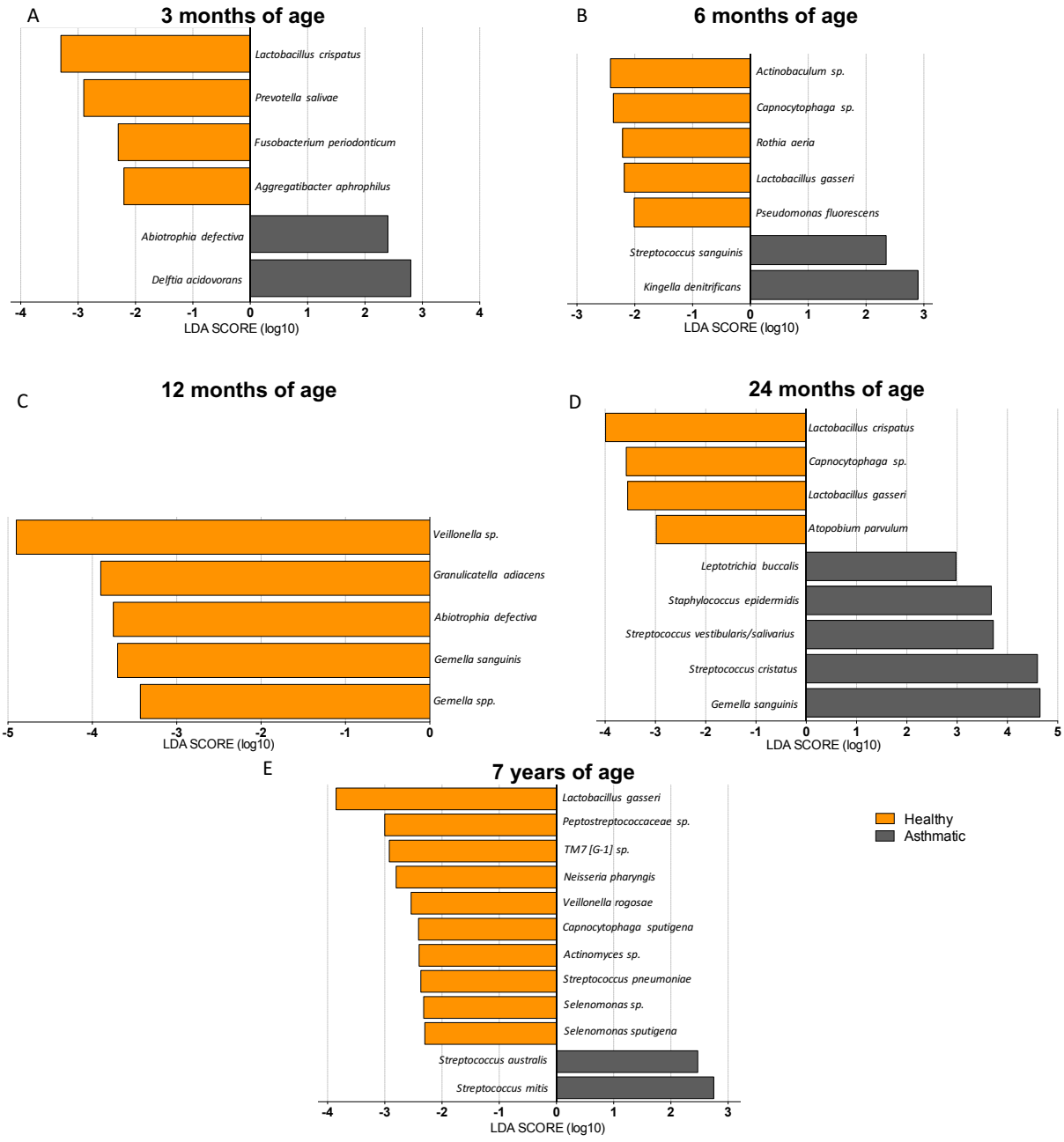

Fig. III-6. Salivary bacterial OTUs associated with asthma development during the first 7 years of life. Bars show bacterial species at 3 **(A)**, 6 **(B)**, 12 **(C)** and 24 **(D)** months and 7 years **(E)** of age increased in abundance in children developing asthmatic symptoms (grey) and children 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 ($N_{Healthy}$=28; $N_{Asthmatic}$=20), 6 months ($N_{Healthy}$=31; $N_{Asthmatic}$=20), 12 months ($N_{Healthy}$=27; $N_{Asthmatic}$=16), 24 months ($N_{Healthy}$=25; $N_{Asthmatic}$=15) and 7 years of age ($N_{Healthy}$=32; $N_{Asthmatic}$=15).

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 characterize 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 [168, 265, 266], 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.[48] 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.[11, 264] They provide many beneficial effects to the host, including breakdown of complex dietary carbohydrates and modulation of mucosal glycosylation, and immune maturation.[478] However, *Bacteroides* is not a common oral inhabitant under healthy circumstances.[11] 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*[479], possibly leading to aberrant immune development. *Gemella* is a predominant genus of the mucosal epithelium [11, 480] 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[481], 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.[48] Most oral streptococci are commensal, frequently acquired during breastfeeding [19], although some are known to cause infective endocarditis when disseminated through the blood stream.[358] We observed that *S. parasanguinis*, a member of viridans streptococci [482], and *S. lactarius*, belonging to the *S. mitis* group [389], 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 [480] and *S. lactarius* isolated from breastmilk of healthy women.[405] 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.[475] 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 [483], tongue, oral mucosa and dental plaque.[480] 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.[484] Members of this genus have been shown to utilize carbohydrates and metabolize lactate and acetate, likely maintaining pH homeostasis in a healthy oral biofilm.[403]

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 [55], and vagina [485], and may promote health by their influence on biofilm microbial composition, or by stimulating the host immune responses.[263] *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 [486], and it has also been evaluated as a possible treatment of allergic rhinitis [487],

demonstrating that supplementation with *L. gasseri* may be beneficial because of its effect on nasal blockage [487], and decreased nasal clinical symptoms scores in children suffering from allergic rhinitis.[488] As *L. crispatus* and *L. gasseri* were also observed to suppress allergic responses [489] and reduce mite-induced airway inflammation and hyperresponsiveness in mice models [490], 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 [13], and several studies have found associations between these factors and allergy development.[263] 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 III-I), 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. III-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 [317], associated with reduced incidence of IgE-associated eczema in infancy.[327] *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.[491] 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.

CONCLUSIONS

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.

Chapter III- Supplementary Information

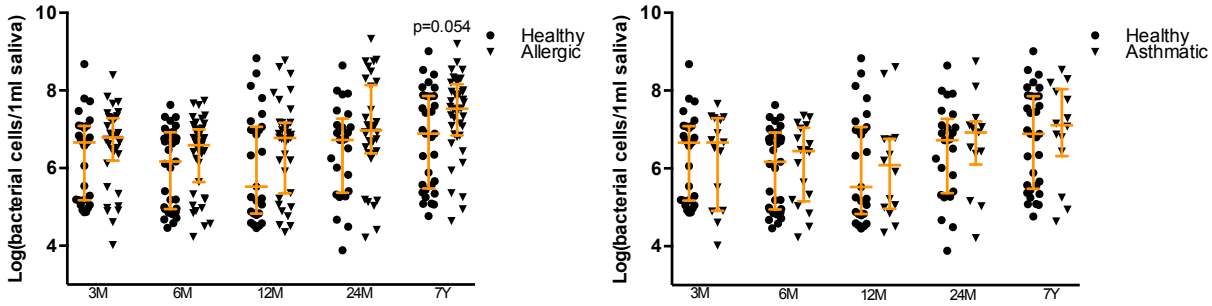

Figure III-S1. Bacterial load in infant saliva samples. Bacterial density in salivary samples, obtained at different time points until 7 years of age, was determined by qPCR using universal primers targeting the 16S bacterial gene. Figures show number of bacterial cells/ml in allergic **(A)** and asthmatic 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 ($N_{Healthy}$=28; $N_{Allergic}$=36; $N_{Asthmatic}$=20), 6 months ($N_{Healthy}$=31; $N_{Allergic}$=45; $N_{Asthmatic}$=20), 12 months ($N_{Healthy}$=27; $N_{Allergic}$=35; $N_{Asthmatic}$=16), 24 months ($N_{Healthy}$=25; $N_{Allergic}$=34; $N_{Asthmatic}$=15) and 7 years of age ($N_{Healthy}$=32; $N_{Allergic}$=40; $N_{Asthmatic}$=15).

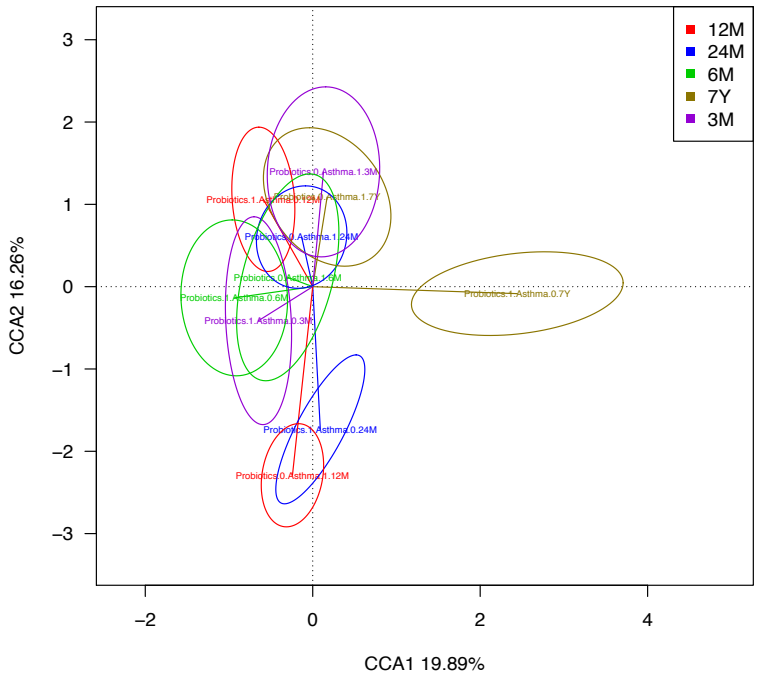

Figure III-S2. The effect of probiotic supplementation with *Lactobacillus reuteri* on the oral microbiota composition in relation to asthma development. Constrained correspondence analyses (CCA), here used to emphasize variations in microbiota species-level patterns, show compositional characteristics 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 ($N_{Probiotics}$=14; $N_{Placebo}$=9), 6 months ($N_{Probiotcs}$=16; $N_{Placebo}$=12), 12 months ($N_{Probiotcs}$=13; $N_{Placebo}$=7), 24 months ($N_{Probiotics}$=10; $N_{Placebo}$=8); 7 years of age ($N_{Probiotics}$=15; $N_{Placebo}$=10).

Chapter IV – RELATIONSHIP BETWEEN BREASTMILK IgA-COATED MICROBIOTA, PROBIOTIC SUPPLEMENTATION AND ALLERGY DEVELOPMENT IN CHILDHOOD

- Majda Dzidic, Alex Mira, Alejandro Artacho, Thomas R. Abrahamsson, Maria C. Jenmalm, Maria Carmen Collado; Relationship between breastmilk IgA-coated microbiota, probiotic supplementation and allergy development in childhood; *Submitted to Pediatric Allergy and Immunology*, June 2019.

Abstract

Early colonization with a diverse microbiota seems to play a crucial role for appropriate immune maturation during childhood, and breastmilk microbiota is one important source of microbes for the infant, transferred together with maternal IgA antibodies. We previously observed that allergy development during childhood was associated with aberrant IgA responses to the gut microbiota already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breastfed infants. Here we aimed to determine the microbial composition and IgA-coated bacteria in breastmilk in relation to allergy development in children participating in an intervention trial with pre- and postnatal *Lactobacillus reuteri* supplementation. A combination of flow cytometric cell sorting and 16S rRNA gene sequencing was used to characterize the bacterial recognition patterns by IgA in breastmilk samples collected one-month *post partum* from 40 mothers whose children did or did not develop allergic and asthmatic symptoms during the first 7 years of age. The milk fed to children developing allergic manifestations had significantly lower bacterial richness, when compared to the milk given to children that remained healthy. Probiotic treatment influenced the breastmilk microbiota composition. The proportions of IgA-bound bacteria and the total bacterial load were similar in breastmilk between mothers of healthy children and those developing allergies, but the pattern of IgA-coated bacteria tended to appear different. Consumption of breastmilk with a reduced microbial richness in the first month of life may play an important role in allergy development during childhood.

Introduction

The infant's immune system is immature at birth, with reduced innate and adaptive effector cell functions, including limited early production of secretory IgA, an antibody playing a crucial role in maintaining mucosal homeostasis.[492] In addition to learn to appropriately eliminate infectious or foreign stimuli, it is essential that the neonatal immune system develops the ability to distinguish between harmless and potentially dangerous antigens, thus inducing tolerance to prevent development of allergic reactions, autoimmunity, and inflammatory bowel disorders.[64]

Human breastmilk, with its evolving and complex composition of nutrients and bioactive compounds, is considered to be an optimal nutritional source for the immature immune system of the infant.[200, 493] Among the bioactive factors, breastmilk contains various immunological substances, including immunoglobulins, cytokines, chemokines and growth factors that can be transferred to the offspring through breastfeeding.[193] Breastmilk antibodies are believed to reflect the numerous pathogens which the mother has had contact with in the gut and airways, thus providing an important defense against the same pathogens likely encountered by her infant.[196, 197] Although all immunoglobulin isotypes can be encountered in breastmilk, secretory IgA (SIgA) is the dominating isotype and considered most important due to its anti-inflammatory properties and important role in defending the mucous membranes.[198] SIgA acts locally on the newborn's mucosal surfaces, thus regulating the binding and penetration of commensals and pathogenic microorganisms.[196]

Breastmilk hosts a diverse array of microbiota and potential probiotic bacteria that are believed to orchestrate the infant's developing mucosal immune system.[64] Culture-independent techniques, including sequencing and metagenomics analysis, have demonstrated a complex and diverse group of bacteria in human milk, including the presence of *Staphylococcus, Lactobacillus, Pseudomonas, Streptococcus*, and *Acinetobacter,* with large degree of individual-specific profiles.[18, 19, 67, 68] In addition to these microbes, prebiotic oligosaccharides are also present.[494] Human breastmilk thus contributes directly to the colonization of the infant intestinal microbiome[18, 65, 72, 74], and multiple studies have observed the sharing of several microbial strains between breastmilk and infant stool.[78–80]

Allergic diseases have become a major public health problem in affluent societies.[417] The increasing prevalence of allergy is hypothesized to be caused by reduced intensity and diversity of microbial stimulation.[57, 263, 495] In support of this theory, the gut microbiota differs in composition and diversity during the first months of life in children who later do or do not develop allergic disease.[42, 57, 265, 266, 275] The breastmilk microbiota is one important source of microbes for the infant [18, 65, 496], and is transferred together with maternal IgA antibodies.[264] The secreted antibodies in breastmilk likely shield the neonate from inadequate stimulation of its own mucosal immune system and may thus shape the composition of its acquired microbiota that is important for appropriate immune system

maturation.[107, 197] SIgA can also limit overgrowth of selected species, thus stimulating development of a diverse microbiota.[110, 423]

We have observed aberrant IgA responses to the gut microbiota during the first year of life in children developing allergies and asthma.[474] Allergy development during childhood was associated with divergent patterns of IgA recognized bacteria in the gut already at 1 month of age, when the IgA antibodies are predominantly maternally derived in breast-fed children.[474] However, the identities of the bacterial taxa targeted by IgA in the breastmilk and what role they may play in immune and allergy development are unknown. In this study, we aimed to characterize the composition and IgA coating pattern of the breastmilk microbiota from mothers whose children developed allergic symptoms during early childhood or stayed healthy.

