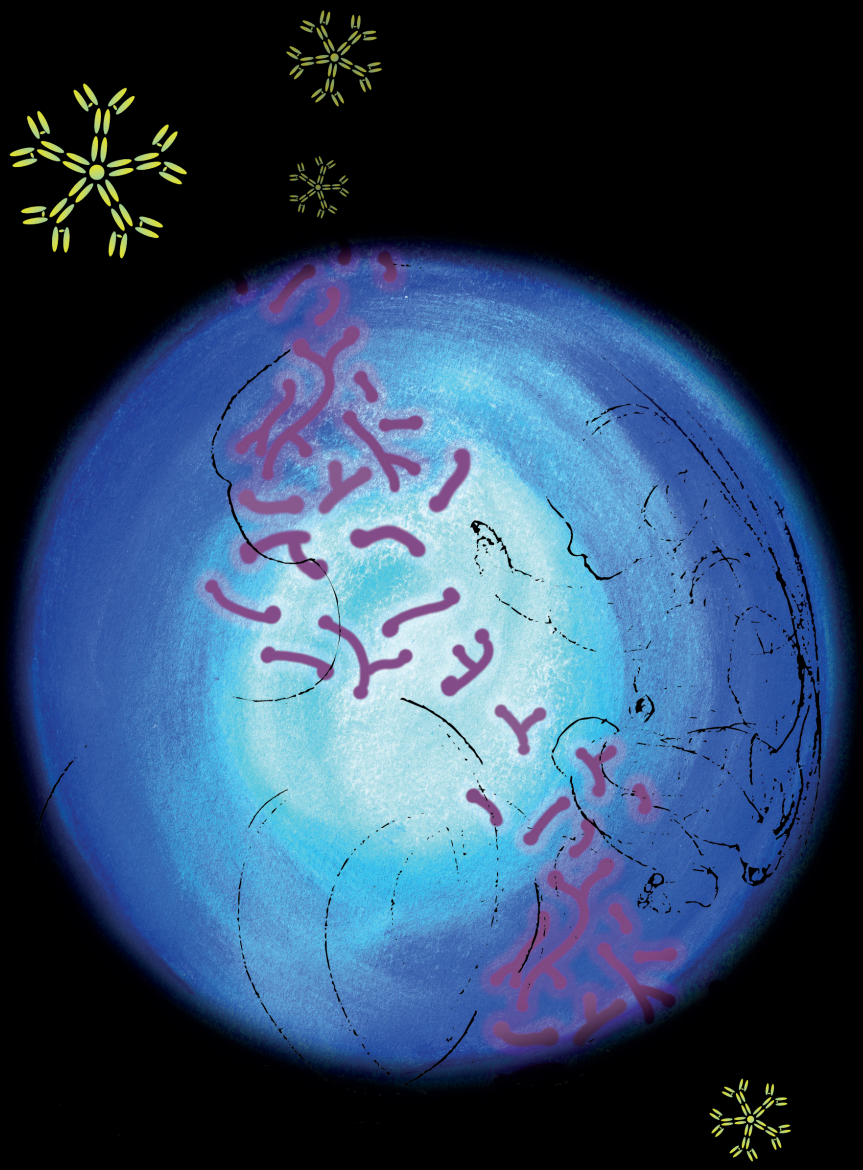
The early-life microbiota in relation to mucosal immune maturation and respiratory infections in children



Emma de Koff

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The early-life microbiota in relation to mucosal immune maturation and respiratory infections in children

De relatie tussen het microbioom in het vroege leven, de ontwikkeling van de mucosale immuniteit en luchtweginfecties bij kinderen
(met een samenvatting in het Nederlands)

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Voetje voor voetje
Zonder de top te bereiken
naar een boek van Paolo Cognetti

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CHAPTER 1

General Introduction

OF MICROBES AND MEN

Bacteria were among the first known forms of life on earth, while *Homo sapiens* evolved billions of years later. Humans have therefore always co-existed with bacteria, which have shaped the phenotype of our ancestors (1). The total of living microorganisms inhabiting our bodily surfaces together comprise the human microbiota. The term microbiome refers not only to the microbial community members but also encompasses their activity. Over the last decades, technological advances led to new sequencing techniques that enabled us to characterize human microbial communities at an increasing level of detail, including the microbiota of the respiratory tract and the gut (2). These early studies revealed an unanticipated diversity including a wealth of unknown bacterial species that had never been detected with conventional culture methods. Overall, gut microbial communities have been the most extensively studied. Nonetheless, the respiratory tract has also been shown to host a low-dense microbial community, spanning from the anterior nares all the way down to the lungs (3, 4).

After researchers catalogued the composition of the human microbiota, and the bacterial component in particular, they quickly shifted their focus to the essential functional benefits that microbes have for human health. Mechanisms by which local microbial communities affect the host include competition with potential pathogens for niche space and nutrients, production of immunomodulatory metabolites such as short chain fatty acids, and direct interaction with epithelial receptors (5–8). Colonizing microbes are in close contact with immune cells residing in the mucosa and underlying lymphoid structures in both the intestinal and the respiratory tracts, providing ideal circumstances for microbial-immune crosstalk (9). The extent to which the microbiome may consequently contribute to health and disease becomes most apparent from the long list of infectious, auto-immune and auto-inflammatory diseases that have been linked to changes to the microbial community composition compared with healthy individuals (10). Conversely, the microbial community composition may also adapt to host signals, for example in response to inflammation (11). This raises the typical chicken-and-egg conundrum: does the microbial community change in response to disease development, or do alterations to the microbial community predispose to disease?

To unravel a potential causal link between the microbiome and the origin and progression of chronic diseases with childhood onset, we need to start by understanding microbial community development from birth onwards, and align this process with health over time. The first 1000 days from conception until the second birthday form a critical developmental window, when life events and environmental exposures can significantly affect a child's path towards health or disease. Birth marks the transition from a sterile environment inside the womb to an extrauterine world swarming with microbes. The assembly of the early-life microbiota and their development from this point onwards are driven by many perinatal, lifestyle and environmental factors including, but not limited to, delivery mode, feeding type, infections and medical treatment like antibiotics (12–16). At the same time, the early-life microbiome is involved in instructing the infant immune system how to distinguish friend from foe (17, 18). Improved understanding of this crucial impact of the microbiome on immunity and child health may pave the way for opportunities for prevention if we are able to safeguard critical early-life microbes.

EARLY-LIFE MICROBIOTA AND IMMUNE MATURATION

The incidence of immune-related diseases with childhood onset such as asthma, allergies, and diabetes, is increasing worldwide (19). The theory of the 'disappearing microbiota' suggests that as a side-effect of modern life with high hygienic standards, altered nutrition and easy access to medical interventions, essential microbes for normal immune maturation are less frequently transmitted to infants and young children with potentially profound health consequences (20). This is supported by epidemiological studies showing that important drivers of the early-life microbial community composition, such as birth by caesarean section, lack of breastfeeding and antibiotic treatment in early life, are indeed associated with a higher risk of developing asthma and allergies in childhood (21–23). The broad involvement of the early-life microbiome in immune maturation and host health has for instance been convincingly demonstrated in experimental studies using infant mouse models (24–27). However, longitudinal studies in human infants that directly link microbial community development from birth to relevant immunological parameters, such as vaccine responses and

total antibody concentrations, remain scarce. Studies focusing on mucosal immunity are especially uncommon, even though mucosal surfaces form the primary site where host-microbiota interactions occur.

EARLY-LIFE MICROBIOTA AND RESPIRATORY INFECTIONS

Lower respiratory tract infections (LRTIs) remain a leading cause of childhood morbidity and mortality worldwide (28). From epidemiological evidence, it is well-known that children who have experienced a severe LRTI in the first years of life are also at increased risk of developing persistent wheeze and asthma later on (29, 30), but the biological underpinnings of these associations remain unclear at this point. The polymicrobial pathophysiology of childhood LRTI was elucidated by recent microbiota-based studies that challenged the longstanding one pathogen-one disease paradigm by revealing significant aberrations to upper and lower respiratory tract microbial communities combined with a higher frequency of specific respiratory viruses compared with healthy individuals (31–33). The developmental trajectory of the respiratory tract microbiota from birth onwards has also been associated with the risk of long-term respiratory morbidity such as a higher frequency of RTIs in the first year of life and the chronic disease asthma (14, 34, 35). Tying these findings together, it follows that studying the respiratory microbiota during and following severe LRTIs in early childhood may improve our understanding of the biological mechanisms underlying subsequent persistent wheeze and asthma development and open new avenues for preventative interventions.

RESEARCH QUESTIONS AND OUTLINE OF THIS THESIS

In this thesis, we address the research gaps mentioned above and study the early-life microbiota in relation to mucosal immune maturation and respiratory infections in children. In addition, we expand on the potential of using saliva for determining antibody concentrations and presence of pathogens.

The following research questions are addressed:

1. What is the current evidence for the influence of the early-life microbiota on respiratory health?
2. What are methodological challenges that researchers face when designing and executing a respiratory microbiota study?
3. Are maternal mucosal antibodies in early life associated with the assembly of the nasopharyngeal microbial community in healthy infants, and subsequently, is early-life nasopharyngeal microbiota development associated with the induction of intrinsic mucosal antibody responses?
4. Is the early-life development of the gut microbial community associated with the antibody response to childhood vaccinations and what is the influence of delivery mode?
5. How do salivary antibody responses to pneumococcal conjugate vaccination compare between two different immunization schedules?
6. Is the nasopharyngeal microbial community composition during and following a severe LRTI associated with recurrence of respiratory symptoms after the acute infection, and how do the nasopharyngeal microbiota recover after the infection?
7. How do the nasopharyngeal, salivary and lower respiratory microbiota develop during and following mechanical ventilation for a very severe LRTI?
8. How does the sensitivity for detection of common respiratory pathogens in children with RTI symptoms compare between saliva, nasopharyngeal swabs and oropharyngeal swabs?

In **chapter 2**, we review pre-existing evidence on the influence of the early-life microbiota on respiratory health, thereby providing an extensive rationale for pursuing additional knowledge on host-microbiota interactions in young children. In **chapter 3**, we briefly discuss major methodological challenges faced when studying the low-dense respiratory microbiota, and provide recommendations on how to overcome these issues. Next, we focus on associations between the early-life nasopharyngeal and gut microbial community in healthy infants and mucosal antibody levels and vaccine responses, respectively, which were measured in saliva. We use a birth cohort of healthy infants (the MUIS study (36)) with intensive follow-

up in the first year of life including microbiota characterization, providing a unique opportunity to align microbial community development from birth with immunological outcomes. Specifically, in **chapter 4**, we align the dynamics of total salivary antibody levels and the nasopharyngeal microbial community composition over time to investigate bidirectional antibody-microbiota associations within the upper respiratory tract. In **chapter 5**, we investigate whether the gut microbial community composition in the first weeks of life, which is strongly driven by mode of delivery, are associated with subsequent differences in vaccine responses. To further explore the potential of saliva for research into mucosal antibody responses, we then compare salivary antibody responses to pneumococcal vaccination between two different immunization schedules in **chapter 6**. Next, we build upon previous knowledge on aberrations to the respiratory microbiota during severe LRTIs (31), to explore microbial community recovery from the acute infection onwards. In **chapter 7**, we use a case-control study of young children hospitalized for a LRTI (the MOL study (31)) to study the recovery of the nasopharyngeal microbial community composition after clinical symptoms of the acute infection had resolved. We also study associations between the microbial community composition at time of the acute infection and recurrence of respiratory symptoms during the 2 months follow-up period. In **chapter 8**, we explore changes to the upper and lower respiratory microbiota during and following mechanical ventilation for a very severe LRTI in pediatric intensive care patients participating in the MEREL study (31). Finally, in **chapter 9**, we compare the sensitivity of saliva, nasopharyngeal and oropharyngeal swabs for the detection of common respiratory pathogens in children presenting to the hospital emergency department with symptoms of a RTI. To conclude, in **chapter 10**, we discuss the main findings of these individual chapters in light of the current literature, and formulate recommendations for future research.

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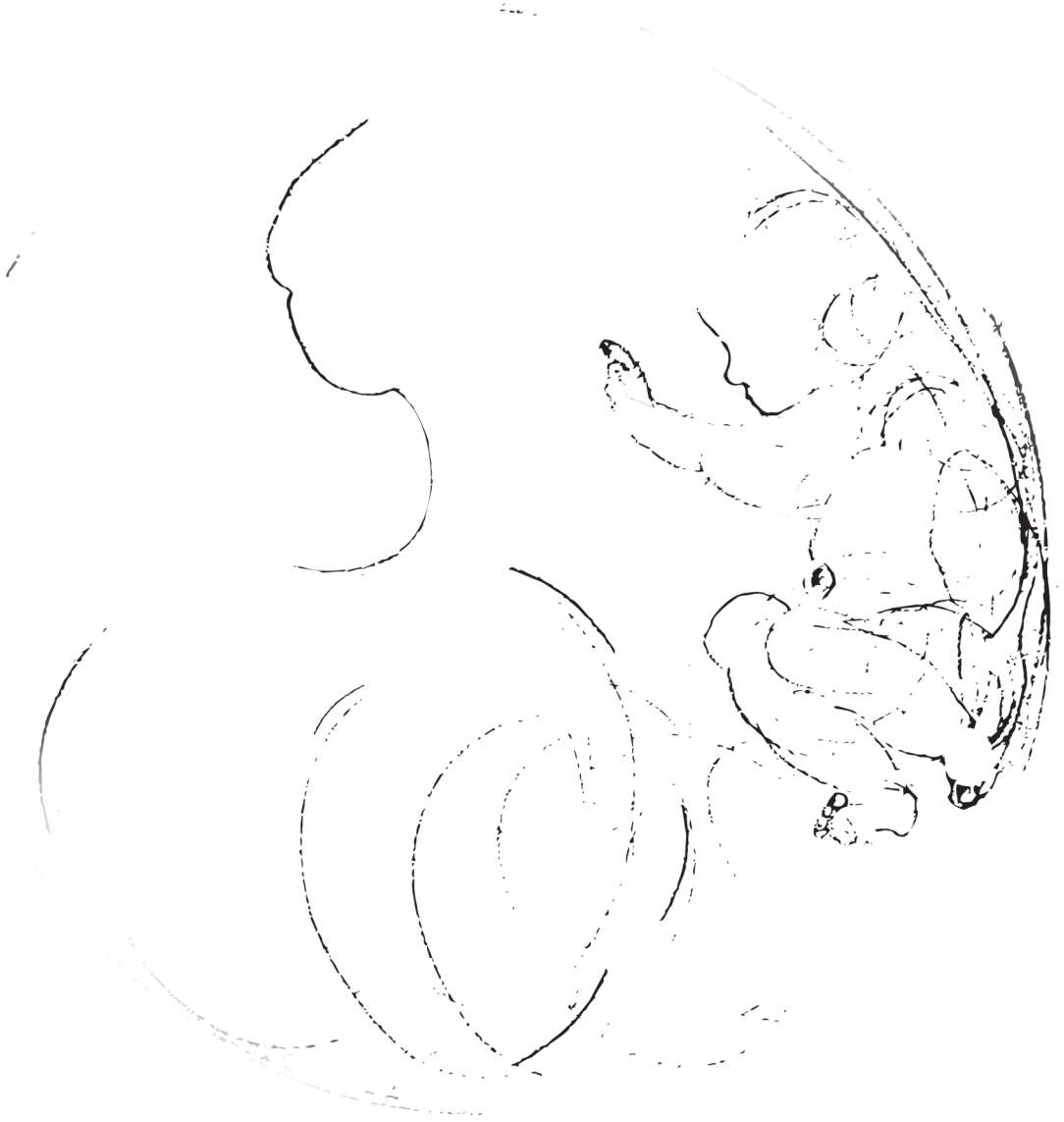

CHAPTER 2

The early-life microbiome: the key to respiratory health?

Emma M. de Koff*, Celine Pattaroni*, Benjamin J. Marsland#, Debby Bogaert#

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ABSTRACT

Microbial colonisation of mucosal surfaces starts at birth and diversifies within the first months of life. This process is mainly driven by niche-specificity but also by early environmental exposures, ultimately shaping the composition of the microbiome. Early-life microbiota likely perform important functions, among which are respiratory tract morphogenesis, pathogen resistance, and immune system development. Microbial dysbiosis or imbalance, instigated by altered exposure to lifestyle factors including antimicrobial treatment, is coupled to a dysregulated immune response, possibly leading to microbial overgrowth, infection and inflammation. Shifts in microbial communities have been associated with the early stages of (respiratory) diseases including acute infection, chronic wheeze and asthma, causing a paradigm shift in our current understanding of disease pathogenesis. Mechanistic insight obtained from animal, *in vitro*, and computational models, slowly start to highlight key host-microbiome-environment interactions contributing to disease. In the future, a systems science approach integrating microbiome data with host and environment characteristics may contribute to novel interventions to better prevent, diagnose and treat respiratory diseases.

INTRODUCTION

Children represent the future, and health problems in early life can significantly influence their well-being in adolescence and later adulthood. Childhood respiratory tract infections (RTIs) remain a major global health concern; pneumonia is the leading cause of death in children under five years old, with most deaths occurring in low-income countries, whereas upper RTIs including acute otitis media (AOM), rhinitis and pharyngitis are amongst the most common causes of childhood illnesses worldwide (1). Antibiotic treatment can be lifesaving in this respect, but adds to the ever-increasing antimicrobial resistance among respiratory pathogens. Analogously, incidences of atopic disease, allergies and asthma have been on the rise for several decades, with allergic sensitisation rates among school children worldwide estimated at 40-50% (2).

Advanced sequencing technologies have revealed that the human body is home to an intricate ecosystem of bacteria, fungi, parasites and viruses, called the microbiome, which might be key to pathogenesis of a broad range of paediatric respiratory diseases. Most work to date has focused on the bacterial component referred to as the microbiota. Acute and chronic respiratory infections in childhood have been related to discrete compositional shifts in the bacterial communities residing in the respiratory tract (3-5). Furthermore, atopy, chronic wheeze and asthma have been associated with changes in the intestinal and airway microbiota in the first year of life, well before clinical disease onset (4, 6). A plausible explanation could be that within the respiratory tract, the healthy microbiome maintains a symbiotic relationship with the mucosal surfaces, together cooperatively ensuring local immune homeostasis and pathogen resistance. On the other hand, a state of microbial dysbiosis triggered by endogenous or exogenous factors can disrupt host-microbiome interactions and consequently allow for infectious and inflammatory disease and vice versa (7).

The neonatal phase is critical for healthy immune development. Birth marks the abrupt transition from the apparently sterile womb to the outside world, where the neonatal mucosal surfaces are immediately exposed to environmental, including microbial, stimuli. Immune cells are present at early stages of foetal development, but their functionality is directed at

tolerance of self- and maternal antigens: the post-natal immune system therefore needs to be 'educated' by its environment to find the balance between immune activation against enemies and tolerance to innocuous agents. The same environmental exposures in infancy including birth mode, feeding type and presence of other children in the household, seem to instruct appropriate immune maturation and shape respiratory microbiota development (8-11) – could the early-life microbiome be the primary instructor of immune maturation? This question drives an active area of research, as an affirmative answer would provide a promising window of opportunity to influence the immune system through manipulation of the microbiome, with potentially profound benefits for later-life health. In this chapter, we will first describe drivers of microbial acquisition and development from birth into childhood, and provide a detailed overview of key microbial occupants in the different respiratory tract niches and functions of the respiratory microbiota. Next, we will discuss possible causes and consequences of altered microbial development, including antimicrobial treatment, acute infection and chronic inflammatory disease. We will further elaborate on *in vitro* and *in vivo* techniques to study the microbiome. Finally, we will present our perspective on future applications of current knowledge in research and clinical practice.

THE HUMAN MICROBIOME AND PHYSIOLOGICAL FUNCTIONS

Initial microbiome acquisition: A window of opportunity

Baby's first microbes generally reflect the first microbial encounters, and are similar across different body sites including the respiratory tract (12). The commonly accepted notion that infants are born sterile was challenged by recent work suggesting that a distinct placental microbiome exists and that mother-to-infant transmission may begin *in utero* (13), although this finding remains controversial (14). One universal and inevitable exposure is birth itself, and the encountered microbes largely depend on mode of delivery; vaginally born children are primarily seeded with the faecal and urogenital microflora of the mother during birth, whereas caesarean born children initially carry typical skin-dwelling bacterial species (10, 12, 15).

Initial colonisation is mostly transient, and over time natural selection occurs of those microorganisms best fit to local micro-environmental conditions like pH and temperature, ultimately forming complex, niche-specific communities (10, 16). Throughout early life this process is progressively influenced by new environmental encounters, including dietary changes, infections, vaccination, pet keeping and exposure to other children, i.e. crowding, at day care facilities or at school (Figure 1) (8, 9, 17-19). Breastmilk in particular is renowned for conferring significant protection against respiratory and other infections to infants, and contains immunomodulatory proteins, prebiotic oligosaccharides and microbes that are each thought to contribute to microbial development in infancy (20).

Health-associated early-life environmental exposures thus likely create local conditions that a plethora of commensal microbes thrive in, while potentially harmful pathogens are kept at bay, thereby constituting a healthy equilibrium between host and microbiota. By contrast, microbial dysbiosis is characterised by overgrowth of potential pathogens, underrepresentation of beneficial commensals, altering bacterial behaviour and predisposing the host to disease. Dysbiosis may be instigated by changes in host lifestyle or environment or a consequence of interspecies interactions, which is likely modulated by the resident microflora (21, 22). Microbiota resilience may be vital to maintaining microbial symbiosis and a healthy equilibrium with the host. In contrast to the gut microbiome, it remains unclear whether microbial diversity plays any role in resilience, or whether it is merely a function of the presence of keystone genera or species (23). Profound understanding of host-bacterial interplay may ultimately inform the development of microbiota-oriented interventions, restoring a healthy equilibrium between microbiome and host during the critical early-life period, thereby preventing a path towards disease.

Respiratory microbiome composition and development in early life

The respiratory tract is composed of the upper respiratory tract (URT) and the lower respiratory tract (LRT), that are themselves divided into multiple anatomical structures. The main function of this complex organ system is

the exchange of oxygen and carbon dioxide. To optimise the gas exchange process occurring in the alveoli, the conducting airways first filter, warm and humidify inhaled air, and hereby also provide a first-line defence against potential pathogens. Microorganisms that are still aspirated from the URT become entrapped in the mucus covering the respiratory tract and are moved towards the oral cavity through ciliary action, from where they are cleared. Nonetheless, overlapping microbial ecosystems inhabit the entire surface of the respiratory tract, with a wide range of mostly aerobic and facultative anaerobic microbes forming specialised communities with large compositional differences between individuals (16, 17, 24, 25) (Figure 2).

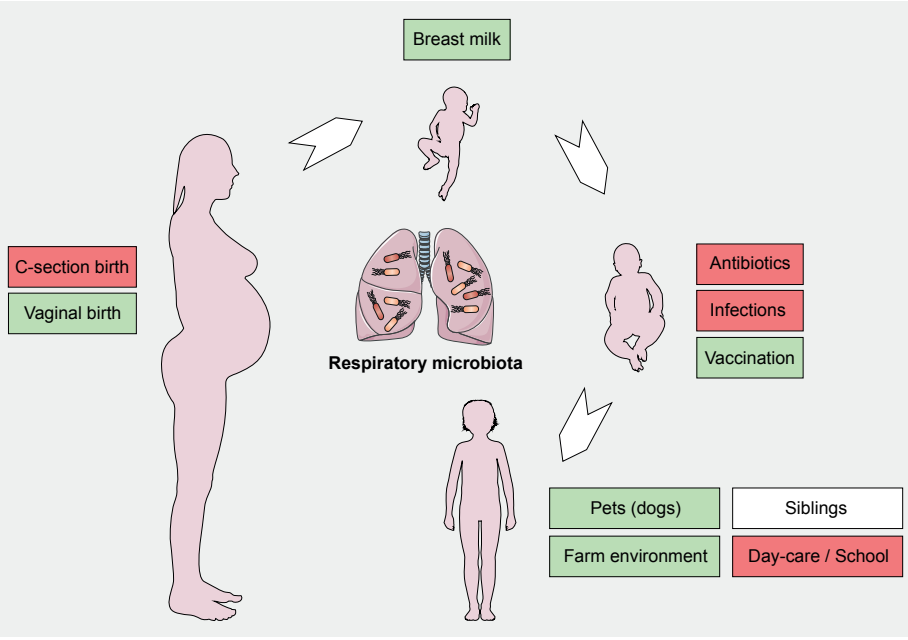

Figure 1 | Environmental factors influencing early-life respiratory microbiota composition. Several factors have been shown to induce shifts in early-life respiratory microbiota composition. These include the very first seeding of microbes during vaginal or C-section deliveries. Postnatal factors include diet, treatments, infectious events and/or familial and environmental exposures. Factors that have been shown to have a positive or negative impact on microbiota development and/or host immunity are depicted in green or red, respectively.

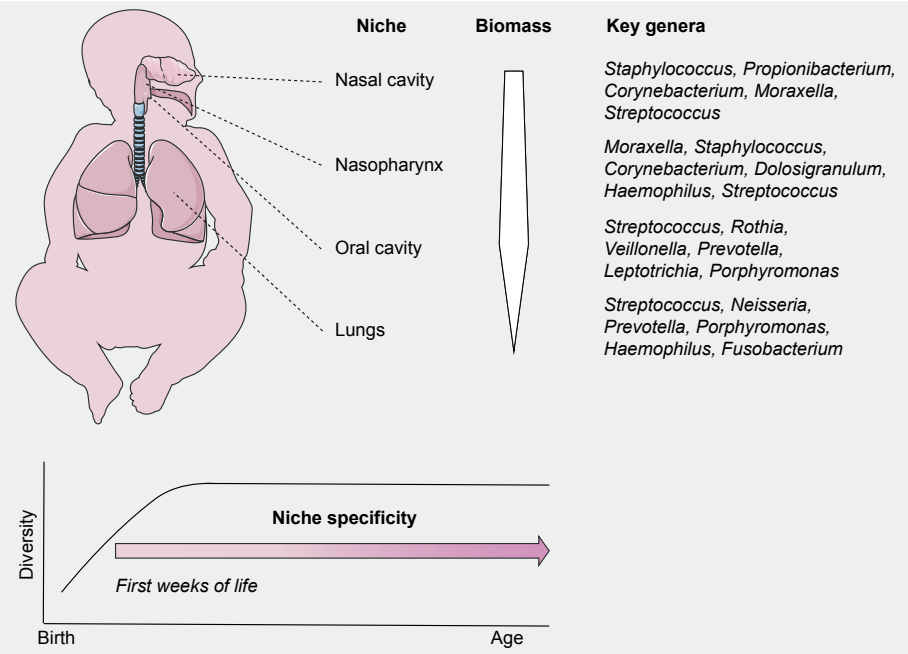

Figure 2 | Early-life microbiota composition along the respiratory tract. Changes in physiological parameters along the respiratory tract create unique ecological niches, which ultimately shape the composition of the microbiota in early-life. This niche-specific microbial diversification probably occurs during the first 2 to 3 postnatal months of life in humans. The upper respiratory tract is an important source of microbes for the lungs, probably as a result of micro-aspiration. This results in a gradient in both the composition and the total biomass of the microbial communities with lower bacterial density at the distal part of the airways. Adapted from: (24).

Even the traditionally considered sterile lungs contain extremely diverse low-density microbial communities (26), which seem in generally healthy individuals the result of microbial migration from the URT, as explained by the island biogeography model (24, 27). This model proposes that the oropharyngeal and - particularly in children - the nasopharyngeal niches form the 'mainland' from where microbes migrate to the 'islands', i.e. the lungs, where the community composition depends on microbial migration, elimination and reproduction, though still is connected with those in the URT (28-30). On the other hand, in advanced stages of chronic lung diseases like cystic fibrosis (CF), the populations ultimately become segregated as a result of alterations to local environmental growth conditions and/or differential

selection of microbes by (repeated) antibiotic treatment (31). Nonetheless, the easily accessible URT is commonly used as a proxy for studying the LRT microbiota, which seems valid for paediatric studies of both chronic and acute lung infections (28, 32), though further validation is required.

Anterior nares and nasal cavity

The anterior nares form the transition from the facial skin to the mucosal surfaces of the respiratory tract; hence, the microbial composition bears close similarity to the skin microbiota. The skin-like epithelial lining contains glands that secrete sebum, an oily matter promoting establishment of lipophilic skin colonisers, such as the genera *Staphylococcus* and *Corynebacterium* (25, 33). Other frequent occupants of this niche include *Streptococcus* spp.*, Dolosigranulum* spp., and *Moraxella* spp. (25, 33). Age appears to be an important driver of the nasal microbial ecosystem, as members of *Streptococcaceae* and *Moraxellaceae* families were relatively more abundant in healthy children compared to healthy adults (34). Furthermore, mode of delivery seems to drive the nasal microbial community composition, with in caesarean born infants increased abundance of *Staphylococcus* spp. and decreased abundance of *Corynebacterium* spp. compared to vaginally born infants, as well as mode of feeding, with in formula-fed infants higher levels of *Moraxella* spp. than breastfed infants (33).

Nasopharynx

The nasopharynx clearly represents the most extensively studied niche of the early-life respiratory microbiome as it is considered the ecological niche for most respiratory pathogens. Predominant bacterial genera in the nasopharyngeal niche overlap considerably with those of the anterior nares, but the community composition becomes much more complex (4, 17, 33). Distinct nasopharyngeal bacterial communities are established in the first weeks of life. On the first day post-partum, most children show a *Streptococcus*-dominated bacterial profile, but niche-specificity develops rapidly, with brief *Staphylococcus aureus* predominance at one week of age often preceding differentiation towards distinct communities dominated by *Corynebacterium pseudodiphtheriticum/propinquum, Dolosigranulum pigrum, Moraxella catarrhalis/nonliquefaciens, Streptococcus pneumoniae,*

and/or *Haemophilus influenzae* over the first six months of life (10). These initial bacterial profiles likely set the infant on a microbial developmental trajectory; early *Corynebacterium/Dolosigranulum* predominance was often succeeded by stable *Moraxella*-dominated colonisation, whereas profiles dominated by *S. pneumoniae* or *H. influenzae* showed large fluctuations over time (17). *D. pigrum* is a lactic acid-producing bacterium, and this group of bacteria are generally considered to contribute to healthy microbiota and to have probiotic properties (35). *Dolosigranulum* and *Corynebacterium* often co-occur, and the hypothesis is that lactic acid production of *Dolosigranulum* decreases the pH, which selects for and supports *Corynebacterium* outgrowth (7).

Microbial encounters in early life heavily influence the earliest developmental phases of the nasopharyngeal microbiota. In caesarean born children, a delay in microbial succession patterns with prolonged *S. aureus* predominance was observed; moreover, these children showed decreased abundance of *Corynebacterium* and *Dolosigranulum* (10). Feeding type also seems an important determinant of early microbial succession patterns: in six-weeks-old exclusively breastfed infants, *Corynebacterium/Dolosigranulum*-dominant profiles typically predominated, whereas *S. aureus* was highly abundant in formula-fed infants (9). Furthermore, exposure to other children in the household or at day-care facilities was associated with increased abundance of *Haemophilus, Streptococcus* and *Moraxella* and decreased abundance of *Staphylococcus* and *Corynebacterium* (4). Further development from a composition dominated by only few taxa into an adult-like, more diverse, microbial community structure continues over the first years of life, although it remains unclear when the nasopharyngeal microbiota reaches full maturity (16).