Methods

Study design

The subjects included in this study were part of a larger randomized double-blind trial in South-eastern Sweden, recruiting participants between 2001 and 2003, where the potential allergy preventive effect of probiotic *Lactobacillus reuteri* ATCC 55730 in the infants with family history of allergic disease was evaluated.[327, 328] The mothers were supplemented with *L. reuteri* during pregnancy from postmenstrual week 36+0 to delivery and the infants from day 1-2 of life until 12 months of age. Among the 184 mothers, out of 232 recruited, of children that completed the 7-year follow up in the original study, breastmilk samples from 24 mothers whose children did not and from 26 mothers whose children did develop allergic disease during early childhood, were randomly selected for flow cytometry based-sorting of IgA-coated bacteria in the current study. Subsequently, 16S rRNA gene characterization was performed on the IgA-coated and IgA-free fractions of breastmilk bacteria from 20 mothers whose children did not and from 20 mothers whose children did develop allergic manifestations as well as from total, non-sorted breastmilk samples from the same mothers. Selection of the samples used for 16S rRNA sequencing in this study, was based on the sample availability and a clear allergy diagnosis (based on proven symptoms to allergy provocation) of the child. There were no differences regarding potential confounders, such as sex, mode of delivery, birth order, maternal atopy, breastfeeding, antibiotics, and probiotic supplementation, between the infants who did or did not have allergic manifestations. Allergic disease included eczema, gastrointestinal allergy, asthma, allergic rhinoconjunctivis and allergic urticaria. The criteria of these diagnoses are described in detail in [327, 328]. All children with allergic disease in the current study were also sensitized (*i.e.* they had at least one positive skin prick test (evaluated at 6, 12 and 24 months and 7 years of age) and/or detectable circulating allergen specific-IgE antibodies (assessed at 6, 12 and 24 months), while the healthy children were non-sensitized.

Breastmilk samples were collected one-month *post partum* by the mother at home. They were immediately placed in the freezer and brought to the hospital and stored at −70°C within 3 days. All the children included in the current study were exclusively breastfed during the first month of life. Total IgA levels were measured by ELISA in a study by Böttcher *et al.*[497]

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.

Table IV-I. Descriptive data of children compare in this study.

Children	Healthy (% [no.]) n=20	Developing allergic disease (% [no.]) n=20	P value*	Developing asthma (% [no.]) n=10	P value*
Girls	50(10)	60(12)	0.53	60(6)	0.71
Older siblings	40(8)	35(7)	1.00	30(3)	0.70
Caesarean delivery	20(4)	20(4)	1.00	20(2)	1.00
Furred pets	15(3)	10(2)	1.00	10(1)	1.00
Breastfeeding					
1 month exclusive	90(18)	95(19)	1.00	90(9)	1.00
3 months exclusive	85(17)	70(14)	0.45	50(5)	0.08
12 months partial	20(4)	25(5)	1.00	0(0)	0.27
Maternal atopy	85(17)	80(16)	1.00	70(7)	0.37
Antibiotic treatment (1-12 m)	35(7)	30(6)	1.00	50(5)	0.46
Day care (1-12 m)	10(2)	5(1)	1.00	10(1)	1.00
Probiotics-*L. reuteri*	45(9)	55(11)	0.53	60(6)	0.70

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

Sample preparation and flow cytometry-based sorting

After thawing, the breastmilk fatty layer and whey were removed by centrifugations. The resulting sample fraction was further suspended in sterile saline solution (autoclaved H_2O; NaCl Sodium Chloride 99.5% PA-ACS-ISO; Panreac, Barcelona, Spain; reference 131689.1211) with 5% BSA (Sigma-Aldrich, St Louis, Mo; reference A7030-100gr) to prevent nonspecific antibody binding. The samples were stained with goat anti-mouse IgA labelled with fluorescein isothiocyanate (FITC), used as an isotype control corresponding to unspecific binding (Sigma; reference SLBD9273), or with goat anti-human IgA labelled with FITC (Life Technologies; reference A18782), according to the manufacturer's instructions. The sorting of the bacterial cells according to whether they were IgA-coated or IgA-free was performed with the MoFlo XDP Cell Sorter (Beckman Coulter, Brea, Calif), according to the procedures of Simon- Soro *et al.*[413]

DNA Extraction

DNA from sorted breastmilk bacteria, both IgA+ and IgA-, as well as the total milk sample (2 ml) was isolated by using the MasterPure complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, Wis), according to the manufacturer's instructions, with a previous glass bead beating (0.17 mm in diameter) and an additional enzymatic lysis step with lysozyme (20 mg/mL, 378C, 30 minutes; Thermo-mixer comfort, Eppendorf, Hamburg, Germany), lysostaphin (2000 units/mg protein, 37 °C, 60 min; Sigma-Aldrich, Madrid, Spain) and mutanolysin (4000 units/mg protein, 37 °C, 60 min; Sigma-Aldrich).

16S rDNA gene amplification and sequencing

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

An Illumina amplicon library was performed following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part #15044223 Rev. A). The gene-specific primer sequences used in this protocol were selected from Klindworth *et al.* [367], and target the 16S rRNA gene V3 and V4 regions, resulting in a single amplicon of approximately 460 bp. After 16S rRNA gene amplification, the DNA was sequenced on a MiSeq Sequencer according to manufacturer's instructions (Illumina) using the 2 × 300 bp paired-end protocol. Sequences supporting the conclusions of this article are publicly available at European Nucleotide Archive database (ENA) with accession number PRJEB30065.

Total bacterial load

Total bacterial load (number of bacterial cells per ml breastmilk) was measured by quantitative PCR. Amplifications were performed in duplicates on a LightCycler 480 Real-Time PCR System (Roche Technologies) by using annealing temperatures of 60 °C. Each reaction mixture of 10mL was composed of SYBR Green PCR Master Mix (Roche), 0.5 mL of the specific primer (concentration 10 mmol/L), and 2 mL of DNA template. The universal forward and reverse primers were 5′-CGTGCCAGCAGCCGCGG-3′ and 5′- TGGACTACCAGGGTATCTAATCCTG-3′, targeting a 293bp long region of the bacterial 16S rRNA gene. The obtained Ct values were transformed in bacterial cell numbers by a calibrated standard curve.[183]

Bioinformatics

The PRINSEQ program was used for a sequence quality assessment.[368] Sequences of <250 nucleotides in length were discarded; sequence end-trimming was performed by cutting out nucleotides with a mean quality of <30 in 20-bp windows. Chimeric 16S sequences were filtered out using USEARCH program.[369] OTUs were built at 97% of identity by using vsearch program and Qiime modules (version qiime2-2017.12) were used for taxonomic annotation.[498] In order to assign taxonomy up to species level to each OTU's centroid, we classified them using a naïve bayes classifier model previously fitted against the Green Genes database version 13.5.

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

α-diversity analyses (presented here as Shannon and Chao1 indices), were utilized to estimate the samples' diversity and richness at the 97% OTU level using the R-package Vegan.[375] Constrained correspondence analysis (CCA) was used here to emphasize variation and bring out strong patterns in the dataset. This analysis was performed by R software ade4 package together with permutational multivariate analysis (Adonis) determining the differences in variance between groups.

For analyzing IgA coating patterns, the threshold used for including the genera was 0.5 % or greater in relative abundance in either the IgA positive or IgA negative fractions. A pseudocount that was equal to 0.001 was added to every genus dedicated in both the IgA positive or IgA negative fractions, thus avoiding the fractions with a value of zero. The abundance proportions of a given genera were log-transformed before calculating the ratio between the IgA positive or IgA negative fractions, resulting in the IgA index.[140, 474] The IgA index (calculated according the formula log(IgA+/IgA-)) score reflects the degree of mucosal immune responsiveness to the microbiota, where the positive values represent the genera predominantly found IgA-coated while the negative values the bacterial genera predominantly found IgA uncoated.

The MaAslin multivariate statistical framework was used in this study in order to evaluate if the confounding factors, including probiotics supplementation during pregnancy and maternal atopy, could influence microbial community abundance.[499] Statistical analyses were performed in R version 3.2.2 and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA, Version 6.1f), where $p<0.05$ was

considered significant. Specific statistical tests (including Mann–Whitney U-test/ Wilcoxon rank-sum test for nonparametric comparisons together with false discovery rate control giving the adjusted p-value) are stated in figure legends.

Results

IgA proportions in breastmilk

On average, approximately 40% of bacteria in breastmilk appeared to be IgA-coated. Proportions of IgA-coated bacteria were similar in breastmilk samples of mothers whose children did or did not develop allergic (Fig. IV-1A) and asthmatic symptoms (Fig. IV-1B) during the first 7 years of life. Furthermore, IgA proportions observed did not seem to be influenced by the total IgA levels in breastmilk samples of these mothers (n=29; Spearman correlation test r=0.32, p=0.095).

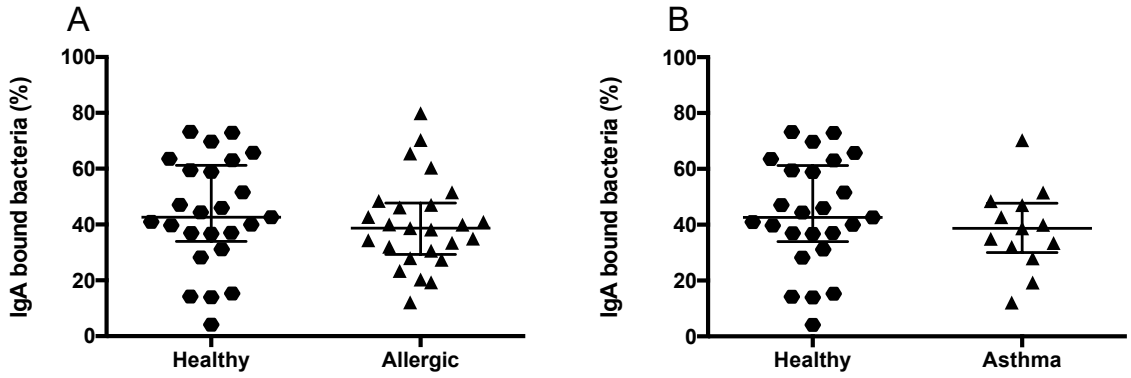

Fig. IV-1. IgA responses towards breastmilk microbiota in samples collected one-month *post partum*. Proportion of breastmilk IgA-bound bacteria (here presented as medians and interquartile ranges) in mothers whose children stayed healthy (N=24, circles) or developed allergic symptoms (N=26, **A**) and/or asthmatic symptoms (N=13, **B**) during the first 7 years of life.

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

The overall species richness (as determined by Chao1 index) in total non-sorted breastmilk samples was significantly higher (p=0.02, Fig. IV-2A) in mothers with healthy children, although the bacterial load (Fig. IV-S1) and species diversity (Shannon index, Fig. IV-2B) were similar in breastmilk samples of mothers whose children did/did not develop allergic manifestations. The total species richness also tended to be higher in breastmilk from mothers whose children stayed healthy than from mothers whose children developed asthmatic symptoms (p=0.066, Fig. IV-2A). However, no significant differences between mothers of healthy and allergic subjects were observed upon comparing the richness and diversity of the IgA-bound fractions in breastmilk (Table IV-SI). Additionally, no differences in bacterial load, species diversity or richness was observed between the total non-sorted breastmilk from the mothers treated with probiotics and placebo (data not shown).

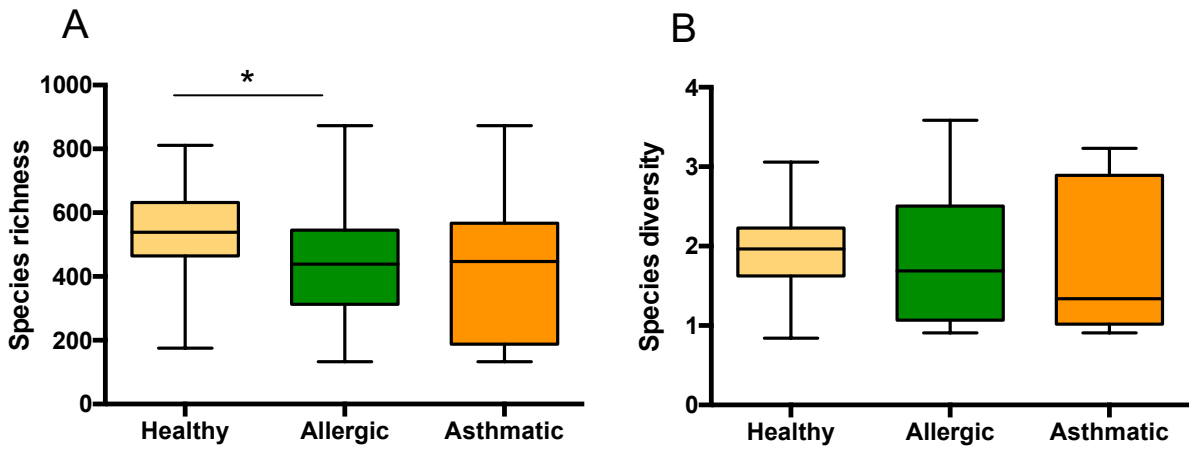

Fig. IV-2. Bacterial richness **(A)** and diversity **(B)** at OTU's species level, as described with Chao1 and Shannon indices, respectively, of the total non-sorted breastmilk samples from mothers whose children stayed healthy up to 7 years of age (N=20), mothers whose children developed allergic manifestations (N=20) and/or asthmatic symptoms (N=10). Medians and interquartile ranges are indicated. * p-value <0.05, Mann-Whitney U test.

Bacterial composition in allergy development and probiotic supplementation

Bacterial 16S rRNA gene sequencing, of the total non-sorted milk and IgA-coated/uncoated fractions, was performed in order to determine the milk microbial composition and to assess IgA responses towards specific bacteria. After quality filtering and removal of chimeric sequences, 40 total non-sorted breastmilk samples and 80 IgA separated fractions remained with 2,000,107 and 1,525,770 high quality reads, respectively. Sequencing of total non-sorted breastmilk samples resulted in an average of 48,987±2725 (SEM) reads per sample, while the IgA separated breastmilk fractions had an average of 20,322±1660 (SEM) sequence reads per sample.

The relative abundance of genera in total breastmilk, i.e. non-sorted samples, is presented in Fig. IV-3A and 3B. Infants developing allergies during the first 7 years of life appeared to have higher, although not statistically significant, abundance of the genera *Enterococcus* (p=0.01; adj. p-value=0.18; Fig. IV-S2A) and *Pseudomonas* (p=0.01; adj. p-value=0.18; Fig. IV-S2B). The genus *Enterococcus* was found in significantly higher abundance (p=0.001; adj. p-value= 0.02) in breastmilk given to children that developed asthmatic symptoms, when compared to breastmilk given to the children that remained healthy (Fig. IV-3B, Fig. IV-S2A). No effect of the confounding factors, such as probiotic supplementation during pregnancy, maternal atopy, sex and the delivery mode influenced the microbial abundance between the groups, as determined with the MaAslin multivariate statistical logarithm.