Oropharynx and oral cavity

The oral cavity and oropharynx form the route of entry to the gastro-intestinal as well as the respiratory tracts, and are thus exposed to a wide range of food- and airborne bacteria as well as colonisers from connected niches. Unsurprisingly, the oropharynx harbours the most diverse bacterial assemblages of all URT niches (36). The oral and oropharyngeal

microbiomes are subject to similar environmental selective pressures, and consequently, their compositions are also similar (7). Interestingly, contrary to the nasopharynx, young children and adults already show comparable oropharyngeal microbial community composition, mostly composed of (facultative) anaerobic genera like *Streptococcus*, *Prevotella*, *Neisseria*, *Veillonella*, *Porphyromonas* and *Rothia* (16). *Streptococcus* spp. are amongst the earliest colonisers of the oral niche and clearly predominate the resident bacterial community, although their abundance decreases slightly when other genera emerge, i.e. *Gemella*, *Granulicatella*, *Haemophilus* and *Rothia* from three months of age, and subsequently *Actinomyces*, *Porphyromonas*, *Abiotrophia* and *Neisseria* after the first year of life (37). The childhood oral microbiome composition is, similar to other URT habitats, shaped by mode of delivery (12), feeding type, with breastfeeding linked to increased levels of lactobacilli (38) and *Streptococcus* (37), and transfer between household members (39). More specific to the oral niche, eruption and health status of teeth also affects local microbiome development (40).

The lower respiratory tract

As one progresses distally in the LRT, the airway mucosa cell composition gradually shifts resulting in a unique microenvironment characterised by high levels of phospholipids in the form of surfactant (41) whose levels are known to gradually increase from 30 weeks of gestational age (42). Dysbiotic lower airways microbiota composition has been observed in neonates born prematurely (before 30 weeks of gestational age) when compared to term neonates of the same age suggesting that the in premature infants impaired phospholipid-rich environment could support the formation of a more diverse microbiota (43). Due to the invasive nature of LRT sample acquisition, studies in human neonates are very limited, mostly targeting premature, cystic fibrosis or chronic lung disease populations (30, 44-46). However, a recent study reported that the LRT microbiota in healthy infants forms within the first two postnatal months of life with diversity gradually increasing (43) consistent with the timing observed in the URT (47) and other body sites (48). Gestational age was strongly linked with immune maturation status, which correlated with differential microbiota profiles. The strongest gene expression signals upregulated with gestational age were related

to IgA pathway and IL-33 production, a cytokine linked with susceptibility to asthma in humans and disease exacerbation in mice (49). Notably, the same study identified *Streptococcus* and *Neisseria* spp. as key for neonatal LRT bacterial community structure. Interestingly, mode of delivery impacted microbiota composition in preterm but not term born neonates suggesting that immature lungs may represent a distinct microhabitat. Although lower airways bacterial communities of both children (30, 43) and adults (50-52) share common genera like *Streptococcus*, *Veillonella*, *Neisseria*, *Porphyromonas* and *Prevotella* with the URT, in particular with the oral cavity, differences remain (29). This suggests the presence of a selective pressure unique to the lower airways that may also explain the lower bacterial load typically observed in at this site (29, 53). Multiple factors, notably the presence of innate immunity proteins, such as surfactant protein A, lactoferrin, and defensins or adaptive secretory immunoglobulin A (sIgA) (43) may contribute to early-life LRT microbiota selection and homeostasis (54).

Homeostasis between the respiratory microbiome and host mucosal surfaces

Respiratory tract organogenesis

At birth, respiratory tract morphogenesis is not complete, in fact alveolarization is exponential in the first 2 years of life and continues until early adulthood (55). Early evidence from animal models indicates that the microbiota can influence lung development as germ-free mice have been reported to have lower numbers of mature alveoli. Intriguingly, experimentally introducing *Lactobacillus* spp. in the nasal cavities of germ-free pups can normalise alveolar architecture, suggesting causality of the lack of microbial exposure (56). The nasopharyngeal-associated lymphoid tissue in mice is in a strategic location for detecting incoming pathogens, and subsequently orchestrates immune responses, analogous to Waldeyer's ring in humans. In neonate rodents this structure is nearly absent, which suggests that its development depends on environmental signals, possibly including the resident microbiota (57). These examples demonstrate that early respiratory microbiota acquisition likely contributes to adequate development of anatomical structures critical for gas exchange and local immunity.

Pathogen resistance

The URT forms a major reservoir for opportunistic pathogens, and is an important gatekeeper for respiratory health (24). Colonisation of the URT with respiratory bacterial pathogens necessarily precedes local and invasive infection (58), but the same bacterial pathogens commonly inhabit the URT in the absence of disease. Bacterial species that are frequently isolated from respiratory specimens of patients suffering from RTI include *S. pneumoniae, S. aureus, M. catarrhalis* and *H. influenzae*, and these species can also be measured in up to 93% of asymptomatic children under two years of age (59). Mutualistic and competitive interspecies interactions underlie the co- and anti-occurrence patterns observed in culture-based studies (21, 59), and likely reflect that potential pathogens have evolved to keep each other at bay within the human host. These observations challenge Koch's first postulate that each pathogen is exclusively linked to a distinct clinical disease, and supports the idea that RTIs are polymicrobial and result from aberrant interplay between (multiple) pathogens and their ecological surroundings. Therefore, viewing colonisation resistance and pathogen containment from an ecological perspective likely provides deeper understanding of respiratory health and disease.

A balanced ecosystem elicits homeostatic host-microbiome interactions and withstands colonisation by incoming pathogens. Once a microbial community has established within the URT, a diverse palette of microorganisms thrives on locally available nutrients, thereby depleting the resources that other species including pathogens need to colonise the niche successfully. Decreased community diversity, defined as the number of different bacterial species (richness) that are present at high abundance (evenness), has been associated with respiratory infections (3, 60, 61), and lack of competition might facilitate pathogen colonisation, but this association is not as clear-cut as in studies of the gut microbiome (62). Certain community members called keystone species that compete with invading pathogens appear critical to maintain a balanced community composition (7). For example, high abundance of *Dolosigranulum* spp. and *Corynebacterium* spp. in the nasopharyngeal niche has been associated with respiratory health, and active exclusion of the renowned respiratory pathogen *S. pneumoniae* by the latter has been observed (17, 60, 63, 64). Furthermore, the oropharyngeal

microbiota has been suggested to prevent bacterial invasion through direct effects on the epithelial barrier, inducing mucus and antimicrobial peptide production and promoting tight junction integrity (65).

Once pathogenic species have settled in the URT, interactions with the resident microflora can constrain their pathogenicity. For instance, *S. aureus* is a highly abundant coloniser of the URT in early life, but rarely causes disease in infants. Both synergistic and antagonistic interactions might explain this: for example, co-occurrence with *Corynebacterium* spp. can inhibit *S. aureus* virulence (66), while presence of several other *Staphylococcus* spp. can limit *S. aureus* outgrowth through secretion of bactericidal substances (67, 68). Apart from interspecies interactions keeping facultative pathogens in check, pathogenic species' behaviour can shift towards commensalism. In such a state, *S. aureus* produces natural antimicrobials that can eliminate bacterial competitors (69). RTI caused by *S. pneumoniae* is more likely to occur soon after the host acquires a previously unmet strain (70). However, when prolonged colonisation exists, a state of symbiosis with the host will develop, where the colonising strain can exclude other strains of its own species that the immune system may not tolerate.

The chances that an opportunistic respiratory pathogen successfully causes infection may thus depend on the URT microbiome composition, as in a healthy state resource scarcity, health-promoting commensals and competing pathogens will counteract the intruder's attempts.

Immune-system development

Development of the immune system continues after birth, when its primary function switches from tolerance to maternal and self-antigens, to protection against disease-causing agents. Consequently, the immune system is likely the most malleable in the earliest life phase, at the same time that the respiratory microbiome develops. In continuous cross-talk between the local microbiota and innate and adaptive immune cells, microbes provide crucial cues that teach the immune system to distinguish friend from foe (71) (Figure 3). Immunoglobulin A (IgA) secreted by B cells is present in the mucus layer covering the URT, where it selectively entraps microorganisms, precluding them from interacting with epithelial

receptors to colonise the mucosal surfaces. In this way, IgA is believed to regulate the commensal microbiota and orchestrate mutualistic host-microbe interactions. This homeostatic role of IgA was most extensively investigated in the gastrointestinal tract (72) but recent findings suggest that similar mechanisms exist in the human airways (43). Specifically, neonatal gene expression in the lower airways linked with IgA production pathway and correlated with predicted microbial IgA proteases function.

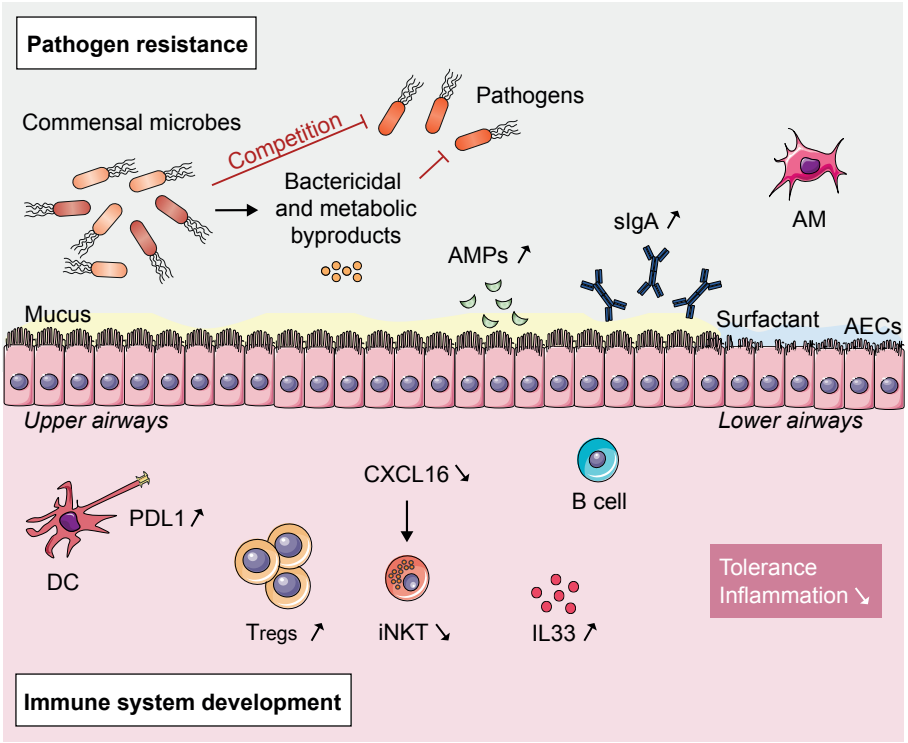

Figure 3 | The roles of the respiratory microbiota in early-life. Early-life colonization of the airways by commensal microbes play a central role in the resistance against invading pathogen (upper panel) and the induction of immune tolerance (lower panel). The protection against harmful pathogens can be mediated by both direct (e.g. niche competition) and indirect mechanisms (e.g. release of microbial by-products). The sensing of microbes and/or their by-products by the resident mucosal cells (dendritic cells, alveolar macrophages, alveolar epithelial cells) induces the activation and the recruitment of immune cells systemically (Tregs, B cells, iNKTs), a process key for long-term immune tolerance and regulation of inflammation. DC, dendritic cell; Tregs, regulatory T cells; iNKTs, inducible natural killer T cells; AECs, alveolar epithelial cells; AMPs, antimicrobial peptides; sIgA, secretory immunoglobulin A.

Dendritic cells (DCs) are important conductors of cell-mediated immunity, by constantly sampling their environment before moving to draining lymph nodes to present microbe-derived antigens either to T cells that can mount an effective immune response or to regulatory T cells that can induce tolerance. Mouse studies demonstrated that the expression of PDL-1 in neonatal lung DCs was a key process for tolerance to aeroallergens during the critical time window when lung microbiota establish (73). Invariant natural killer cells may also play an important role in mucosal tolerance, as perinatal microbial stimulation was needed to prevent exaggerated accumulation of these cells in both the respiratory and gastrointestinal tracts

Finally, although these mechanisms still require validation in humans, it is now evident that early-life microbial colonisation plays a major role in orchestrating peripheral tolerance in mucosal tissues.

The gut-lung axis

Moreover, although the above findings implicate a crucial role of the microbiome in immunity, it does not make a distinction between the role of the local (respiratory) microbiome, and the microbiome present at distal sites such as the gut. The bacterial gene pool of the gut greatly outnumbers that of the respiratory tract, and therefore, it is no surprise that the gut microbiota has shown important for respiratory health as well. The critical role of early microbial colonisation of the gut in regulating host immune responses was for example shown by a study in germ-free mice, where lack of microbial stimuli in infancy led to decreased immune activation to bacterial respiratory pathogens aggravating infection (74). Furthermore, allergic hyperresponsiveness and RSV-induced inflammation of mice airways was attenuated by an airway allergen challenge with dog-associated house dust through increased abundance of, amongst others, *Lactobacillus johnsonii* in the gut (75). In humans, it was shown that specific changes in intestinal bacterial communities in infants with CF, were followed by worsening of the clinical status of the patients, including pulmonary exacerbations and *Pseudomonas aeruginosa* acquisition in the respiratory tract (76). Although the exact mechanisms by which gut microbiota composition affects respiratory health are under active investigation, recent work showed that transport

of gut microbial antigens to the lung seems to directly modify alveolar macrophage function, and improve their host defences against bacterial pathogens (77, 78). Furthermore, gut microbiota changes induced by high-fibre diets led to increased systemic levels of short-chain fatty acids, which seemed to modulate haematopoiesis and DC and macrophage functionality, conferring increased protection against asthma and lung tissue damage caused by viral infection (79, 80). However, alternative pathways suggested for this phenomenon are that microbial selection in the gut and lung occur simultaneously under similar selective pressures, or that these findings are merely a bystander effect resulting from systemic inflammatory processes. Therefore, future research into the gut-lung axis seems important to unravel (parallel) protective immune-modulating pathways, since these could serve as basis for future (dietary) interventions modulating respiratory health through manipulating the gut microbiome.

DISEQUILIBRIUM BETWEEN THE HOST AND THE MICROBIAL WORLD

Ecological equilibrium between the host and its microbial population is believed to promote health by limiting pathogen overgrowth and dampening inflammation. By contrast, microbial dysbiosis, defined as microbial imbalance or maladaptation, may tip the equilibrium out of balance, possibly leading to infectious or inflammatory disease. In the following, we will explain how microbial dysbiosis may result from altered exposure, virus acquisition, antibiotic elimination or inflammatory selection and how it may enable acute infection, chronic wheeze and asthma.

Causes of dysbiosis

Altered exposure: Caesarean section and formula feeding

The effects of mode of birth and infant feeding type on the respiratory microbiome composition were outlined in the previous section. Generally, birth by caesarean section and formula feeding are associated with colonisation patterns characterised by decreased abundance of health-associated commensals like *Corynebacterium* and *Dolosigranulum,* and increased abundance of potential pathogens like *Haemophilus* and

Staphylococcus. Although the microbiome seems to recover with age (37), initial bacterial profiles likely determine bacterial succession patterns, which may impact short- and long-term disease susceptibility. Stable microbial colonisation in the first half year of life has been associated with exclusive breastfeeding and with fewer parent-reported upper RTIs, suggesting that breastfeeding may exert its protective effects through the microbiome composition (9, 17). Moreover, epidemiological evidence linking life style factors including mode of delivery and infant feeding type to development of chronic inflammatory diseases such as inflammatory bowel disease, asthma and allergies, further underscores the importance of this key period (81-83).

External influence: Virus acquisition

Like bacteria, viruses can reside in the respiratory tract asymptomatically: while they are considered the main cause of (childhood) RTI, they are also detected in approximately 70% of healthy children (59, 84). Common benign respiratory viruses include rhinovirus, bocavirus, polyomaviruses, adenovirus and coronavirus. Viral-bacterial interplay may affect the resident microbiota composition and trigger the transition of colonising potential pathogens into disease-causing agents (85). Presence of respiratory viruses appears to increase rates of *S. pneumoniae, H. influenzae* and *M. catarrhalis* colonisation in asymptomatic children; for example, rhinovirus and respiratory syncytial virus were associated with increased risk of *H. influenzae* colonisation (59). Moreover, influenza A virus infection was followed by pneumococcal release from biofilm growth in the airways of mice, which led to severe respiratory disease (86). Potential mechanisms underlying virally induced alterations to bacterial colonisation were investigated in *in vitro* and animal studies and include enhancing bacterial adhesion and translocation through direct damage to the epithelial barrier (87-89), downregulating various components of the innate immune system (90-92), and increasing expression of host-derived nutrient sources (93). It therefore seems plausible to abandon the one-pathogen-one-disease theory, instead study RTIs from an ecological perspective. In a recent study, we attempted to classify children with a moderate severe LRTI and healthy matched controls based on their total ecological community including bacteria and viruses: this indeed yielded a more complex classification model which included viruses, potential

pathogenic bacteria as well as (the presence or absence of) multiple other commensal bacteria (28). Studies detailing simultaneous development of the respiratory bacterial and viral microbiota in young children in a longitudinal fashion are scarce, and would likely yield important novel insight into processes leading to RTIs. This would be of particular interest in the context of rhinovirus- and/or respiratory syncytial virus-associated LRTI in the first year of life, as these viruses are associated with later-life persistent wheeze and asthma (94).

Elimination: Antibiotics

Antibiotics represent one of the greatest advances in modern medicine, but due to their lack of selectivity, they have a broad impact on many constituents of the 'healthy' microbiota. Epidemiologically, antibiotic use in early childhood is also linked to an increased risk of later-life immune-mediated diseases, and it has been proposed that this effect is mediated by microbiome changes resulting in impaired immune education (reviewed in (95)). Importantly, antibiotics are most frequently taken in the first two years of life, which can have important side effects on microbiome maturation (96).

Antibiotic use affects respiratory microbiome composition through loss of biodiversity and decreased abundance of keystone species like *Dolosigranulum* and *Corynebacterium*, leaving the niche partially vacant for opportunistic pathogens to grow or intrude and potentially cause a vicious cycle of disease (3-5). Indeed, risk of recurrent AOM was increased after recent antimicrobial exposure, probably due to diminished pathogen resistance (60). After completion of an antibiotic course and clinical recovery from infection, microbial communities reform to a state comparable but not always equal to before the infection, indicating to certain extent the microbiome's resilience (3, 5). On the other hand, children taking antibiotics in the first two years of life were shown to harbour compositionally different oral microbial communities at least up to seven years of age, with decreased abundance of commensals such as *Granulicatella* and increased abundance of genera associated with oral disease such as *Prevotella* (37). Lasting perturbations of the microbiome suggest that dysbiosis induced by early

antibiotic exposure is not temporary, which could explain the association with later-life susceptibility to inflammation-mediated diseases, though the exact pathophysiological mechanisms require further study.

We propose two possible explanations for incomplete community resilience after antibiotic treatment. Microbiome re-formation may depend on the level of mucosal homeostasis prior to infection onset. Coming from a state of symbiosis between host and microbiota, microbiome species may be better able to sustain microbial balance, promoting their own survival and reconstitution after a disruptive event. Alternatively, selective elimination of commensals that are critical to a balanced microbial ecosystem may itself lead to long-term microbial dysbiosis, with a consecutive risk of pathogen overgrowth and inflammation. In this scenario, the damage done by antimicrobial therapy may depend on the antibiotic regimen, though the disruptive effect of different types of antibiotics on the respiratory microbiome is currently not yet known.

Selection: Inflammation

Inflammation may select for bacterial species that thrive in a pro-inflammatory environment, and thereby elicit microbial dysbiosis. Local inflammation may be involved in pathogen invasion and infection or instigated by external stimuli such as exposure to air pollution. The respiratory microbiome during infection is less diverse and contains increased levels of pathogenic species *S. pneumoniae, H. influenzae*, and *M. catarrhalis* compared to healthy controls (3, 5, 62, 97), supporting the hypothesis that host pro-inflammatory responses to pathogen invasion mediate selective bacterial colonisation. Moreover, it has been shown that following virus-associated RTI, an individual more readily develops a *Moraxella*-dominated bacterial profile and is more susceptible to future infections, strengthening the idea that the inflammatory state accompanying viral infection may modulate the respiratory microbiota long-term (4). Conversely, protective microbes, for example high nasal abundance of *Corynebacterium* and *Dolosigranulum*, is associated with decreased infection rates, possibly by limiting the host pro-inflammatory response to a viral or bacterial pathogen and maintaining

the balance between asymptomatic colonisation and infection (4, 63). The quality of inhaled air is affected by particles including microbes, but also allergens, dust, and fine particulate matter that come in direct contact with the respiratory mucosal surfaces and affect host-microbe interactions (98-100). Individuals are variably and often chronically exposed to inhaled particles because of urbanisation, the working environment or proximity to domestic and/or farm animals, which can either prevent or induce inflammation. For instance, living near poultry farms was associated with an increased risk of community-acquired pneumonia, and during infection patients had an imbalanced oropharyngeal microbiota with higher abundance of *S. pneumoniae* (101). It was suggested that inhalation of emissions from agricultural activities affects mucosal immune responses, creating a pro-inflammatory environment that instigates microbial dysbiosis, although a direct microbiome-modulating effect cannot be excluded. On the other hand, early-life outdoor and indoor allergen exposure appears to protect from development of persistent wheeze, asthma and allergies, possibly through modulation of the immune response and more diverse microbiota (11, 19).

The question that remains is a typical chicken-and-egg conundrum: does mucosal inflammation lead to microbial dysbiosis with overgrowth of bacterial pathogens that prosper in the inflammatory milieu (102, 103), or does an imbalanced microbiome primarily drive inflammation and consequently induce infection (104, 105)?

Role of dysbiosis in disease

Acute respiratory tract infection

Respiratory pathogens generally enter the respiratory tract through the nose and nasopharynx, where they first encounter the resident microbiota and compete for resources required for successful colonisation of the mucosal surfaces. Since the nasopharynx and oropharynx are connected, colonising microbes may then also become member of the oropharyngeal microbiome (24). The nasopharynx is considered the primary niche from where potential bacterial and viral pathogens migrate to the sinuses, middle ears as well as the meninges (106). By contrast, dissemination to the lungs

is believed to occur primarily by aspiration from the oropharynx, although in young children, the nasopharynx seems to be an alternative source due to anatomical differences, preferred nasal breathing and increased nasal secretions (24). Lower RTIs are far less frequent than upper RTIs, which suggests that oropharyngeal pathogen containment may be highly effective, possibly resulting from the diverse microbial mixture of respiratory, gastro-intestinal and skin bacteria.

Alterations to the respiratory microbiome associated with infectious disease include increased abundance of respiratory pathogens like *Haemophilus* and *Streptococcus* spp. and decreased abundance of health-associated commensals like *Corynebacterium, Dolosigranulum* and *Moraxella* spp. (28, 107). Interestingly, changes to the microbiota seem to precede clinical signs of infection, suggesting causal involvement. Longitudinal follow-up of young children showed that microbial development trajectories of children experiencing more RTI episodes already deviated from normal development from the first month of life, with pronounced shifts in the nasopharyngeal microbial composition characterised by prolonged enrichment with oral-type bacteria before RTI onset (108). However, merely a pro-inflammatory bacterial community is insufficient to cause clinical disease, and environmental stimuli, irritants, allergens, or viral infections seem required to tip the balance. For instance, viral invasion may enhance potential bacterial pathogen colonisation by damaging epithelial cells, modulating innate immune responses, and promoting bacterial proliferation through direct effects on the mucus layer, thereby predisposing to secondary bacterial infection (reviewed in (7)). Besides infection-promoting changes in the micro-environment, pro-inflammatory responses elicited by environmental triggers may result in tissue swelling, which may cause translocation of bacteria from the respiratory tract to other sites, such as sinuses or the Eustachian tube where they may subsequently cause, sinusitis or AOM (109). In this way, microbial disequilibrium occurring in parallel with an additional infectious or non-infectious trigger may incite acute infections.

The microbial composition of the URT appears to not only influence RTI susceptibility, but also modulate RTI severity. Hospitalisation for severe RSV infection was associated with nasopharyngeal bacterial profile enrichment

by *S. pneumoniae* and *H. influenzae*, whereas high abundance of *S. aureus* was related to mild disease (110). Infants carrying high levels of *S. pneumoniae* and/or *H. influenzae* in their noses showed upregulation of genes involved in pro-inflammatory pathways, which probably underlies the enhanced disease severity (110). Mechanistic evidence was obtained in mice: the airway microbiota was shown to protect from influenza-related death through dampening the antiviral immune response and limiting lung tissue damage (111). Further support is derived from metabolome analysis of infants with viral-associated LRTI, in which increased abundance of *Streptococcus* in the nasopharynx was associated with increased levels of pro-inflammatory sphingolipids and increased disease severity (112). In a similar study, high *Haemophilus* abundance was related to increased levels of N-acetyl amino acids, of which the functional potential remains unclear (113). Together these results again suggest that the resident respiratory microflora play an important role in acute RTI pathogenesis, even in infections of presumed viral origin.

Chronic wheeze and asthma

Asthma is a chronic inflammatory condition that affects one in ten children in westernised countries. Virus-associated LRTIs are a major risk factor for its development, particularly in case of rhinovirus and respiratory syncytial virus (94, 114). Furthermore, it is generally accepted that atopy at a young age, characterised by a bias towards Th2 response, is a risk factor for asthma development in children. The mechanisms driving this Th2 imbalance are still poorly understood but evidence points towards early-life microbial exposures deriving either from host's microbiota and/or its environment leading to immune dysregulations that translate into allergic disorders later on.

Murine studies have again identified a critical neonatal period, during which the presence of microbial signals had protective effects against allergic sensitisation. Airway microbial colonisation was shown to be required for the induction of regulatory T cells via PDL1 expression on lung dendritic cells in the first weeks of life conferring protection against aeroallergen-induced airway inflammation in mice (73). Similarly, eradication of the

microbiome by broad-spectrum antibiotic treatment resulted in increased airway inflammation that correlated with reduced Tregs in the gut; an effect observed only if the antibiotics were administered perinatally (115). Invariant natural killer T cells were also reported to play a role in this process, as early life microbial colonisation prevented their accumulation in both the lungs and the gut and reduced exaggerated airway responsiveness (116).

This so-called window of opportunity in the neonatal period of mice is just starting to be explored in humans and seems to fall within the first three months of postnatal life. Multiple studies have associated changes in gut microbial diversity (6) or composition (117-119) early in life with later development of allergy and asthma. In the respiratory tract, asymptomatic hypopharyngeal colonisation with *S. pneumoniae*, *M. catarrhalis*, *H. influenzae*, or a combination of these species in one-month-old infants using traditional bacterial culture was associated with increased asthma prevalence at the age of five in children born to asthmatic mothers (120). In addition, nasopharyngeal colonisation characterised by high levels of *Streptococcus, Moraxella* and *Haemophilus* in the first two years of life, as defined by 16S-based taxonomic profiling, correlated with increased prevalence of chronic wheeze at five years of age even after correcting for LRTI (114, 121). However, the impact of such dysbiosis and the mechanisms driving host-microbial interactions in the early stages of human life is largely unknown, calling for future studies.

MODELS FOR STUDYING THE ROLE OF THE EARLY-LIFE MICROBIOME IN RESPIRATORY HEALTH

In humans, early-life microbiota studies have mostly been descriptive as they typically rely on bacterial communities taxonomic and functional characterisation using next generation sequencing technologies. Studies integrating the host component in the (neonatal) airways are limited (43) and conclusions are often drawn based on available clinical data. Moving from descriptive to mechanistic approaches targeting host-microbiome-environment interactions is crucial when aiming to predict or influence respiratory health.

In vitro functional studies in controlled conditions could provide important mechanistic insights of direct host-microbe interactions. This later approach was used to characterise the impact of specific bacteria/viruses on cell lines of human bronchial epithelial cells upon infection (122) or monocyte-like cells in the context of lung transplantation (123). While these models can be useful to address specific host-microbe interactions, caution must be exercised in extrapolating the results as it may miss important complexity at both microbial and host system levels. This becomes even more challenging when studying early life, a dynamic period during which changes rapidly occur due to both intrinsic developmental factors and external environmental cues.

A substantial number of animal studies have supported the concept for a role of the early-life microbiota. In particular, the use of germ-free mice, lacking exposure to any microorganism, provided key mechanistic evidence for the link between early-life microbial colonisation of the lungs and allergic disease development (73, 116). Although human and murine lung microbiota seem to share some similarities at least at the phylum level (73, 124, 125), important differences at higher taxonomic levels have been observed not only among these two mammalians but also between different mouse strains, which is also true for the gut (126), and between different vendors (127). Germ-free or antibiotic treated mice may also serve as recipients for human microbiota xenografts, making extrapolation of data towards humans slightly easier. Studies targeting the gastrointestinal tract have used this approach, where murine gut microbiota has been reconstituted either using fresh faecal samples or a defined number of bacterial species detected in early life (117). However, the final degree of community recolonisation appears limited as the murine diet and intestinal microenvironment presumably do not support colonisation by all microbial constituents of the human microbiome.

Finally, computational approaches represent a promising avenue to model early-life microbiota establishment and interactions with the host. Advances in next generation sequencing technologies have enabled the generation of elaborate datasets, which could serve as a basis for *in silico* modelling. Such mathematical approaches have been used to explore lung

microbiota structure and stability, notably during chronic infections in cystic fibrosis patients (128, 129). This approach is particularly relevant for early-life microbial ecology where the community gradually matures after initial seeding while responding to invading pathogens. The challenge now resides in the integration of both host and microbiota multi-omics datasets, requiring the development of new algorithms to capture interactions within heterogeneous datasets.

CONCLUSION AND FUTURE PERSPECTIVES

Our expanding knowledge of the form and function of the airway microbiome has highlighted it as a potentially key determinant in both the development and progression of respiratory diseases. However, the majority of our knowledge is still limited to associations, with very little mechanistic insight available. The mechanisms of host-microbial crosstalk need to be approached from a system science perspective in order for the field to be able to distinguish cause from consequence and to develop rational intervention strategies (Figure 4). Mechanistic insight can come from animal models, *in vitro* studies and new bioinformatics approaches capable of integrating multiple highly dimensional datasets. In addition, the vast majority of research to-date has focused solely upon the bacterial component of the microbiome, disregarding the impact of resident viruses, fungi and phages – constituents whose role in host-microbe interactions are likely significant as well. Finally, the impacts of the microbiome upon immune cell function, immune development and respiratory diseases will depend on the stage of life, with early life representing an opportunity for shaping the health trajectory of an individual. Overall, this burgeoning area of research into the airway microbiome is providing novel insights into disease development and progression, and may pave the way for novel preventative and therapeutic approaches to restore symbiosis, for example by (i) reducing the burden of potentially pathogenic species by targeted vaccination or antibiotic treatment which is current practice, (ii) increasing species diversity, for example by dietary adjustments or (iii) stimulating or supplementing specific beneficial microorganisms by pre- and probiotic products. The latter, interestingly, may be achieved either

directly or indirectly through the immune-modulating properties of the gut microbiome. However, much work remains to be done, and future efforts will build on the currently existing body of evidence summarised in this chapter.

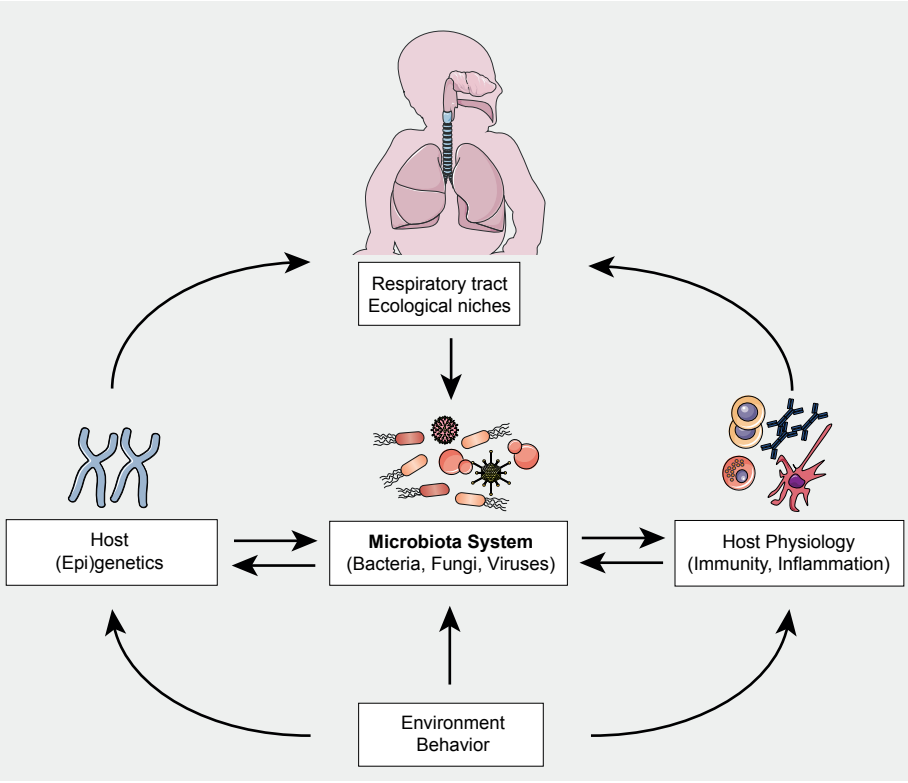

Figure 4 | Exploring early-life respiratory microbiota using a system science approach. The study of early-life microbiota should be approached in a holistic manner, integrating information from both the host and its environment in a temporal order. Arrows indicate the different type of interactions that may occur at different levels of the system and lead to its perturbation.

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CHAPTER 3

Characterizing the respiratory microbiome

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INTRODUCTION

Over the last decade, researchers have begun to unravel the causes and consequences of variation within the respiratory microbiota, developing a more profound understanding of its role in the pathogenesis of pulmonary disease to improve clinical management. Developments in culture-independent identification of bacterial species have provided faster and more cost effective methods to characterize niche-specific microbial ecosystems. Historically, the gut has been the niche of focus for human microbiome research, but recent studies have revealed an unexpected diversity of bacteria in both the upper and lower airways, linking community composition to a number of respiratory diseases, including cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and acute infections (1). Monitoring temporal changes in community composition of the respiratory microbiota can reveal the influence of host and environmental drivers on ecosystem behavior, as well as the consequences of infection susceptibility or severity, and treatment effects. Here, we outline current best practices and upcoming developments for respiratory microbiome research and potential clinical applications.

STUDY DESIGN

So far, in respiratory microbiome study design, we have learned that crucial elements in generating valid, useful results include clear research questions, power calculations, enrolment of sufficient numbers of subjects and controls, robust sampling and exhaustive patient information collection. Although this applies to any well-designed population-based or clinical study, we also need to carefully consider possible confounding effects of a broad range of environmental and host characteristics on microbiome composition (1, 2). The first pioneering cross-sectional studies linked altered microbial community structure and composition to disease state (3, 4), but longitudinal sampling is needed to fully understand the causes and long-term clinical outcomes of variation in respiratory microbiota. For example, recent well-characterized healthy birth cohorts have shown the dynamics of nasopharyngeal microbiota development in relation to lifestyle factors (5, 6), and have revealed marked shifts in microbial community composition associated with acute respiratory infections (7, 8). Intensive follow-up of CF (9, 10) and COPD (11) patients

demonstrated changes in the airway microbiome composition preceding symptom onset, suggesting that dysbiosis coupled to a dysregulated host immune response could be at the basis of disease progression (12). Support for the potential role for the respiratory microbiome in early disease pathogenesis is evident in early childhood, as microbial communities with fewer commensals and more potential pathogens are associated with consecutive wheeze and asthma (8). So far, every study of respiratory microbiota in relation to any lung disease, has revealed clear aberrations of microbial community composition from the healthy state, redefining commonly accepted pathophysiological concepts in respiratory disease pathogenesis (12).

SAMPLE COLLECTION

With respect to anatomy and site of sampling, the respiratory tract is not a single uniform system, but consists of interconnected niches harboring distinct microbial communities that depend highly on local microenvironmental conditions. Therefore, when designing a new microbiome study, the appropriate sampling niche will largely depend on research question, hypothesis and target population. Key procedural practicalities also require consideration; for example, sampling the lower respiratory tract (LRT) requires invasive bronchoscopic procedures, limiting sample size, age-groups to be studied, and frequency of repeated sampling. To overcome this lack of access to the lungs, many studies use the easily accessible upper respiratory tract (URT) which is considered the likely source community of the lungs as well as a reservoir for most respiratory pathogens (12, 13). In healthy adults, microbial colonization of the LRT is assumed to originate from micro-aspiration of the oropharyngeal 'flora', and hence, the oropharynx can be used, albeit imperfect, as a proxy for the lungs. In children, however, both the nasopharynx and oropharynx are likely sources of microbial seeding to the LRT, probably resulting from anatomical differences, nasal breathing, and higher production of nasal secretions by children (14, 15), further limiting result extrapolation. In chronic lung diseases such as CF and COPD, the URT and LRT communities appear to become segregated with increasing disease duration. This is probably due to chronic inflammation, failure of lung clearance mechanisms, and repeated

antimicrobial treatment resulting in localized selection and evolution of independent communities, the latter rendering LRT sampling from multiple sites mandatory to obtain meaningful results (16, 17).

SAMPLE PROCESSING

An important aspect to consider throughout the design and execution of a respiratory microbiome study is the risk of and control for contamination. The respiratory tract harbors low-density bacterial communities, with microbial densities dropping along the way from the URT to the LRT (14, 18). As a result, environmental DNA introduction during sample collection and processing becomes a likely threat, and can entirely overrule the true microbial signal (18). Sampling of the LRT particularly carries a high risk of microbial carryover from the URT, and so accurate sampling should be undertaken by well-trained and consistent personnel to reduce the risk of contamination. During transportation, samples should be kept cooled in appropriate storage media, and then processed and stored at -80 °C as soon as possible to prevent selective bacterial outgrowth. Additionally, contamination from the laboratory environment and the reagents used for sample processing can significantly influence results from low-biomass microbial communities (19). Implementing proper 'negative' controls for all sampling, storage and laboratory procedures allows for later comparison and identification of potentially confounding environmental signals (for more details see (20)). Variations in methodology and batches can also affect results, highlighting the importance of clean working during DNA extraction and using fully optimized methods for the specific sample type. In addition to contamination, the extraction method can also affect the quality of the data and care should be taken to use methods which do not bias the bacteria extracted from the samples (18). Including 'positive' controls in the form of mock communities, will allow for adequate control and comparison between sequencing runs, laboratories and institutes (13).

SEQUENCING PLATFORMS

Regarding sequencing platforms, amplicon sequencing is currently the most commonly used method for determining the microbial community

composition and targets the bacterial 16S ribosomal RNA (rRNA) gene, containing highly conserved as well as hypervariable regions. This targeted approach has revealed a wealth of information regarding community composition and dynamics. However, the taxonomic resolution provided by 16S rRNA sequencing is limited due to the short target region length, complicating accurate species- and strain-level identification. In comparison, metagenomic sequencing captures the entire microbial genomic content, including bacteria, viruses and eukaryotes, and allows for microbial characterization at the deepest taxonomic levels as well as functional potential profiling. However, applying this technique to low-biomass respiratory samples is challenging, as genome assembly requires high numbers of sequencing reads per sample, which makes detection of low-abundant species difficult, and increases the risk of contamination (21).

DATA HANDLING

Once data is generated, the bioinformatics and statistical methods required to analyze the large amounts of raw DNA reads generated by sequencing can be daunting. Initially, raw reads are filtered to remove sequencing errors and are assembled into complete sequences, after which the sequences are grouped based on similarity and assigned taxonomic names to reveal their identities. Several bioinformatics pipelines are freely available for data pre-processing, including Qiime (22) and mothur (23). Each resulting microbial profile shows the abundance of individual species relative to the entire bacterial population within a sample, and contains many zero abundances, demanding nonparametric statistical methods developed specifically for handling microbiome data (24). Characterizing microbial development over time requires multiple measurements of the same individual, further complicating data analysis, but several approaches have been proposed to correct for repeated measures (25, 26). The increasing application of machine-learning techniques that perform predictive modelling of clinical outcomes from microbial profiles combined with host and environmental characteristics, is a promising development (27). However, the study of temporal microbiome dynamics, especially while accounting for confounding factors, remains in its infancy (24).

CLINICAL APPLICATION

In the era of the 100,000 Genome Project and the launch of the NHS Genomic Medicine Service, it is clear that sequencing techniques are not only more accessible but are also becoming more integral to the clinical environment. In the clinic, identification by culture still dominates pathogen detection, and although quantitative methods such as qPCR are increasingly available, applications of sequencing technologies are lacking. Cost effectiveness and efficiency of sample and data processing are currently being improved to enable clinical implementation of sequencing methods. Single-use sequencing applications are being developed, as are faster methods of DNA extraction and library preparation (28). Technological and bioinformatic advances are in the pipeline to improve detection of subtle strain-specific variation within the target region (24). For applying sequencing at the point of care, the portable, low cost, real-time DNA sequencer Oxford Nanopore MinION has real potential with its ability to rapidly sequence the bacterial 16S gene, even up to strain-specific resolution (29). The emergence of real-time sequencing technologies could dramatically influence diagnostic methods through accurate species identification and quantification within a clinically relevant time frame.

RESEARCH PRIORITIES

To move closer towards clinical applications, comparative and meta-analyses must combine results from different cohorts to define actionable thresholds of microbial abundance. Current methodological heterogeneity restricts comparability across institutes, and so by underlining essential aspects of study design including consistent sample collection and processing, adequate contamination controls, and longitudinal sampling (summarized in the Figure and Box 1), we hope to encourage reaching a consensus on solid, robust methodology for respiratory microbiota research. Our increased understanding of respiratory disease pathogenesis will contribute to reshaping clinical diagnostic, preventative and therapeutic strategies. Important challenges remain to integrate the advances within microbiota research into everyday medical practice, and future efforts should prioritize standardization of protocols and analysis, adaptation of technology for

application in the field including remote settings, and collaboration across countries and disciplines (Box 2). However, current progress in respiratory microbiota research certainly provides a promising platform for the clinical application of culture-independent techniques in the future.

Box 1 | Essentials for respiratory microbiome studies

- Longitudinal study design
- Appropriate power calculations
- Consistent sampling
- Appropriate niche (proxy)
- Minimise contamination at all stages
- Contamination controls at all stages
- Robust quality checks
- Consistent bioinformatics processing
- Suitable analysis techniques (complex data)

Box 2 | Research priorities for future studies

- International platforms for communication
- Uniform sampling and transport protocols
- Standardized controls across laboratories
- Agreement on handling complex data
- Adapt technology for remote settings
- Collaboration between research disciplines (clinics, microbiology, molecular biology, ecology, bioinformatics)
- Invest in (interdisciplinary) training

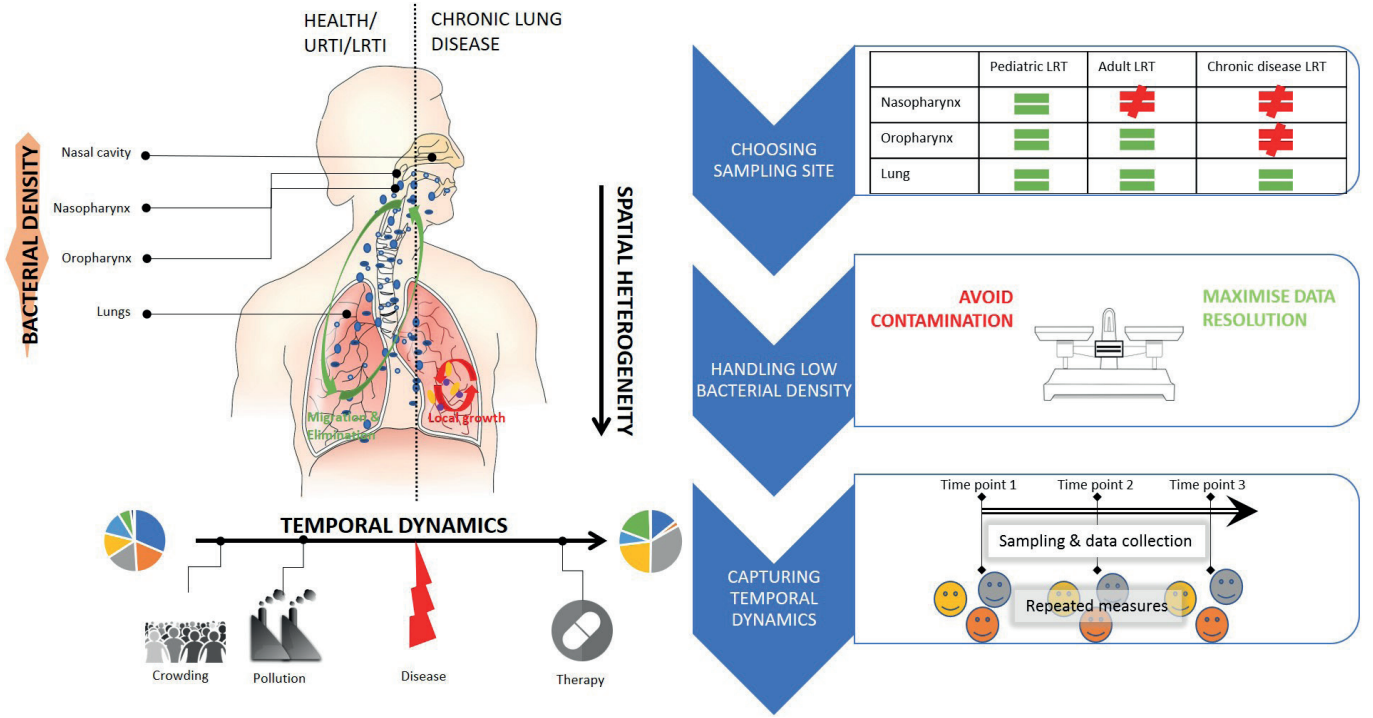

Figure | Challenges in characterizing the respiratory microbiome. Niche-specific communities reside in the different parts of the URT and LRT, and therefore sampling site should depend on the research question and population studied. During health and acute URTI or LRTI, the LRT is transiently colonized with microbes from the URT (oropharynx for adults, naso- and oropharynx for children), while in chronic lung diseases, over time local selection and community assembly leads to differences between URT and LRT assemblages. In general, the local bacterial density in the respiratory tract is low, further decreasing when descending towards the LRT. Therefore, working with low-biomass samples requires careful sampling procedures and laboratory handling, including appropriate negative and positive controls to acquire reliable results. Microbial development over time is affected by environmental stimuli including crowding factors and pollution, and is altered in various acute and chronic diseases, so repeated sampling and exhaustive data collection of the same subjects over time is required to study cause-consequence relationships and estimate environment-induced variation. Appropriate bioinformatic processing of the sequencing data is required before robust statistical analysis is executed, which preferably accounts for covariates and repeated measures where relevant. Abbreviations: URT = upper respiratory tract; LRT = lower respiratory tract; URTI = upper respiratory tract infection; LRTI = lower respiratory tract infection.

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CHAPTER 4

Interplay between the early-life respiratory microbiota and mucosal antibody development is associated with infection susceptibility

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Submitted

ABSTRACT

Objective: To investigate associations between mucosal immunoglobulin (Ig) dynamics, nasopharyngeal microbiota development, and RTI frequency in the first year of life.

Materials and Methods: We collected nasopharyngeal and saliva samples from 127 healthy infants at 11 timepoints between birth and 12 months of age, together with data on perinatal and lifestyle factors, breastfeeding, and RTIs. Salivary IgG, IgA and IgM concentrations were measured using multiplex immunoassays. Nasopharyngeal gene expression profiles were determined in 43/127 infants using microarray. The nasopharyngeal microbial community was characterized in 114/127 infants using 16S-rRNA-sequencing.

Results: We observed strong upregulation of Ig production pathway gene expression preceding increases in salivary IgA and IgM at 1-2 weeks, and in salivary IgG at 6-9 months. High IgA concentrations directly after birth were observed exclusively in breastfed infants, and were associated with fewer RTIs in the first year of life as well as with lower bacterial biomass in the first days of life and earlier predominance of *Corynebacterium* and *Dolosigranulum* in the nasopharynx. Vice versa, early predominance of *Moraxella* was associated with higher subsequent IgA (2 weeks) and IgG concentrations (6 months), which were in turn associated with higher RTI frequency.

Conclusion: Our results suggest that breastmilk-associated IgA directly after birth may affect microbiota assembly and development, and potentially thereby infection risk. Conversely, early pathogen exposure appears to boost subsequent mucosal Ig production and increase RTI susceptibility. Further studies are required to determine cause-effect relationships and mechanisms underpinning these results.

INTRODUCTION

Local immunoglobulin (Ig)G, IgA and IgM at mucosal surfaces lining the respiratory tract is critical to the first-line defence against infections. T lymphocytes that can induce antibody production by B cells, are already present in nasopharynx-associated lymphoid tissue during gestation (1). However, directly after birth, regulatory T cells actively repress these cells to ensure tolerance to microbial colonization, which occurs at the cost of heightened infection susceptibility (2). The neonatal defence against infections therefore depends on maternal Ig acquired via transplacental transfer (IgG) and breastfeeding (predominantly IgA). Subsequent development of intrinsic mucosal antibody responses during infancy and their role in host protection remain poorly understood.

Microbial colonization of respiratory surfaces in infancy is influenced by host and environmental factors, including gestational age, mode of delivery, breastfeeding, antibiotic treatment, season and crowding (3–7). In turn, the early-life microbiota developmental trajectory is associated with long-term health, including susceptibility to respiratory tract infections (RTIs) (7, 8). Host-microbiota interactions in the early-life respiratory tract likely shape the local immune environment including the mucosal antibody repertoire (3). For example, IgA-secreting plasma cells remain scarce in germfree mice, but increase in number only after bacterial exposure (9). Conversely, mucosal antibodies influence microbial function. Secretory IgA coats and retains commensals to the mucosa, dampens pro-inflammatory immune responses and limits bacterial growth (10, 11). IgM likely has overlapping functions with IgA (12). By contrast, mucosal IgG mostly binds to potentially pathogenic invaders and appears to especially increase in response to infections (10, 13).

To date, antibody-microbiota interplay has mostly been studied in the gut (14–17) and much less in the respiratory tract. Here, we demonstrate in a prospective, healthy birth cohort that early-life IgA is associated with nasopharyngeal microbiota assembly in the first days of life, and nasopharyngeal microbial community development is associated with mucosal antibody levels in the first year of life as well as RTI frequency.

METHODS

Microbiota and gene expression data that support the findings of this study were deposited in the National Centre for Biotechnology Information GenBank database (accession numbers PRJNA740120 and GSE152951).

Study design and sample collection

Study design and inclusion criteria were previously published (6). In short, 127 healthy, full-term infants were enrolled in a prospective birth cohort study, and saliva and nasopharyngeal swabs were collected during 11 visits in the first year of life: within 2 hours after birth, on days 1, 7 and 14, and at 1, 2, 3, 4, 6, 9 and 12 months of age. Sampling procedures are described in the supplementary materials. The nasopharyngeal microbiota (*n*=114 infants, *n*=1156 samples) and gene expression data (*n*=43 infants, *n*=286 samples) were previously published (18). For the current analyses, we added data on salivary IgG, IgA and IgM concentrations (*n*=127 infants, *n*=1099 samples; Figure S1). In addition, data were obtained on perinatal, environmental and lifestyle factors and experienced RTIs, defined as parent-reported respiratory symptoms and/or fever between 2 consecutive study visits. Infants were subdivided into groups with 0-2, 3-4, or 5-7 RTIs (7). Ethical approval was granted by the Dutch national ethics committee. Both parents provided written informed consent.

Salivary antibody measurement

IgG, IgA and IgM concentrations in saliva were quantified using fluorescent bead-based multiplex immunoassays (details in supplementary materials). Anti-human IgG, IgA or IgM were coupled to carboxylated beads (Luminex). Saliva was thawed and centrifuged, and the supernatant was diluted 1:200, which was applied to a well containing the beads, allowing the antibodies in the sample to bind to the bead surface. R-phycoerythrin-labelled mouse anti-human kappa and lambda solution (ITK Diagnostics) was added. Samples were acquired on a BioPlex 200 apparatus and concentrations interpolated using BioPlex software (Bio-Rad Laboratories). Samples were re-analysed at a 1:1000 dilution when the measured concentration was too high to accurately interpolate. Concentrations below the lower limit of detection were set at half the limit.

Nasopharyngeal microbiota characterization and viral qPCR

Bacterial DNA extraction and library preparation for the V4 region of the bacterial 16S-rRNA-gene and sequencing of amplicon pools with the Illumina Miseq platform were previously described (7). Bioinformatic pre-processing of raw reads was performed using DADA2 (details in supplementary materials) (18, 19). Each Amplicon Sequence Variant (ASV) was assigned taxonomic annotation with a unique rank number. Contaminant ASVs were identified and removed using the *decontam*-package (20). Raw and relative abundance ASV tables were used in downstream analyses. Total bacterial DNA was quantified using 16S-rRNA-gene quantitative (q)PCR (7). Viral qPCR specific for 17 respiratory viruses was performed as described elsewhere (18).

Nasal gene expression profiling

RNA extraction followed by microarray analysis (Affymetrix Clariom S Human Pico array) was performed as previously described (18). For the current study, eigengene expression of genes included in the Ig production pathway (Gene Ontology database) was calculated as the first principal component of the expression matrix (21).

Statistical analysis

R (version 4.1.2) was used for statistical analysis. Ig concentrations were analysed as continuous variables (log_{10}-transformed) or categorized into high, middle and low terciles depending on the type of analysis. We considered p-values <0.050 or Benjamini-Hochberg (BH-)adjusted q-values <0.100 statistically significant. Z-scores of Ig concentrations, eigengene expression and relative abundances were generated by standardizing to all values.

Associations between host factors and Ig concentrations were assessed using linear (mixed-effects) models. Group differences in Ig concentrations or log-transformed bacterial density over time were assessed using linear mixed-effects models with subject identifier as random effect and an interaction term between timepoint and the variable of interest as fixed effect, allowing for *post-hoc* extraction of pairwise contrasts per timepoint (*emmeans*-package).

Differences in microbial community composition were evaluated using permutational analysis of variance on the Bray-Curtis dissimilarity matrix (PERMANOVA, *vegan*-package). Microbiota cluster membership was allocated to each sample using average linkage hierarchical clustering as previously described (18). Differences in time to cluster transitions were evaluated with Cox proportional hazard models (*survival*- and *survminer*-packages). Associations of ASVs (present in >25% of samples tested) with Ig concentrations/terciles per timepoint were tested using Microbiome Multivariable Association with Linear Models 2 (MaAsLin2; *MaAsLin2*-package). Differential abundance of individual ASVs (present in >10% of samples tested) over time between Ig terciles was tested using smoothing spline (ss-)ANOVA (*metagenomeSeq*-package). Associations were tested between specific ASVs presence/absence (defined as >0.10% relative abundance) or relative abundance (log-transformed after adding a pseudocount of 1) and Ig levels in linear models.

RESULTS

Salivary antibody dynamics in the first year of life

Study population characteristics are summarized in Table S1. We distinguished 3 developmental phases with specific salivary antibody concentration dynamics within the first year of life (Figure 1A). In the first 2 weeks of life, total IgA and IgM varied between individuals but generally increased (early phase), with a peak at month 1 followed by a subsequent decrease in concentrations (middle phase). Salivary IgG dynamics differed from IgA/IgM with the highest levels directly after birth, followed by a gradual decrease until 6 months of age. From 6 months onward, IgG levels increased (late phase), while IgA/IgM levels remained stable.

Salivary IgA/IgM increases were preceded by upregulation of genes involved in Ig production in the nasopharynx at day 1 (eigengene expression; Figure 1B-C). IgG increases followed a second upregulation of Ig production eigengene expression between months 4 and 6. These findings suggest that intrinsic salivary IgA/IgM production started already in the first 2 weeks of life, while intrinsic IgG production only substantially increased after 6 months. Therefore, we focused our analyses on IgA/IgM in the first 4 months of life and IgG in the second half-year.

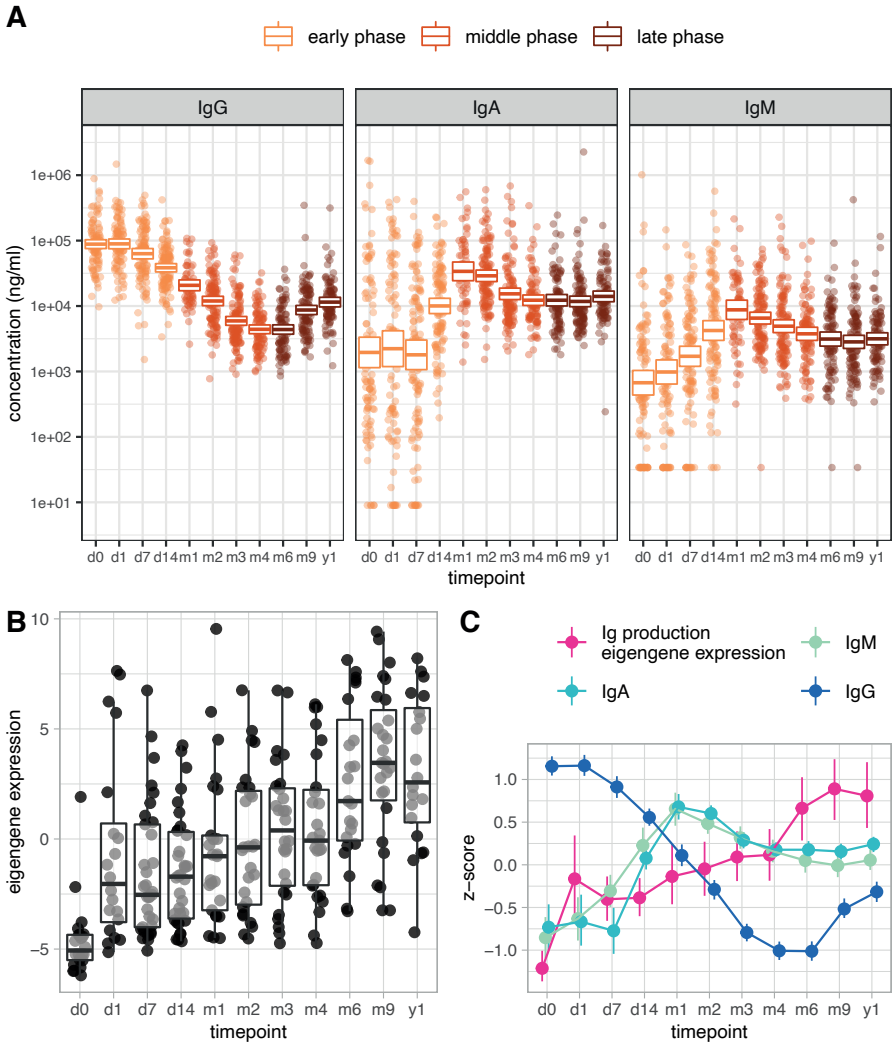

Figure 1 | Mucosal IgGAM development in the first year of life. (A) Salivary immunoglobulin (Ig)G, IgA and IgM concentrations in healthy infants (n=127) at 11 timepoints in the first year of life (d=day, m=month, y=year). Boxes represent geometric mean concentrations (GMC) with 95% confidence interval (CI). (B) Eigengene expression of genes included in the Gene Ontology (GO) pathway for immunoglobulin production over time. Boxes show median with interquartile ranges (IQR). (C) Z-scores of Ig eigengene expression and salivary IgG, IgA and IgM concentrations over time showing strong upregulation of Ig production pathway gene expression preceding increases in salivary IgA and IgM at 1-2 weeks, and in salivary IgG at 6-9 months. Dots and lines show means with 95% CI.

Early salivary IgA levels were associated with RTI susceptibility

We studied antibody dynamics in relation to cumulative RTI frequency in the first year of life (0-2, 3-4 or 5-7 RTIs; Figure 2). IgA concentrations at day 1 were higher in infants with 0-2 compared with 5-7 RTIs in the first year of life ($q<0.001$). Conversely, IgA concentrations at day 14 were higher in infants who ultimately developed 5-7 RTIs ($q=0.082$). This association remained after adjusting for breastfeeding (day 1: $q<0.001$; day 14: $q=0.038$). IgM concentrations showed similar patterns. Regarding IgG, infants with 5-7 RTIs showed higher levels at 6 months of age than infants with 0-2 RTIs ($q=0.014$), suggesting that IgG responded to stimulation by the first infectious events in the preceding months.

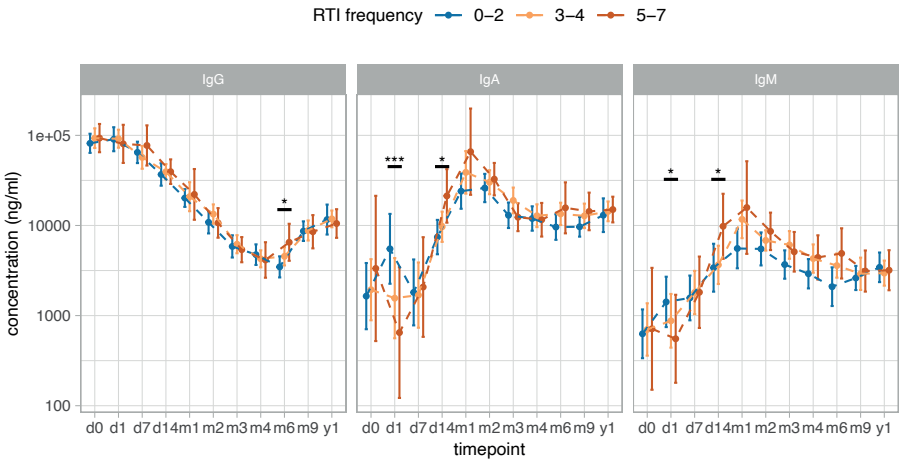

Figure 2 | Salivary antibodies in the first year of life according to the respiratory tract infection frequency. IgG, IgA and IgM geometric mean concentrations (GMC) per timepoint (d=day, m=month, y=year) for infants with 0-2, 3-4 and 5-7 respiratory tract infections (RTIs) in the first year of life. Error bars represent 95% CI. Dashed lines connect GMCs at consecutive timepoints per RTI group. Significance was assessed using linear mixed-effects models over time followed by post hoc extraction of pairwise contrasts per timepoint, and is denoted by ***: $q<0.001$; *: $q<0.100$.

Early salivary IgA levels were associated with breastfeeding and nasopharyngeal microbiota assembly

We then investigated the effects of feeding type, mode of delivery, siblings, pets and season of birth on IgA/IgM concentrations in the early and middle developmental phases using multivariable linear mixed-effect models. In the early phase, breastfeeding was strongly associated with higher salivary IgA/

IgM concentrations (Figure 3A). IgA/IgM concentrations were the highest in exclusively breastfed infants, followed by infants receiving both formula- and breastfeeding, and the lowest in exclusively formula fed infants (Figure 3B). Notably, at day 7, we observed a higher Ig production eigengene expression in formula fed infants compared with breastfed infants (Figure 3C), which might suggest earlier induction of intrinsic Ig production in infants lacking breastmilk-derived IgA. In the middle phase, formula feeding was associated with higher IgM but no longer with IgA concentrations.