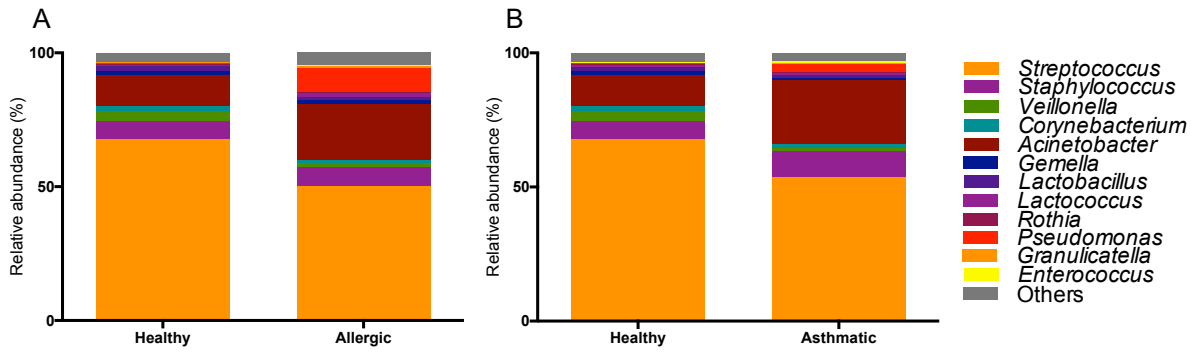

Fig. IV-3. Microbiota composition of most dominant bacterial genera in total non-sorted milk samples. **(A)** The relative abundance (>0.5 % of the total) of dominant bacterial genera in 20 mothers whose children stayed healthy and in 20 mothers whose children developed allergic manifestation during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

Probiotic supplementation during the last month of pregnancy were associated with reduced proportions of IgA-coated bacteria (p=0.04; Fig. IV-4A), and particularly in breastmilk of probiotic supplemented mothers whose children stayed healthy (p=0.02; Fig. IV-4B). Moreover, microbiota composition patterns, of total non-sorted breastmilk, were significantly different (p=0.04 Adonis testing) between mothers that received, or not, probiotic supplementation (Fig. IV-4C) with significantly increased relative abundance (p=0.03, Wilcoxon rank-sum test adjusted p-value) of the genus *Rothia* in mothers treated with placebo. However, upon comparing IgA pattern recognitions of bacteria in mothers who were treated, or not, with probiotics supplementation during the last month of the pregnancy, no statistically significantly different IgA responses could be observed for bacterial genera presented (Fig. IV-4D).

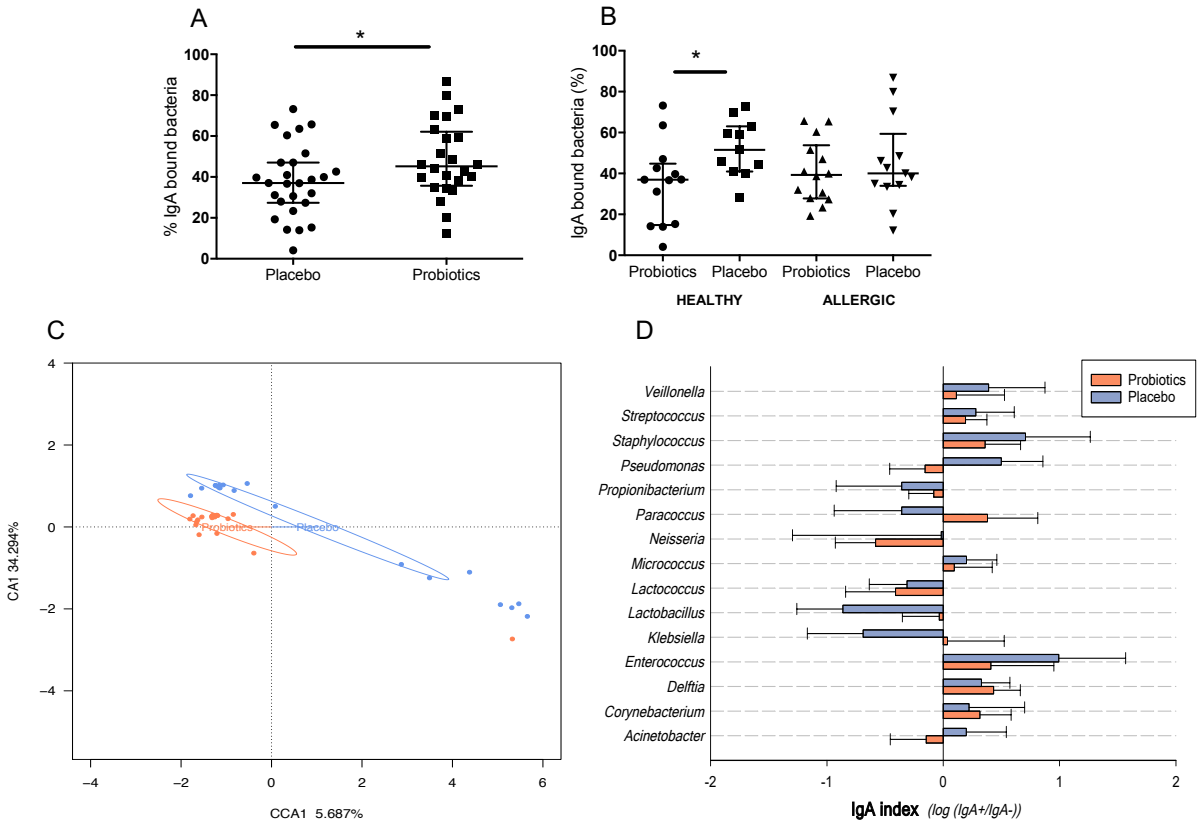

Fig. IV-4. IgA-coating responses in breastmilk of mothers treated, or not, with probiotics. Breastmilk microbiota composition and IgA-coating patterns of the dominant genera (>0.5% of total), in probiotic supplementation. **(A)** Proportions of IgA bound bacteria in breastmilk (medians and interquartile ranges) of mothers treated with *L. reuteri* (N=24) or placebo (N=27). **(B)** Proportions of IgA-bound bacteria in breastmilk of mothers, treated with probiotics or not, whose children stayed healthy (p=0.02, $N_{Placebo}$=11, $N_{Probiotics}$=13) or developed allergic diseases (nPlacebo=13, nProbiotics=14). *p<0.05. **(C)** Constrained correspondence analyses (CCA) based on microbiota patterns in breastmilk of mothers treated with probiotics *L. reuteri* or placebo. The percentage of variation explained by constrained correspondence components is indicated on the axes. p value for CCA plots were determined by Adonis (p=0.04) and indicate if the factor provided (in this case probiotics) can significantly explain data variability. **(D)** Plots represent IgA coating patterns (defined by IgA index, reflecting the ratio in IgA bound and IgA free breastmilk microbiota) to dominant genera in breastmilk samples collected at one month *post partum* from mothers whose were treated with probiotic *L. reuteri* or placebo. For a given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA bound fraction, to negative values (genera found dominantly in the IgA free fraction). $N_{Probiotics}$=20, $N_{Placebo}$=20.

IgA responses towards milk microbiota in allergy development

Although the analysis of the relative abundance of dominant bacterial genera in breastmilk, and the composition patterns of sorted IgA fractions (Fig. IV-S3) were generally similar between the IgA-bound and IgA-free fractions, upon considering the health status of the children, some differences at genus level were observed when analyzing the bacterial targets of IgA responses, represented as the IgA index here. The value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA positive fraction, to negative values (genera found dominantly in the IgA negative fraction). Breastmilk given to children that developed allergic symptoms had higher levels of IgA-coating, although with non-

significant tendencies, for genera *Rothia Acinetobacter, Granulicatella* and *Veillonella*. On the contrary, milk fed to children that stayed healthy appeared to have greater IgA coating of *Pseudomonas*, although the results were statistically non-significant (Fig. IV-5A).

Regarding the breastmilk given to children that developed asthmatic symptoms, the genera *Acinetobacter*, *Rothia, Granulicatella* and *Veillonella* tended to be predominantly found as IgA-bound, when compared to the milk given to children that remained healthy (Fig. IV-5B). On the contrary, *Veillonella* and *Rothia* were predominantly IgA-free in breastmilk fed to children that stayed healthy until 7 years of age (Fig. IV-5B).

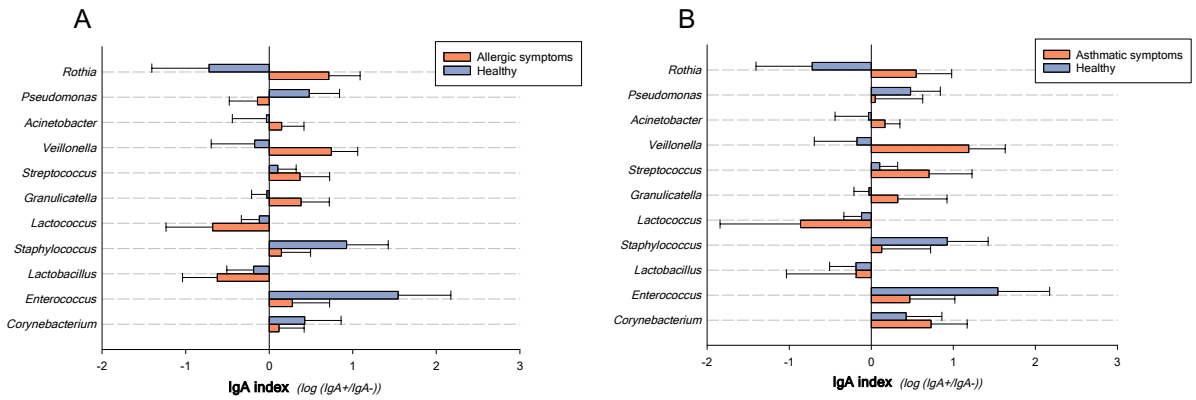

Fig. IV-5. IgA responses towards breastmilk microbiota. Plots represent IgA-coating patterns to dominant genera (>0.5 % of total) from mothers whose children stay healthy (N= 20) compared to children that develop allergic (**A**, N=20) or asthmatic (**B**, N=10) symptoms. For a given genera, the value of the IgA index can range from positive values, reflecting genera found dominantly in the IgA-bound fraction, to negative values (genera found dominantly in the IgA-free fraction). Means with SEs are indicated.

Discussion

The data presented in the current study demonstrate that total non-sorted breastmilk from mothers whose children developed allergic symptoms during early childhood had lower bacterial richness, when compared to milk fed to children staying healthy. While no differences in bacterial composition of the total non-sorted milk samples, nor the IgA proportions between the mothers whose children develop allergies or not could be observed, IgA-coating patterns of several bacterial genera, tended to be different. Additionally, IgA proportions observed were not influenced by the total IgA levels in breastmilk samples of these mothers.[497]

The influence of breastmilk composition on later allergy development appears to be linked to higher richness of bacterial species and not to the relative abundance of specific bacteria. For instance, intestinal bacteria can stimulate the formation of intestinal blood vessels, B cell development in Peyer's Patches and increase the production of mucosal IgA.[95, 500] Previously, low total diversity of the gut and oral microbiota have been associated with atopy and asthma development during early childhood.[265–267, 421, 501] Moreover, aberrant IgA immune responses towards gut microbiota were observed as early as 1 month *post partum*, in exclusively breastfed children who subsequently developed allergies.[474] At this time point, the IgA antibodies are predominantly maternally derived in exclusively breastfed children, as the levels of endogenously produced IgA in the baby during this period is limited.[58, 426] Therefore, any divergent responses observed at this time suggest that immunological interactions between mother and infant may play an important role in subsequent immune development.

Streptococcus, *Acinetobacter*, *Staphylococcus* and *Veillonella* were the most commonly found bacterial genera in milk samples used in this study. Furthermore, lactic acid bacteria *Lactococcus*, *Lactobacillus* and *Enterococcus* as well as oral inhabitant *Gemella* were also detected, in agreement with previous reports.[19, 21, 73] Various studies have demonstrated that there is a mother-to-infant transfer of bacterial genera including *Lactobacillus*, *Staphylococcus*, *Enterococcus* and *Bifidobacterium* through breastfeeding.[78–80, 88, 502–504] The constant intake during lactation of breastmilk bacteria leads to the establishment of an intestinal microbiota that deeply impacts on the newborn's immune maturation.[60, 496]. An interesting finding is the significantly higher levels of the lactic acid bacteria *Enterococcus* in breastmilk fed to children developing asthma. Work from other authors have shown that *Enterococcus* is a common inhabitant of breastmilk microbiota and one of the first microbes to colonize the infant gut after birth[265, 505], being more abundant in the gut of atopic infants, from the same cohort, at 12 months of age.[265] Moreover, *Enterococcus faecalis has been shown to* regulate inflammatory responses in the host.[506] However, whether our finding here reflects an overgrowth of Enterococcus due to the absence of competing species, or whether Enterococcus is suppressing the growth of allergy protecting bacteria remains to be addressed.

Independently of the original source of bacteria, breastmilk, with its hundreds of bacterial genera, influences the colonization of infant gastrointestinal tract. It has been proposed that exposure of the breastfed infant to a variety of bacterial antigens may favor the infant's protection against gastrointestinal and respiratory infections.[21, 179, 507] Furthermore, breastfed newborns have been shown to possess a more stable intestinal bacterial population and controlled mucosal immune response as compared to formula fed neonates.[188, 508]

An important function of SIgA is immune exclusion, a mechanism where this antibody binds to commensals, through its recognition of multiple antigenic epitopes on the surface, encountered in gut lumen and prevents their attachment to mucosal barrier.[197] IgA coating patterns appeared to be different, although mainly not statistically significant, between mothers having allergic and healthy children in this study. Typical oral inhabitants, including *Streptococcus*, *Veillonella*, *Rothia* and *Granulicatella* [386], appeared to be more IgA-bound in the allergic or asthmatic samples compared to the healthy group. This contrasted with the higher levels of IgA-free *Lactococcus* and *Lactobacilli* in the healthy group (Figure 4). However, these differences were not statistically significant and larger studies are needed in order to evaluate possible distinctive IgA-coating patterns. Also, the full function of IgA coating is still not completely understood. An interesting hypothesis is that the IgA binding does not necessarily only cover neutralizing function and exclusion of bacteria but also that this antibody can promote bacterial adhesion to the mucosa, as shown *in vitro* and *in vivo* [108, 144, 145], thus enriching for the growth of particular microbial strains.[430] In this study, we were not able to observe significant differences in the patterns of IgA-binding in breastmilk fed to children staying healthy and children developing allergies but the possibility that the true differences are at the lower bacterial resolution (for instance species and even different strains), should not be excluded. The fact that the majority of the mothers included in this study, and also in the original study of this cohort, suffered from allergic disease, should be considered as it also may affect both the IgA-coating patterns and the microbiota transferred to the child.

IgA-coating proportions of breastmilk bacteria were higher in mothers treated with placebo, compared to those treated with probiotic *L. reuteri*. As the mothers were treated with probiotics until delivery, likely the changes in the microbial composition were more significant in colostrum than in breastmilk samples extracted at one month *post partum*, as in the present study. We have previously shown that Lactobacilli colonization was significantly increased in colostrum of the mothers treated with *L. reuteri*, but not in samples obtained one month *post partum*.[330] This could in turn suggest that part of the mother´s IgA responses, while treated with probiotics, were directed towards *L. reuteri*, but that the ending of probiotic supplementation was reflected in diminished targeting of this bacterial species and decreased proportions of IgA-bound microbiota. However, this requires to be address further in larger studies. Additionally, microbiota patterns of total non-sorted milk were also significantly different between mothers treated with probiotics and those with placebo, with the genus *Rothia* significantly increased in the placebo group. This further confirms that the probiotic supplementation during pregnancy has an effect on mother´ breastmilk

microbiota that in turn may aid transfer of probiotic bacteria at delivery and during infancy, along with immunomodulatory antibodies [264], to the infant. However, results obtained here needs to be further confirmed in larger cohorts and the role of *Rothia*, needs to be further evaluated. *Rothia*, a common oral inhabitant associated with good oral health, is an important nitrate reducer that in combination with other nitrate reducing microbes has been shown to give rise to nitric oxide, which is involved in vasodilation in cardiovascular system and inflammation in different tissues.[509, 510]

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

CONCLUSIONS

Consumption of breastmilk with a reduced microbial richness in the first month of life correlates with the risk for allergy development during childhood. In addition, our data show that probiotic supplementation during pregnancy alters the breastmilk microbiota composition and the proportion of IgA coated bacteria. This opens the possibility of modulating breastmilk microbiota and its interaction with antibodies as a strategy to promote a healthy microbial colonization with the purpose of reducing allergy risk.

Chapter IV – Supplementary Information

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

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

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

***** Mann-Whitney U test.

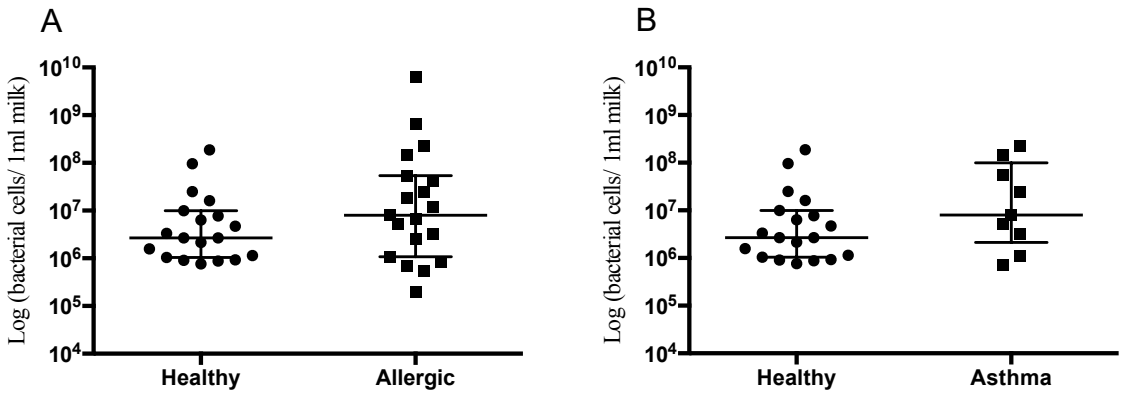

Fig. IV-S1. Bacterial load in breastmilk collected at one month *post partum*. Quantification of bacterial numbers was obtained by using qPCR detection with universal primers targeting the 16S rRNA bacterial gene. $N_{Healthy}$=19; and $N_{Allergic}$= 19; $N_{Asthmatic}$ =9. Media with interquartile ranges are indicated.

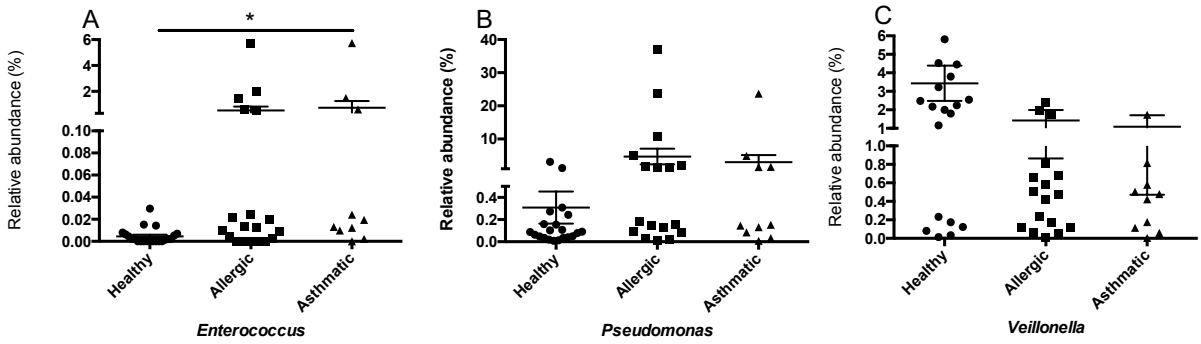

Fig. IV-S2. Selected bacterial genera found in milk of mothers whose children stay healthy or develop allergic and/or asthmatic manifestations. Mean with SEM are presented. * $p < 0.05$, Mann-Whitney U test, FDR adjusted p-value.

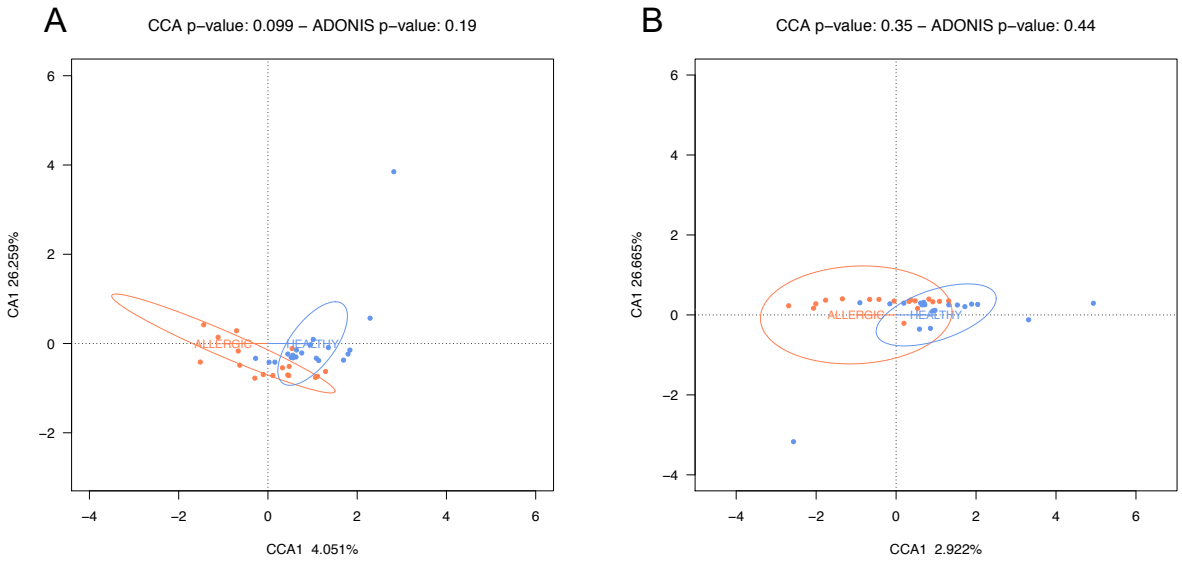

Fig. IV-S3. Breastmilk microbiota composition patterns of sorted IgA fractions, IgA-coated and IgA-free, in allergy development. Constrained correspondence analyses (CCA) based on breastmilk microbiota patterns of the dominant bacterial genera (>0.5% of total) coated with IgA **(A)** or not coated with IgA **(B)**, from mothers whose children did or did not develop allergic diseases.

GENERAL DISCUSSION

In this thesis, we have used longitudinally collected infant stool and oral samples in order to investigate the importance of microbiota maturation in relation to health and allergy development. We described that colonization of the oral cavity during early childhood is transitional, increasing in complexity with time, and several external factors appear to greatly influence oral microbiota maturation, having either a short or a long-term impact. We have also shown that early changes in oral microbial composition influence immune maturation and allergy development in childhood, and that the presence of specific bacterial species may be important for this progress. Furthermore, altered IgA responses towards the gut microbiota during infancy were detected to preceded asthma and allergy manifestations during the first 7 years of age, and we observed that consumption of breastmilk with a reduced microbial richness in the first month of life may play an important role in allergy development during childhood. However, there are some methodological aspects to consider in more detail that could introduce biases and errors in the interpretation of the data.

The Power (and Limitations) of High-throughput Sequencing

During the last decade, high-throughput next generation sequencing (NGS) technologies have contributed to a step change in the sequencing of human and bacterial genomes, among other organisms.[511] This has been received by the scientific community as a way to improve the limited classical culture techniques for describing complex microbial environments. A pioneer second-generation technique, 454 pyrosequencing, delivers longer read lengths, in comparison to Illumina technique, but lower coverage, as well as errors in homopolymeric tracts (i.e. difficulties to detect the actual number of bases in repetitive regions, for example AAAAA).[322, 512, 513] On the other side, MiSeq generates shorter reads but higher throughput coverage per run and lower error rate of the instrument (Table I). Also, when performing amplicon sequencing of the 16S rRNA gene, an overlap between the forward and reverse sequences can be obtained if a paired-ends strategy is used (as with Illumina in the current thesis), allowing for more stringent quality control.[513]

Vertical transmission of bacteria from the mother´s body and breastmilk to her offspring has gained attention as a significant source of early microbial colonization.[91, 171, 514] Therefore, it is of interest to study possible transmission of shared bacterial species between mother and the offspring. Moreover, assessment of bacterial composition between different niches, at comparable time-points, allows to obtain a global overview of colonization patterns and timing of bacterial settlement during childhood. In this

dissertation, both 454 pyrosequencing and Illumina MiSeq technologies were used in separated manuscripts, in order to describe composition of gut, oral and breastmilk microbiota in the same cohort of children. However, by sequencing two different regions of the 16S rRNA gene (V3-V4 and V3-V5 for Illumina and 454 pyrosequencing, respectively) of stool and breastmilk microbial communities, together with distinctive pre-sequencing amplification protocols (and PCR primers), we found it inaccurate to compare the findings between the studies. Nevertheless, upon characterization of the same microbial community samples, using an identical sequencing region and the same set of primers, both Illumina MiSeq and 454 pyrosequencing were shown to be reliable for quantitatively assessing genetic diversity, by revealing similar microbial operational taxonomic units.[512] Production of kits for 454 pyrosequencing was stopped by the manufacturing company during the course of this thesis, not allowing the use of the same sequencing platform for the different chapters. For future studies, in order to more accurately compare data between studies, it is required that the same region of 16S rRNA gene is targeted by preferably using the same sequencing platform and PCR primers, which would minimize differences in bacterial composition estimates due to methodological biases. Additional limitation was that the collection of breastmilk samples and first saliva samples in infants was performed at two different time-points (one month *post partum* and at three months of age, respectively). As it has been shown that human milk microbiota changes over lactation [91], we did not consider to search for potential shared bacteria.

Table I. Characteristics of 454 pyrosequencing and Illumina MiSeq sequencing platforms.

Sequencing method	Characteristics
454 pyrosequencing	• Read lenght: 400 - 800bp • Coverage: > 1 million reads/run) • Errors in homopolymeric tracts • Obsolet
Illumina MiSeq	• Read length: 250 - 300bp (400bp in paired-ends libraries) • Throughput coverage: > 25 million reads/run • Moderate cost per base sequences • Paired-ends strategy sequencing -> Stricter quality control • Quality diminishes with sequence length

16S ribosomal RNA NGS is a common sequencing method used to identify and compare microbial populations present within a given sample. 16S rRNA, encoded by a ~1,500 base pair gene [515, 516], is a widely used housekeeping genetic marker gene that is present in almost all bacteria with a function that has not changed over time, implying that random sequence alterations in the gene are an accurate measure of evolution.[517] The gene has several conserved segments, which are therefore used as targets where PCR and DNA sequencing primers anneal, and a highly variable region that serve as a species-specific mark for taxonomic purposes.[515] Thus, variations within the 16S rRNA sequences facilitate identification of bacteria at the genus [518] (and in many cases at the species [519]) level and, therefore,

the sequencing of this gene is an ideal tool for bacterial taxonomic studies.[520] Nevertheless, different organisms contain different 16S gene copy numbers, which might lead to biased read count estimates.[521]

Partial (approximately 500 base pair) 16S rRNA gene sequencing is a commonly used approach today to fast and accurately identify a wide variety of aerobic and anaerobic bacterial communities.[522–524] However, while a non-full length sequencing of 16S rRNA gene allows for rapid identification, it also limits the taxonomic resolution of the gene, as specific hypervariable regions dictate taxonomic coverage.[367] Commonly, 16S rRNA gene sequencing provides genus identification in >90% of the cases but an important limitation is its inability to discriminate among all bacterial species.[517] In the majority of cases this is due to recognition of taxa under-represented in databases, or to high similarity of 16S rRNA sequences between neighbouring species, such as the species within the *Streptococcus* genus that we discuss in chapter I.[517]

There is a general consensus for the definition of species boundaries via 16S rRNA gene sequencing and commonly the interval of within-species match includes 97-99% similarity (1-3% divergence).[439] However, some authors consider that this is not a standardized approach between studies within this field, as mentioned by Janda and colleagues [517], and these threshold values may not be sufficient in all instances to assure an accurate species identification, particularly in cases when only 30% of the total gene is targeted. In chapter I and III, where we described the oral microbiota at the species level (including the species within *Streptococcus* and *Lactobacillus* genera) we intended to avoid this problem by using 100% of similarity for species identification. However, we could observe that there were differences in only one base pair between closely related species, making it difficult to accurately distinguish between them. The length of the sequenced 16S rRNA reads in this case was approximately 400 bp and likely longer fragments, ideally >1,000 bp, would be more convenient for accurate species identification. This will be possible in the near future by the use of single-molecule, third generation sequencing such as PacBio (*Pacific Biosciences*) or Nanopore technologies (*Oxford Nanopore technologies*), which have longer sequencing reads spanning the full length of the 16S rRNA gene.[525]

Other concerns in 16S rRNA sequencing technologies involve DNA extraction methods, PCR amplification and possible chimeric molecule formation. All of these difficulties, to some extent, affect final bacterial identifications.[517] For all the studies presented in this thesis, an additional PCR amplification of extracted bacterial DNA was performed, in order to increase the bacterial yield prior to the sequencing. PCR amplification is not flawless since it may introduce both biases and new hybrid, or incorrect sequences, into the pool of amplified DNA molecules.[526] Commonly used PCR primers are designed to amplify many different 16S rRNA gene sequences, from as wide a range of organisms as possible. However, the use of different primers changes the inferred bacterial composition, due to the selective amplification of some bacterial groups.[527] In addition, careful selection of primers is needed in

order to, for instance, amplify bacteria with high CG-content (such as bifidobacteria) that generally might be underrepresented in 16S rRNA surveys of diversity due to poor primer matching.[434] Also, for adequate discrimination of bifidobacterial, V3-V5 sequencing region of 16S rRNA gene appears to be optimal.[434] Due to their beneficial role in shaping the gut microbiome of breastfed infants, bifidobacterial prevalence in infant's gut has been studies frequently. For instance, they appear to reduce inflammation, enhance gut mucosal barrier integrity and direct the immune system towards tolerance during weaning period, thus potentially influencing the development of allergic diseases.[528] Therefore, we used modified, bifidobacteria-universal primers in order to ensure effective detection of this taxa too.[434] Furthermore, PCR might also have a large impact on sequence representation after PCR amplification basically due to stochastic events. This means that in the cases when the sequences are present at very low copy numbers, stochastic amplification may have a significant impact on sequence representation.[529] However, we aimed to limit the abovementioned mentioned errors by keeping the cycles of the amplification process as low as possible and by maintaining a low annealing temperature in order to reduce amplification bias.[366] We also tried to improve the confidence of the data by advanced bioinformatics tools and quality filtering criteria in order to limit the errors, presence of chimeras or misassignment.

An important general limitation of this thesis is that we focused exclusively on explaining the bacterial taxonomic abundance of the samples, without exploring the potential function of the bacteria present. Indeed, mapping the microbial composition of the different habitats during the early childhood, before and after the development of different disease (in this case allergy), is crucial for reaching an overview of the target environment and establishing a foundation for further studies within this field. For establishing the repertoire of functions encoded by complex microbial communities, an alternative to 16S rRNA studies is direct shotgun metagenomic sequencing.[530] This type of pipeline would also be able to accurately track mother-to-infant vertical transmission of microbes, and its influence on post-infancy microbiome development, as shown by Ascnicar *et al.*[531] Also, characterization of gene expression profiles by transcriptomics methods may enhance our understanding of the core activities involved in disease conditions.[532] Inference of biological functions and potential pathways, based on the 16S composition, has also been suggested [533], but its accuracy may be limited by the enormous intra-specific genomic variability of the bacterial pan-genome.[534] Therefore, we consider the work presented here an important first step for future studies that will be able to answer more functional or mechanistic questions about the microbiota in health and allergy. The significance of the 16S rRNA data presented here for understanding potential interactions with the immune system is stronger when combined with flow cytometry based-cell sorting, as described in the following section.