Next, we studied whether salivary IgA levels directly after birth were associated with nasopharyngeal microbiota assembly in the first 2 weeks of life. Analyses were focused on IgA, the most abundant mucosal antibody, which strongly correlated with IgM at each timepoint (Spearman's ρ 0.72-0.84, $q<0.001$). We compared infants with high (n=39), middle (n=38) and low (n=39) IgA concentrations at day 0 (or day 1 for 14 infants missing day 0 data) in both unadjusted and feeding type-adjusted analyses. First, infants with low day 0/1 IgA had significantly higher nasopharyngeal bacterial density at day 7 compared with infants with middle (unadjusted q=0.010, adjusted q=0.032) or high day 0/1 IgA (unadjusted q=0.007, adjusted q=0.031; Figure 4A). The microbial community composition at day 7 in infants with high day 0/1 IgA was also significantly different compared with infants with low day 0/1 IgA (PERMANOVA: unadjusted R^2=3.4%, p=0.047; adjusted R^2=3.9%, p=0.027) or with middle day 0/1 IgA (unadjusted R^2=4.4%, p=0.020; adjusted R^2=4.1%, p=0.025; Figure 4B), while no significant differences were observed at days 0 and 1 themselves ($p>0.050$ for all comparisons). At the individual ASV level, infants with high day 0/1 IgA showed higher *Dolosigranulum* (5) abundance (p=0.034, q=0.39) and lower *Staphylococcus* (3) abundance (p=0.043, q=0.39) at day 7 than infants with low day 0/1 IgA (9 ASVs tested, MaAsLin2-analysis; Figure 4C). Similarly, infants with high day 0/1 IgA showed higher *Dolosigranulum* (5) abundance (p=0.012, q=0.21) and a trend towards higher *Moraxella* (2) abundance (p=0.067, q=0.33) as well as lower *Staphylococcus* (3) abundance (p=0.012, q=0.210) at day 7 than infants with middle day 0/1 IgA (10 ASVs tested; Figure 4D). Because *Dolosigranulum* and *Corynebacterium* abundances are strongly correlated, we performed a sensitivity analysis excluding either of these ASVs, which confirmed that infants with high day 0/1 IgA showed trends towards higher abundance of *Corynebacterium* (5) at day 7 when omitting *Dolosigranulum* (7), and *vice versa* ($p<0.100$, Figure S2).

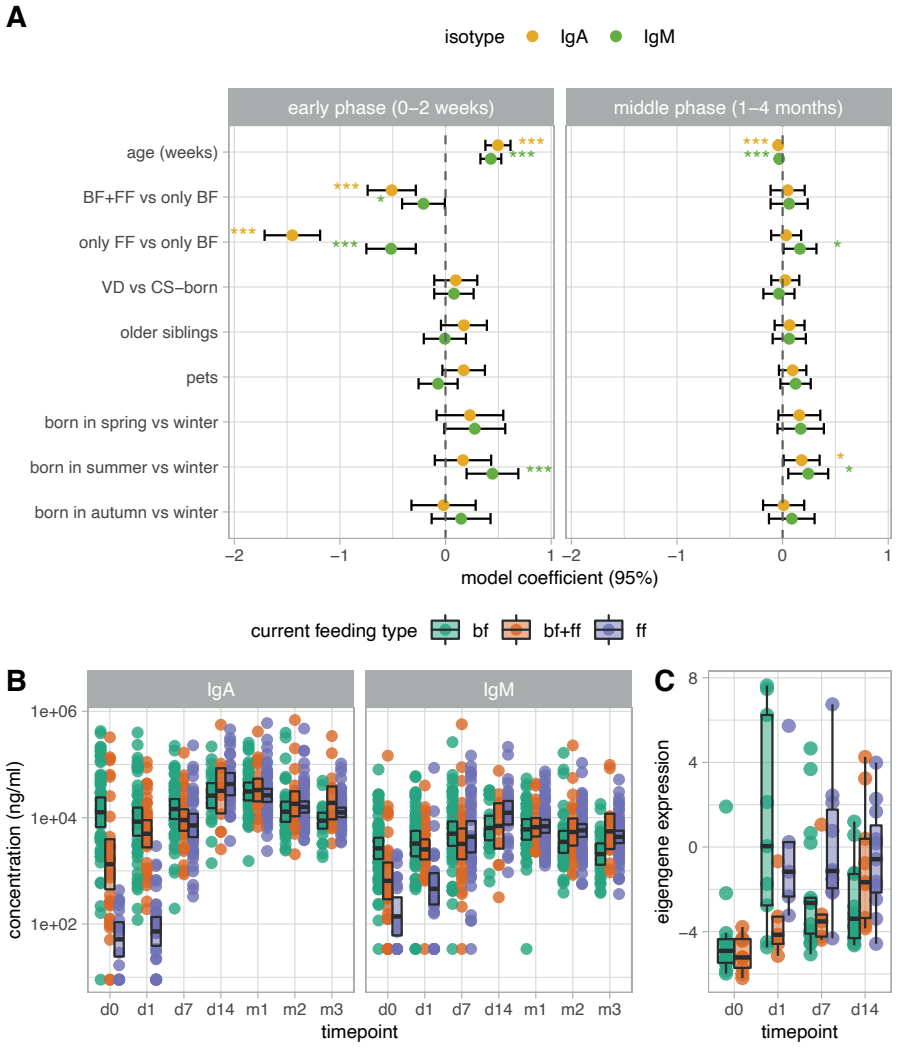

Figure 3 | Early-life IgA and IgM are driven by feeding type. (A) Linear mixed-effect models showing correlations between early-life factors and IgA and IgM concentrations in the early phase (days 0-14) and the middle phase (months 1-4). Dots and error bars denote model coefficients with 95% CI per antibody isotype. Significant associations are shown by the colored asterisks (***: p<0.001; *: p<0.050). (B) IgA and IgM concentrations in saliva over time stratified by feeding type per timepoint, i.e. exclusive breastfeeding (bf), mixed breast- and formula feeding (bf+ff) and exclusive formula feeding (ff). Boxes denote geometric mean concentrations with 95% CI. (C) Nasopharyngeal eigengene expression of the Ig production pathway in the first 2 weeks of life according to (current) feeding type. Boxes show medians with IQR and whiskers extend to 1.5*IQR.

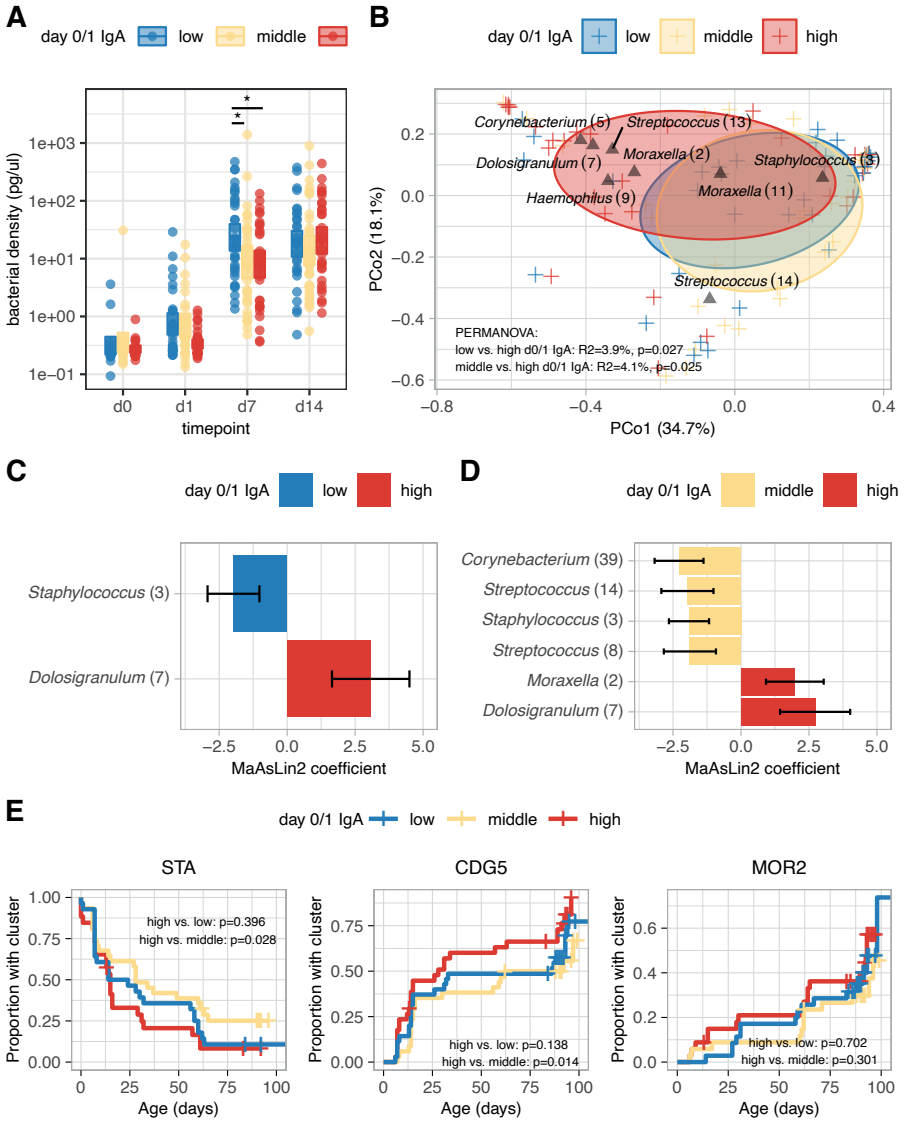

Figure 4 | Associations between day 0/1 IgA and nasopharyngeal microbiota assembly in early life. (A) Overall bacterial density in the first 2 weeks for infants stratified by low, middle and high day 0/1 IgA concentrations. A linear mixed-effects model was used to assess significance of the association between day 0/1 IgA group and log-transformed bacterial density over time, and contrasts were extracted by timepoint. Boxes indicate means with 95% CI. Significance is denoted by *: q<0.100. (B) Principal coordinate (PCo) analysis based on Bray–Curtis dissimilarities showing the nasopharyngeal microbial community composition at day 7 for infants with high, middle and low day 0/1 IgA. Percentages in brackets denote the total variance

Figure 4 | Continued

explained by the first 2 principal coordinates. Each data point (cross) indicates a nasopharyngeal sample colored by day 0/1 IgA group. Ellipses denote the standard deviation of data points per group. In addition, the 8 highest-ranked ASVs at the day 7 timepoint were visualized (triangles). Significance was assessed using permutational analysis of variance (PERMANOVA) adjusted for feeding type. (C,D) Differentially abundant ASVs at day 7 between infants with low and high day 0/1 IgA (C) and infants with middle and high day 0/1 IgA (D). ASVs present in at least 25% of samples tested were analyzed and ASVs that showed a trend towards significance after adjusting for feeding type but without adjusting for multiple testing are shown ($p<0.100$). Bars are colored according to the day 0/1 IgA group they are associated with. Lengths of the bars correspond with the MaAsLin2-model coefficient, which is related to the strength of the association. Error bars denote standard errors. (E) Kaplan-Meier curves depicting high, middle and low day 0/1 IgA in relation to the age at which a given infant first transitioned from a *Staphylococcus*-cluster (STA; left), to a *Corynebacterium*/*Dolosigranulum*- (CDG5; middle) or to a *Moraxella*-cluster (MOR2; right) within the first 3 months of life. Significance was assessed using Cox proportional hazards models adjusted for feeding type, with the high day 0/1 IgA group as a reference.

Although significance of these findings was lost after adjusting for multiple testing, temporal ss-ANOVA comparing infants with high and low day 0/1 IgA across the first 2 weeks of life revealed similar associations (Table S2).

To investigate whether day 0/1 IgA was also associated with long-term microbial outcomes, we compared the time to cluster transitions within the first 3 months of life using survival analyses. Clusters were previously allocated to each sample using hierarchical clustering (18). Generally, the nasopharyngeal microbiota transitioned from the typical early-life *Staphylococcus* (STA)-cluster via a *Corynebacterium* (5)*/Dolosigranulum* (CDG5)-cluster to a stable *Moraxella* (2) (MOR2)-cluster (Figure S3). Infants with high day 0/1 IgA were younger than infants with middle day 0/1 IgA when they transitioned from the STA-cluster (Cox proportional hazard models, high day 0/1 IgA as reference: unadjusted $p=0.056$, adjusted $p=0.028$), and to the CDG5-cluster (unadjusted $p=0.028$, adjusted $p=0.014$) but not to the MOR2-cluster ($p>0.050$). Differences between infants with high and low day 0/1 IgA were not significant (Figure 4E).

Early *Moraxella* acquisition was associated with higher IgA levels

We next studied associations between the nasopharyngeal microbial community in the first 2 weeks of life and salivary IgA at day 14. At day

14, IgA was comparable between breastfed and non-breastfed infants, so these analyses were not adjusted for breastfeeding. However, supervised analyses of the biomarker ASVs of the highly abundant early-life STA-, CDG5- and MOR2-clusters, showed that *Moraxella* (2) presence (p=0.057) and relative abundance (β=1.33 (95% CI 0.18-2.48), p=0.042) at day 7 were associated with higher IgA at day 14 (Figure 5A-B), whereas presence but not relative abundance of *Dolosigranulum* (7) at day 7 was associated with lower day 14 IgA (p=0.008), which persisted at month 1 (p=0.016; Figure 5C-D). *Corynebacterium* (5) and *Staphylococcus* (3) presence and relative abundance at day 7 were not associated with day 14 IgA. Infants subsequently transitioning to the MOR2-cluster between days 7 and 14 (n=6) had significantly higher IgA at day 14 than infants with different cluster membership (p=0.038), which was no longer observed at later timepoints (Figure 5E). Conversely, infants who transitioned to the CDG5-cluster between days 7 and 14 (n=38) showed a trend towards lower IgA at day 14 than infants with different cluster membership (p=0.051). In line, at day 14, relative abundance of *Corynebacterium* (5) negatively correlated with IgA (β=-0.95 (95% CI -1.58- -0.32), p=0.004), while relative abundance of *Moraxella* (2) positively correlated with IgA (β=0.90 (95% CI 0.044-1.76), p=0.040), while no significant correlations were found for *Dolosigranulum* (7) or *Staphylococcus* (3). Subsequent transition to the MOR2- or CDG5-cluster between day 14 and month 1 was not associated with month 1 IgA (Figure 5F). Taken together, these findings suggest an association between nasopharyngeal *Moraxella* (2) acquisition within the first 2 weeks of life and higher salivary IgA at day 14.

RTIs, viral exposure and early pathobiont enrichment were associated with an earlier increase in IgG levels

Next, we studied associations of host and microbial factors with salivary IgG concentrations at 6 months of life, when IgG concentrations started to increase. In univariable analysis, breastfeeding, mode of delivery and presence of siblings were not found to be associated with salivary IgG concentrations. However, environmental and infectious exposures like day-care attendance, a recent positive viral PCR at the preceding timepoint month 4, and experiencing a RTI between 4 and 6 months were significantly associated with higher IgG concentrations at month 6 (Figure 6A).

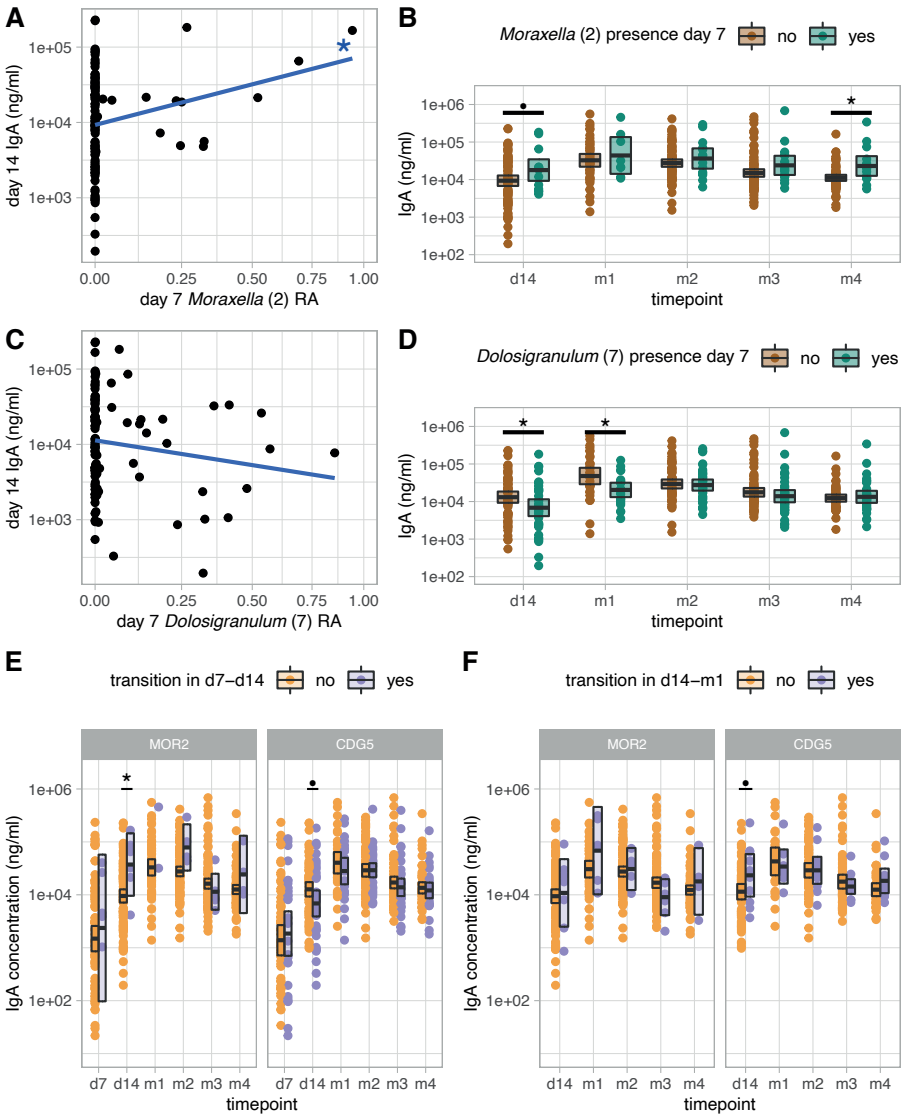

Figure 5 | Correlates between the nasopharyngeal microbial community composition and early IgA levels. (A,C) Correlations between log-transformed relative abundances (RA) of *Moraxella* (2) (A) and *Dolosigranulum* (7) (C) at day 7 and $\log_{10}$-transformed IgA at day 14. Significance was assessed using linear models and denoted by *: p<0.050. (B,D) IgA concentrations over time (day 7 (d7)-month 4 (m4)) for infants who did versus infants who did not acquire *Moraxella* (2) (B) or *Dolosigranulum* (7) (D) at day 7. Significance was assessed with linear mixed-effects models over time and post-hoc extraction of pairwise contrasts per timepoint, and was denoted by •: p<0.10; *: p<0.050. (E,F) IgA concentrations over time for infants who did compared to infants who did not acquire the *Moraxella* (2)-cluster (MOR2)

Figure 5 | Continued

(left) and for infants who did compared to infants who did not acquire the *Corynebacterium* (5)/*Dolosigranulum*-cluster (CDG5) (right) between days 7 (d7) and 14 (d14) (E) and between day 14 and month 1 (m1) (F). Significance was assessed with linear mixed-effects models over time and post-hoc extraction of pairwise contrasts per timepoint, and was denoted by •: p<0.10; *: p<0.050.

Indeed, month 6 IgG was significantly higher in infants with a recent RTI in combination with day-care attendance (p=0.010), viral detection at month 4 (p=0.001) or both (p<0.001), and in infants with day-care attendance and viral detection at month 4 (p<0.001), compared with infants without these factors (Figure 6B). Regarding nasopharyngeal microbiota development in the first 6 months of life, infants with low month 6 IgG had higher abundances of *Streptococcus* (13) (days 15-179, q=0.009) and *Moraxella* (2) (days 35-190, q=0.014), and lower abundance of *Staphylococcus* (3) (days 49-190, q=0.031) from month 1-2 onward than infants with low month 6 IgG (14 ASVs tested, SS-ANOVA; Figure 6C, Table S3). At 6 months of age, infants with middle/high month 6 IgG also had higher bacterial density (high vs low month 6 IgG: q=0.008; high vs middle month 6 IgG: q=0.040; Figure 6D) and a different overall microbial community composition (PERMANOVA: high vs. low R^2=4.9%, p=0.008; low vs. middle R^2=4.2%, p=0.034; Figure 6E) than infants with low month 6 IgG, while these differences were not found at preceding timepoints. These findings suggest that besides (viral) infectious pressure, (accompanying) bacterial factors may contribute to the induction of endogenous IgG concentrations in infants.

Mucosal antibodies contribute to respiratory health in infants by preventing pathogen invasion while tolerating commensal colonization. We here describe the dynamics of salivary IgG, IgA and IgM concentrations in healthy infants across the first year of life. Endogenous IgA/IgM secretion into saliva appeared to start in the first weeks of life, while IgG concentrations only began to increase several months later. Sharp increases in IgA/IgM and IgG concentrations at 2 weeks and 6-9 months of age, respectively, were both preceded by increases in host Ig production gene expression, and may thus reflect induction of intrinsic antibody production around these ages. Up to 2 weeks of age, IgA/IgM were strongly associated with breastfeeding,

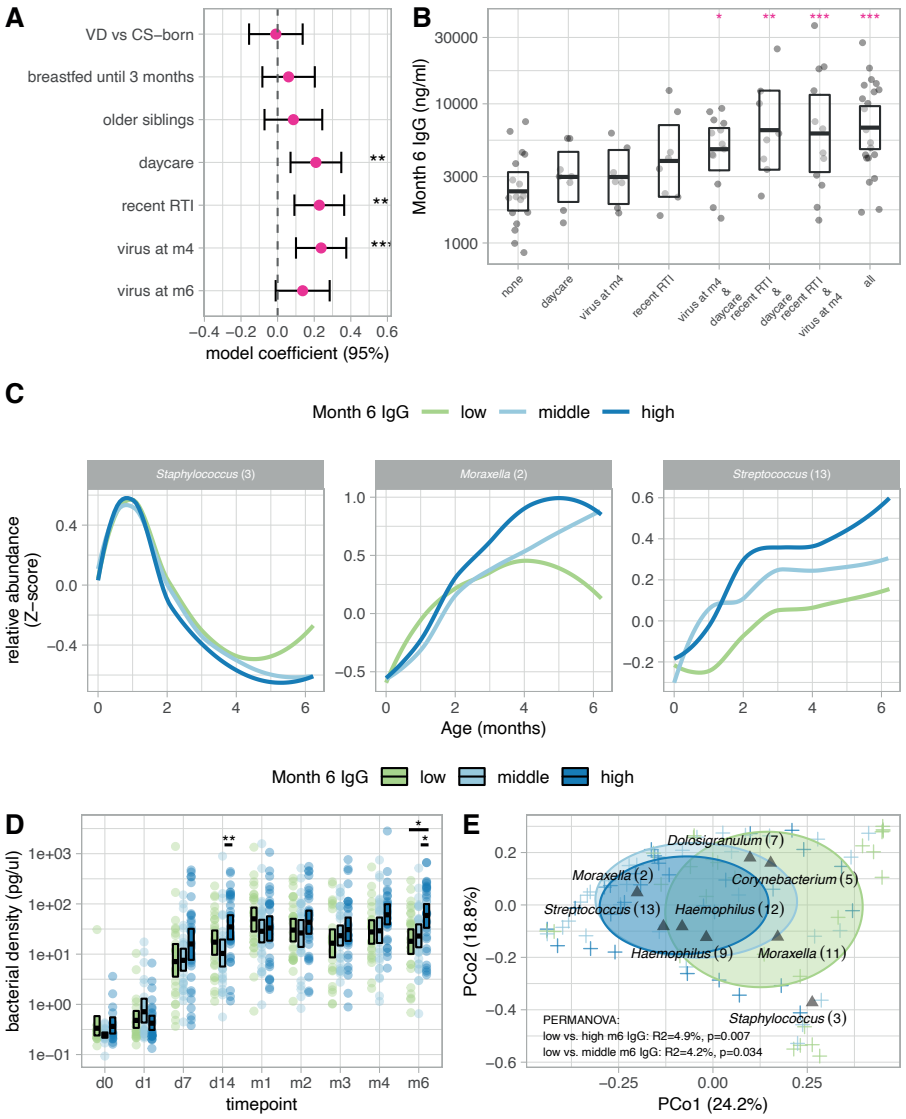

Figure 6 |Host and microbial factors associated with IgG production at month 6. (A) Univariable models of correlations between host and environmental factors and month 6 IgG concentrations. Dots and error bars denote model coefficients with 95% CI. Significant associations are denoted by the colored asterisks (***: p<0.001; *: p<0.050). (B) Infants were stratified according to daycare attendance at month 6, viral detection at the preceding month 4 (m4) timepoint and/or recent RTI between months 4 and 6, and associations with month 6 IgG concentrations were assessed in a linear model, using infants with none of these characteristics as a reference. Significance was denoted by ***: p<0.001; **: p<0.005; or *: p<0.050. (C) Z-scores of relative abundances of ASVs that were identified as significantly different between

Figure 6 | Continued

infants with high versus low month 6 IgG by smoothing spline-ANOVA. (D) Bacterial density over time up to month 6 for infants with low, middle and high month 6 IgG. Boxes show median and IQR per timepoint. Significance was assessed using linear mixed-effects models with contrasts extracted post-hoc per timepoint, and indicated by **: q<0.005; *: q<0.100. (E) Principal coordinate (PCo) analysis based on Bray–Curtis dissimilarities showing the nasopharyngeal microbial community composition at 6 months of age for infants with high, middle and low month 6 IgG. Percentages in brackets denote the total variance explained by the first two principal coordinates. Each data point (cross) indicates a nasopharyngeal sample colored by month 6 IgG group. Ellipses denote the standard deviation of data points per group. In addition, the 8 highest-ranked ASVs at the month 6 timepoint were visualized (triangles). Significance of differences between groups was assessed using permutational analysis of variance (PERMANOVA).

which is in line with breastmilk being the primary source of these antibodies at the beginning of life. An early increase in intrinsic IgA/IgM production may serve to compensate for their rapidly declining levels in breastmilk with time (22). Ig production also seemed to be induced even earlier in formula fed infants than in breastfed infants, which might reflect the body attempting to compensate for the lack of breastmilk-derived IgA. Furthermore, we found high IgG concentrations in saliva directly from birth, which likely reflects maternal IgG, passively derived from the systemic circulation (23). Intrinsic IgG synthesis was previously shown to start in the first week of life already (24). However, we here observed an overall decrease in salivary IgG in the first 6 months, suggesting that the natural decline of maternally-derived IgG prevailed over endogenous production in this period.

DISCUSSION

Interestingly, high breastfeeding-derived IgA levels directly after birth were associated with limited bacterial outgrowth, early replacement of the initial colonizer *Staphylococcus* with niche-specific commensals *Dolosigranulum* and *Corynebacterium,* and, importantly, a lower susceptibility to RTIs in the first year of life. Breast milk contains microbial communities of its own as well as bioactive components like oligosaccharides and lactoferrin, which are known to influence the gut microbial community composition (25, 26). Nonetheless, the associations between IgA and the nasopharyngeal microbiota persisted after adjusting for feeding type. Secretory IgA was previously shown to

shape the gut microbial community (27). For instance, a study comparing IgA-deficient patients with healthy individuals revealed differences in the gut microbiota composition, including increased abundance of pathobionts and underrepresentation of normally IgA-bound commensals (12). Our results suggest that reduced levels of salivary IgA directly after birth may similarly affect the nasopharyngeal microbial community. Given that the early-life nasopharyngeal microbiota is associated with later-life respiratory morbidity (8, 28), the ability of breastfeeding-derived IgA to shape the infant microbiota from early on may partially explain the positive effect of breastfeeding on respiratory health even beyond the breastfeeding period (29).

Nasopharyngeal microbial communities characterized by higher abundance of pathobionts like *Moraxella* and *Streptococcus*, and lower abundance of commensals like *Dolosigranulum* and *Corynebacterium* from early in life were associated with elevated IgA levels at day 14 and IgG levels at month 6. Experimental mouse studies have shown that in the gut, bacterial exposure induces IgA (30, 31) and IgG (32) repertoires adapted to the commensal microbiota. Based on our observations, the nasopharyngeal microbial community may analogously contribute to mucosal antibody induction in the upper respiratory tract. Enrichment of the nasopharyngeal microbiota with pathobionts has previously also been associated with a more pro-inflammatory milieu in the respiratory tract (33), and we speculate that local inflammation may boost the antibody response. Alternatively, an impaired mucosal barrier function might underlie aberrant antibody responses to the local microbiota (34). In turn, elevated IgA levels at day 14 and IgG levels at month 6 were associated with a higher frequency of RTI episodes in the first year of life.

Strengths of this study include the highly detailed participant information and the prospective design with frequent sampling in early life. Furthermore, the highly sensitive multiplex immunoassay allowed us to measure antibodies in very low saliva volumes.

However, limitations include that for practical reasons, antibody levels and microbial community composition were determined in different, adjacent compartments of the upper respiratory tract. Since the nasopharynx-

associated lymphoid tissue appears to be an important site for activation of B cells that subsequently migrate to the salivary glands (23), we considered this comparison to be valid. Furthermore, the study population consisted exclusively of healthy, term-born infants, and infants with risk factors for (severe) RTIs, such as prematurity or smoke exposure, were either absent or underrepresented, which might explain the more nuanced findings.

Taken together, our findings suggest that the respiratory microbial community directly after birth may be shaped by breastfeeding-derived, maternal antibodies, and in turn affect intrinsic mucosal antibody responses and respiratory infection susceptibility. Although cause-effect relationships cannot be determined based on our observational data, we believe that host-microbe-environment interactions in early life may be key to mucosal and microbial homeostasis, and respiratory health in the first year of life.

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SUPPLEMENTARY METHODS

Study design and sample collection

We collected saliva and nasopharyngeal samples from healthy, full-term infants who participated in a prospective birth cohort study. Details on study design and in- and exclusion criteria were previously published (1). Samples were collected at 11 predefined timepoints: within 2 hours and 24-36 hours after birth, at 7 and 14 days and 1, 2, 3, 4, 6, 9 and 12 months of age. Saliva for antibody measurement was collected from 127 infants by rubbing an absorbent sponge (Malvern Medical Developments) on the gums, cheek pouches and tongue for approximately 1 minute, which was immediately transferred to an EDTA tube (BD Vacutainer) with protease inhibitor (Roche). Nasopharyngeal swabs for microbiota profiling were obtained from 114 infants as previously described (1), and stored in 10% glycerol/0.1% DEPC water or, for a subset of 43 infants, in RNA protect Cell Reagent (Qiagen), allowing for additional gene expression profiling (2). Samples were transported on dry ice and stored at -80˚C until laboratory analysis. Extensive questionnaires on perinatal, environmental and lifestyle factors were obtained as well as data on experienced RTIs, defined as parent-reported respiratory symptoms and/or fever between two study visits. Infants were subdivided into groups with 0-2, 3-4, or 5-7 RTIs, as previously described (1). Ethical approval was granted by the Dutch national ethics committee (METC Noord-Holland). Both parents provided written informed consent.

Salivary antibody measurement

Total immunoglobulin G (IgG), IgA and IgM in saliva were quantified using a fluorescent bead-based multiplex immunoassay (MIA). Anti-human IgG (clone JDC-10, mouse isotype IgG1 kappa, Southern Biotech, Cat. No. 9040-01S), IgM (clone SA-DA4, mouse isotype IgG1 kappa, Southern Biotech, Cat. No. 9020-01) or IgA (clone MH14-1 mouse isotype IgG2b kappa, Progen, Cat. No. 11000) were individually conjugated to carboxylated microspheres (Luminex) using a two-step carbodiimide reaction (3). Saliva was thawed and centrifuged at 3000g at 4˚C for 5 minutes, and 5 µl of the supernatant was diluted 1:200 in PBS supplemented with 0.1% Tween-20

and 3% bovine serum albumin (grade V). From each diluted sample, 25 µl was applied to a well containing an equal volume of beads, allowing the anti-human IgG, IgM or IgA on the bead surface to capture antibodies in the sample. In addition, blanks (serum dilution buffer), standard human serum IgG (6 mg/ml), IgA (1 mg/ml) and IgM (1 mg/ml; Sigma), and quality control human serum (NIBSC) and intravenous immunoglobulin (IVIg) (Sanguin) were included on every plate. R-phycoerythrin labeled mouse anti-human kappa and lambda solution diluted 1:100 (ITK Diagnostics) was added to each well. Samples were acquired on a BioPlex 200 (Luminex) apparatus and concentrations interpolated using the 5-parameter logistic fit in the BioPlex software package version 6.2 (Bio-Rad Laboratories). When the measured concentration was too high to accurately interpolate using criteria built into the software, samples were re-measured at a 1:1000 dilution. Concentrations below the lower limit of detection (IgG: 76 ng/ml, IgA: 17 ng/ml, IgM: 67 ng/ml), were set at half the limit. Concentrations were given in ng/ml.