Combining Fluorescence Activated Cell Sorting and 16S rRNA Sequencing

In chapters II and IV of this dissertation, fluorescence activated cell sorting was used in combination with 16S rRNA sequencing as a purpose to characterize the IgA responses towards the gut and milk microbiota in relation to allergy development. This type of approach has been used in several other reports up to date [451, 452, 535–537], and it will likely become increasingly common in future studies of the microbiota and its interactions with the host.

Our data clearly illustrate the utility of this methodology as a way to functionally categorize intestinal/breastmilk bacteria based on their interactions with and recognition by the host immune system. However, it is important to mention that the fluorescence activated cell sorting may introduce contaminants in the system (including the contamination with environmental bacteria and other contaminants present in the instrument) which could bias the results. This was mainly problematic in breastmilk samples, which had lower bacterial load in comparison to stool samples, and several typical environmental contaminants in both IgA coated and non-coated fractions were detected here.[538, 539] This problem was limited by sequencing the whole breastmilk sample (beside the IgA+/- fractions), and by using powerful bioinformatic tools in order to eliminate the contaminants. Cell sorting appeared to reduce bacterial diversity and richness (see chapter IV), when compared to unsorted breastmilk samples, which is logical since only the extreme IgA-coated/non-coated populations (mainly IgA coated or mainly not IgA coated, as detected with the fluorescence) were collected upon sorting. The bacterial populations sorted have, most likely, the expected IgA-status; however, there is a risk that a fraction of targeted cells (mainly those coated with IgA) are not collected due to restrictive gating strategies. Nevertheless, the same procedure is done between all the samples, thus making the data obtained comparable.

An alternative method to fluorescent activated cell sorting is the magnetic bead-based separation of immunoglobulin - recognized bacteria.[540] In this approach, anti-human immunoglobulin-coated beads are incubated with biological samples in order to separate immunoglobulin-coated bacterial cells from non-coated ones, which resemble the method described above. Compared to FACS, and beside the fact that this approach needs less preparation in form of proper calibration and a dedicated machine operator, this methodology may reduce the risk of contamination that can be obtained upon separation with the FACS instrument, although appropriate protocols still need to be developed. While the samples are commonly fixed with paraformaldehyde prior to FACS, thus avoiding the contamination in the system, the magnetic bead-based separation (or Magnetic Activated Cell Sorting) would allow to maintain the cells viable and use them for further analyses, including bacterial isolation and other *in vitro* and *in vivo* model experiments. However, the bead-based separation method is not a quantitative method (e.g. not giving any information about the proportions of coated/non-coated bacteria) and it does not provide a real-time image of the sorted fractions. Moreover, it is difficult to predict the appropriate cellular load/density of the samples in order to avoid saturation of the system.

In conclusion, fluorescence activated cell sorting is a powerful and reliable method for separating (bacterial) cells according to a specific immunological status (like IgA coating in this thesis) in samples with medium and high microbial load. However, this approach may cause contamination, causing biases (with subsequent PCR and sequencing methodology), particularly in the case of samples with low bacterial density, although this might be avoided by the use of appropriate controls. In addition to the information about the proportions of IgA coating immune responses towards microbiota, bacterial load quantification, as described in the following section, can explain if the IgA-coated proportions detected partly depend on the bacterial density of the samples investigated.

Bacterial Load Measurements

In general, most of the studies published nowadays are focusing on bacterial composition while bacterial load (the density of microorganisms in a sample) in different habitats, its levels under health-disease conditions or how external factors influence it, are still understudied. The information of total bacterial numbers could be useful in understanding bacterial behavior at the interface of host immunity, but also for establishing the standard levels in healthy conditions and compare them with alterations that may occur during infectious and inflammatory disorders. For instance, increased bacterial load in bovine and human milk has been positively associated with clinical signs of mastitis [541–543], an inflammation of the breast tissue that commonly occurs during the time of breastfeeding. Moreover, increased bacterial density has also been associated in airway inflammation in human patients with chronic bronchitis [544, 545] and similarly with the severity of ileal Crohn's disease.[546] In order to better understand the progress of bacterial density through the children's age, the bacterial load (defined as bacterial cells/ml saliva) in the oral cavity has been measured in two different studies within this thesis. Oral development, presented in the chapters I and III, was accompanied by a steady increase in oral bacterial load from 3 months to 7 years of age, probably reflecting the influence of environmental interactions and the emergence of teeth. While there were no statistically significant differences between children developing allergies/asthmatic symptoms and children staying healthy, children developing allergic disease tended to have higher levels of bacterial load at 7 years of age ($p=0.054$). The patterns observed could not be attributed to the confounding factors studied (including sex, probiotics and antibiotics intake, breastfeeding habits *etc.*) and future studies should focus on whether the physicochemical properties of saliva and other potentially influencing factors, including treatment with asthma medication and oral hygiene habits, may influence cell density.

We detected that gut bacterial load decreased with age, with significantly higher levels at 1 month than at 12 months of age. Furthermore, children developing allergic diseases had significantly lower

bacterial density at 12 months of age, when compared to healthy children. However, the bacterial load at 1 month of age showed the opposite, although not significant, pattern with higher density in children developing allergic symptoms. Likely, the differences observed at this age were influenced by breastmilk as all the children included in this study were exclusively breastfed until 1 month of age. Moreover, while the bacterial load in healthy children appeared stable from 1 to 12 months of age, children developing allergic manifestations had significantly higher bacterial load at 1 month of age when compared to 12 months of age. We further observed the bacterial levels in the breastmilk of corresponding mothers, at one month post pregnancy (chapter IV). Although no statistically significant differences could be observed, bacterial levels appeared to be higher in breastmilk of mothers whose children developed allergic, and asthmatic, symptoms during childhood. This might explain the higher levels of gut bacterial density, at 1 month of age, in children developing allergic diseases (chapter II). Perhaps, bacterial transmission through breastfeeding might be important for the differences observed, and larger studies are necessary to evaluate this further.

Traditional culture-dependent methods (measurements of the colony forming units (CFU)/ml samples) for determining bacterial density are known to underestimate the bacterial diversity simply because all bacteria are not culturable with current methods and growth media.[547] During the last decades, real time quantitative PCR (qPCR), in combination with 16S rRNA primers with broad interspecies specificity, have been frequently used as a reliable molecular technique to describe bacterial numbers in complex communities.[19, 548–552] However, an important limitation is the varying number of 16S rRNA operons in any given bacterial species that may bias the total bacteria numbers.[553] The use of a single-copy protein coding gene, such as fusA [554], would tackle this problem, making it a more accurate target for bacterial load estimations compared to the 16S rRNA gene.[19] However, these genes are not as widely used and tested as the 16S rRNA gene and they might underestimate the load of some bacterial taxa.[553] Another solution that we applied in our studies was the use of specific standard curves for each particular type of biological sample where bacterial load was quantified. The standard curves consisted of a combination of the most common bacteria encountered in a pool of representative samples (for instance breastmilk or saliva samples). Shortly, bacterial isolates obtained by traditional microbiological methods were sorted and quantified by a MoFlo XDP cytometer. Standard curves were then generated by using serial 10-fold dilutions of DNA extracted from a known number of sorted bacteria.[19, 413]

No statistically significant differences of bacterial density in the oral cavity, considering the allergy status, could be detected in the current thesis, indicating that this feature does not have an early predictive value. It should be born in mind that we are investigating the microbial composition and density before the allergic symptoms appeared. If changes in bacterial load are produced later in life or at the time of inflammation should be studied in the future.

In conclusion, quantification of bacterial load in biological samples is a useful method to estimate the bacterial density both under normal as well as infectious conditions, thus providing an overall image of the alterations that might occur in different inflammatory diseases. Moreover, determination of bacterial levels by qPCR is a great complement to 16S rRNA composition analysis, as it provides the information about bacterial cells/ml samples, and this approach has been extensively used in the studies presented in this thesis. We assume that the bacterial load measurements should be performed at the time of an inflammation, and not as part of a predictive analysis, where this approach could be used as a screening tool to detect problems rapidly, for instance in situations of nursing mothers suffering from mastitis. However, the methodology has various caveats (for instance the viability of bacteria is not taken into account) that should be controlled with traditional microbiological procedures and other molecular techniques. Additionally, the selection of representative samples is important and will be discussed in the following section.

Microbiota Beyond the Gut

Sample selection for representing different habitats

Over the past decade, microbiota studies have been an attractive topic in the biomedical field. The findings describing bacterial composition have been carried out in both healthy subjects and patients affected with different disorders, exploring various microbial habitats. However, accurate analysis of microbial composition requires appropriate selection of representative samples, where a worthy example is provided by gut microbiota studies. The gut microbiota is probably the most complex bacterial community in the human body [555], playing an important contributing role in nutrients uptake and development of the immune system, among many other functions.[556, 557] The majority of gut microbiota studies, including the study presented in the chapter II of this thesis, are based on the examination of stool samples, simply because they are easily collected in a non-invasive manner. However, the microbial composition of stool samples reflect mainly luminal colonic microbiota, and not necessarily bacterial communities located in the small intestine, where a major part of the gut immune system is located.[9, 558, 559] Even though our observations were limited to stool samples, we were able to detect differences in IgA responses towards gut microbiota between children staying healthy and children developing allergic manifestations. Nevertheless, we are not excluding the possibility that a more severe dysbiosis might be occurring in the small intestine and this deserves to be evaluated further.

Similarly, it is important to consider that the bacterial communities can vary between different collection sites in the oral cavity [560] and both culture and molecular based approaches have shown that the tongue, teeth, mucosa, palate, and gingiva harbor a distinctive composition.[386] In the chapters I and III of this thesis, saliva samples were used to address the maturation of microbial communities in the oral cavity of

children through age. Several studies have reported that saliva serves as the optimal oral compartment to detect microbial differences in various human diseases, as it reveals the closest true representative microbiota in the oral cavity.[561–563] However, in chapter III, where we evaluate the importance of oral microbiota maturation for allergy and asthma development during childhood, additional analyses of buccal mucosa would be of a great interest as they would likely reflect the microbial communities located in the near proximity to the host immune system.

Overall, it may be said that the oral and gastrointestinal tract microbiome represent the major part of the overall human microbial load, thus providing unique perspectives for advancing human health prognosis, diagnosis, and therapy development. Most of the studies published today are focusing on the importance of the gut microbiota for allergy and asthma development but since the oral cavity is the first site of encounter between the bacteria and the host immune system, it is plausible to believe that oral microbiota plays a significant role in early establishment of microbial communities and subsequent allergic disease development. Another strength of the studies presented here, particularly in the case of the oral microbiota, included the blinded, controlled design with a long-term, closely monitored follow-up of a birth cohort, longitudinal sample collection and well-documented clinical records, which is still uncommon in many pediatric studies. However, in the future, it would be ideal to expand the study size to children without an allergy family history and confirm if our observations in Swedish children can be translated to children in other regions of the world. Moreover, the role of other parts of microbiota, including skin, lung and nasopharyngeal niches, in allergy development during childhood should be further addressed.

Other important microbiota niches

We have exclusively focused on describing the bacterial compartments of oral, gut and breastmilk microbiota but the human microbiota consists also of other organisms as archaea, viruses, and fungi that build a highly complex network of interactions between each other and the host.[564] However, the members of these microbial community have rarely been assessed up to date. Filamentous fungi and yeasts are common members of the gut microbiota [565] and their role in the development of allergic airway disease has been reported in murine models. Manipulation of the mycobiota (*i.e.* fungal microbiota), resulted in increased severity of murine lung inflammation and specific fungal species overgrowth in the gut.[566] When it comes to human studies, the importance of fungal gut dysbiosis for the development of atopic wheeze in babies, has been reported in few studies.[277, 567] For instance, high relative abundance of *Candida* and *Rhodotorula*, together with decreased abundance of *Bifidobacterium*, *Akkermansia* and *Faecalibacterium* have been associated with neonates with the highest risk to develop atopy and asthma.[277] It is also known that during the first 3 months of life, fungi are present at a significantly higher diversity than later in life [277], which is likely due to elevated transmission or growth promotion

through breastmilk.[568] However, now that the bacterial composition has been widely mapped, the importance of fungi (as well as viruses and archaea) for the early establishment of the infant microbiota and immune system development needs to be further evaluated. Taxonomic composition of archaea and fungi, however, can also be studied by amplicon sequencing of rRNA genes or the fungal ITS region.[569, 570]

Microbiota and Immune Maturation During Childhood

Microbial colonization of the gut and oral cavity

Early microbial colonization of the neonate exerts a major effect on host future health status. The selective growth and timing of early colonizing pioneers, is determined by various factor, including breastfeeding.[571] Microbial colonization observed in the gut and oral cavity in the studies presented here, was likely influenced by breastfeeding as the majority of the children were exclusively breastfed during the first month of age. Therefore, the colonization pattern might reflect the bacteria encountered in the breastmilk of the mothers and be influenced by the presence of HMOs, which may act as prebiotics favouring the growth of specific microorganisms.[572] Deeper sequencing technologies, with higher resolution, are required for establishing the bacterial species transmission and stimulation through breastmilk.[531]

Generally, the *Streptococcus* genus was the primary, and the most abundant, commensal to colonize both the oral cavity at 3 months of age and the infant gut at 1 month of age. This genus, together with *Staphylococcus* and *Pseudomonas*, was also a main constituent of the breastmilk microbiota collected at 1 month of age, which has also been reported before.[19, 571, 573] *Streptococcus* is a facultative anaerobe that commonly colonizing the infant already during the vaginal delivery where, together with *Escherichia coli* and *Staphylococcus* species, they produce an environment that favor the thriving of strict anaerobes, including *Bacteroides* and *Bifidobacterium* spp. [574] Although we were not able to catch this transmission, as the stool samples were collected at 1 month of age, the families *Staphylococcaceae* and *Bacteroidaceae* were observed as dominant members of gut microbiota by this age. Moreover, *Veillonella*, a common oral commensal associated to acidic pH (it metabolizes organic acids such as lactate), was found to colonize both the gut microbiota at 1 month of age and the oral cavity at 3 months of age, probably due to transmission by breastmilk in these children. Similarly, a colonization of *Lactobacillus* in the oral cavity at 3 months of age was likely due to breastfeeding, as species belonging to this genus have been frequently isolated from breastmilk.[573]

In chapter II, we used stool samples, obtained at 1 and 12 months of age, to describe the microbial gut maturation during the first year of life. We observed that beside increased bacterial diversity and richness during this period, *Streptococcaceae*, that dominated during the first month of life, was later accompanied by the bacterial families *Lahnospiraceae* and *Ruminococcaceceae*. The *Lachnospiraceae* family, which includes the *Roseburia*, *Blautia* and *Lachnospiraceae incertae sedis* genera, is known to participate in the breakdown of carbohydrates into SCFAs [575] and increased abundance of this bacterial family has been associated with transition from milk-based feeding to solid foods-diet.[75] Moreover, these colonization events during the first years of age were also in agreement with an European study including infants from five different countries, demonstrating that consistent compositional changes characterized by an increase in *Ruminococcaceae* and *Lachnospiraceae* species (and decreased *Bifidobacteriaceae*, *Enterobacteriaceae* and *Clostridiaceae*) occurred with the introduction of solid foods [189], including dietary fibers and protein from cheese and meat.[576]

Oral development (chapter I), from 3 months to 7 years of age, was accompanied by a steady increase in diversity and richness of the oral microbiome in this study, especially between the first and second year of life. The abundance of bacterial genera varied through age, starting with early pioneers that were replaced by steady levels of constant colonizing bacteria (including *Gemella* and *Granulicatella)*, associated with emergence of the teeth and dental plaque formation. We also observed that there were several bacterial genera, here referred to as "late colonizers", that became dominant in the oral cavity at a later stage, approximately after the first year of life.