Nasopharyngeal microbiota characterization and viral qPCR

Bacterial DNA was extracted, after which the V4-region of the 16S-rRNA-gene was amplified using the 515F/806R primer pair (4, 5). Amplicon pools were sequenced with the Illumina MiSeq platform (1). Bioinformatic pre-processing of raw reads was performed using DADA2 (v1.16.0; maxEE = 2; truncLen = 200/150) as previously described (2, 6). Each Amplicon Sequence Variant (ASV) was assigned taxonomic annotation using the naïve Bayesian classifier and the Silva v138 (version 2) reference database (7) and a unique number. Contaminant ASVs were identified using the *decontam*-package (8) (v1.12.0; default parameters; combined method using per-batch detection) and removed. Samples with <3000 reads were excluded. We filtered ASVs with at least 0.1% relative abundance in at least two samples (1411 ASVs; excluding 9971 ASVs [87.6%] corresponding to 1.0% of reads). Relative abundances were obtained from raw read counts by total sum scaling. Both the raw and relative abundance ASV tables were used in downstream analyses. Total bacterial DNA was quantified using quantitative (q)PCR targeting the 16S-rRNA-gene as previously described (1). Viral qPCR specific for 17 respiratory viruses was performed on all nasopharyngeal samples as described elsewhere (9).

Nasal gene expression profiling

RNA extraction from nasopharyngeal cells followed by microarray (Affymetrix Clariom S Human Pico array) for nasal gene expression profiling was performed as previously described (2). For the current study, we calculated the eigengene expression of the Ig production pathway from the Gene Ontology (GO) database (10) as the first principal component of the expression matrix, limited to genes included in the pathway (ID GO:0002377).

Statistical analysis

Statistical analyses were performed in R version 4.1.2. Antibody concentrations were analyzed either as continuous variable (log_{10}-transformed) or categorized into high, middle and low terciles. We considered p-values <0.050 or, in case of multiple comparisons, false discovery rate-adjusted q-values <0.100 (Benjamini-Hochberg procedure) as statistically significant. Z-scores of Ig levels, eigengene expression and relative abundances were generated by standardizing to all values.

Associations between host factors and antibody concentrations were assessed using linear (mixed-effects) models. Group differences in Ig concentrations or bacterial density over time were assessed using linear mixed-effects models with subject identifier as random effect and an interaction term between timepoint and the variable of interest as fixed effect. This interaction term allowed for post-hoc extraction of pairwise contrasts per timepoint using the *emmeans*-package (11).

Differences in overall microbial community composition according to Ig tercile were evaluated using permutational analysis of variance on the Bray-Curtis dissimilarity matrix (PERMANOVA, *adonis/adonis2*-function, *vegan*-package (12)) and visualized with principal coordinate analysis. Microbiota cluster membership was allocated to each sample using average linkage hierarchical clustering on the Bray-Curtis dissimilarity matrix as previously described (2). The optimal number of clusters was determined based on clustering indices (Silhouette and Calinski-Harabasz indices). We only considered clusters with at least 10 samples. Differences in time to cluster transitions were evaluated with Cox proportional hazard models and visualized using Kaplan-Meier curves (*survival*- and *survminer*-packages (13, 14)).

On the individual ASV level, cross-sectional associations with Ig concentrations/terciles per timepoint were tested using Microbiome Multivariable Association with Linear Models 2 (MaAsLin2; *MaAsLin2*-package) (15) using default parameters, limited to ASVs present in >25% of samples tested. Differential abundance of ASVs over time between Ig terciles was tested using smoothing spline (ss-)ANOVA (*fitTimeSeries*-function, *metagenomeSeq*-package (16)), limited to ASVs present in >10% of samples tested. This method allows for the detection of differentially abundant ASVs and also identifies the timeframe when significant differences exist. Default normalization and transformation methods were applied to the raw ASV table prior to running these analyses, i.e. cumulative sum scaling for ss-ANOVA and total sum scaling for MaAsLin2, followed by log-transformation. In addition, associations were tested between specific ASV presence/absence (defined as >0.10% relative abundance) or relative abundance (log-transformed after adding a pseudocount of 1) and Ig levels in linear models.

SUPPLEMENTARY TABLES

Table S1 | Characteristics of the study population.

n	**127**
Demographic characteristics	
Female (%)	67 (52.8)
Season of birth (%)	
Winter	27 (21.3)
Spring	28 (22.0)
Summer	47 (37.0)
Autumn	25 (19.7)
Perinatal	
Vaginal delivery (%)	76 (59.8)
Birth weight, grams (median [IQR])	3530.0 [3141.2, 3851.2]
Gestational age, weeks (median [IQR])	39.0 [38.0, 40.0]
Environment	
Breastfeeding, days (median [IQR])	53.5 [3.0, 225.0]
Feeding type from birth (%)	
Exclusive formula feeding	23 (18.9)
Breast- and formula feeding	22 (18.0)
Exclusive breastfeeding	77 (63.1)
Number of siblings (median [IQR])	1.0 [0.0, 1.0]
Siblings <5 years of age (%)	70 (56.9)
Pets (%)	56 (45.9)
Daycare during first year of life (%)	81 (63.8)
Parents highly educated (%)	92 (74.8)
Inhouse smoking (%)	3 (2.4)
Respiratory infections	
Respiratory infections, frequency (median [IQR])	3.0 [2.0, 4.0]
Respiratory infections, group (%)	
0-2	50 (39.4)
3-4	57 (44.9)
5-7	20 (15.7)

Table S2 | Associations between high compared with low day 0/1 IgA and individual ASV abundances over time in the first 14 days of life.

					Unadjusted		Adjusted for feeding type	
ASV	**Associated with day 0/1 IgA**	**Interval start (days)**	**Interval end (days)**	**Area**	**p-value**	**q-value**	**p-value**	**q-value**
Moraxella (2)	high	9	16	14.2	0.079	0.082	0.079	0.079
Corynebacterium (5)	high	4	10	12.7	*ns*	*ns*	0.041	0.074
Dolosigranulum (7)	high	4	12	20.4	0.018	0.061	0.016	0.051
Staphylococcus (3)	low	9	16	-13.3	*ns*	*ns*	0.033	0.074
Granulicatella (155)	high	11	16	6.0	0.017	0.061	0.017	0.051
Corynebacterium (235)	low	6	8	-1.5	0.067	0.082	0.067	0.075
Actinomyces (314)	high	0	1	0.8	0.068	0.082	0.067	0.075
Noviherbaspirillum (464)	high	0	4	2.9	0.082	0.082	0.067	0.075
Corynebacterium (495)	high	0	7	5.2	0.026	0.061	0.017	0.051

Individual ASV abundances (present in >10% of samples tested) over time in the first 14 days of life were compared between infants with day 0/1 (d0/1) IgA in the highest and lowest terciles using ss-ANOVA. The analysis was executed both unadjusted and adjusted for feeding type. All significant findings after adjusting for multiple testing, in either the unadjusted or adjusted analysis, are shown (q<0.100). ns=not significant.

Table S3 | Associations between individual ASV abundances in the first 6 months of life and high compared with low month 6 IgG concentrations.

ASV	**Associated with high/ low m6 IgG**	**Interval start (days)**	**Interval end (days)**	**Area**	**p-value**	**q-value**
Moraxella (2)	high	35	190	431.1	0.003	0.009
Staphylococcus (3)	low	49	190	-228.8	0.021	0.031
Streptococcus (13)	high	15	179	380.7	0.002	0.009
Streptococcus (14)	low	27	119	-137.1	0.006	0.012
Corynebacterium (21)	high	23	110	128.4	0.077	0.092
Corynebacterium (39)	low	28	62	-25.3	0.097	0.097

Individual ASV abundances (n=14, present in >10% of samples tested) over time in the first half year of life were compared between infants with month 6 (m6) IgG in the highest and lowest terciles using ss-ANOVA. All significant findings after adjusting for multiple testing are shown (q<0.100).

SUPPLEMENTARY FIGURES

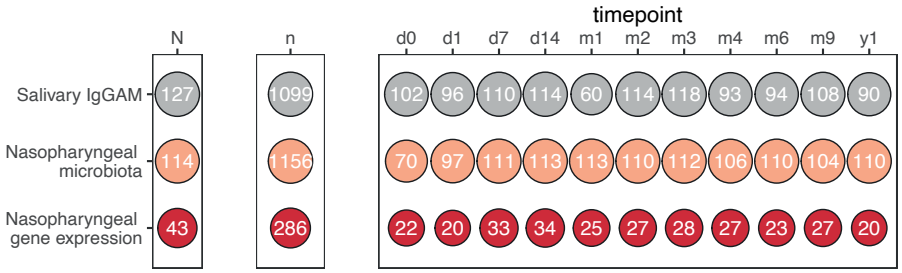

Figure S1 | Numbers of samples available per dataset. Sample availability is shown per dataset per timepoint (d=day, m=month, y=year). N=number of infants with at least one datapoint in a dataset. n=total number of samples in a dataset.

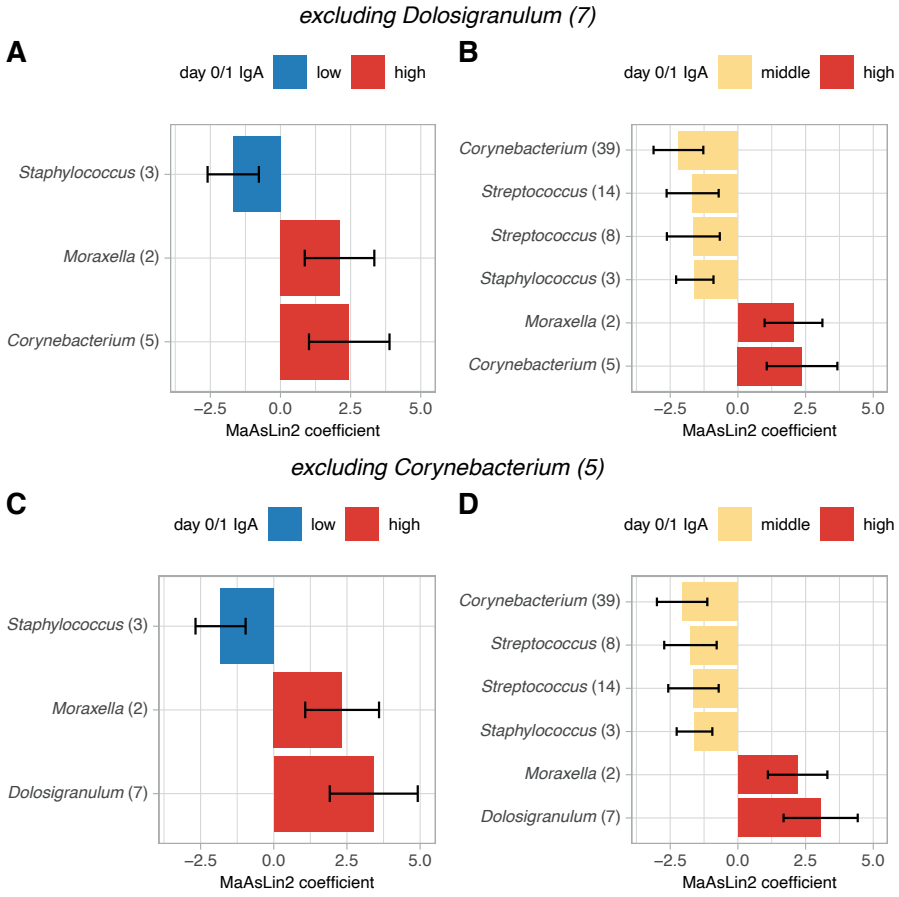

Figure S2 | Differentially abundant ASVs at day 7 according to day 0/1 IgA category. (A,B) Differentially abundant ASVs at day 7 between infants with low and high day 0/1 IgA (A) and infants with middle and high day 0/1 IgA (B) after excluding Dolosigranulum (7) from the analysis. (C,D) Differentially abundant ASVs at day 7 between infants with low and high day 0/1 IgA (C) and infants with middle and high day 0/1 IgA (D) after excluding Corynebacterium (5) from the analysis. ASVs present in at least 25% of samples tested were analyzed and ASVs that showed a trend towards significance after adjusting for feeding type but without adjusting for multiple testing, are shown ($p<0.100$). Bars are colored according to the day 0/1 IgA group they are associated with. Lengths of the bars correspond with the MaAsLin2-model coefficient, which is related to the strength of the association. Error bars denote standard errors.

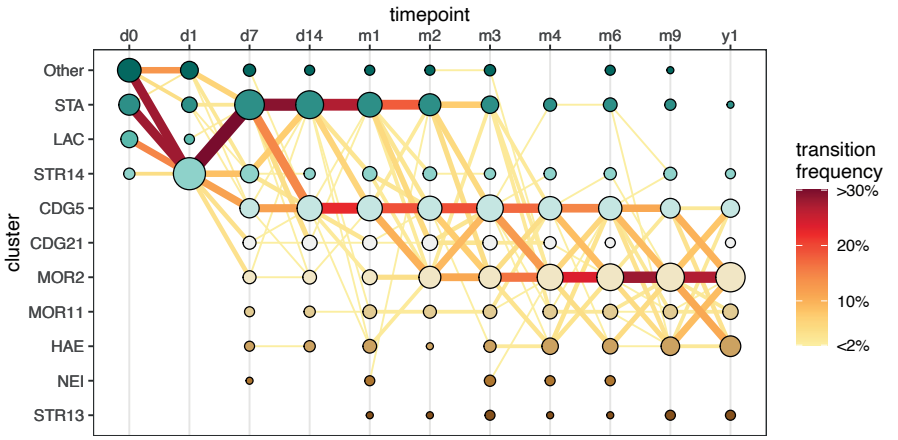

Figure S3 | Nasopharyngeal microbiota cluster membership succession. Dot flow diagram showing the progression of microbiota cluster membership over time. Clusters were characterized by predominance of *Corynebacterium* (5) with *Dolosigranulum pigrum* (7), (CDG5; n=268), *Staphylococcus* (3) (STA; n=231), *Moraxella* (2) (MOR2; n=266), *Streptococcus* (14) (STR14; n=124), *Haemophilus* (9/12) (HAE; n=74), *Moraxella* (11) (MOR11; n=53), *Corynebacterium* (21) with *Dolosigranulum pigrum* (7) (CDG21; n=44), *Lactobacillus* (49) (LAC; n=14), *Neisseria* (51) (NEI; n=10) or *Streptococcus* (13) (STR13; n=10). 62 samples were not assigned a (sufficiently large) cluster. Transitions between clusters are plotted. Transition frequency is determined by dividing the number of transitions towards a given clusters by the total number of transitions within each time window. Edges and nodes are scaled by the number of available samples.

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CHAPTER 5

Mode of delivery modulates the intestinal microbiota and impacts the response to vaccination

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ABSTRACT

The gut microbiota in early life, when critical immune maturation takes place, may influence the immunogenicity of childhood vaccinations. We assess the association between mode of delivery, gut microbiota development in the first year of life, and mucosal antigen-specific antibody responses against pneumococcal vaccination in 101 infants at age 12 months and against meningococcal vaccination in 66 infants at age 18 months. Birth by vaginal delivery is associated with higher antibody responses against both vaccines. Relative abundances of vaginal birth-associated *Bifidobacterium* and *Escherichia coli* in the first weeks of life are positively associated with anti-pneumococcal antibody responses, and relative abundance of *E. coli* in the same period is also positively associated with anti-meningococcal antibody responses. In this study, we show that mode of delivery-induced microbiota profiles of the gut are associated with subsequent antibody responses to routine childhood vaccines.

INTRODUCTION

Vaccination in early childhood is estimated to save millions of lives each year (1). Vaccine-induced protection is mediated through a combination of innate, humoral and cellular immunity, and is often quantified by measuring antigen-specific antibody titers (2). Large interindividual variation in antibody responses to vaccines administered in early life may limit vaccine effectiveness, leaving some fully vaccinated infants unprotected against serious infectious diseases (3). Factors that influence vaccine responses include, among others, genetics, sex, perinatal characteristics like gestational age, birth weight, maternal antibodies, and feeding type, but also more general factors like geographical region (reviewed in (4)). Recent research has shown that the gut microbiota, i.e. the sum of all microorganisms residing in the human intestinal tract, also plays a role in immune responses to vaccination (5–11). This offers a potentially modifiable target to improve immunogenicity of childhood vaccines.

The gut microbiome is seeded at birth and rapidly develops over the first months of life under the influence of mode of delivery, breastfeeding, antibiotic administration and nutrition (12–15). Timely exposure to specific microbes within the critical window of opportunity in early infancy shapes the immune system (16–18), including the B cell and immunoglobulin repertoire (19, 20). Microbial imprinting on the immune system in early life may in turn explain part of the variation in vaccine responses. In support of this hypothesis, it has been shown that antibiotic-induced microbial perturbances in an infant mouse model led to impaired antigen-specific immunoglobulin G (IgG) responses against five common childhood vaccines including the meningococcal group C (MenC) conjugate vaccine and the 13-valent pneumococcal conjugate vaccine (PCV-13) (21). Microbiota perturbance due to antibiotic exposure also resulted in impaired immune responses to seasonal influenza vaccination in healthy adults without pre-existing immunity, suggesting that primary responses are more sensitive to microbiota changes than recall responses (7). In human infants, the composition of the microbial community pre-vaccination has been correlated with systemic immune responses to oral rotavirus vaccine, oral poliovirus vaccine, *Bacillus* Calmette-Guérin, hepatitis B, and tetanus vaccines (5, 6, 10, 11, 22, 23). However, the temporal relationship between 1. early-life exposures, 2. gut microbiota composition, and 3. subsequent childhood vaccine responses has not yet been studied.

Here, we demonstrate in a healthy birth cohort that mode of delivery-induced differences in microbial colonization patterns in the gut in early life are associated with antigen-specific IgG responses to the 10-valent PCV (PCV-10) and the MenC conjugate vaccine in saliva. For these vaccines, mucosal IgG has been shown to confer vaccine-induced protection against infection (24). These findings are key for the design of intervention strategies that modulate the gut microbiota to enhance vaccine immunogenicity in infants.

RESULTS

We investigated associations between early-life exposures, gut microbiota development in the first year of life and its effect on vaccine responses later in life in a birth cohort of 120 healthy, term born infants (25). Follow-up of the infants and sample inclusion for gut microbiota characterization by 16S rRNA gene sequencing and salivary antigen-specific IgG measurement by multiplex immunoassay are shown in Supplementary Figure 1. Basic, lifestyle and environmental characteristics were previously published (26), and are briefly summarized in Table 1. Infants received routine vaccinations according to the Dutch National Immunization Program (NIP). Serotype-specific anti-pneumococcal IgG concentrations were measured in routinely collected saliva of 101 infants at the age of 12 months median 28 days [IQR 21-33] after the PCV-10 booster dose). Anti-MenC IgG concentrations were measured in routinely collected saliva of 66 infants at the age of 18 months (median 116 days [IQR 105-120] after MenC vaccination). Geometric mean concentrations (GMC) of IgG concentrations against the different pneumococcal vaccine serotypes ranged from 7.33 ng/ml (95% CI 5.75-9.33 ng/ml) for serotype 23F to 27.30 ng/ml (95% CI 22.14-33.67) for serotype 19F. The anti-MenC IgG GMC was 10.64 ng/ml (95% CI 8.64-13.11 ng/ml) (Figure 1A). IgG concentrations against the 10 pneumococcal vaccine serotypes strongly correlated with each other (Pearson's ρ 0.56-0.86, adjusted $p<0.001$ for all pairwise correlations), and not with anti-MenC IgG antibodies (Pearson's ρ 0.12-0.31, adjusted $p>0.397$ for all pairwise correlations) (Figure 1B). As serotype-specific anti-pneumococcal IgG concentrations were strongly correlated, we focused our analyses on serotype 6B, which shows relatively weak antigenic properties, and is commonly found during (severe) pneumococcal disease (27). Significant findings were validated for the other serotypes.

Table 1 | Cohort description.

	PCV-10 response	MenC response
n	**101**	**66**
Sex, female (%)	54 (53.5)	35 (53.0)
Perinatal characteristics		
Mode of delivery, vaginal (%)	58 (57.4)	42 (63.6)
Antibiotics during birth (%)	2 (2.0)	1 (1.5)
Exclusive formula feeding (%)	17 (16.8)	10 (15.2)
Breastfeeding, days (median [IQR])	55.0 [3.0, 248.0]	114.0 [3.0, 289.8]
Environmental characteristics		
Presence of siblings (%)	68 (67.3)	46 (69.7)
Number of siblings (median [IQR])	1.0 [0.0, 1.0]	1.0 [0.0, 0.1]
Pets in the household (%)	46 (45.5)	36 (54.5)
Antibiotic treatment		
Antibiotics in the first 3 months (%)	13 (12.9)	4 (6.1)
Antibiotic courses* (median [IQR])	0.0 [0.0, 1.0]	0.0 [0.0, 1.0]

Participant characteristics are summarized for all infants who had anti-pneumococcal immunoglobulin G (IgG) responses available (n=101; left column), and the subset of infants who had anti-meningococcal type C (MenC) IgG responses available (n=66; right column). *The number of antibiotic courses is given up to the time that vaccine responses were measured, so up to 12 months in the left column and up to 18 months in the right column.

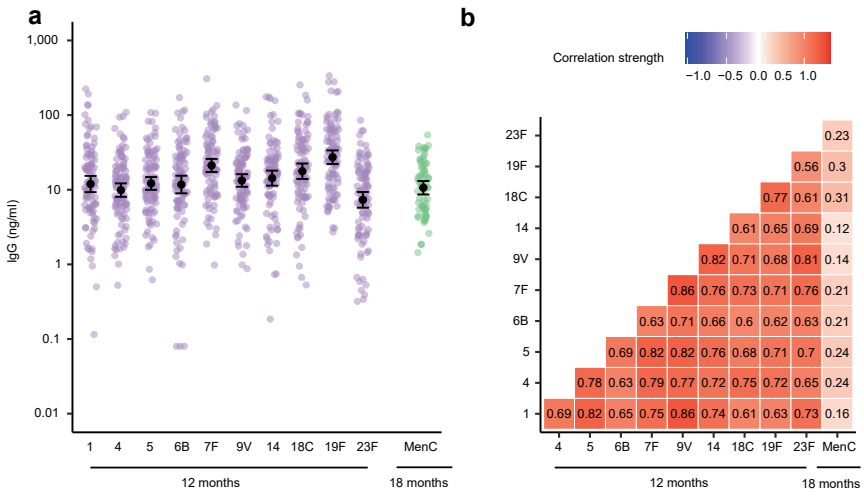

Figure 1 | Anti-pneumococcal and anti-MenC IgG concentrations following vaccination. A) Immunoglobulin G (IgG) concentrations against 10 pneumococcal vaccine serotypes (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F; n=101) and meningococcus type C (MenC; n=66) following vaccination. Black dots and error bars represent geometric mean concentrations with 95% confidence intervals (CI). B) Correlation plot of IgG concentrations against the 10 pneumococcal vaccine serotypes and against MenC following vaccination. Numbers indicate the correlation strength, which was evaluated using Pearson's correlation coefficients.

Mode of delivery was associated with vaccine responses

We first investigated whether early-life host characteristics previously associated with differences in gut microbiome development and/or vaccine immunogenicity, were related to anti-Ps6B and anti-MenC IgG responses. Mode of delivery, feeding type, sex, antibiotics use in the first 3 months of life, and pets in the household were related to vaccine responses against one or more serotypes in univariate analysis, while having older siblings, the number of antibiotic courses, and daycare attendance were not. These variables were included in multivariable linear models, including an interaction term between mode of delivery and feeding type due to the interdependency of these variables. Vaginal delivery (in contrast to caesarean (C-)section birth) was independently associated with higher anti-Ps6B IgG concentrations (β=0.51 [95% CI 0.043-0.97], p=0.033; Figure 2A). However, we also observed a negative interaction between vaginal delivery and exclusive formula feeding on anti-Ps6B responses (β=-1.32 [95% CI -2.43 - -0.21], p=0.021), suggesting that the positive effect of vaginal birth was diminished by subsequent formula feeding. Similar associations were found for IgG responses to most of the other pneumococcal vaccine serotypes (Supplementary Table 1). Stratified analyses confirmed that, within the vaginally delivered group, the anti-Ps6B IgG GMC of breastfed infants (n=51) was 3.5-fold higher compared to formula fed infants (n=7; adjusted p=0.070); similarly, within the breastfed group, the anti-Ps6B IgG GMC of vaginally delivered infants (n=51) was two-fold higher compared to C-section born infants (n=33), although this difference was not significant (adjusted p=0.51). Anti-Ps6B IgG concentrations did not differ between feeding types within the C-section born group (Figure 2B). Likewise, for MenC, vaginal delivery was also associated with higher IgG concentrations compared to C-section delivery (β=0.42 [95% CI 0.016-0.83], p=0.042), which was independent of feeding type (Figure 2A). In a stratified analysis, vaginally delivered infants (n=42) showed a 1.7-fold higher anti-MenC IgG GMC compared to C-section delivered infants (n=24; p=0.002; Figure 2B). Mode of delivery and feeding type were thus the only early-life factors significantly associated with IgG responses against Ps6B and MenC, while sex, antibiotic use, and having pets were not. We concluded that mode of delivery and feeding type are likely microbiome modulators from birth onward (26), and therefore considered them as such for our downstream analysis.

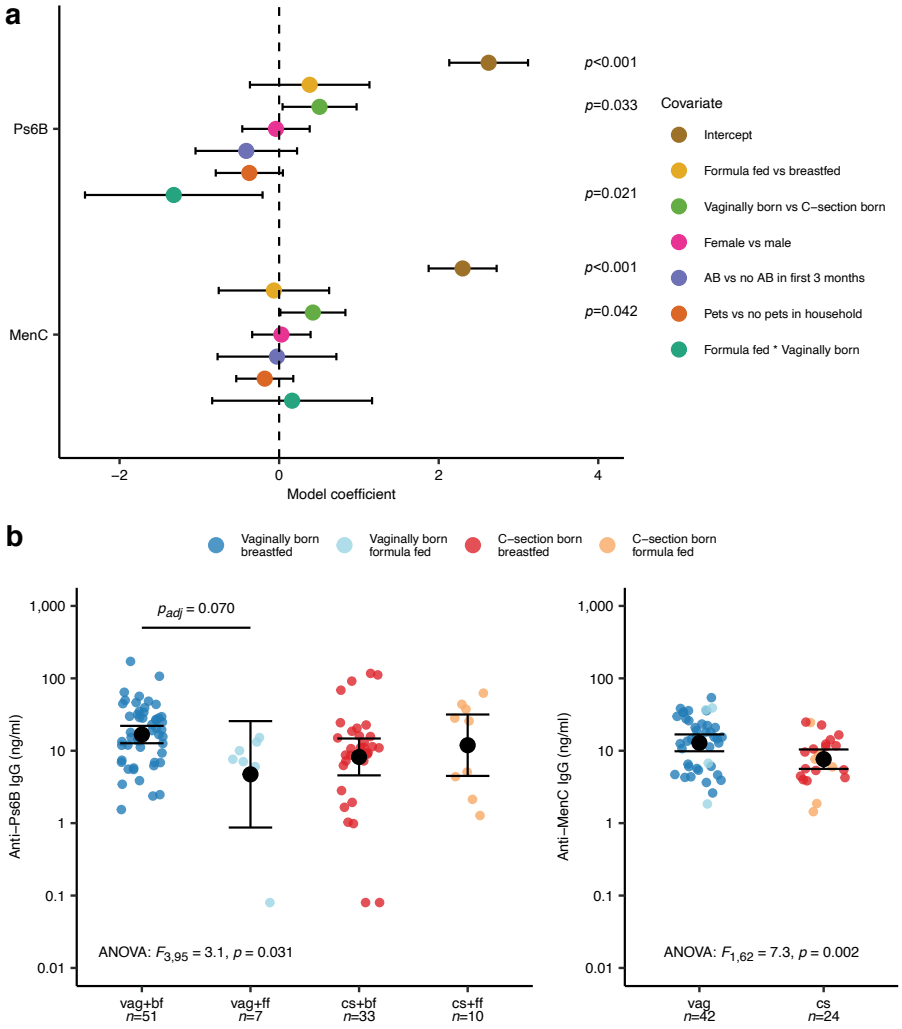

Figure 2 | Associations between early-life exposures and anti-pneumococcal and anti-MenC IgG concentrations following vaccination. A) Data are presented as model coefficients with 95% CI per covariate computed with two-sided multivariable linear regression with log-transformed anti-Ps6B (n=101) or anti-MenC (n=66) IgG concentrations as dependent variable. The analysis was not adjusted for multiple comparisons. C-section=caesarean section; AB=antibiotics. B) anti-pneumococcal serotype 6B (anti-Ps6B) IgG responses for vaginally born, breastfed (vag+bf, n=51), vaginally born, formula fed (vag+ff, n=7), C-section born, breastfed (cs+bf, n=33), and C-section born, formula fed (cs+ff, n=10) infants (left) and anti-meningococcus type C (anti-MenC) IgG responses for vaginally born (vag, n=42) and C-section born (cs, n=24) infants (right). Black dots and error bars represent geometric mean concentrations (GMCs) with 95% CI. Significance was assessed using two-sided analysis of variance (ANOVA) on log-transformed IgG concentrations followed by a post-hoc Tukey-Kramer test, correcting for time between vaccination and IgG measurements. Padj = FDR-adjusted p-value.

Gut microbial community composition at one week of age was associated with vaccine responses

We then studied whether gut microbiota development in the first year of life was associated with anti-Ps6B and anti-MenC IgG responses. Overall, 1,052 out of 1,156 fecal samples (91.0%) passed quality control for 16S rRNA gene-based sequencing, and were included in further analyses (Supplementary Figure 1). We have previously shown in this cohort that the gut microbiota composition of infants born by C-section was significantly different compared to vaginally delivered infants, with lower relative abundance of *Bifidobacterium* and *Escherichia coli*, and enrichment of *Enterococcus faecium* and *Klebsiella*, from birth persisting up to the age of two months (26). From the age of two months onward, the gut microbiota composition remained comparable between mode of delivery groups.

We first studied associations between the alpha diversity measures, including Shannon diversity and the observed number of species, at each time point and vaccine responses. No association was found between alpha diversity and anti-Ps6B or anti-MenC IgG concentrations at any time point, with the exception of an inverse correlation between the observed number of species at the age of two months and anti-Ps6B IgG concentrations (β=-0.029 [95% CI -0.049- -0.0087], adjusted p=0.082). This association was not observed for the other pneumococcal vaccine serotypes.

We compared the overall microbial community composition between infants with above and below median anti-pneumococcal and anti-meningococcal IgG responses using permutational analysis of variance (PERMANOVA) on the Bray-Curtis dissimilarity matrix per timepoint, and found no significant differences. As a measure of gut microbiota stability, we calculated the Bray-Curtis similarity (1-Bray-Curtis dissimilarity) between consecutive timepoints within individuals. Microbiota stability between day one and week one, and between week one and week two correlated with higher anti-Ps6B IgG concentrations (day one-week one: β=1.66 [95% CI 0.44-2.88], adjusted p=0.074; week one-week two: β=1.22 [95% CI 0.22-2.22], adjusted p=0.077), which was not observed for any other time interval. Microbiota stability in the first two weeks of life was also significantly positively associated with IgG concentrations against all other pneumococcal vaccine serotypes (adjusted $p \leq 0.083$, Supplementary Table 2). In contrast, no significant associations were found between microbiota stability and anti-MenC IgG concentrations.

The first two weeks of life, where gut microbiota stability was associated with anti-pneumococcal IgG concentrations, is compatible with the time frame when we previously found the largest difference in gut microbial composition between vaginally born and C-section born infants in this cohort (at the age of one week) (26). In addition, this time frame coincides with the 'window of opportunity' when the gut microbiota primes the maturation of the immune system (16–18). Therefore, we decided to focus on the microbial community composition in 'week one' samples, where we identified three distinct community state types (CSTs) (Supplementary Figure 2). PERMANOVA confirmed that these CSTs differed considerably in community composition (R^2=34.8%, p<0.001). Infants with CST1 (n=55) were characterized by a microbial community with low relative abundances of both *Bifidobacterium* and *E. coli*, while infants with CST2 (n=48) had profiles with high relative abundances of *Bifidobacterium,* and infants with CST3 (n=16) had high relative abundances of *E. coli* (Figure 3A). Species-level microbial community composition obtained by shotgun sequencing of a subset of 20 'week one' samples confirmed that samples assigned to CST2 had high relative abundances of *Bifidobacterium breve* and/or *Bifidobacterium longum*, and samples assigned to CST3 had high relative abundances of *E. coli*, while samples assigned to CST1 mostly lacked these species (Supplementary Figure 3).

We then studied whether these CSTs were associated with anti-Ps6B and anti-MenC IgG concentrations following vaccination. Infants with CST1 had the lowest IgG concentrations against both Ps6B and MenC (anti-Ps6B IgG: GMC 7.84 ng/ml [95% CI 4.88-12.60]; anti-MenC IgG: GMC 8.28 ng/ml [95% CI 5.93-11.56]) (Figure 3B). Compared with infants with CST1, anti-Ps6B IgG concentrations were approximately two-fold higher in infants with CST2 (GMC 17.05 ng/ml [95% CI 12.64-23.00], adjusted p=0.096) as well as in infants with CST3 (GMC 14.85 ng/ml [95% CI 7.36-29.97], adjusted p=0.202), though only the comparison of anti-Ps6B responses between CST1 and CST2 infants was significant. We observed similar overall associations between week one CSTs and IgG responses against most other pneumococcal vaccine serotypes, but differences between CST1 and CST2 were not significant (Supplementary Table 3). By contrast, anti-MenC IgG concentrations in infants with CST3 were nearly two-fold higher (GMC 15.76 ng/ml [95% CI 7.25–34.26], adjusted p=0.054) than in infants with CST1.

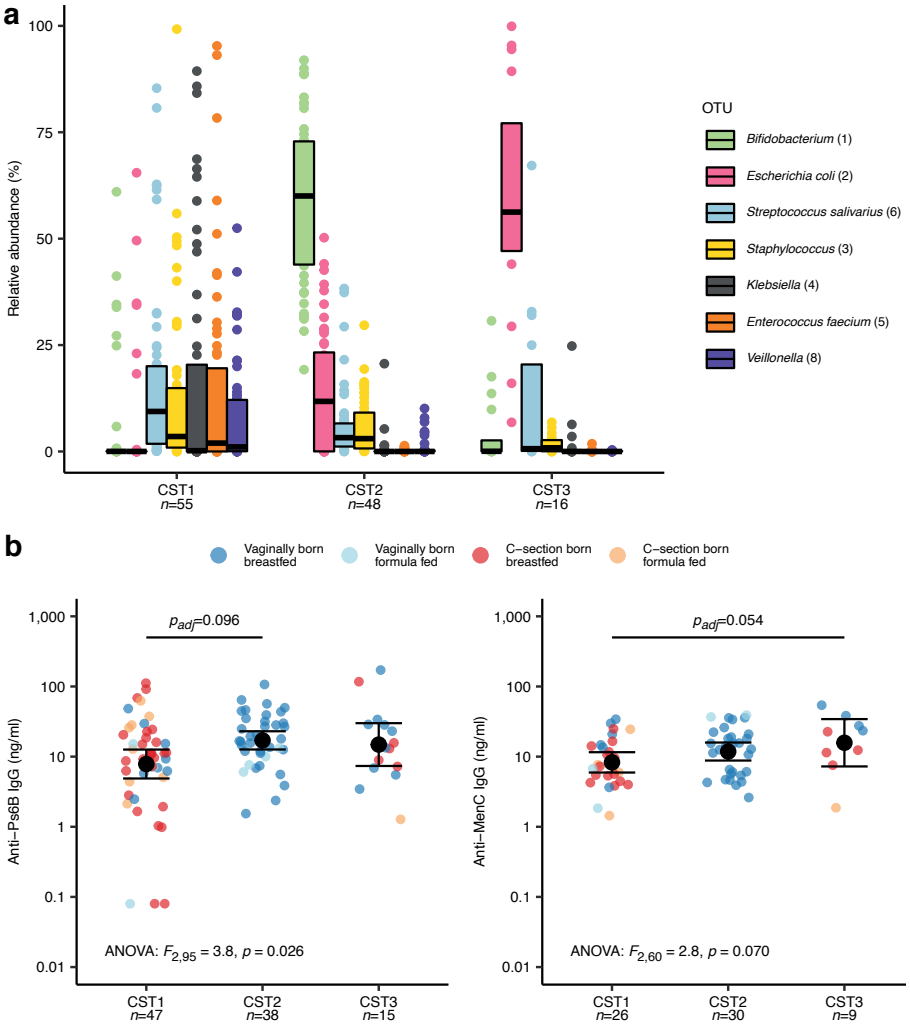

Figure 3 | Gut microbial community state types at week 1 and anti-Ps6B and anti-MenC IgG concentrations.

(A) Boxplot of relative abundances of the top 7 operational taxonomic units (OTUs) per community state type (CST) defined at 1 week of age. Boxes show medians with interquartile ranges. (B) CSTs are plotted against anti-Ps6B IgG concentrations (left) and anti-MenC IgG concentrations (right). Dots are colored according to mode of delivery and feeding type from birth. Black dots and error bars represent GMCs with 95% CI. Significance was assessed using two-sided ANOVA on log-transformed IgG concentrations followed by post-hoc Tukey-Kramer tests, correcting for time between vaccination and IgG measurements. padj = FDR-adjusted p-value.

Mode of delivery was a strong driver of week one CSTs. All infants with CST2 were vaginally born, which was significantly more than infants with CST1 (29.1%; Fisher's exact test, adjusted $p<0.001$), or CST3 (62.5%, adjusted $p<0.001$). Vaginal birth was also overrepresented in infants with CST3 compared to CST1 (adjusted $p=0.020$). In contrast, feeding type (breastfeeding vs. exclusive formula feeding) was not significantly different between these CSTs. A post-hoc analysis revealed that the association between mode of delivery and anti-Ps6B IgG responses disappeared with the addition of week one CST as an independent variable, indicating that the positive effect of vaginal delivery on anti-Ps6B IgG depended fully on the CST. In contrast, vaginal delivery remained significantly associated with anti-MenC IgG responses, regardless of week one CST, suggesting an independent effect (Supplementary Table 4).

To evaluate whether observed differences in early-life microbial community composition were sustained for a prolonged time, including time points closer to vaccination, temporal development of the gut microbiota according to week one CST was assessed using PERMANOVA. The microbial community composition of children according to their CST defined at week one converged over time, resulting in no differences between samples belonging to the CST groups from month six onward (Figure 4A). In pairwise comparisons, the observed differences in microbial community composition disappeared between infants with CST1 and CST3 by month one, between infants with CST2 and CST3 by month four, and between infants with CST1 and CST2 by month six. Similarly, relative abundances of *Bifidobacterium* and *E. coli* converged over time between CST groups (Figure 4B). At the age of 12 months, we identified two distinct CSTs, which were not significantly associated with anti-Ps6B or anti-MenC IgG responses, confirming that early-life microbiota were more strongly related to vaccine responses than the microbiota close to time of vaccination (Supplementary Figure 4).

Early-life dynamics of individual OTUs were related to vaccine responses

Next, we investigated differences in individual OTU succession patterns within the first two months between high and low vaccine responders (stratified along the median antigen-specific IgG response). Higher abundances of *E. coli* (days 0-41, adjusted $p=0.013$) and *Bifidobacterium* (days 0-5, adjusted $p=0.027$) were associated with high anti-Ps6B responses (confirmed for 7/9 other pneumococcal vaccine serotypes, Supplementary Table 5).

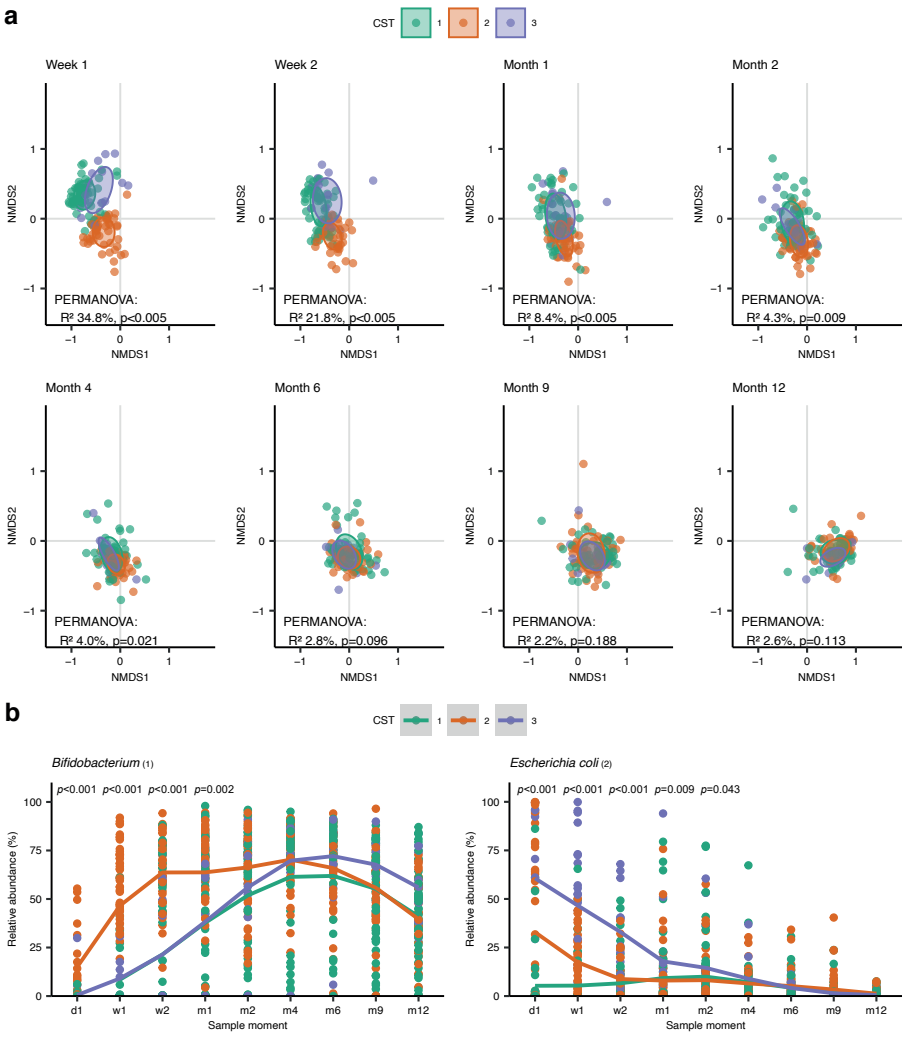

Figure 4 | Temporal gut microbial composition development according to week 1 CST. (A) Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity, depicting the gut microbial composition per timepoint. Each dot represents the microbiota composition in a single participant's sample. Infants are stratified according to week 1 CST. Ellipses represent the standard deviation of data points for each CST. Effect sizes (R2) calculated by permutational analysis of variance (PERMANOVA) and corresponding p-values are shown in the plots. (B) Relative abundances of *Bifidobacterium* (1) (left) and *Escherichia coli* (2) (right) over time according to week 1 CST. Significance of differences according to week 1 CST was assessed using Kruskal Wallis tests.

This was also observed for several *Bacteroides* OTUs, whereas *Clostridium, Prevotella* and *Streptococcus pyogenes* were associated with low responses (adjusted $p<0.050$).

Higher *E. coli* abundance (days 0-13, adjusted $p=0.072$) was also associated with high anti-MenC responses (Supplementary Table 6). Because the MenC vaccination is administered at the age of 14 months, which is much later in life than the pneumococcal vaccinations, we extended the analysis to 12 months to allow for identification of associations with OTUs that colonize later in life. In high anti-MenC responders, we observed significantly higher abundances of multiple low abundant OTUs belonging to the Lachnospiraceae family, including *Fusicatenibacter saccharivorans* (days 101-381, adjusted $p=0.080$), *Pseudobutyrivibrio* (days 125-381, adjusted $p=0.036$) and several *Blautia* and *Roseburia* OTUs (Supplementary Table 7).

Species-specific validation using targeted qPCR

Finally, we performed a targeted species-specific qPCR to validate the presence and abundance of *E. coli*, *Klebsiella* spp. and *Enterococcus* spp. in all samples obtained at one week of age ($n=119$). The relative abundance of *E. coli* showed a strong inverse correlation with *E. coli* Ct-values (Spearman's $\rho=-0.88$, $p<0.001$), and the same was observed for *Klebsiella* spp. Ct-values (Spearman's $\rho=-0.41$, $p<0.001$) and for *Enterococcus* spp. Ct-values (Spearman's $\rho=-0.88$, $p<0.001$), corroborating our 16S rRNA gene sequencing-based data. In line with our findings, *E. coli* presence was more often detected by qPCR in infants who would subsequently have high anti-Ps6B IgG responses (34/49, 69%) than in infants with low anti-Ps6B IgG responses (25/50, 50%; $p=0.078$). *E. coli* was also more often detected in week one samples of infants who were born by vaginal delivery (54/74, 73%) than in C-section born infants (20/44, 45%; $p<0.001$). Presence of *Enterococcus* spp. or *Klebsiella* spp. were not associated with the anti-Ps6B IgG response. Also, none of the species identified by targeted qPCR were associated with the anti-MenC IgG response (Supplementary Table 8).

DISCUSSION

We studied interactions between early-life exposures, gut microbial community development in the first year of life, and subsequent antibody responses in saliva against pneumococcal and meningococcal conjugate vaccination in a healthy birth cohort. A stable gut microbial community with high relative abundances of potentially beneficial bacteria in the first weeks of life, including *Bifidobacterium* and *E. coli*, was associated with high antibody responses to pneumococcal vaccination at 12 months of life. Furthermore, high *E. coli* abundance in early life was associated with high antibody responses to meningococcal vaccination at 18 months of life. Vaginal delivery was associated with high antibody responses to both vaccines, and, as we previously showed in this cohort (26), with the early-life gut microbiota colonization patterns that we now associated with high antibody responses. Previous studies on associations between gut microbiota composition and serum antibody responses have focused on the microbiota near the time of vaccination (5, 6, 10, 11, 22). However, our findings suggest that especially early-life gut microbiota development may set the stage for robust immune responses to childhood vaccinations.

The period in which we identified associations between the gut microbiota composition and vaccine responses coincides with the critical window of opportunity spanning the first 100 days of life, when immune maturation is most affected by the early-life gut microbiota (28). In mice, the detrimental effects of antibiotic-induced gut microbiota disruption on host immunity, including vaccine responses, metabolism and even lifespan were shown to be particularly potent when exposure occurs in early life (21, 29, 30). Relevant to the capacity to mount an effective antibody response to vaccination, the early-life gut microbiota have been implicated in the shaping of the systemic B cell and immunoglobulin repertoire (19, 20, 31). For instance, deficiency of IgA and IgG1 production in germ free mice can be restored by microbial exposure (32). In line, a culture-based study executed in human infants showed that the presence of *E. coli* and bifidobacteria in the gut in the first weeks of life was related to higher numbers of circulating $CD27^+$ memory B cells at four and 18 months of life (33). In a recent microbiota-based study, lack of early bifidobacterial colonization was also linked to immune

dysregulation at the age of three months, showing reduced levels of circulating plasmablasts, and naïve and transitional B cells (17). This suggests that bacterial colonization patterns in early infancy drive B cell maturation, and have a lasting effect on, among others, adaptive immunity which may, for instance, be reflected in differences in antibody responses to infant and childhood vaccinations. In line with this concept, we found associations between gut microbiota community state types (CSTs) characterized by high relative abundances of *E. coli* and/or *Bifidobacterium* and low relative abundances of, among others, *Streptococcu*s, *E. faecium* and *Klebsiella* in one-week-old infants, with higher antibody responses to vaccination months later in childhood.

Our study adds to an existing body of evidence for a positive effect of *E. coli* and *Bifidobacterium* on the immune response to vaccination. For instance, higher relative abundances of Gram-negatives including *E. coli* were associated with an adequate immune response against oral rotavirus vaccines (5). Another study showed that treatment with the probiotic *E. coli* Nissle in a pig model enhanced the immune response to human rotavirus infection (34), providing a causal link. A potential mechanism whereby *E. coli* may influence vaccine responses was pinpointed by a study demonstrating that impaired antibody responses to seasonal influenza vaccination in germ-free or antibiotic-treated mice were restored through TLR5-signaling by flagellated, but not unflagellated, *E. coli* (8), suggesting strain- and antigen-specific immune enhancement. Furthermore, early-life absence of *Bifidobacterium* has been associated with reduced systemic immune responses to *Bacillus* Calmette-Guérin, polio virus, tetanus and hepatitis B vaccination (11, 22), which we also found for pneumococcal conjugate vaccination. *Bifidobacterium* species produce short chain fatty acids (SCFAs) known to interact with host immune cells. For instance, early-life reductions in fecal SCFAs have been linked to an increased risk of asthma (28), but effects of such metabolites on vaccine responses have not yet been studied. Conversely, we also found associations between other taxa such as *Clostridium, Prevotella* and *S. pyogenes* and lower vaccine responses, and it remains open to investigation whether these associations reflect a potential negative effect on the maturing immune system. Although the exact mechanisms remain to be unraveled, we

hypothesize that very early-life microbiota-host crosstalk at the intestinal mucosa imprints on systemic immunity, and may thereby affect vaccine responses.

Vaginal delivery and breastfeeding are important drivers of early-life *Bifidobacterium* and *E. coli* abundance (13, 26, 35), whereas antibiotic treatment in the neonatal period has shown to dramatically reduce these bacteria (36). Our results reveal an association between mode of delivery-induced early-life microbiota profiles and anti-pneumococcal and anti-meningococcal vaccine responses, underlining that discouraging the increasing application of C-section in the absence of medical urgency may be important to preserve the microbiota-immune axis in infants. Whether antibiotic-induced microbiota disruption is associated with reduced vaccine responses has not yet been studied in infants (7, 21). Nonetheless, preterm infants, who generally receive antibiotic treatment in the first weeks after birth, have been shown to generate lower antibody levels following vaccination compared to term-born controls (37). In our healthy, term-born cohort, very few infants were exposed to maternal antibiotics or required antibiotic treatment themselves in the first weeks of life, and further studies are required to compare our findings to (preterm) infants who received antibiotics as neonates.

We observed stronger associations of specific gut colonization patterns in early life with antibody responses to pneumococcal vaccination than with antibody responses to meningococcal vaccination. Furthermore, antibody responses against pneumococcal serotypes were not correlated to those against MenC, suggesting antigen-specific associations between the early-life microbiota and vaccine responses. A more likely explanation is that pneumococcal and meningococcal vaccinations are administered at different ages. When meningococcal vaccination is administered at 14 months of age, the immune system has been exposed to other factors, and is already more mature and possibly more resilient to microbiota-related cues than when the first pneumococcal vaccination is administered at two months of age (16). Notably, we associated higher abundances of members of the Lachnospiraceae family, including butyrate-producing taxa, with higher anti-meningococcal antibody responses. The abundance of these bacteria in the

gut typically increases following the cessation of breastfeeding (35, 38), and are generally found to be also beneficial for the developing immune system (39).

Perturbed gut microbial colonization patterns may contribute to reduced vaccine effectiveness across certain populations and settings (9). Methods to modulate the gut microbiota following perturbations such as C-section birth are being investigated, and range from probiotic administration (40) to maternal fecal microbiota transplants (41), but it remains unknown if such interventions confer any long-term health benefits including enhanced vaccine immunogenicity. Our findings provide a rationale for investigations into potential interventions that modulate the infant gut microbiota to improve vaccine immunogenicity. Our results also suggest that different interventions should be considered for vaccinations given earlier in life compared to later in life in future studies.

Strengths of our work include the dense sampling at different timepoints, especially in the beginning of life. The extensively documented epidemiological data and microbiota composition of our cohort allowed us to establish associations between gut microbiota and vaccine responses in healthy infants. Furthermore, with the sensitive multiplex immunoassay technology, we could accurately measure antigen-specific antibody concentrations, even in very low volumes of saliva. Limitations of our work include using saliva for antibody measurements rather than serum for practical and ethical reasons. However, both anti-pneumococcal and anti-MenC vaccine-induced IgG concentrations in saliva were shown to correlate with serum concentrations (42, 43), and are, therefore, a valid proxy for systemic IgG. Furthermore, while pneumococcal and meningococcal vaccination protect from infection primarily through neutralizing IgG, we did not assess other parameters of immunity such as IgA, antibody affinity, and T cell responses. Future studies could employ a multi-omics approach to obtain a complete overview of the mechanisms that underlie interindividual variation in vaccine responses (2, 44). Our observational study was also not primarily designed to study relationships between drivers, microbes and health outcomes such as antibody responses to vaccination, which limited our power to detect significant associations. Finally, the time between vaccination of the infants

and sampling was variable and antibody measurement was not always performed within the optimal time window of 2-6 weeks after vaccination, which despite that we corrected for this in our analyses, may still have affected our results.

In conclusion, we demonstrate that mode of delivery-induced differences in the gut microbiota in the first weeks of life, including differences in *E. coli* and *Bifidobacterium* relative abundances, are associated with anti-pneumococcal and anti-MenC IgG responses to vaccination. Incorporating antibody responses to vaccination as a parameter in future trials of early-life microbiota modulation could offer opportunities to assess beneficial outcomes on the microbe-mediated training of the immune system. Improved understanding of the microbial factors driving immune maturation and vaccine immunogenicity is key to improve vaccine performance and combat infectious diseases in children.

METHODS

Study population and sample collection

Fecal samples, saliva and questionnaires were collected from a healthy birth cohort in which 120 healthy, full-term infants were enrolled. This study was primarily designed to investigate the effect of mode of delivery on early-life microbiota development independent of intrapartum antibiotics, and therefore, routine peri-operative antibiotic administration to mothers delivering by C-section was postponed until after umbilical cord clamping. The current analysis of associations between host and microbial factors and antibody responses to vaccination entails a secondary goal of the study. Details on study design were previously published (26, 45). For the current analyses, we expanded our dataset with data and salivary samples up to 18 months from 78 (65%) subjects, who participated in the follow-up study beyond the first year of life. Both parents provided written informed consent. Ethical approval was granted by the Dutch national ethics committee (METC Noord-Holland, M012-015), and the study was registered in the Netherlands Trial Register under number NTR3986. Participants received no financial compensation. Study visits were conducted within 2 hours post-partum, 24-36 hours after

birth, at 7 and 14 days and at 1, 2, 4, 6, 9, 12 months and, for those who participated in the follow-up study, 18 months of age. Saliva for antibody measurement was collected at the ages of 12 and 18 months. An absorbent sponge (Malvern Medical Developments Ltd., Worcester, UK) was rubbed on the gums, cheek pouches and tongue for 1 minute. Saliva was immediately transferred to a tube containing EDTA (BD Vacutainer, New Jersey, USA) with protease inhibitor (Roche, Basel, Switzerland). Fecal samples for gut microbiota profiling were collected by the parents prior to each visit using sterile containers, and were directly stored in the home freezer, until collection by research personnel. Saliva and feces were transported on dry ice and stored at -80˚C awaiting subsequent laboratory analysis. Directly after birth, information on prenatal and perinatal characteristics was obtained. Glean Study Manager was used to build a database for data collection (Sidekick-IT). At each subsequent home visit and additionally at the age of three months, extensive questionnaires including vaccination dates were collected. Infants received all routine childhood vaccinations from healthcare professionals at well-baby clinics according to the Dutch national immunization program (NIP), independent from the study. Ten-valent pneumococcal conjugate vaccine (PCV-10) was administered to infants born before September 2013 (52/120 participants) at the ages of 2, 3, 4, and 11 months, and to infants born from September 2013 (68/120 participants) at the ages of 2, 4, and 11 months due to changes in the NIP. Meningococcus group C (MenC) conjugate vaccination was administered at the age of 14 months.

Measuring antibody responses to vaccination

Antigen-specific IgG against the capsular polysaccharides of pneumococcal vaccine serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F was measured in saliva obtained at 12 months of age (approximately 1 month after the final PCV-10 dose), and IgG against MenC polysaccharide in saliva obtained at 18 months of age (approximately 4 months after vaccination). Antibodies were quantified using fluorescent bead-based multiplex immunoassays (MIA) (46–48). Carboxylated microspheres (Luminex, Austin, TX) were coated with the respective polysaccharide antigens. To this end, antigens were first linked to Poly-L-lysine, and then the complex was bound to the

microspheres in a reaction using EDC with sulpho-NHS. Standard reference sera with previously assigned concentrations of serotype-specific IgG were an in-house intravenous immunoglobulin (IVIG) for pneumococcal serotypes (Sanquin, Amsterdam, The Netherlands), calibrated on the WHO international standard 007sp (NIBSC), and CDC1992 for MenC (NIBSC, Ridge, United Kingdom) (49). Saliva was thawed and centrifuged, and supernatants were diluted 1:2 and 1:10 using phosphate buffered saline (PBS; pH=7·2) with 5% antibody-depleted human serum (Valley Biomedical, Winchester, VA) and with 15 ug/ml multi cell wall polysaccharide (Statens Serum Insititut, Copenhagen, Denmark). From each dilution, 25 µl was mixed with an equal volume of beads. R-phycoerythrin conjugated goat anti-human IgG solution diluted 1:200 (Jackson ImmunoResearch, West Grove, PA) was added to each well. Analysis of the beads was performed on a BioPlex 200 apparatus using the BioPlex software package version 6.2 (Bio-Rad Laboratories, Hercules, CA). IgG concentrations were determined based on averaging results of both dilutions. When the concentrations differed more than twofold (coefficient of variation >47%), the result of the 1:10 dilution was used when in standard range. IgG concentrations were expressed in ng/ml. IgG concentrations below the lower limit of detection, which ranged from 0.08 ng/ml for pneumococcal serotype 4 to 0.37 ng/ml for pneumococcal serotype 14, and was 0.21 ng/ml for MenC, were set at half the lower limit of detection.

DNA isolation and sequencing

For bacterial DNA extraction and microbiota profiling, fecal samples were first thawed and vortexed. Approximately 100 µl raw feces from each sample was added to 300 µl lysis buffer (Agowa Mag Mini DNA Isolation Kit, LGC ltd, UK), 500 µl 0.1-mm zirconium beads (BioSpec products, Bartlesville, OK, USA) and 500 µl phenol saturated with Tris-HCl (pH 8.0; Carl Roth, GMBH, Germany) in a 96-wells plate. The fecal samples were mechanically disrupted with a Mini-BeadBeater-96 (BioSpec products, Bartlesville, OK, USA) at 2100 oscillations per minute for 2 minutes. DNA purification was performed with the Agowa Mag Mini DNA Isolation Kit following the manufacturer's recommendations. Finally, the extracted DNA was eluted in 60 µl elution buffer (LGC Genomics, Germany). Adaptations in the standard DNA isolation procedure were applied

for samples collected directly postpartum and on day 1, which were presumed to have low bacterial abundance and diversity (26). The amount of bacterial DNA was determined by a quantitative polymerase chain reaction (qPCR), as described elsewhere (50), using primers targeting the bacterial 16S rRNA gene (forward: CGAAAGCGTGGGGAGCAAA; reverse: GTTCGTACTCCCCAGGCGG; probe: 6FAM-ATTAGATACCCTGGTAGTCCA-MGB) on the 7500 Fast Real Time system (Applied Biosystems, CA, USA). Samples with a minimum bacterial DNA yield of >0.3 ng/ul above the concentration in negative isolation controls were included in the sequencing protocol. The V4 hypervariable region of the 16S rRNA gene was amplified using F515/R806 primers (30 amplification cycles), and amplicon pools were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA) in 17 runs along with isolation and PCR blanks as negative controls.

Bioinformatic processing

Sequences were processed in our bioinformatics pipeline (25). We applied an adaptive, window-based trimming algorithm (Sickle, version 1.33) to filter out low quality reads below a Phred score threshold of 30 and/or a length threshold of 150 nucleotides (51). Sequencing errors were corrected with BayesHammer (SPAdes genome assembler toolkit, version 3.5.0) (52). Sets of paired-end sequence reads were assembled using PANDAseq (version 2.10) and demultiplexed (QIIME, version 1.9.1) (53, 54). Singletons and chimeras (UCHIME) were removed. Operational taxonomic unit (OTU) picking was performed with VSEARCH abundance-based greedy clustering of reads at 97% similarity (55). Taxonomic annotation of OTUs was performed with the Naïve Bayesian RDP classifier (version 2.2) and the SILVA (version 119) reference database (56, 57). The resulting OTU table contained 6690 taxa. We selected OTUs that were present at a confident level of detection, i.e. representing at least 0.1% of all reads in at least two samples (excluding 0.4% of all reads). This abundance-filtered dataset contained 623 OTUs, and is referred to as the raw OTU table. We performed normalization by total sum scaling to obtain the relative abundance OTU table. Both OTU tables were used for downstream analyses.

Whole genome sequencing for validation of OTU taxonomic annotations

Taxonomic annotations of the 16S rRNA gene sequences were validated, using whole genome shotgun sequencing (WGS) on a subset of 20 week one samples (ten from vaginally delivered infants, and ten from C-section born infants). For library preparation, the Truseq Nano gel free kit was used. From the libraries, 150 base paired-end sequence data were generated using a NovaSeq instrument to yield 750M+750M reads in two runs. Reads were trimmed to remove amplicon adapter sequences and to maintain a quality threshold of 30 and a minimum read length of 35 base pairs using Cutadapt (58) (version 1.9.dev2). SAM files were generated per sample and per run with Bowtie2 (59). SAM files from different runs were merged per sample using Picard (60), and were used as input to MetaPhlAn2 (61) for profiling and annotating the microbial communities within each sample (default parameters). The relative abundances of the top five 16S rRNA gene sequencing-based OTUs *Bifidobacterium* (1), *E. coli* (2), *Staphylococcus* (3), *Klebsiella* (4) and *E. faecium* (5) were shown to correlate strongly with the WGS species-level relative abundances of *B. breve, B. longum* and *B. adolescentis* (combined; Pearson's ρ=0.95, adjusted p<0.001), *E. coli* (Pearson's ρ=0.95, adjusted p<0.001), *Staphylococcus epidermidis* (Pearson's ρ=0.86, adjusted p<0.001), *Klebsiella oxytoca* (Pearson's ρ=0.83, adjusted p<0.001) and *E. faecium* (Pearson's ρ=0.92, adjusted p<0.001), respectively, confirming their taxonomies.

Species-specific qPCR

Species-specific qPCR was performed on all week one samples (n=119) to confirm the presence and abundance of *E. coli*, *Klebsiella* spp., and *Enterococcus* spp., using the VetMAX™ MastiType Multi Kit (Applied Biosystems™, CA, USA) according to the manufacturer's instructions. The qPCR test results were analyzed with the recommended Animal Health VeriVet Software, available on Thermo Fisher Cloud. One sample was excluded from statistical analysis because its Internal Amplification Control did not pass the Ct-value criteria in three out of the four mixes.