Early infant colonization sets the stage for the complex and more stable adult microbiome.[577] Generally, early commensals of both the oral cavity and the gut are likely having an ecological advantage over the bacteria arriving later [40], and their presence can contribute to environmental changes that benefit, but also limit, the growth of arriving bacterial communities. Moreover, these pioneers educate and stimulate the developing immune system, thus protecting the colonizing microbes against systemic immune responses [578], as discussed in the next section. The early process of microbial succession and increased diversity is likely influenced by several perinatal factors during the important window of opportunities.[61] As lactation ends and dentition begins, bacterial communities will adapt to handle solid foods which in turn will result in selection of bacterial populations with relevant metabolic activities, as we could observe in the oral microbiota. However, the real key features of colonizing microbiota, and inter-species interactions, deserve to be further evaluated, as the majority of the studies available focus on the descriptive composition of body sites. Do individuals respond to the same microorganism in the same way? Moreover, longitudinal sampling and prospective studies are crucial to describe important phenomena of the infant colonization process. These efforts will in turn help us to understand the alterations occurring from health to disease conditions and directs us towards discovering preventive interventions and therapies.

The effect of perinatal factors on oral microbiota

Delivery mode, breastfeeding and antibiotics use, among other factors, have been shown to influence the initial development and maturation of the neonatal microbiota [571], thus playing an important role in development of allergic diseases.[571, 579] We found that the overall bacterial composition development in the oral cavity was influenced by delivery mode, breastfeeding habits and antibiotics intake during the first years of age (chapter I). The impact of delivery mode was reflected in differences in oral bacteria composition at 3 and 6 months of age, while the effect disappeared at later time points. Hence, we assume that the delivery mode might have a short-term effect that is due to the direct transmission of initial bacteria from mother to newborn, favoring the establishment of specific pioneers. This would in turn regulate further settlement of specific bacterial species but, likely, the clear effect that we observe at early age will fade later due to the influence of other external factors and immunological events. It is also well known that early events occurring during critical window of initial microbial colonization and immune development, may have long-standing consequences on immune-related diseases, as allergies, during childhood.[235] The early differences detected here is in line with previous studies showing that microbial oral colonization in 3-month-old infants delivered vaginally and those delivered by C-section was different.[55] Similar findings of an early impact, but also more long-term effects [343, 380], have been reported for the microbiota of the lower gastrointestinal tract [171, 172], and increased incidence of allergic disease due to disruption of early bacterial colonization caused by C-section.[580–584] However, we did not observe any effect of the mode of delivery on microbial composition, in relation to allergy development, in any of the allergy-related studies presented in the thesis. The possibility that the differences were masked by low statistical power in C-section group of this cohort, should not be excluded. It has also been proposed that the human milk microbiota is shaped by mode of delivery [91], but this has been debated [20] and we were not able to detect any effect either.

The clinical data of the cohort used in this thesis allowed us to access the influence of partial breastfeeding, compared to no breastfeeding until 12 months of age, and antibiotics intake in early life (first and second year) on the developing oral microbiota. We identified that partial breastfeeding, as well as antibiotics intake, had a long-term effect on overall composition of oral microbiota at 2 and 7 years of age. We assume that the effect of breastfeeding observed at later age, and not in bacterial colonization at early age (3, 6 and 12 months) that appeared to be similar which, is due to the fact that the majority of the infants in this cohort were breastfed during their first months of life. Interestingly, partial breastfeeding was also associated with lower bacterial diversity through age, and particularly at 2 years of age, when compared to children which abandoned breastfeeding before 12 months of age. Similar finding have been reported in gut, where children not being breastfed had higher bacterial diversity [77, 163, 378], likely due to introduction of solid foods. As findings presented here suggest that variation in the initial oral

microbial communities may result in differences in the bacterial succession patterns that persist over time, further work should therefore address the impact of formula feeding on the microbiome.

We also detected that the repeated use of antibiotics (at least one treatment per year, during the first 2 years) during infancy was associated with significant increase in abundance of several bacterial species at 7 years of age, and many of them (including *Fusobacterium* and *Actinomyces*) has previously been associated with oral diseases (chapter I). It is important to mention that we lack the clinical follow-ups (additional antibiotics courses that have not been registered) and salivary samples between year 2 and 7 of age might affect the results observed here. However, given that the first year of age represents the crucial "*window of opportunity*" for microbial development and immune system regulation, we hypothesize that the changes observed here can have long-term consequences for microbiota development.

In conclusion, colonization and development of the oral microbiota in this longitudinal study is dynamic and follows an ecological succession influenced by delivery mode, breastfeeding duration and antibiotics courses, factors that together shape the microbial composition and have a crucial role in long-term health of the child. Our data suggest that this succession pattern may be critical for appropriate immune development and subsequent risk of allergic disease.

IgA-coating of Gut and Breastmilk Microbiota in Relation to Allergy Development

SIgA antibodies at the mucosal surfaces target and regulate pathogenic bacteria invasion, but they also target common resident commensal microbes that inhabit the epithelial mucosa or live free in suspension (such as in the gut lumen).[107] Although it has been suggested that this immunoglobulin influences microbiota composition during homeostasis, the exact importance of this immunoselection remains controversial and poorly understood. However, lack of IgA against specific bacterial epitopes has been shown to result in innate immune system activation in the intestine [141], confirming the importance of IgA for protection from mucosal inflammation.

Breastmilk counteracts early deficiency of IgA production in neonatal gut, as this antibody is the principal Ig (>90%) in human milk.[64] Endogenous IgA production is induced during the first month of life and increases during infancy, while the IgA concentration in breastmilk decreases with time.[585, 586] At one month of age, approximately 50% of stool microbiota was IgA-coated, while the proportions decreased significantly to 20% at 12 months of age. The proportions observed during the first month of age are likely reflecting both the endogenous production of IgA as well as IgA transmitted through breastmilk, compared to 12 months of age where the proportions are dependent on the infant's own production. These findings were further confirmed upon measuring SIgA levels in stool samples, by ELISA immune

assay, as the levels were three-fold higher at 1 month of age than at 12 months of age. Interestingly, the overall proportions of IgA-coated bacteria in breastmilk, collected one-month *post partum*, were comparable to the percentages of coated gut bacteria (≈40% *vs* 50%), indicating that breastmilk may be responsible for initial bacterial targeting with IgA. The slightly increased proportions observed in stool samples are likely reflecting the endogenous IgA production by infants. In the future, this could be further confirmed by studying IgA coating levels and IgA bound bacterial patterns in formula fed children, as those would not have any maternal contribution of IgA. As a pilot test, we tested IgA coating levels in a sample of a formula-fed 1-month old baby and observed that proportions of bacterial coating were extremely low (approximately 4%, Fig. 10). This confirms the importance of vertical transmission, from mother to offspring, of immunomodulatory components that may aid and direct early mucosal immunity development. Indeed, these findings should be further confirmed in larger studies.

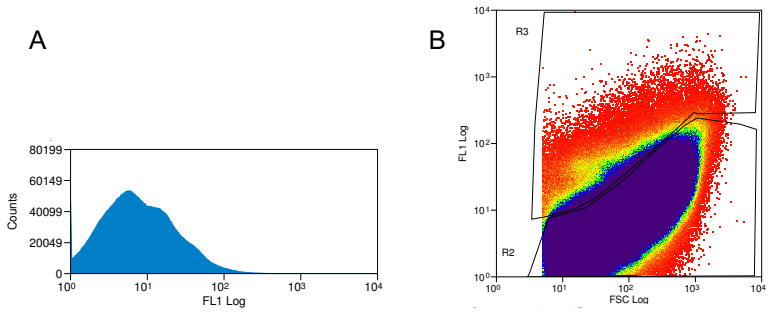

Fig. 10. Proportion of the stool bacteria bound to IgA or not at 1 month of age in a formula-fed infant. **(A)** The histogram shows the green fluorescence intensity on the horizontal axis and the number of events is shown on the vertical axis. **(B)** Scatter plot showing the proportion of IgA bound fecal bacteria where the Y-axis corresponds to the green fluorescence, which is related to the number of bacteria bound to IgA (here 3.98%), and the X-axis is showing the intensity of forward light scatter, correlating with the cell size.

Proportions of IgA-coating differed between healthy children and children that developed allergic manifestations later in life. Children that remained healthy during the first 7 years of age had significantly higher proportions of IgA-coated bacteria at 12 months age, compared to children developing allergic and asthmatic symptoms. However, no statistically significant differences could be observed at 1 month of age, which is consistent with the findings in IgA-coated breastmilk microbiota of corresponding mothers. Possibly, the initial transmission of IgA via breastmilk mainly provides a protective coating of the mucosal barrier, independently of allergy status, while the infant is developing its own nascent immune system, which plays a role in subsequent allergy development. This in turn implicates that additional factors, influencing during the first year of life, are important for an appropriate immune system development. Moreover, it is important to mention that the majority of mothers included in the study (80%) had been diagnosed with allergic disease, which could bias the results. Cohorts following children without a clinical history of allergic disease in the family would be important in this aspect, as well as additional stool

samples collected at earlier (for instance at 6 months of age) and later (around 2 or 3 years of age) time points, when IgA production resembles adult-like levels.[587]

An important question to address is the selectivity of the bacterial taxa targeted by IgA. Is IgA useful as a marker of potentially immunomodulatory taxa on mucosal surfaces? In a study by Palm and colleagues, 35 different bacterial species were selectively IgA-coated in patients with inflammatory bowel diseases and upon colonization of germ-free mice with this bacterial population, these animals were more prone to display signs of colitis, when compared to mice colonize with IgA-free taxa.[452] The selectivity can also depend on immune-location of IgA induction and secretion. Mouse experiments have shown that IgA responses are selectively focused on microorganisms located in the small intestine, as this antibody is dominantly produced there.[140] In contrast, bacteria found primarily in the colon are not major targets of IgA. In this way, IgA can protect the thin layer of small intestinal mucus from proinflammatory exposures, in contrast to thicker mucosal barrier of the colon that is less vulnerable.[100]

In the studies presented in this thesis, we observed that IgA recognition patterns of gut microbiota differed between healthy children and children with allergic symptoms, with divergent coating patterns already found at 1 month of age. For instance, two important human gut symbionts [453], *Faecalibacterium* and *Bacteroides,* were mainly IgA-free at 1 and 12 months of age in children with allergic manifestations but were predominantly IgA-coated in healthy children, which could reflect lower stimulation of the mucosal immune system in infants with allergic diseases. In contrast, allergy development was associated with increased IgA responses at 12 months of age to *Lachnospiraceae*, gram positive barrier associated microbes that colonize the inner mucus layer, staying in close contact with host mucosa. This might imply that increased IgA coating of these bacteria in children with allergies might be an indication of an altered mucosal barrier function.[464, 465] Even though we speculate that different IgA coating patterns of bacteria may play a distinctive role in mucosal barrier function, it is inevitable to doubt the actual function of IgA-targeting in health and allergy development. An interesting hypothesis is that the IgA-coating is not necessarily solely involved in neutralization of microorganisms and their exclusion but it can also endorse bacterial adhesion to the mucosa, as shown *in vitro* and *in vivo*.[108, 144, 145] As a consequence, IgA and gut microbes co-colonize the epithelial outer mucus, thus enriching for the growth of particular microbial strains.[145, 430] Therefore, the function of IgA bacterial coating would support colonization by or growth of symbiotic species, while limiting expansion by pathogens and growth of other commensal bacteria.[588]

An interesting question here is how a single antibody can mediate that many functions? Since IgA is passed from mother to child in breastmilk, possibly symbionts that are targeted by the mother´s IgA can be vertically transmitted to the infant, thus favoring colonization by specific bacterial species. As proposed by Schofield *et al.*[588], this event may protect against colonization in the infant by environmental strains of the same species, distinguishing between commensals and symbionts, and permitting co-development

of host–microbe symbiosis. In line with this, we could observe that IgA antibody responses at 1 month of age, in exclusively breastfed infants, were already divergent between children developing allergies and children that remained healthy until 7 years of age. To investigate if immunological interaction during breastfeeding influence allergy development later in life, we explored the IgA responses towards breastmilk bacteria in the corresponding mothers. However, no similarity in IgA-coating patterns could be detected between stool and breastmilk samples but, as discussed above, this could mainly be due to use of two different sequencing methods to address this problem. Except the methodological issue, it is important to consider that only a fraction of the milk SIgA is absorbed by the infant´s intestine after birth. Much of the immunoglobulins transferred through breastmilk are moderately resistant to digestion as they pass throughout the intestinal tract. This is especially relevant concerning IgG, which is less resistant towards digestion than IgA.[197] Also, to which extent can SIgA survive enzymatic digestion needs to be further investigated, as most of the studies have been performed on the IgG in animal models.

The approach for studying IgA responses towards bacteria, in different body sites, is relatively novel and it provides information that is important for understanding the basis of mucosal immune system responses. We cannot exclude the possibility that the differences observed in IgA-coating patterns between healthy and allergic children could be due to specific bacterial strains, and this should be explored further. For instance, specific isolates from breastmilk and gut microbiota should be addressed in order to see if they vary in their immune-inflammatory responses. Moreover, it is also required to complement this kind of data with methodologies that identify the specific antigen targets of secretory IgA at high-resolution. Beside SIgA, IgM might also play a role in immune responses towards commensals, as it has been observed that SIgM co-target with SIgA mucus-embedded commensals thus increasing diversity and richness in the gut.[151] Therefore, the understanding of this immunoglobulin in influencing the growth of common commensals should be broadened, and the fluorescent activated cell-sorting and sequencing approach has also been set up for IgG- and IgM-coated bacteria.[413]

The Influence of Environmental Factors on Microbiota Development in Health and Allergy

Neither delivery mode, nor breastfeeding or antibiotics intake during early period of life appeared to have an impact on microbial composition, in relation to allergy development, in any of the allergy-related studies presented in the thesis. However, it is logical to believe that all these environmental factors may influence early microbial development and immune system maturation, as reported in other studies.[60] For instance, delivery by C-section imposes on microbial establishment, diversity and immune system maturation simply due to the lack of exposure to the vaginal and stool microbes of the mother.[571]

Although we were not able to detect any effect of C-section delivery on allergy development, likely due to limited statistical power, we do detect the importance of this process for the oral microbiota maturation in longitudinally collected saliva samples throughout childhood. Therefore, large intervention studies where neonates delivered by C-section are exposed to bacterial populations found in the mother's vagina and stool would be really important for understanding the consequences that delivery mode may cause.

When it comes to the protective role of breastfeeding in asthma and allergic disease in children, the topic is still controversial since it is not possible to make a definitive conclusion regarding this relationship.[186] Like discussed in a recent systematic review [186], the interaction between breastmilk, the infant intestinal ecosystem and the developing immunity is highly complex to study and comprehend. Besides the genetic factors, mother's diet and health status, the heterogeneity in study design complicates the interpretation of the data. Likely, some factors in breastmilk may protect the infant from developing allergic disease while others have the opposite effect. For instance, breastmilk of atopic mothers have higher concentration of cytokines IL-4, IL-5, and IL-13 that are closely related to IgE production and eosinophil induction.[589] Therefore, it is important to direct future research on interactions between microbiota and the immune system and not only to study these areas separately.[590]

Similarly, the association between early antibiotic use and increased risk of allergic disease and asthma has been controversial.[216, 590, 591] The negative impact of antibiotics on the gut microbiota is often referred to as potential induction of bacterial dysbiosis, benefiting the growth of opportunistic pathogens, that subsequently leads to development of allergic disease. Retrospective studies have mainly showed the relation between infant antibiotic use and allergy development while the results from prospective studies have varied.[169, 592, 593] The age of initial exposure might be crucial since maternal use of antibiotics during pregnancy has been shown to increases the risk of allergy development in children.[594] However, the information about prenatal antibiotics intake was not available in the cohort studied here. Other studies have revealed that the risk of asthma development increases with the number of antibiotic courses administered during the first year of life [595–597] and that an association with asthma is stronger upon broad-spectrum antibiotic use.[596, 597] The lacking effect of antibiotic use on gut microbiota development in allergy in the study presented here might likely be influenced by the small sample size, but also the fact that we observe IgA-coated microbiota and not the total, unsorted microbial composition. It is also unclear to what extent antibiotic use can alter oral microbiota in relation to allergy and asthma development and this needs to be studied in more detail. Another important problem to address in this type of studies is occurrence of reverse causation – is the treatment administered for a condition that in fact may be an early symptom for the outcome (for instance eczema) measured?