Statistics and reproducibility

Microbiome data were excluded from the analysis if fecal samples had insufficient bacterial DNA available (*n*=104). Antibody measurements were excluded from the analyses if infants did not receive their vaccinations in time (*n*=8 at month 12, *n*=1 at month 18), or if the saliva sample did not have a sufficient volume for laboratory analysis (*n*=8 at month 12, *n*=11 at month 18; Supplementary Figure 1). The study sample size was originally calculated to detect differences in the microbiota composition between infants born by vaginal delivery and by C-section (26). For the current study, no statistical method was used to predetermine sample size. Data analysis was performed in R version 4.0.3 within RStudio version 1.3.1093 (62). All statistical tests were two-tailed, and *p*-values below 0.050 or Benjamini-Hochberg adjusted *p*-values below 0.100 were considered statistically significant. IgG responses were analyzed as continuous log-transformed variables or stratified along the median into high and low responses. All analyses were adjusted for time between vaccination and saliva collection using a second degree polynomial to account for the natural kinetics of the antibody response.

Concordance between IgG concentrations was evaluated using Pearson's correlations. Associations between early-life host characteristics (mode of delivery, feeding type, sex, antibiotic use in the first three months, number of antibiotic courses, daycare attendance, having siblings and having pets) and IgG concentrations were assessed using univariate linear models, and factors with a $p<0.050$ for one or more serotypes were included in multivariable models. IgG geometric mean concentrations (GMCs) were compared between groups defined by mode of delivery and feeding type, using ANOVA followed by post-hoc Tukey-Kramer tests to account for unequal group sizes (HSD.test-function, *agricolae* package [version 1.3-5] (63), parameter 'unbalanced' set to TRUE). We tested the assumptions of normality and homogeneity of variance of the ANOVA test by inspecting the distribution of the residuals and with Levene's test, respectively.

Gut microbiota alpha diversity was assessed by the number of observed species and the Shannon diversity index (*phyloseq* package [version 1.38.0] (64)). Associations between alpha diversity measures per timepoint and IgG

concentrations were tested using linear models. Permutational multivariate analysis of variance (PERMANOVA) on the Bray-Curtis dissimilarity matrix was used to test for overall differences in the microbial community composition per timepoint between infants with high and low IgG responses (adonis2-function, *vegan* package [version 2.5-7] (65)). Stability of the microbial community composition over time was calculated as the Bray-Curtis similarity (1–Bray-Curtis dissimilarity) between consecutive samples from the same individual, where a higher similarity indicates higher stability.

Dirichlet multinomial mixture models were used to group infants into community state types (CSTs) based on gut microbiota composition at week one and at month 12 separately (*DirichletMultinomial* package [version 1.36.0] (66)). For this analysis, the raw OTU table was filtered, retaining OTUs present in >10% of the samples included in the analysis. The optimal number of CSTs was set at the number of Dirichlet components representing optimal model fit, testing a range of one to seven components. Model fit was based on the Laplace approximation to the negative log model, where a lower value indicates a better fit. Differences in the gut microbial community composition according to CST were evaluated using PERMANOVA (adonis-function, *vegan* package [version 2.5-7] (65)). Differences in IgG GMCs according to week one and month 12 CSTs were evaluated using ANOVA and post hoc Tukey-Kramer tests, as described above.

Smoothing-spline analysis of variance (SS-ANOVA, fitTimeSeries-function, *metagenomeSeq* package [version 1.36.0] (67, 68)) was used to detect differences in individual OTU abundances in the first two months of life between infants with responses above and below the median antigen-specific IgG concentration. For the anti-MenC IgG response, this analysis was repeated for the entire 12 month follow-up period. For this analysis, the raw OTU table was filtered, retaining only OTUs present in >10% of all samples included in the analysis. This method detects differentially abundant OTUs, and identifies the time intervals in which significant differences exist.

Correlations between the relative abundances of *E. coli, E. faecium* and *Klebsiella* at the age of one week and the species-specific Ct-values from targeted qPCR were evaluated with Spearman's rank-order correlations.

Chi-square tests were used to assess differences in presence of species identified by targeted qPCR between infants with above and below median IgG responses and between mode of delivery groups.

Data and code availability

Sequence data that support the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) database with BioProject ID PRJNA481243 [https://www.ncbi.nlm.nih.gov/bioproject/481243], and PRJNA555020 [https://www.ncbi.nlm.nih.gov/bioproject/555020]. Taxonomic annotations were based on the Silva reference database (version 119). All R code used to run the statistical analysis is publicly available at https://gitlab.com/EMdK/muis_vaccine_responses.

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SUPPLEMENTARY TABLES

Supplementary Table 1 | Validation of associations between early-life characteristics and anti-pneumococcal IgG concentrations following vaccination.

	Formula feeding vs breastfeeding		Vaginal birth vs C-section birth		Female vs male		AB vs no AB in first 3 months		Pets vs no pets in household		Formula feeding * vaginal birth	
Ps	***β*** **(95% CI)**	***p*-value**	***β*** **(95% CI)**	***p*-value**	***β*** **(95% CI)**	***p*-value**	***β*** **(95% CI)**	***p*-value**	***β*** **(95% CI)**	***p*-value**	***β*** **(95% CI)**	***p*-value**
1	0.57 (-0.19-1.33)	0.142	0.48 (0.01-0.96)	0.046	-0.09 (-0.52-0.34)	0.682	-0.26 (-0.91-0.39)	0.429	-0.19 (-0.62-0.24)	0.384	-1.87 (-3.00- -0.73)	0.002
4	-0.17 (-0.82-0.47)	0.596	0.26 (-0.14-0.66)	0.194	-0.01 (-0.37-0.36)	0.971	-0.29 (-0.83-0.26)	0.302	-0.38 (-0.74- -0.02)	0.041	-0.80 (-1.76-0.16)	0.100
5	0.35 (-0.27-0.97)	0.263	0.49 (0.11-0.87)	0.013	0.06 (-0.29-0.40)	0.751	-0.25 (-0.78-0.27)	0.340	-0.15 (-0.50-0.20)	0.394	-1.37 (-2.28- -0.45)	0.004
7F	0.35 (-0.29-1.00)	0.281	0.41 (0.01-0.81)	0.045	0.38 (0.02-0.75)	0.040	-0.32 (-0.87-0.23)	0.250	-0.25 (-0.62-0.11)	0.171	-1.46 (-2.42- -0.50)	0.003
9V	0.36 (-0.26-0.99)	0.246	0.36 (-0.02-0.75)	0.065	0.03 (-0.32-0.38)	0.874	-0.40 (-0.92-0.13)	0.140	-0.19 (-0.54-0.16)	0.291	-1.23 (-2.16- -0.31)	0.009
14	0.41 (-0.31-1.12)	0.262	0.32 (-0.12-0.76)	0.154	0.16 (-0.24-0.57)	0.422	-0.23 (-0.84-0.37)	0.444	-0.37 (-0.77-0.03)	0.070	-1.50 (-2.56- -0.44)	0.006
18C	-0.49 (-1.23-0.25)	0.191	0.05 (-0.40-0.51)	0.820	0.09 (-0.33-0.50)	0.674	-0.72 (-1.34--0.09)	0.025	-0.52 (-0.94--0.11)	0.014	-0.28 (-1.38-0.81)	0.610
19F	0.27 (-0.44-0.99)	0.452	0.29 (-0.16-0.73)	0.203	0.16 (-0.24-0.56)	0.434	-0.53 (-1.14-0.08)	0.087	-0.29 (-0.69-0.11)	0.154	-1.32 (-2.38- -0.26)	0.015
23F	0.10 (-0.60-0.80)	0.778	0.39 (-0.04-0.83)	0.076	-0.01 (-0.40-0.39)	0.978	-0.40 (-0.99-0.20)	0.187	-0.26 (-0.66-0.13)	0.186	-0.79 (-1.83-0.25)	0.135

Model coefficients (β) with 95% CI and p-values were computed with two-sided multivariable linear regression on log-transformed anti-pneumococcal concentrations per serotype (Ps; n=101). All analyses were corrected for time between vaccination and IgG measurement, but not for multiple comparisons.
Abbreviations: C-section = caesarean section; AB = antibiotics

Supplementary Table 2 | Validation of associations between Bray-Curtis similarity and anti-pneumococcal IgG levels.

Ps	time interval	*β* (95% CI)	adjusted *p*-value
1	d1-w1	1.20 (-0.03 - 2.42)	0.065
	w1-w2	0.86 (-0.05 - 1.78)	0.065
4	d1-w1	1.07 (0.08 - 2.06)	0.055
	w1-w2	0.75 (-0.02 - 1.51)	0.055
5	d1-w1	1.00 (0.05 - 1.95)	0.040
	w1-w2	0.80 (0.09 - 1.51)	0.040
7F	d1-w1	0.72 (-0.29 - 1.73)	0.162
	w1-w2	0.96 (0.22 - 1.70)	0.024
9V	d1-w1	1.22 (0.29 - 2.15)	0.011
	w1-w2	1.02 (0.33 - 1.72)	0.009
14	d1-w1	1.25 (0.18 - 2.33)	0.023
	w1-w2	1.25 (0.43 - 2.08)	0.006
18C	d1-w1	1.77 (0.72 - 2.82)	0.002
	w1-w2	0.77 (-0.10 - 1.65)	0.083
19F	d1-w1	1.14 (0.13 - 2.15)	0.056
	w1-w2	0.41 (-0.38 - 1.20)	0.307
23F	d1-w1	1.01 (-0.11 - 2.13)	0.077
	w1-w2	1.15 (0.27 - 2.03)	0.023

Model coefficients (β) with 95% CI and adjusted p-values were computed with two-sided multivariable linear regression on log-transformed anti-pneumococcal concentrations per serotype (Ps; n=101). All analyses were corrected for time between vaccination and IgG measurement and for multiple comparisons. Abbreviations: Ps = pneumococcal serotype; d1=day 1; w1=week 1; w2=week 2.

Supplementary Table 3 | Validation of association between week 1 CST and IgG concentrations against pneumococcal serotypes.

	ANOVA		Post-hoc Tukey-Kramer tests					
	All CSTs		CST 1 vs. CST 2		CST 1 vs. CST3		CST 2 vs. CST 3	
Ps	*F* (2,95)	*p*-value	difference	adjusted *p*-value	difference	adjusted *p*-value	difference	adjusted *p*-value
1	3.2	0.043	-0.554	0.239	-0.765	0.068	-0.211	0.808
4	2.9	0.062	-0.450	0.267	-0.595	0.102	-0.145	0.869
5	3.6	0.031	-0.537	0.114	-0.469	0.188	0.067	0.965
7F	2.5	0.084	-0.481	0.203	-0.382	0.361	0.098	0.934
9V	3.1	0.049	-0.468	0.188	-0.512	0.135	-0.045	0.985
14	3.0	0.056	-0.540	0.204	-0.596	0.145	-0.056	0.982
18C	3.1	0.050	-0.580	0.179	-0.598	0.161	-0.018	0.998
19F	1.0	0.386	-0.297	0.576	-0.300	0.571	-0.002	1.000
23F	3.0	0.056	-0.642	0.139	-0.356	0.538	0.286	0.670

All analyses were two-sided and corrected for time between vaccination and IgG measurement. Abbreviations: CST = community state type; Ps = pneumococcal serotype.

Supplementary Table 4 | CST as a mediator between mode of birth and anti-Ps6B and anti-MenC IgG concentrations.

	Anti-Ps6B IgG				**Anti-MenC IgG**			
	Model without CST		**Model with CST**		**Model without CST**		**Model with CST**	
	***β* (95% CI)**	***p*-value**	***β* (95% CI)**	***p*-value**	***β* (95% CI)**	***p*-value**	***β* (95% CI)**	***p*-value**
(Intercept)	2.13 (1.71-2.55)	<0.0001	2.05 (1.62-2.48)	<0.0001	2.03 (1.71-2.35)	<0.0001	1.93 (1.59-2.27)	<0.0001
Vaginal birth	0.53 (-0.02-1.08)	0.060	0.04 (-0.74-0.83)	0.911	0.52 (0.12-0.91)	0.012	0.68 (0.14-1.22)	0.015
CST2 vs 1	*NA*	*NA*	0.72 (-0.12-1.57)	0.091	*NA*	*NA*	-0.14 (-0.69-0.40)	0.602
CST3 vs 1	*NA*	*NA*	0.60 (-0.25-1.45)	0.165	*NA*	*NA*	0.48 (-0.11-1.06)	0.107

Model coefficients (β) with 95% CI and adjusted p-values were computed with two-sided multivariable linear regression on log-transformed anti-Ps6B (n=101) and anti-MenC concentrations. All analyses were corrected for time between vaccination and IgG measurement. Abbreviations: CST = community state type; NA = not applicable.

Supplementary Table 5 | Differentially abundant OTUs in the first 2 months of life between infants with above vs. below median anti-Ps6B IgG levels.

OTU	Interval number	Interval start	Interval end	Area	Association	*p*-value	Adjusted *p*-value	Validated for Ps*:
Bifidobacterium (1)	interval:1	0	5	7.2	Above median	0.013	0.027	1, 4, 5, 7F, 9V, 19F, 23F
Escherichia coli (2)	interval:1	0	41	82.0	Above median	0.003	0.013	1, 4, 5, 7F, 9V, 18C, 23F
Ruminococcus gnavus (9)	interval:1	0	16	20.5	Above median	0.016	0.031	7F
Pseudobutyrivibrio (13)	interval:1	0	20	-14.3	Below median	0.050	0.071	1, 4, 5, 7F, 9V, 23F
Anaerostipes (20)	interval:1	0	17	-8.6	Below median	0.064	0.084	5, 7F, 9V
Clostridium sensu stricto 1 (21)	interval:1	0	31	-46.0	Below median	0.023	0.040	5, 7F, 23F
Prevotella (25)	interval:1	0	23	-22.4	Below median	0.001	0.010	1, 4, 5, 7F, 9V, 14, 18C, 19F, 23F
Streptococcus pyogenes (26)	interval:1	0	33	-21.4	Below median	0.013	0.027	1, 9V, 14
Dorea (32)	interval:1	0	4	-5.2	Below median	0.070	0.089	4, 5, 7F, 9V
Bacteroides (53)	interval:1	0	48	42.7	Above median	0.002	0.010	1, 4, 5, 7F, 9V, 19F, 23F
Streptococcus (55)	interval:1	0	43	-50.3	Below median	0.024	0.040	1, 4, 9V
Lactococcus lactis (80)	interval:1	0	33	-35.7	Below median	0.001	0.010	1, 5, 7F, 9V, 14, 18C, 19F, 23F
Bifidobacterium (147)	interval:1	0	19	18.0	Above median	0.002	0.010	1, 4, 5, 7F, 19F, 23F
Escherichia/Shigella (185)	interval:1	0	36	46.7	Above median	0.002	0.010	1, 7F, 18C
Pseudomonas fluorescens (236)	interval:1	0	5	-3.7	Below median	0.088	0.099	5, 7F, 9V
Bacillales (255)	interval:1	0	39	-38.7	Below median	0.001	0.010	4, 5, 7F, 9V, 19F, 23F
Enterococcus (256)	interval:1	0	45	-48.8	Below median	0.002	0.010	1, 4, 5, 7F, 9V, 18C, 19F, 23F
Enterobacteriaceae (345)	interval:1	0	15	7.4	Above median	0.020	0.038	
Staphylococcaceae (382)	interval:1	0	58	-52.1	Below median	0.001	0.010	4, 5, 7F, 9V, 19F, 23F
Streptococcus gallolyticus (18)	interval:1	2	44	45.0	Above median	0.013	0.027	14
Bacteroides (433)	interval:1	3	8	2.0	Above median	0.023	0.040	4, 9V, 19F, 23F

Supplementary Table 5 | Continued

OTU	Interval number	Interval start	Interval end	Area	Association	*p*-value	Adjusted *p*-value	Validated for Ps*:
Streptococcus (502)	interval:1	3	49	-42.3	Below median	0.002	0.010	1, 5, 7F, 9V, 23F
Bacteroides (19)	interval:1	4	47	58.0	Above median	0.021	0.039	1, 4, 5, 7F, 9V, 18C, 19F, 23F
Streptococcus (189)	interval:1	4	26	-26.4	Below median	0.007	0.019	1, 4, 5, 9V
Gardnerella (333)	interval:1	11	60	-51.8	Below median	0.004	0.014	1, 5, 9V, 14, 18C
Veillonella (366)	interval:1	11	62	-44.3	Below median	0.004	0.014	1, 5, 7F, 9V, 14, 23F
Bacteroides (35)	interval:1	12	47	42.3	Above median	0.009	0.022	1, 4, 5, 18C, 19F, 23F
Bilophila wadsworthia (136)	interval:1	14	70	52.7	Above median	0.006	0.018	1, 4, 5, 9V, 14, 23F
Subdoligranulum (38)	interval:2	15	30	13.4	Above median	0.006	0.018	1, 4, 14
Bifidobacterium bifidum (11)	interval:1	16	70	-101.4	Below median	0.026	0.041	9V, 14, 18C, 19F
Rothia (113)	interval:1	17	29	-9.1	Below median	0.062	0.084	4, 9V, 23F
Peptostreptococcus (168)	interval:1	17	70	50.8	Above median	0.081	0.095	9V, 23F
Bacteroides (249)	interval:1	17	70	39.4	Above median	0.002	0.010	4, 5, 7F, 9V, 19F, 23F
Streptococcus (69)	interval:1	18	55	-35.0	Below median	0.074	0.090	
Lactobacillus fermentum (75)	interval:1	18	30	-6.0	Below median	0.074	0.090	4
Veillonella (179)	interval:1	19	32	-7.3	Below median	0.029	0.045	1, 4, 5, 7F, 9V, 19F
Enterococcus faecium (5)	interval:1	20	23	-3.8	Below median	0.046	0.067	5, 7F, 23F
Bifidobacteriaceae (309)	interval:1	21	48	-14.5	Below median	0.089	0.099	4, 7F, 9V, 18C, 23F
Bifidobacterium animalis (41)	interval:1	22	64	-54.7	Below median	0.015	0.031	1, 5, 9V, 14, 18C, 19F, 23F
Bacteroides (48)	interval:1	24	41	14.6	Above median	0.065	0.084	14, 23F
Bacteroides (12)	interval:1	26	70	81.1	Above median	0.007	0.019	7F, 23F
Bifidobacterium (229)	interval:1	29	70	-40.3	Below median	0.005	0.017	9V, 14, 18C, 19F
Blautia (10)	interval:1	33	59	140.0	Above median	0.003	0.013	1, 4, 19F, 23F

Supplementary Table 5 | Continued

OTU	Interval number	Interval start	Interval end	Area	Association	*p*-value	Adjusted *p*-value	Validated for Ps*:
Enterobacteriaceae (242)	interval:1	33	70	-42.4	Below median	0.002	0.010	9V, 18C, 19F, 23F
Klebsiella (4)	interval:1	34	70	-80.7	Below median	0.004	0.014	
Carnobacteriaceae (311)	interval:1	38	70	22.6	Above median	0.061	0.084	14, 19F
Bifidobacterium animalis (175)	interval:1	39	70	-27.0	Below median	0.082	0.095	14
Citrobacter sedlakii (288)	interval:1	46	70	-23.1	Below median	0.026	0.041	9V
Finegoldia (203)	interval:1	50	70	-20.0	Below median	0.034	0.051	
Blautia (7)	interval:1	57	70	-23.5	Below median	0.012	0.027	4, 5, 7F, 9V, 18C
Corynebacterium propinquum (79)	interval:1	66	70	-11.6	Below median	0.009	0.022	

Differential abundance analysis was performed using two-sided smoothing spline ANOVA (ss-ANOVA). All analyses were corrected for time between vaccination and IgG measurements and for multiple comparisons.
* Validation for other pneumococcal vaccine serotypes 1, 4, 5, 7F, 9V, 14, 18C, 19F and 23F was performed using the same method. OTUs were considered validated for a given serotype if they were significantly differentially abundant (adjusted p<0.100) and were associated with the same response category (above or below median). Abbreviations: Ps = pneumococcal serotype

Supplementary table 6 | Differentially abundant OTUs in the first 2 months of life between infants with above vs. below median anti-MenC IgG levels.

OTU	Interval number	Interval start	Interval end	Area	Association	*p*-value	Adjusted *p*-value
Escherichia coli (2)	interval:1	0	13	23.2	Above median	0.062	0.072
Veillonella (8)	interval:1	0	7	-9.2	Below median	0.037	0.055
Peptostreptococcaceae (46)	interval:1	0	7	9.4	Above median	0.021	0.054
Bacteroides (65)	interval:1	0	26	25.8	Above median	0.014	0.054
Streptococcus (69)	interval:1	0	14	-18.5	Below median	0.023	0.054
Rothia (113)	interval:1	0	43	-52.8	Below median	0.027	0.054
Bifidobacteriaceae (299)	interval:1	0	16	-7.9	Below median	0.005	0.036
Lachnospiraceae (96)	interval:1	1	51	47.4	Above median	0.003	0.029
Collinsella (16)	interval:1	3	20	23.7	Above median	0.037	0.055
Veillonella (85)	interval:1	3	16	-20.9	Below median	0.002	0.029
Klebsiella (252)	interval:1	4	12	-10.8	Below median	0.001	0.029
Veillonella (368)	interval:1	5	14	-7.7	Below median	0.013	0.054
Bifidobacterium (218)	interval:1	6	42	-25.1	Below median	0.025	0.054
Clostridium sensu stricto 1 (24)	interval:1	7	20	-19.7	Below median	0.017	0.054
Escherichia Shigella (185)	interval:1	10	24	12.3	Above median	0.043	0.059
Bifidobacteriaceae (309)	interval:1	11	53	34.6	Above median	0.060	0.072
Enterococcaceae (251)	interval:1	13	39	-28.1	Below median	0.012	0.054
Veillonella (160)	interval:1	15	41	-29.0	Below median	0.034	0.055
Lactobacillus (49)	interval:1	21	55	-47.0	Below median	0.062	0.072
Bilophila wadsworthia (136)	interval:1	21	69	-54.8	Below median	0.022	0.054
bacterium NLAE zl C558 (45)	interval:1	26	69	54.9	Above median	0.068	0.076

Supplementary Table 6 | Continued

OTU	Interval number	Interval start	Interval end	Area	Association	*p*-value	Adjusted *p*-value
Lactobacillus fermentum (75)	interval:1	33	69	-49.0	Below median	0.037	0.055
Lachnospiraceae (30)	interval:1	35	69	-39.3	Below median	0.023	0.054
Bifidobacterium breve (261)	interval:1	38	69	30.6	Above median	0.072	0.077
Streptococcus salivarius (6)	interval:1	40	69	-42.4	Below median	0.028	0.054
Enterobacteriaceae (281)	interval:1	45	62	-11.9	Below median	0.054	0.071
Clostridium butyricum (33)	interval:1	62	69	26.2	Above median	0.038	0.055

Differential abundance analysis was performed using two-sided smoothing spline ANOVA (ss-ANOVA). All analyses were corrected for time between vaccination and IgG measurements and for multiple comparisons.

Supplementary Table 7 | Differentially abundant OTUs of the Lachnospiraceae family in the first 12 months of life between infants with above vs. below median anti-MenC IgG levels.

OTU	Interval number	Interval start	Interval end	Area	Association	*p*-value	Adjusted *p*-value
Lachnospira (89)	interval:1	32	169	60.8	Above median	0.013	0.052
Lachnospiraceae (30)	interval:1	82	134	-49.9	Below median	0.013	0.052
Pseudobutyrivibrio (132)	interval:1	90	381	144.2	Above median	0.030	0.066
Blautia (67)	interval:1	100	381	313.6	Above median	0.020	0.053
Fusicatenibacter saccharivorans (15)	interval:1	101	381	439.7	Above median	0.050	0.080
Pseudobutyrivibrio (13)	interval:1	125	381	353.8	Above median	0.004	0.036
Lachnospiraceae (306)	interval:1	130	381	130.4	Above median	0.023	0.054
Roseburia (77)	interval:1	155	381	284.2	Above median	0.019	0.053
Dorea (54)	interval:1	156	381	185.8	Above median	0.075	0.094
Blautia (28)	interval:1	204	286	230.8	Above median	0.027	0.062
Lachnospira (117)	interval:1	207	357	242.1	Above median	0.002	0.021
Roseburia (133)	interval:1	257	381	150.6	Above median	0.002	0.021
Blautia (47)	interval:1	277	381	198.6	Above median	0.023	0.054
Moryella (71)	interval:1	317	381	85.6	Above median	0.065	0.092
Blautia (28)	interval:2	362	381	87.5	Above median	0.015	0.053

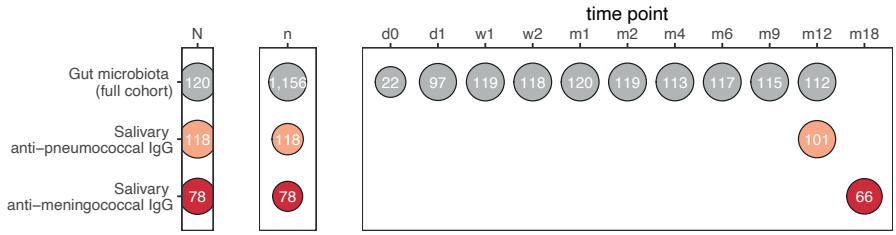
Differential abundance analysis was performed using two-sided smoothing spline ANOVA (ss-ANOVA). All analyses were corrected for time between vaccination and IgG measurements and for multiple comparisons.

Supplementary Table 8 | Validation of 16S rRNA gene sequencing-based results by targeted qPCR.

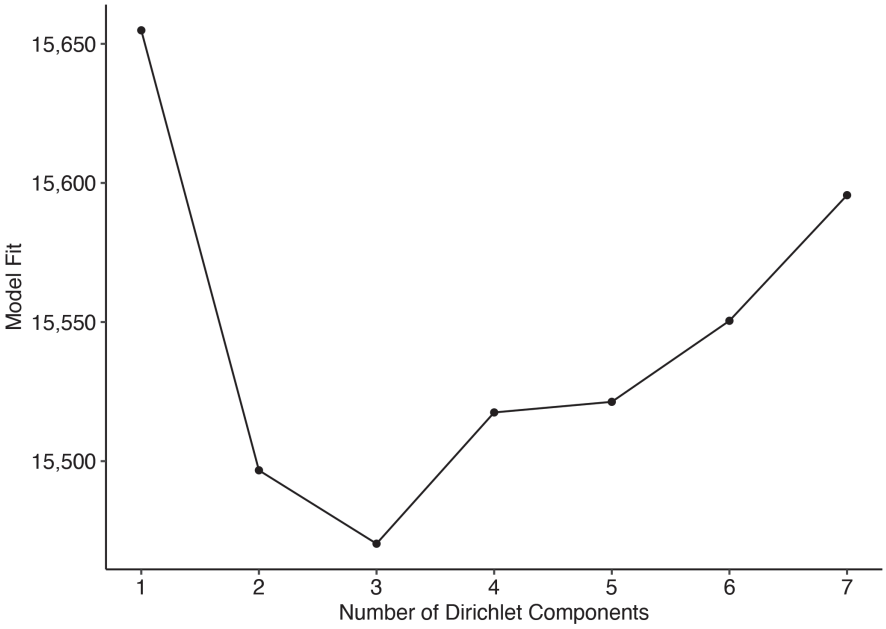
	Anti-Ps6B IgG response			Anti-MenC IgG response		
	High	**Low**	***p*-value**	**High**	**Low**	***p*-value**
n	**49**	**50**		**33**	**31**	
Escherichia coli presence (%)	34 (69.4)	25 (50.0)	0.078	23 (69.7)	19 (61.3)	0.657
Klebsiella spp. presence (%)	23 (46.9)	21 (42.0)	0.770	16 (48.5)	13 (41.9)	0.783
Enterococcus spp. presence (%)	29 (59.2)	37 (74.0)	0.177	22 (66.7)	19 (61.3)	0.851

Presence of *E. coli, Enterococcus* spp. and *Klebsiella* spp. identified by quantitative polymerase chain reaction (qPCR) on all week 1 samples, for infants with high and low anti-Ps6B and anti-MenC IgG responses. p-values were calculated with two-sided chi-square tests.

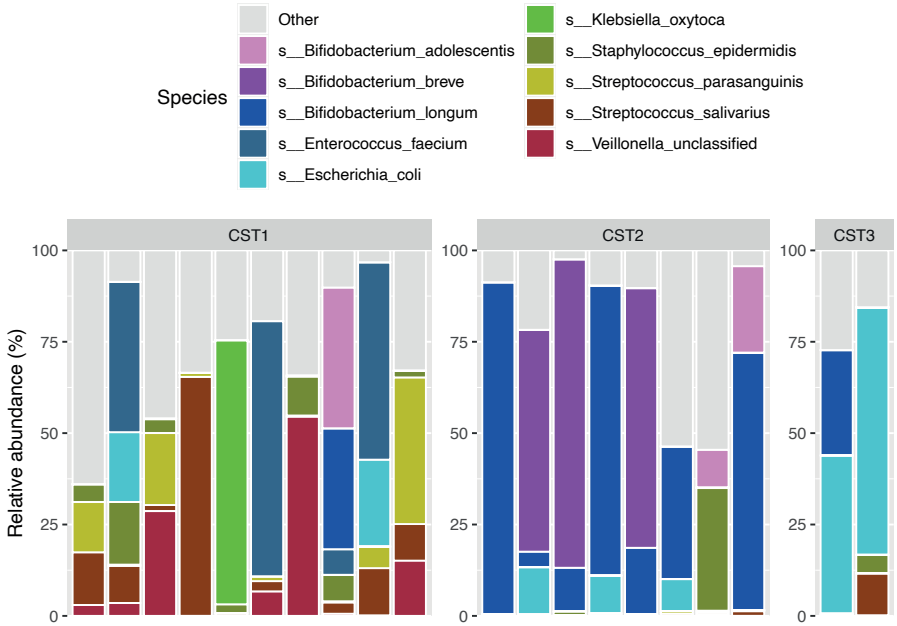
SUPPLEMENTARY FIGURES



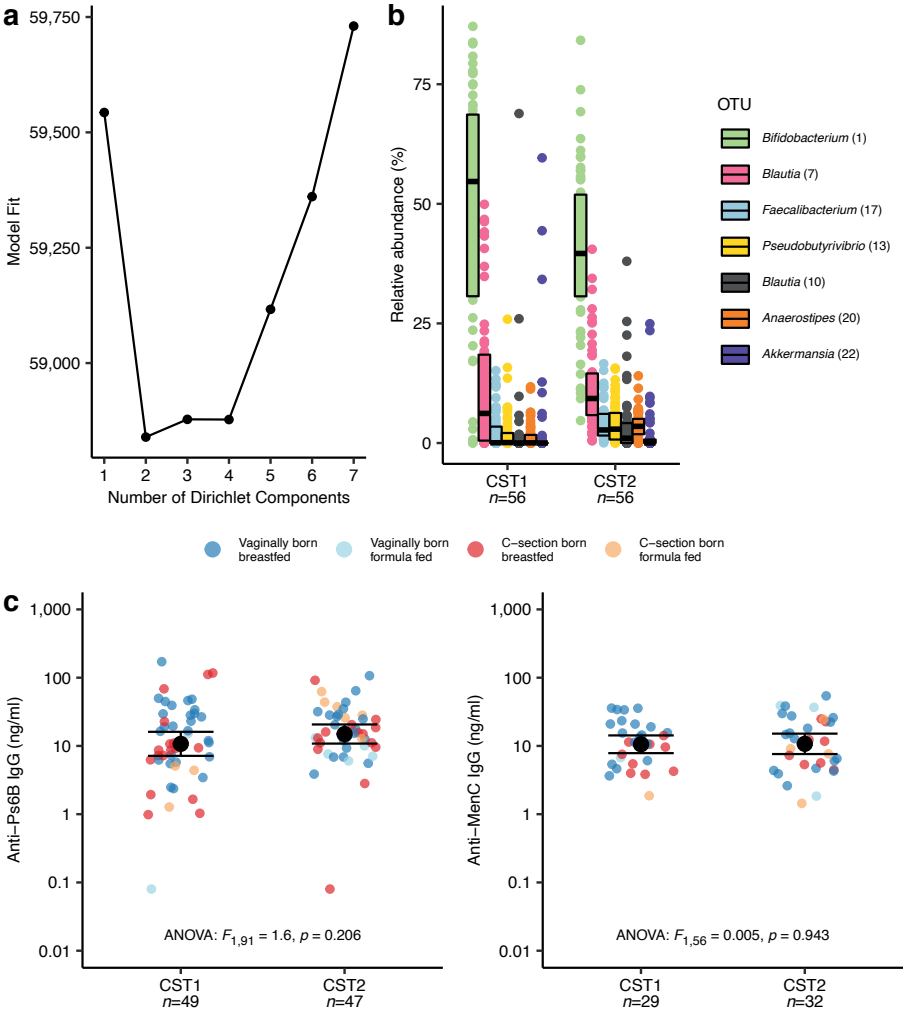
Supplementary Figure 1 | Sample overview. Fecal samples were collected for gut microbiota characterization from 120 healthy infants at days (d)0 and 1, weeks (w)1 and 2, and months (m)1, 2, 4, 6, 9 and 12. Fecal samples were excluded from the analysis if they had insufficient bacterial DNA available (n=104). Saliva was collected from 118 infants at the age of 12 months for measuring anti-pneumococcal immunoglobulin G (IgG) and from 78 infants at the age of 18 months for measuring anti-meningococcal IgG. Saliva samples were excluded if infants did not receive their vaccinations in time (n=8 at month 12, n=1 at month 18), or if the saliva sample did not have a sufficient volume for laboratory analysis (n=8 at month 12, n=11 at month 18). N=total number of infants from which samples were collected; n=total number of available samples.