In conclusion, we assume that the discovered microbiota differences between children staying healthy and developing allergies are mainly driven by health status. However, larger studies, preferably with

longitudinally collected samples and clinical follow-up until school age, are required to further investigate and confirm the role of the environmental factors discussed here, in allergic disease development. Nevertheless, microbiota studies endure high inter-individual variation and, even by using large study groups, we cannot exclude the possibility that the differences will be apparent only upon observation at the individual level. Also, because of the multivariate nature of the human microbiota data, and difficulties to create robust study design, potential effects on microbial composition and allergy outcome by any of these factors might get masked by different variables confounding each other. For instance, by performing multivariate analyses we could observe that breastfeeding duration was the main factor driving oral microbiota differences through age and that the other factors were partly influenced by it. In addition, it needs to be determined whether our findings can be replicated in cohorts of other geographic origins and with a different family history of allergic disease. A next step would of course be to investigate if probiotic treatment during early life can be used as an intervention strategy to restore microbial composition.

Modifying the Microbiota to Promote Immune-related Health

Probiotics and allergy – Can we control the microbial colonization during early life?

An altered microbial exposure, C-section delivery, short breastfeeding habits and antibiotics use are some of the factors that may be partly responsible for microbial dysbiosis in children and subsequent allergy development.[271] Probiotic supplementation both during pregnancy and early life has been suggested as a potential strategy for both modulating the initial microbiota colonization but also for restoring microbial changes that can lead to development of allergic diseases.[308]

Children and mothers included in the publications of this thesis were part of a larger prospective study where 232 families with allergic disease were recruited (see *Method section* and [327, 328] for more details). The aim of the original study was to evaluate the potential allergy preventing effect of the probiotic *Lactobacillus reuteri* during early childhood. The mothers started taking *L. reuteri* ATCC 55730, or placebo, four weeks before term and continued until delivery, while infants continued with the same treatment as the mother (probiotic or placebo), daily up to 12 months of age. *L. reuteri* is a gram-positive rod that has been isolated from gastrointestinal tract in several mammals, including humans [598–600], and this species has been reported to influence immune responses, possessing an anti-inflammatory effect, in both *in vitro* and animal studies.[601, 602] Briefly, the original studies reported that the *L. reuteri* treated infants had higher prevalence of this bacteria in stool samples (as determined by bacterial culturing) during the first year of life, when compared to placebo group.[330] Moreover, the *L. reuteri* group had a lower incidence of IgE- associated allergic disease, with lower prevalence of IgE-related eczema and decreased allergen responsiveness during the first two years of life.[317, 327] Colostrum of *L. reuteri* treated mothers had

reduced levels of TGF-β2 [497], which has been associated with less allergic sensitization. However, the probiotic treatment was not effective in preventing respiratory allergies.[328]

The original studies were analyzed from a more immunological perspective with conventional cultivation methods, while 16S rRNA gene sequencing was not performed. Surprisingly, in the studies considering gut and breastmilk bacterial composition presented in this thesis, we were not able to identify this particular *Lactobacillus* species in our sequencing data, and this could be due to amplification bias, as our primers do amplify this genus when present. Likely, since the breastmilk samples were obtained at one-month *post partum*, and the mothers were treated only until delivery, this species may no longer be able to colonize milk. When we studied gut microbiota composition (chapter II), the genus *Lactobacillus* had a very low abundance and we were not able to detect *L. reuteri*. As mentioned before, we were only checking cell-sorted fractions of bacteria, which could have influenced the detection of this strain. It is also important to consider that certain strains of probiotics are less efficient colonizers than others, even after direct administration to the infant. A possibility to explore in the future is whether the probiotics administered favor co-colonization of other beneficial bacteria for immune system development, even though the probiotics themselves are not able to significantly colonize and expand.

Supplementation with *L. reuteri* during the first year of life seemed to influence the association between oral microbiota composition and asthma development (chapter III). 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. In the original study, the probiotic intervention was associated with lower prevalence of IgE-related eczema during the first 2 years of age, while *L. reuteri* did not directly reduce asthma development in the cohort.[328] A benefit of probiotics for primary prevention of eczema, but not significant effect on any other allergic manifestations, was further confirmed by two meta-analyses published in 2015.[603, 604] Cuello-Garcia *et al.* concluded that there was a benefit of probiotics for eczema reduction (until 2 years of age) when administered in the last trimester of pregnancy, when supplemented to mothers during breastfeeding or when administered to infants and/or mothers. Also, no significant effect on eczema was observed when probiotics were administered exclusively to the infant.[604] The long-term preventative effect on eczema using four different strains of specific probiotics has also been reported recently.[605] In this study by Peldan *et al*, they observed that the early use of probiotics was associated with a reduction in the incidence of eczema at 10 years of age. However, the results of their long-term follow-up data was based on solely on questionnaires, and not clinical examinations, which could be a limitation of the study.[605] Intriguingly, in the same study they observed that allergic rhinoconjunctivitis was more prevalent in children, between 5-10 years of age, who had previously been treated with probiotics, when compared to placebo. A similar finding was reported by another study [304], but as authors explain, it can be difficult to distinguish between allergic and viral rhinitis, particularly when the outcome has been based on parent-reported data. Generally, there is very little evidence for a role of

probiotics in prevention of respiratory allergies, including asthma, rhinitis and wheeze but the possibility should not be completely excluded, and further evidence are needed.

Therefore, larger studies including the replication of our findings and the exact role of *L. reuteri* in the oral cavity are required to further investigate and confirm the role of this *Lactobacillus* in allergy development. Interestingly, increased abundance of two other *Lactobacilli* species (*L. gasseri* and *L. crispatus*) in the oral microbiota were observed in children staying healthy until 7 years of age, when compared to children developing allergies (chapter III). As discussed in the corresponding paper, both of these bacteria have been observed to have health-promoting immunomodulating properties with ability to suppress allergic responses and reduce mite-induced airway inflammation and hyperresponsiveness in mice models.[489, 490] Thus, we propose the isolation of these species from the oral cavity of healthy children to be tested for possible beneficial effects. Potential probiotic strains, such as the lactic acid bacteria *Lactobacillus* and *Enterococcus*, are generally isolated from safe sources like fermented dairy products [606], while the use of bacteria identified and isolated in the oral cavity is uncommon. In addition, most of the studies present today are describing the association between early microbial dysbiosis in the gut [39, 263] and the risk of allergic and asthmatic disease. Yet, as the oral cavity is the first line of encounter between the immune system and the majority of foreign antigens, the potential role of oral microbiota in allergy developments needs to be thoroughly evaluated. In has to be born in mind that some bacterial species observed in paper III were clearly associated with allergic conditions even before the appearance of symptoms and therefore their potential immunomodulatory effects deserve further attention and future investigation in longitudinal and animal model studies, where 16S rRNA gene sequencing is complemented with more traditional microbiology.

Wider perspective of probiotics in treatment in allergy prevention

World Allergy Organization (WAO) guidelines suggest administration of probiotics in mothers during gestational and lactating period, and later on in infants, when there is a high risk of allergy development during childhood.[607] Probiotics may have a potential role in the prevention of atopic eczema but studies to date have not been completely conclusive and recommendations today are based on little evidence.[608] The heterogeneity in study design, sample size, intervention age and age at sample collection, methods of analysis of gut microbiome, and geographic location are factors that make the comparisons between studies difficult.[609] Moreover, the hypothesized mechanisms by which the commensal microbiota influences the outcome of the allergic response are manifold [610] but necessary to understand in order to perform an optimal probiotic selection. As mentioned above, a combination of bacterial strains may be important to prevent development of childhood allergic diseases, both due to characteristics of strains that will influence the immune system in different manner but also due to variation on the initial composition of the host's (gut) microbiota. However, it has also been reported that

solely one probiotic strain has been able to induce allergy preventive effect, across the spectrum of allergic disease (although not asthmatic disease), during childhood.[611] The majority of probiotic intervention studies in allergy are focused on the most well-known species (*Lactobacillus*, *Enterococcus*, *Bifidobacterium*) but they represent only a small fraction of the total composition. Therefore, wider understanding of yet unknown species, and their role in colonization and immune system modulation, is needed for improving future probiotic therapy. We should also take advantage of longitudinal studies where changes in microbial composition between health and disease condition (including allergy) have been reported, in order to identify particular biomarkers that might aid in predication of allergy development, as discussed in the next section. As we gain broader perspective about these alterations, microbiota-immune system interactions and the species present, we may be able to develop more effective intervention strategies and probiotic supplements, which will likely have to be individually tailored.

Can Microbiota Composition Predict Allergies and Asthma?

As we gain wider understanding of the relation between the human microbiota composition and a variety of diseases, the thought of using these findings to predict and diagnose disease conditions is truly attractive. Many studies have identified different gut microbiota biomarkers that might predict a variety of disease conditions, including intestinal bowel disease, obesity, diabetes and colorectal cancer.[612–615] Therefore, modification of gut microbiota may be a potential therapeutic strategy to prevent or reverse diseases, as it has been shown in obesity-related studies where transferring healthy microbiota to a second host can reverse and prevent metabolic syndrome.[616] The ability to predict allergic disease at an early age would have many advantages in Western countries where the prevalence of allergies has increased dramatically during the last decades.[617] Due to great advances in gene sequencing technologies, research assessing complete gut microbial communities during early period of life, has augmented our ability to identify important biomarkers that might predict future allergy development. For instance, reduced diversity in gut microbiota, together with decreased abundance of *Bacteroides* genus at 1 month of age, have been associated with the risk of developing atopic eczema at second year of life.[265, 269] Other cohort studies have reported that colonization with *Clostridium difficile* (an opportunistic pathogen), during the first month of life, was associated with atopic sensitization at age 2 [278], and with asthma at 6 to 7 years of age.[618] Moreover, besides reduced bacterial diversity during the first month in children developing asthma [266], low abundance of the bacterial genera *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* has also been associated with risk of asthma in children at school age.[168]

With the study II, we aimed to find potential gut microbiota biomarkers related to allergy development during childhood, by going one step further than in other studies focused exclusively on describing the composition: addressing the interaction between mucosal immunity, represented by IgA, and bacterial composition. Our work here suggested early characterization of IgA-coating proportions and patterns may

represent a novel strategy to identify children with increased risk of allergy and asthma development. The fact that these alterations can be detected already during the first year of life, paves the way for possible clinical interventions that might modulate or delay the dysbiosis, thus preventing children from developing asthma and allergic diseases. Indeed, these findings need to be followed further and confirmed in larger cohorts, as discussed earlier. We speculate that already the transmission of maternal immunity can be used as an even earlier predictive event in allergy development, since breastmilk fed to children developing allergies had significantly lower bacterial richness, when compared to breastmilk fed to children staying healthy. Additionally, the relative amounts of several bacterial species in oral cavity, including increased abundance of *Gemella haemolysans* in children developing allergies and *L. gasseri* and *L. crispatus* in healthy children, were distinctive during early infancy, likely influencing early immune maturation. In conclusion, we have encountered alteration in microbial composition and immune responses towards bacteria, at several bacterial habitats, that influence and precede allergy development during childhood. We reported that the presence of the bacterial species *G. haemolysans* in the oral cavity was associated with the risk of developing allergic diseases while *L. gasseri* and *L. crispatus* were detected as potential probiotics associated with children staying healthy until 7 years of age. The possibility that these early findings could be applied to clinical research and used as potential early biomarkers capable to predict the risk of allergy and asthma should be considered.

In order to translate our findings however, as well as other studies focused on microbiota related biomarkers in health and disease, we must address numerous challenges. Taxonomic changes reported in different studies that focus on a particular disease condition, like allergy in this case, are inconsistent. Distinctive and unstandardized methodological approaches and sample population (size and ethnicity, geographical location and lifestyle), as well as external confounding factors such as diet (for instance breastfeeding duration) may account for relative lack of consistency among different biomarkers. Moreover, the microbiota is highly variable, and it is difficult to define a range of "healthy" composition (healthy core) and diversity of the human microbial ecosystem (gut microbiota for instance). Additionally, many dysbiotic changes, particularly bacterial diversity and richness, are not disease-specific and can be associated with several inflammatory conditions. Another curious thought to study here is – Do the observed microbiota changes reflect cause or consequence of allergy development? This is particularly interesting in case of respiratory allergies where medication administered, for instance asthma inhalators, might affect local microbiota composition. However, in chapter III where we compared bacterial diversity of the oral cavity, at 7 years of age, between children developing allergies (and asthma) and healthy children, we took into account the effect of asthma medication. While a similar trend was observed even after excluding children that were using asthma medication, we were not able to fully evaluate this statistically due to a low subject number. Also, there are good reasons to expect that many other potential influences, including the oral hygiene habits and diet during childhood, should whenever possible be considered, compared and reported in microbiota studies.

When it comes to methodological aspects, it is worth to mention that 16S rRNA gene sequencing is not an optimal approach for identifying microbial communities at species or strain levels, which could provide for example different surface antigens within the same species. This is highly limiting as species- or strain-level differences between individuals can be large and therefore, whole genome sequencing approaches would provide better information about human gut microbes and their functions. Another important challenge is given by the feasibility of developing an easy-to-use test that can be used in the clinical setting or even at home and that is inexpensive. For that, the complex sequence-based approach developed in the current thesis must be considered as an open-ended approach to identify potential biomarkers, but future development must be directed towards selecting the minimum number of biomarkers with high sensitivity and specificity levels which can be translated into simple PCR tests or diagnostic strips that can be manufactured with commercial purposes.

On the other hand, microbiota biomarkers are not expected to be a standalone diagnostic assay and should be used as a complement with routine clinical laboratory testing (like skin prick test and allergen-specific IgE blood levels) and clinical (family) history. Moreover, if the approaches targeting IgA-coating patterns and proportions of bound bacteria are validated to have early diagnostic power, different intervention strategies could be developed in order to prevent allergic disease onset during childhood. Conclusively, our findings need to be replicated in other countries and additional controlled studies, including experiments directed towards understanding the underlying cellular and molecular mechanisms, are required for further understanding of the host-microbiota interactions that seem to play a crucial role in allergy development.

The "Perfect Storm"

Asthma and allergic diseases are complex conditions caused by a combination of susceptibility genes and epigenetics, numerous variable environmental factors and host-microbiota interactions. Therefore, any attempt to study the causes of allergic diseases by focusing on only one of those factors will be incomplete.

Allergy development during childhood consists of a chain of events that are still only partly understood. The intra-uterine environment, influenced by maternal factors, acts as a starting point for early development of the immune system in the fetus. As shown in the present thesis for oral microbiota development, variables like caesarean section and breastfeeding duration are important in directing initial extra-uterine colonization of pioneer bacteria, creating a base for further microbial succession. For these aspects of early microbiota and immune development, there are relatively fixed windows of susceptibility and events occurring here will have a critical role for lifelong host-microbial and immune homeostasis,

possibly predisposing us to conditions, such as atopy. Following this "*window of opportunity",* antibiotic intake, oral hygiene, allergen exposure or number of older siblings are all environmental factors that will direct microbial habitats across the body of the toddler, resulting in an adult-like microbiota complexity by late childhood or even by preadolescence.[13, 45, 46] Apparently, there are several key events in the immune system development and microbial colonization that follow each other, and it is of great interest to understand how they might change the microbial/disease outcome more exactly in order to develop strategies to neutralize the effects. There might come a time when the host is exposed to different microorganisms at different time-points in order to support specific developmental (immune) processes.

Despite accumulating studies in the human microbiota field, we still lack the knowledge to define an optimal early life microbiome and how it can be promoted and maintained. Therefore, significant efforts have been devoted to identify universally shared core microbiota, in contrast to transient colonizers that depend on diet and other external factors.[92] Here we would need to document which specific bacteria, and their metabolites, are critical in early programming of immune system. Once classified and characterized, this core microbiota representing a selected group of health-associated microorganisms, would facilitate relevant strategies to promote or inhibit the microbes which are significant for human health with potential diagnostic and therapeutic features. However, there are various difficulties that need to be overcome, including technical variations between the studies, the cost and practical feasibility of microbiome analyses or the selection of universal biomarkers, if they exist.

We have also observed that early mucosal IgA responses towards gut microbiota might play a role in subsequent allergy and asthma development in childhood, as children developing allergies had lower proportions of bacteria coated with IgA with distinctive coating patterns of several bacterial genera. These findings possibly reflect altered mucosal barrier functions in allergic children and we speculate that early characterization of IgA coating patterns might represent a novel way to identify infants with increased risk of asthma and allergic disease, although this needs to be confirmed in larger cohorts. Moreover, the exact function of IgA requires to be further emphasized, especially in the case of vertical transmission of IgA-coated bacteria from mother to breastfeeding child.

In parallel to microbial colonization and maturation that are important for development of an appropriate and tolerant immune system, there are numerous genes, and epigenetic alterations, involved in allergy predisposition. The significance of epigenetic processes in allergic disease is under active research, as these mechanisms are identified to be at the interface among gene regulation, environmental stimuli and immune-developmental processes, all of which are essential for the pathogenesis for asthma and allergy.[619] In parallel, that early-life microbial colonization influences epigenetic modification of host DNA in a manner that produces life-long effects on the host immune response.[620] Speculatively, in a complex disease, like atopy and asthma, where various factors are involved, microorganisms will have different functions and effects depending on the changing environment and host susceptibility. It is

therefore plausible that a certain microbiota exposure, under specific external or environmental factors, and within genetically susceptible individuals, will cause a "perfect storm", triggering disease development (Fig. 11). From those pillars of disease factors, the genetic predisposition, although a large proportion remains still unexplained [621], will give the clinician important information, while both the environmental and microbial aspects are susceptible to be modified. Therefore, taking into account and integrating these general components of the perfect storm, several therapeutic and preventive interventions could theoretically be implemented in order to reduce disease risk.

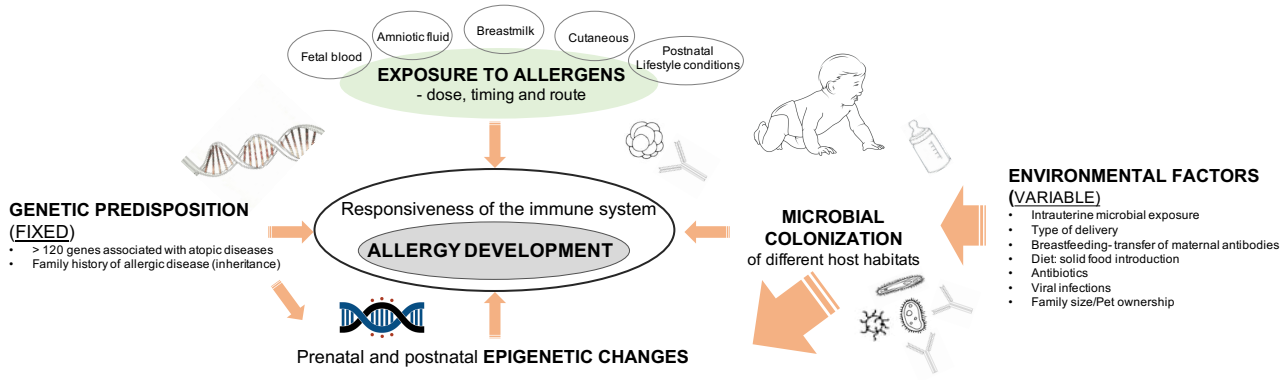

Fig. 11. Influence of different factors on immune responses and allergy development during early life.

Future Perspectives

While substantial gaps in our understanding on the microbial interactions and parallel immune system development in allergy still exist, exploratory studies like those reported in this thesis are crucial for laying the foundations for further scientific investigation and knowledge building. I propose that further research should go beyond characterization of bacterial community composition and investigate the functional mechanisms between early colonizing microorganisms and allergy and asthma development during childhood. Given the difficulties to distinguish between cause and consequence in microbiome studies [622], we should instead move the focus towards providing explanations of causality. With new techniques for microbiota function prediction (with widely agreed standards and protocols), advanced cellular and animal models and novel analytical approaches for studying bacterial interactions, future research should aid to map and clarify the detailed mechanisms between microbiota and infant development, opening up for possible strategies to specifically manipulate different microbial niches, particularly in the earliest stages of life when the immune response is maturing and the microbiome settlement appears most plastic. Hence, by addressing the importance of infant microbiota in health, and allergy and asthma development, at a more profound level, I anticipate that the findings will have a crucial role in clinical research of microbiome-based diagnosis and personalized treatment strategies.

CONCLUSIONS

The studies presented in the current thesis suggest that:

1. Colonization of infant's oral cavity by microorganisms follows a timely manner, increasing in complexity with time.
2. Bacterial density, richness and diversity in the oral cavity increase from 3 months to 7 years of age.
3. Oral microbiome development during childhood is an ecological succession impacted by delivery mode, breastfeeding duration and antibiotics treatment.
4. Low proportions of IgA-coating of gut microbiota during the first year of life are associated with asthma and allergy development during childhood.
5. Early aberrant IgA responses towards bacterial genera in the gut preceded asthma and allergy development and could therefore have diagnostic value of disease risk.
6. Lower bacterial diversity in breastmilk collected at one month *post partum*, appears to be associated with allergy development in children.
7. Probiotic supplementation with *L. reuteri* during pregnancy altered the proportions of IgA-coating of breastmilk bacteria, at one month post pregnancy.
8. Development of allergic disease during childhood, and particularly asthma, was associated with significantly lower oral bacterial diversity and divergent bacterial composition at 7 years of age.
9. Increased abundance of *Gemella haemolysans* in oral microbiota was observed in children developing allergies during the first 7 years of age.
10. Infant oral microbiota composition, including the increased abundance of *Lactobacillus gasseri* and *L. crispatus* in children staying healthy, compared to children developing allergies during the first 7 years of age, is likely influencing early immune maturation and I propose the isolation of these bacteria from healthy individuals for testing as potential probiotics.

ANNEX A – Published version of Chapter I

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ARTICLE

Oral microbiome development during childhood: an ecological succession influenced by postnatal factors and associated with tooth decay

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Abstract

Information on how the oral microbiome develops during early childhood and how external factors influence this ecological process is scarce. We used high-throughput sequencing to characterize bacterial composition in saliva samples collected at 3, 6, 12, 24 months and 7 years of age in 90 longitudinally followed children, for whom clinical, dietary and health data were collected. Bacterial composition patterns changed through time, starting with "early colonizers", including *Streptococcus* and *Veillonella*; other bacterial genera such as *Neisseria* settled after 1 or 2 years of age. Dental caries development was associated with diverging microbial composition through time. *Streptococcus cristatus* appeared to be associated with increased risk of developing tooth decay and its role as potential biomarker of the disease should be studied with species-specific probes. Infants born by C-section had initially skewed bacterial content compared with vaginally delivered infants, but this was recovered with age. Shorter breastfeeding habits and antibiotic treatment during the first 2 years of age were associated with a distinct bacterial composition at later age. The findings presented describe oral microbiota development as an ecological succession where altered colonization pattern during the first year of life may have long-term consequences for child´s oral and systemic health.

Introduction

The development and structure of the neonatal microbiome have been partially elucidated, with a main focus on the microbial population inhabiting the lower intestinal tract, while information about the oral cavity colonization following delivery is still limited [1]. As yet, no published longitudinal studies have characterized oral microbiota development during infancy and childhood with culture independent next-generation sequencing methodologies, particularly in association with tooth decay.

It is believed that by production and excretion of metabolic products of pioneer colonizers (including facultative anaerobes *Streptococcus* and *Actinomyces*), acquired at birth and the following hours, the environment can be altered, thus benefiting and selecting the growth of other species (including more strictly anaerobic genera like *Veillonella* and *Fusobacteria*) [1, 2]. As the baby grows, microbial communities evolve and increase in microbial diversity [3, 4]. During this period, the oral microbiota is characterized by high variability and current knowledge indicates that it reaches adult-like stability around 2 years of age [1].

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ANNEX B – Published version of Chapter II

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

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Background: Although 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 cytometric 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 months of age from children staying healthy or having allergic symptoms up to 7 years of age.
Results: The children with 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 2 groups. Moreover, the bacterial targets of early IgA responses (including coating of the *Bacteroides* genus), as well as IgA recognition patterns, differed between healthy children and children with allergic manifestations. Altered IgA recognition patterns in children with allergy were observed 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. (J Allergy Clin Immunol 2017;139:1017-25.)

Key words: *Allergic disease, asthma, secretory IgA, IgA index, IgA recognition patterns, microbiome composition, gut microbiota, childhood*

Allergic diseases have become a major public health problem in affluent societies.[1] Reduced microbial exposure, both prenatally and postnatally, has been proposed to underlie the increase in allergy development.[2-5] The gut microbiota, which hosts a complex bacterial community, is quantitatively the most important source of microbial stimulation and might provide a primary signal for appropriate immune development.[4] The gut microbiota differs in composition and diversity during the first months of life in children who later do or do not have allergic disease,[2,6-17] although no specific microbes with consistently harmful or allergy-protective roles have yet been identified. Also, we observed that differences in the gut microbiota diversity during infancy between healthy children and children with allergies were mainly related to asthma and not allergic rhinoconjunctivitis (ARC) development.[16] Early establishment of a diverse gut microbiota, with repeated exposure to new bacterial antigens, might be more important than the distribution of specific microbial species in shaping a normal immune mucosal and systemic maturation.[4]

A reduced mucosal barrier function might increase the risk for allergy development,[1] and IgA is the primary mediator of humoral mucosal immunity.[18] IgA is the most abundantly produced antibody in human subjects, with the highest amount of secretion in the intestinal tract.[18,19] Secretory IgA (SIgA) has a crucial role in the gut through its binding to bacterial antigens, thus preventing their direct interaction with the host through immune exclusion and maintaining mucosal homeostasis.[18,20] SIgA can also limit overgrowth of select species, thus stimulating diversity.[18,21] 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.[19] However, in contrast to IgG and IgM levels, generation of this anti-inflammatory antibody is limited during early infancy, and delayed development of mucosal IgA production, for instance in the absence of breast-feeding, might lead to infectious disease in young infants.[22,23] Studies and clinical reports suggest that SIgA that originates from the mother's breast milk is important for

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ANNEX C – Published version of chapter III

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

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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 7 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, were 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, *Gemella haemolysans*, infancy, *Lactobacillus*, oral microbiota

1 | 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 has a symbiotic relationship with the host, although, under certain circumstances, some microbes can overcome host defenses and become pathogenic.[5] At the birth and following hours, the infant's oral cavity is exposed to a large amount of microorganisms

ANNEX D – Gut microbiota and Mucosa Immunity in the Neonate (*Review*)

medical sciences

MDPI

Review

Gut Microbiota and Mucosal Immunity in the Neonate

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Abstract: Gut microbiota colonization is a complex, dynamic, and step-wise process that is in constant development during the first years of life. This microbial settlement occurs in parallel with the maturation of the immune system, and alterations during this period, due to environmental and host factors, are considered to be potential determinants of health-outcomes later in life. Given that host–microbe interactions are mediated by the immune system response, it is important to understand the close relationship between immunity and the microbiota during birth, lactation, and early infancy. This work summarizes the evidence to date on early gut microbiota colonization, and how it influences the maturation of the infant immune system and health during the first 1000 days of life. This review will also address the influence of perinatal antibiotic intake and the importance of delivery mode and breastfeeding for an appropriate development of gut immunity.

Keywords: gut microbiota; postnatal immune development; gut immunity; breastfeeding; probiotics; antibiotics

1. Introduction

Epidemiological studies highlight the relevance of the period from conception to early life in the physiological and structural patterns of infant development, affecting their potential "health programming". The fetus adapts to the intrauterine environment, being able to alter its metabolism in response to external stimuli. The physiological and metabolic adaptations that the fetus undergoes in response to those stimuli could produce permanent changes in the host, which may lead to a higher risk of developing diseases and/or disorders, such as obesity, allergies, diabetes, or cardiovascular diseases, in adult life [1].

The first 1000 days after conception (including the pregnancy period and the first two years of life), which are considered a "window of opportunity", are crucial for the development and health of the future adult, as well as key to the establishment of the intestinal microbiota and immune system maturation. The physiological and immune development of the infant and the establishment of their microbiota occur in parallel throughout this short space of time. This microbiota plays a central role in health, intervening in key host metabolic and immunological functions.

DEFINITIONS

Microbiota: the microbial community in a specific niche/environment.

Microbiome: the total genomic repertoire of a microbial community (microbiota).

ANNEX E – Bugging allergy; role of pre-, pro- and synbiotics in allergy prevention (*Review*)

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Invited review article

Bugging allergy; role of pre-, pro- and synbiotics in allergy prevention

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Abbreviations:
GRADE, Grading of Recommendation Assessment Development and Evaluation; HMO, Human milk oligosaccharide; NCD, non-communicable disease; RCT, randomized controlled trial; RR, relative risk; SCORAD, Scoring Atopic Dermatitis; SCFA, short-chain fatty acid; Treg, regulatory T-cell; TLR, Toll-like receptor; WAO, World Allergy Organization

ABSTRACT

Large-scale biodiversity loss and complex changes in social behaviors are altering human microbial ecology. This is increasingly implicated in the global rise in inflammatory diseases, most notably the "allergy epidemic" in very early life. Colonization of human ecological niches, particularly the gastrointestinal tract, is critical for normal local and systemic immune development and regulation. Disturbances in composition, diversity and timing of microbial colonization have been associated with increased allergy risk, indicating the importance of strategies to restore a dysbiotic gut microbiota in the primary prevention of allergic diseases, including the administration of probiotics, prebiotics and synbiotics. Here, we summarize and discuss findings of randomized clinical trials that have examined the effects of these microbiome-related strategies on short and long-term allergy preventative effects – including new guidelines from the World Allergy Organization which now recommend probiotics and prebiotics for allergy prevention under certain conditions. The relatively low quality evidence, limited comparative studies and large heterogeneity between studies, have collectively hampered recommendations on specific probiotic strains, specific timing and specific conditions for the most effective preventive management. At the same time the risk of using available products is low. While further research is needed before specific practice guidelines on supplement probiotics and prebiotics, it is equally important that the underlying dietary and lifestyle factors of dysbiosis are addressed at both the individual and societal levels.

Introduction

The epidemic rise in allergic diseases and asthma is inexorably linked to complex environmental and modern lifestyle changes. Urbanization and global decline of environmental biodiversity are directly implicated in changes in human commensal microbiota, which are critical for both normal immune maturation and subsequent immune function. While these global effects are likely to vary widely across both macro-scale geographic environments and micro-scale human microbial habitats, there is growing evidence that 'dysbiosis' is a major factor in the global increase in inflammatory non-communicable diseases including allergic disease.[1–3] The ecological pressures on microbial diversity are multifaceted and reflect changes in individual exposures such as nutritional patterns (increased processed foods, less fresh and fermented foods), sedentary indoor living (vitamin D insufficiency, reduced nature relatedness and exposure to environmental biodiversity) as well as the wider social and economic drivers of 'dysbiotic drift'.[4–6]

Thus while it is important to develop strategies for individuals to restore personal biodiversity for disease prevention, as is the subject of this review, it is equally important to address the fundamental drivers of dysbiosis and nutritional supplements must be viewed in this broader ecological context.[7]

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