Supplementary Figure 2 | Dirichlet multinomial mixture model fit. Dirichlet multinomial mixture model identified 3 compositionally distinct community state types (CST) as the best model fit at the week 1 timepoint. Model fit was based on the Laplace approximation to the negative log model where a lower value indicates a better model fit.



Supplementary Figure 3 | Species-level composition of community state types at week 1 of age. Relative abundances of the top 10 species in 20 week 1 samples, determined by whole genome shotgun sequencing. Samples were ordered by week 1 community state type (CST).



Supplementary Figure 4 | Community state types at 12 months of age. (A) Dirichlet multinomial mixture model identified two compositionally distinct community state types (CST) as the best model fit at the month 12 timepoint. Model fit was based on the Laplace approximation to the negative log model where a lower value indicates a better model fit. (B) Boxplot of relative abundances of the top 7 operational taxonomic units (OTUs) per community state type (CST) defined at 12 months of age. Boxes show medians with interquartile ranges. (C) Month 12 CSTs are plotted against anti-Ps6B IgG concentrations (left) and anti-MenC IgG concentrations (right). Dots are colored according to mode of delivery and feeding type from birth. Black dots and error bars represent geometric mean concentrations with 95% confidence intervals. Significance was assessed using two-sided ANOVA on log-transformed IgG concentrations, correcting for time between vaccination and IgG measurements.

CHAPTER 6

Salivary antibody responses to 10-valent pneumococcal conjugate vaccination following two different immunization schedules in a healthy birth cohort

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ABSTRACT

Pneumococcal conjugate vaccines reduce pneumococcal colonization via serotype-specific immunoglobulin G (IgG) at mucosal surfaces. The infant immunization schedule with the ten-valent pneumococcal conjugate vaccine (PCV10) changed from a 3+1 schedule (2-3-4-11 months) to a 2+1 schedule (2-4-11 months) in The Netherlands in 2013. We compared anti-pneumococcal IgG concentrations in saliva between the schedules. IgG was measured using a fluorescent bead-based multiplex immunoassay, at the ages of 6 (post-primary) and 12 (post-booster) months in 51 infants receiving the 3+1 schedule and 68 infants receiving the 2+1 schedule. Post-primary IgG geometric mean concentrations (GMCs) were comparable between schedules for all vaccine serotypes. Post-booster IgG GMCs were significantly lower after the 2+1 schedule for serotypes 4 (p=0.035), 7F (p=0.048) and 23F (p=0.0056). This study shows small differences in mucosal IgG responses between a 3+1 and a 2+1 PCV10 schedule. Future studies should establish correlates of protection against pneumococcal colonization for mucosal antibodies.

INTRODUCTION

Pneumococcal conjugate vaccination in children has drastically reduced the burden of disease caused by *Streptococcus pneumoniae,* ranging from respiratory infections like acute otitis media and pneumonia to invasive disease like septicaemia and meningitis (1,2). Apart from direct protection, a reduction in nasopharyngeal pneumococcal colonization is the second pillar of success of conjugate vaccines (3). Nasopharyngeal colonization can be considered both a prerequisite for infection as well as the source of community spread (4,5). The pneumococcal conjugate vaccine does not only induce long-term immunoglobulin type G (IgG) in serum, but also at mucosal surfaces (6), which were shown to correlate well with serum levels (7). Although the exact mechanisms by which pneumococcal acquisition at the nasal mucosa is prevented following pneumococcal conjugate vaccination remain unclear, mucosal IgG has been suggested as a protective agent (8,9).

The Netherlands introduced the 7-valent pneumococcal conjugate vaccine (PCV7) in the national immunization program (NIP) in 2006, and switched to the 10-valent vaccine (PCV10, Synflorix) in 2011. Initially, immunization was advised in a primary series of 3 vaccines at the ages of 2, 3 and 4 months followed by a booster dose at the age of 11 months (3+1 schedule). In November 2013, the schedule was adapted by dropping the 3-month dose (2+1 schedule). This decision was based on non-inferiority data from a trial investigating the impact of timing and number of doses for the 13-valent vaccine (10). However, less is known for the 10-valent vaccine, which covers fewer serotypes, and differs in carrier proteins and immunogenicity (11,12), and in particular, the impact on mucosal IgG concentrations has not been compared between different immunization schedules. Therefore, we aimed to compare post-primary and post-booster serotype-specific anti-pneumococcal IgG concentrations in saliva following vaccination with PCV10 in a birth cohort of infants who received either the 3+1 (2-3-4-11 months) or the 2+1 (2-4-11 months) immunization schedule.

METHODS

Study population and sample collection

Saliva was available from 119 healthy, full-term infants born between December 2012 and June 2014, who participated in the Microbiome Utrecht

Infant Study (13). Follow-up until the age of 12 months was complete for 117 (98.3%) infants (Figure 1). According to the Dutch NIP, 51 infants born before September 2013 received PCV10 in a 3+1 schedule with vaccinations at 2, 3, 4 and 11 months of age, and 68 infants born from September 2013 received a 2+1 schedule with vaccinations at 2, 4 and 11 months of age. Mothers were not vaccinated with PCV10. Saliva was collected from infants during home visits at 1 month of age (baseline), after finishing the primary series at 6 months of age (post-primary), and after the booster dose at 12 months of age (post-booster). Saliva was collected by placing an absorbent sponge (S10 Oracol, Malvern Medical Developments Ltd., Worcester, UK) in the cheek pouch and under the tongue for 1 minute, so that the sponge became saturated with saliva, and was immediately transferred to a tube containing EDTA (BD Vacutainer, New Jersey, USA) plus protease inhibitor (Roche, Basel, Switzerland). Samples were transported on dry ice and stored at -80°C awaiting laboratory analyses. Ethical approval was granted by the Dutch national ethics committee (METC Noord-Holland, M012-015, NTR3986), and parental informed consent was obtained from all participants. The study was conducted in accordance with the European Statements for Good Clinical Practice.

Laboratory methods

Concentrations of serotype-specific anti-pneumococcal IgG in saliva were measured using a fluorescent bead-based multiplex immunoassay (MIA), which had been validated internally for use in saliva and was previously published (7, 14) In short, 10 sets of carboxylated microspheres (Luminex, Austin, TX) were coated with the pneumococcal polysaccharide antigens 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (ATCC, Manassas, VA). Antigens were linked to Poly-L-lysine, and the complex was bound to the microspheres in a reaction using EDC with sulpho-NHS. The in-house reference serum IVIG (Sanquin, Amsterdam, The Netherlands) with previously assigned concentrations of IgG, determined by calibration with international standard serum, was used in serial dilutions as standard reference (15). Saliva samples were thawed and centrifuged, and supernatants were diluted 1:2 and 1:10 using phosphate buffered saline (PBS; pH=7.2) with 5% antibody-depleted human serum (Valley Biomedical, Winchester, VA) and with 15 ug/ml multi cell wall polysaccharide (Statens Serum Insititut, Copenhagen, Denmark). From each dilution, 25 µl

was mixed with an equal volume of beads. R-phycoerythrin conjugated goat anti-human IgG solution diluted 1:200 (Jackson ImmunoResearch, West Grove, PA) was added to each well. Analysis of the beads was performed on a BioPlex 200 apparatus using the BioPlex software package version 6.2 (Bio-Rad Laboratories, Hercules, CA). Concentrations were determined by averaging results of both dilutions. Results from the two dilutions differed more than twofold (coefficient of variation (CV) >47%) in 23 samples with low IgG concentrations. The 1:10 dilution was considered to be more precise because of less interference from components of the saliva, and therefore, the result of the 1:10 dilution was used when in standard range for samples with a high CV. IgG concentrations were expressed in ng/ml. The lower limit of detection ranged from 0.08 ng/ml for serotype 4 to 0.37 ng/ml for serotype 14. Lower IgG concentrations were set at half the limit.

Statistical analysis

Serotype-specific IgG antibody concentrations in saliva are reported as geometric mean concentrations (GMCs) with 95% confidence intervals (CI). Fold changes were calculated as the ratio between GMCs. Baseline, post-primary and post-booster IgG concentrations were compared with Kruskal-Wallis tests followed by pairwise comparisons, and p-values were adjusted for 3 multiple comparisons using Bonferroni correction. Differences in post-primary and post-booster salivary serotype-specific IgG concentrations between immunization schedules were first assessed using Wilcoxon rank-sum tests. Multivariable linear regression was used to test independent associations of log2-transformed IgG concentrations with vaccination schedule, month 1 (baseline) log2-transformed IgG concentrations, sex, age in days at time of first vaccination, and time between vaccination and sampling as covariates. The antilog (2^x) of model coefficients with their 95% CIs are presented as geometric mean ratios (GMRs). GMRs indicate the relative increase in salivary IgG concentrations that is related to a 1-unit change in the model covariate. Time between vaccination and sampling was included in the model in a second degree polynomial to reflect the natural kinetics of IgG responses to vaccination. P-values below 0.050 were considered statistically significant, and p-values above 0.050 but below 0.100 were considered relevant trends towards significance. Analyses were performed in R version 4.0.3.

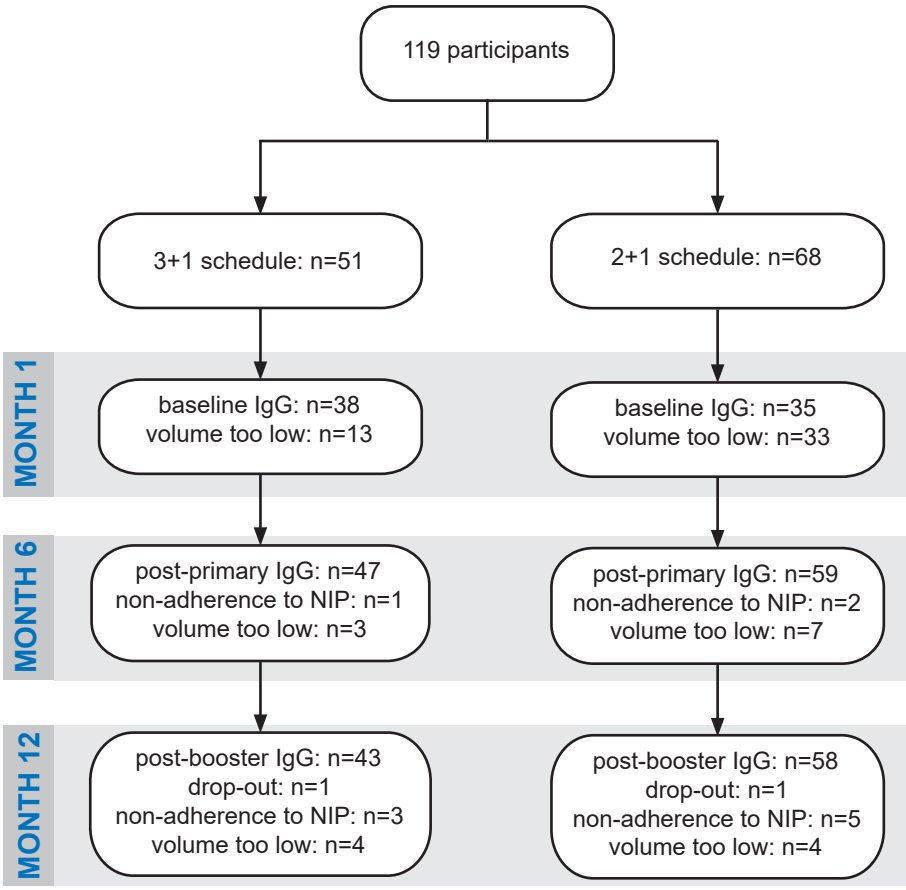

Figure 1 | Flowchart showing number of infants and samples per PCV10 immunization schedule. Numbers of infants in the study and of immunoglobulin G (IgG) measurements at each sampling moment, broken down according to PCV10 immunization schedule (2+1 or 3+1 schedule). Reasons for missing measurements were a too low sample volume for laboratory analysis, the infant not having received the vaccine before the sampling moment (non-adherence to the national immunization program [NIP]) or the infant dropping out of the study.

RESULTS

Salivary serotype-specific anti-pneumococcal IgG concentrations were measured in 73 baseline saliva samples, 106 post-primary samples (obtained on average 62 days after the last priming dose, range 22-89 days) and 101 post-booster samples (obtained on average 27 days after the booster dose, range 5-64 days) (Figure 1). IgG concentrations increased

over time for all vaccine serotypes (Figure 2A). After the primary series, salivary serotype-specific IgG GMCs varied between 1.00 ng/ml (95% CI, 0.75-1.34) for serotype 23F and 4.56 ng/ml (95% CI, 3.67-5.65) for serotype 7F, representing a 1.2 to 5.5-fold change compared with pre-immunization concentrations; this increase was significant for serotypes 1, 4, 5, 6B, 7F, 9V and 18C (all Bonferroni-adjusted p-value<0.050) but not for serotypes 14, 19F, and 23F. After the booster dose, we observed a stronger, significant 3.2 to 9.4-fold increase compared with post-primary salivary IgG GMCs for all vaccine serotypes (all Bonferroni-adjusted p-value <<0.001), resulting in post-booster GMCs between 7.33 ng/ml (95% CI 5.75-9.33) for serotype 23F and 27.30 ng/ml (95% CI, 22.14-33.67) for serotype 19F.

Because the PCV10 immunization schedule changed in 2013, 51 (42.5%) infants who were immunized according to the 'old' 3+1 schedule with priming doses at 2, 3 and 4 months, were compared with 68 (56.7%) infants who received the 'new' 2+1 schedule, dropping the 3-month dose. Regarding post-primary IgG concentrations, there were no differences between the schedules, although we observed a trend towards higher IgG concentrations against serotype 4 in children who received the 2+1 schedule compared with the 3+1 schedule (p=0.064) (Figure 2B). By contrast, post-booster IgG concentrations were significantly lower following the 2+1 in comparison with the 3+1 schedule for serotypes 4 (p=0.035), 7F (p=0.048) and 23F (p=0.0056), with a borderline significant difference for serotype 9V (p=0.058) (Figure 2C).

To investigate independent effects of immunization schedule and other factors known to influence PCV immunogenicity, i.e. age at first immunization, baseline anti-pneumococcal IgG concentrations, and male compared to female gender, on the IgG response to PCV10, we performed a multivariable analysis (Figure 3). These host factors were comparable between subjects receiving different schedules (Table 1). We found no significant associations between immunization schedule and post-primary or post-booster salivary anti-pneumococcal IgG concentrations in multivariable analysis, though for serotype 23F a trend towards lower post-booster IgG concentrations was observed for the 2+1 group (p=0.079). Older age at time of first immunization was associated with higher post-primary IgG concentrations against serotypes 1, 4, 18C and 19F (GMR 1.05-1.08, p<0.030),

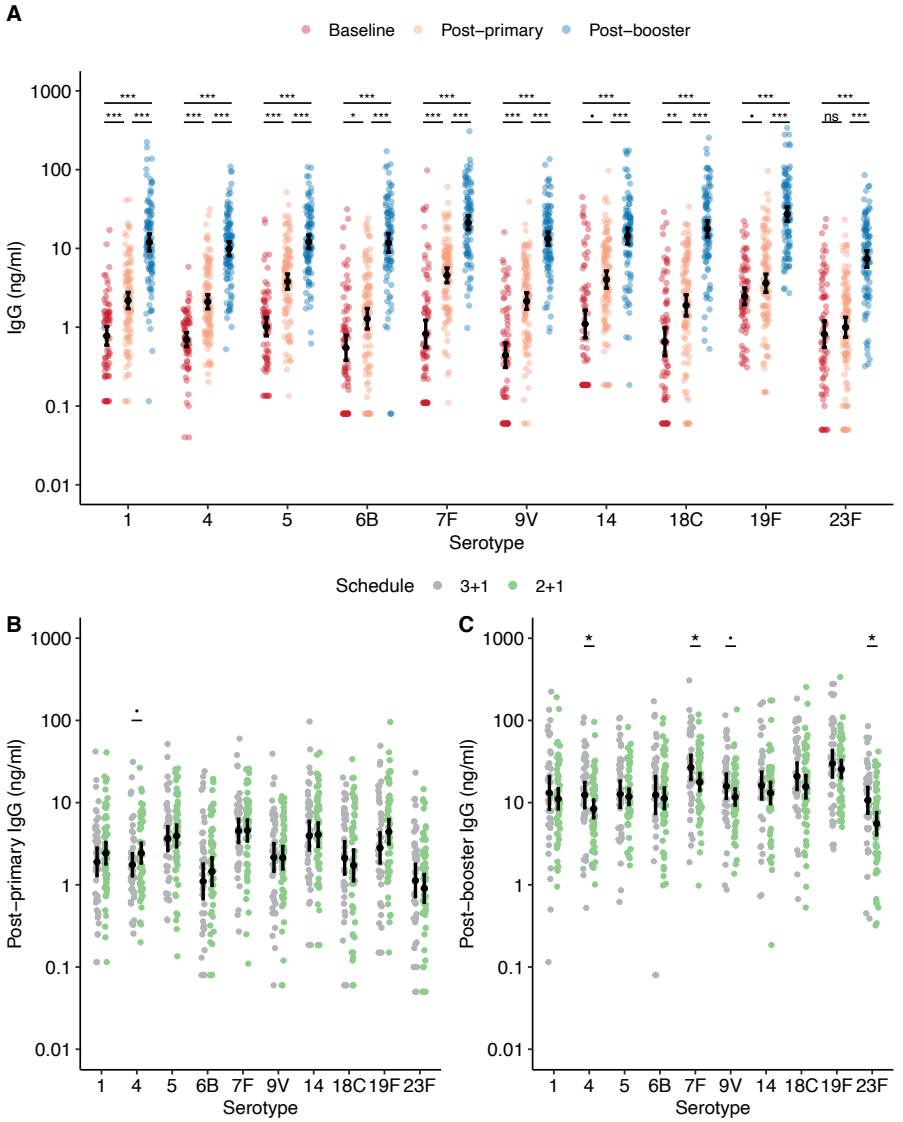

Figure 2 | Serotype-specific anti-pneumococcal IgG responses following the priming doses and the booster dose of PCV10. Serotype-specific anti-pneumococcal IgG concentrations were compared (A.) before PCV10 vaccination, after the primary series and after the booster dose, and between the 3+1 (at 2-3-4-11 months) and the 2+1 (at 2-4-11 months) PCV10 immunization schedule (B.) after the primary series, or (C.) after the booster dose. Black dots and error bars represent geometric mean concentrations with 95% confidence intervals. Significance was assessed by pairwise Wilcoxon rank-sum tests, and was indicated by ***: p<0.001; **: p<0.005; *: p<0.05; •: p<0.10; ns: not significant.

but not with post-booster salivary IgG concentrations. Finally, we observed negative correlations between baseline and post-primary IgG concentrations for serotypes 5, 6B, 7F, 14, 18C and 23F (GMR 0.70-0.86, all p<0.005), and between baseline and post-booster IgG concentrations for serotype 14 (GMR 0.84, 95% CI 0.73-0.96, p=0.014). Male compared to female gender was not associated with IgG responses to PCV10.

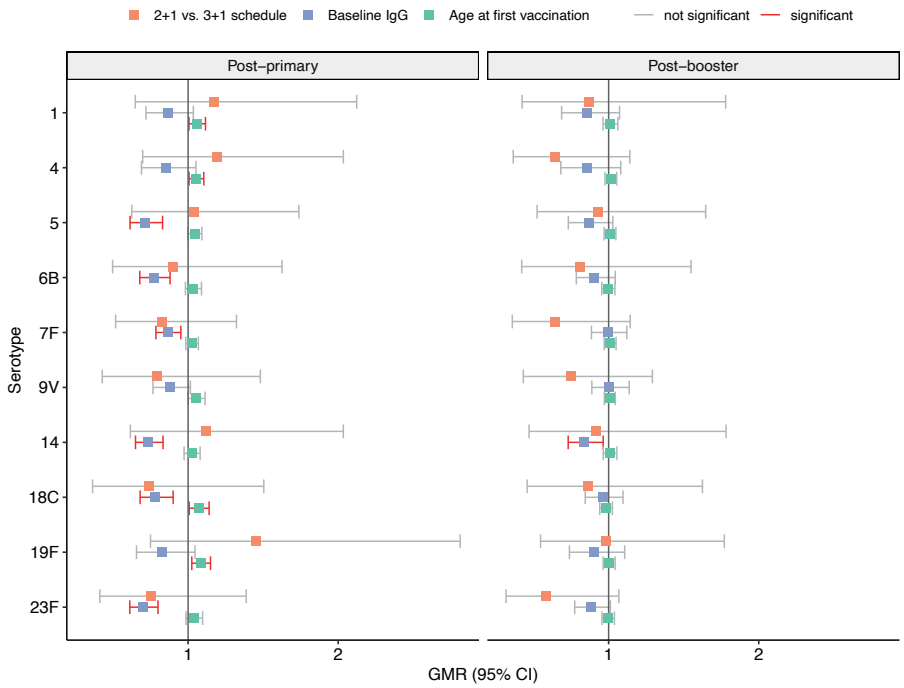

Figure 3 | Independent associations between PCV10 immunization schedule, baseline antibody concentrations, age at first vaccination, and sex and vaccine responses. Significant findings (p<0.050) are shown in red. For PCV10 schedule, geometric mean ratios (GMRs) greater than 1.0 indicate that children who received the new 2+1 (2-4-11 months) schedule, have higher IgG concentrations than children who received the old 3+1 (2-3-4-11 months) schedule after the primary series or the booster dose. For baseline IgG, the GMR indicates the relative increase in IgG concentrations associated with a doubling of baseline IgG concentrations. For age at first PCV10 vaccination, the GMR indicates the relative increase in IgG concentrations associated with a 1 day increase. Male vs. female sex was not significantly associated with serotype-specific IgG concentrations and was therefore not included in the figure. The analyses were also corrected for time between vaccination and saliva collection using a second degree polynomial.

DISCUSSION

To our knowledge, this is the first study that compared serotype-specific anti-pneumococcal IgG in saliva following pneumococcal conjugate vaccination with PCV10 between different immunization schedules. Comparable IgG concentrations were observed 2 months after completion of a 2-dose (2-4 months) and a 3-dose (2-3-4 months) primary series of PCV10. However, IgG concentrations against serotypes 4, 7F and 23F were modestly but significantly lower following the booster dose in children who received PCV10 in a 2+1 schedule. Studies comparing serum IgG responses between the 3+1 and 2+1 PCV10 schedule have not been published, and it remains unclear whether our findings in saliva align with results in serum. However, a large trial comparing schedules with PCV13 showed that post-booster serum responses following the 2+1 schedule were non-inferior to the 3+1 schedule (10).

Table 1 | Gender, age at first immunization, and baseline anti-pneumococcal IgG in infants receiving the 3+1 and the 2+1 PCV10 schedule.

	3+1 schedule	**2+1 schedule**	**p-value**
	51	**68**	
Basic characteristics			
Male (%)	23 (45.1)	33 (48.5)	0.85
Age at first immunization, months (median [IQR])	1.9 [1.8, 2.1]	1.9 [1.8, 2.0]	0.74
Baseline anti-pneumococcal IgG, GMC (95% CI) (ng/ml)			
Ps1	0.75 (0.54-1.06)	0.80 (0.51-1.26)	0.80
Ps4	0.79 (0.60-1.04)	0.61 (0.45-0.83)	0.28
Ps5	0.94 (0.63-1.39)	1.11 (0.76-1.62)	0.52
Ps6B	0.45 (0.26-0.76)	0.69 (0.40-1.17)	0.19
Ps7F	1.08 (0.58-2.01)	0.61 (0.37-1.01)	0.25
Ps9V	0.52 (0.29-0.92)	0.37 (0.24-0.57)	0.62
Ps14	1.05 (0.60-1.84)	1.15 (0.62-2.16)	0.84
Ps18C	0.69 (0.40-1.18)	0.62 (0.32-1.18)	0.80
Ps19F	2.16 (1.50-3.10)	2.86 (2.04-4.00)	0.32
Ps23F	0.82 (0.48-1.40)	0.81 (0.44-1.47)	0.93

Significance was assessed using Wilcoxon rank-sum tests. Data are summarized as n (%); median with interquartile range (IQR); or geometric mean concentrations (GMC) with 95% confidence intervals (CI). Abbreviations: Ps=pneumococcal serotype.

Previous studies have demonstrated strong correlations between saliva and serum anti-pneumococcal IgG (7,16), suggesting that salivary antibodies can be a useful indicator of serum antibody status. Furthermore, IgG at mucosal surfaces may contribute to protection against pneumococcal infection by inhibiting nasopharyngeal colonization. Findings from a human challenge model showed that mucosal IgG effectively prevented acquisition of *S. pneumoniae* through bacterial agglutination (9). Future studies should strive to establish correlates of protection against pneumococcal colonization for mucosal antibodies.

Furthermore, anti-pneumococcal IgG patterns in saliva were comparable to those observed in studies of systemic IgG responses in serum following vaccination with PCV-10, which suggests that saliva may be a good, non-invasive specimen next to serum to monitor IgG responses to vaccination. In line with earlier observations, we confirmed that older age at time of the first PCV10 dose was associated with higher post-primary IgG concentrations (10,17). Likewise, we confirmed that pre-vaccination IgG concentrations at 1 month of age were negatively related to post-primary IgG concentrations. Similar associations were previously reported for serum antibody concentrations, and likely reflect that maternally derived IgG, which predominates in early life, inhibits the IgG response to pneumococcal vaccination (17).

Strengths of our study include the extensive participant data including vaccination dates. The sensitive MIA technology also allowed us to accurately measure IgG concentrations, even in very low volumes of saliva. A limitation of our study is the lack of serum; therefore, salivary IgG could not be correlated with serum levels. Furthermore, the relatively low number of participants limited our power to detect significant differences and may explain why vaccine schedule was not associated with the anti-pneumococcal IgG response in multivariable analysis. We did not correct IgG measurements for the dilution factor in saliva, but earlier work has shown that this has only little effect (7). Moreover, our observational design does not allow to fully preclude the influence of potential confounding factors, including potential differences in circulation of pneumococcal vaccine serotypes in

the population and thereby boosting of mucosal immunity during the study period (7,18). However, since 2011, temporal changes in vaccine serotype prevalence in the paediatric population in The Netherlands have been rare, so we anticipate this to be of limited effect (19).

In conclusion, modest decreases in serotype-specific anti-pneumococcal IgG concentrations in saliva following the booster PCV10 dose were observed after leaving out the middle dose of the primary series, resulting in a 2 (2-4) month interval between the first and second primary immunizations. Future research should investigate whether salivary IgG is a good correlate of protection from nasopharyngeal pneumococcal colonization after immunization. We propose that measuring IgG against vaccine serotypes in saliva next to serum may represent a valid, non-invasive method to quantify mucosal immune responses in studies investigating or monitoring changes to pneumococcal vaccines and schedules.

ACKNOWLEDGEMENTS

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CHAPTER 7

Microbial and clinical factors are related to recurrence of symptoms after childhood lower respiratory tract infection

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ABSTRACT

Childhood lower respiratory tract infections (LRTI) are associated with dysbiosis of the nasopharyngeal microbiota, and persistent dysbiosis following the LRTI may in turn be related to recurrent or chronic respiratory problems. Therefore, we aimed to investigate microbial and clinical predictors of early recurrence of respiratory symptoms as well as recovery of the microbial community following hospital admission for LRTI in children. To this end, we collected clinical data and characterized the nasopharyngeal microbiota of 154 children (4 weeks-5 years old) hospitalized for a LRTI (bronchiolitis, pneumonia, wheezing illness, or mixed infection) at admission and 4-8 weeks later. Data were compared to 307 age-, gender- and time-matched healthy controls. During follow-up, 66% of cases experienced recurrence of (mild) respiratory symptoms. In cases with recurrence of symptoms during follow-up, we found distinct nasopharyngeal microbiota at hospital admission, with higher levels of *Haemophilus influenzae/haemolyticus*, *Prevotella oris* and other gram-negatives and lower levels of *Corynebacterium pseudodiphtheriticum/propinquum* and *Dolosigranulum pigrum* compared to healthy controls. Furthermore, in cases with recurrence of respiratory symptoms, recovery of the microbiota was also diminished. Especially in cases with wheezing illness we observed a high rate of recurrence of respiratory symptoms, as well as diminished microbiota recovery at follow-up. Together, our results suggest a link between the nasopharyngeal microbiota composition during LRTI and early recurrence of respiratory symptoms, as well as diminished microbiota recovery after 4-8 weeks. Future studies should investigate whether (speed of) ecological recovery following childhood LRTI is associated with long-term respiratory problems.

INTRODUCTION

Lower respiratory tract infections (LRTIs) remain a leading cause of illness in early childhood, and are a risk factor for the development of recurrent and even chronic respiratory problems (1–3). For instance, approximately half of infants with bronchiolitis will subsequently experience recurrent wheezing episodes (4), which may persist for years and might eventually develop into asthma (5). Also, health-related quality of life may remain substantially decreased for months or even years following a LRTI in both children (6) and adults (7). Causes of respiratory problems following a LRTI remain largely unknown, but emerging evidence suggests that the upper respiratory tract (URT) microbiota may play a role (8, 9).

As expected, during a LRTI, the microbial communities of the respiratory tract differ strongly from those of healthy, matched controls, with increased presence of potential pathogens ('pathobionts') like *Streptococcus pneumoniae* and *Haemophilus influenzae*, and decreased presence of presumed beneficial bacteria like *Corynebacterium* and *Dolosigranulum* (10–13). However, microbiota 'recovery' following a LRTI is not extensively studied. One case-control study has shown that the abundance of pathobiont *Haemophilus* decreased to normal levels within a month (11). Conversely, a longitudinal study demonstrated persistent enrichment with *Moraxella* up to 6 months after a LRTI (14). Furthermore, in a prospective cohort of infants with bronchiolitis, increased nasal levels of *Moraxella* and *Streptococcus* in the weeks directly following hospitalization were associated with recurrent wheeze at the age of 3 years, suggesting that persistent microbial 'dysbiosis' may contribute to long-term respiratory outcomes following a LRTI (8).

Using a matched case-control design, we have previously demonstrated significant aberrations of the nasopharyngeal microbial community at time of hospital admission for childhood LRTI, when compared to asymptomatic age-, gender- and time-matched controls (13). Currently, we extend our analysis to 4-8 weeks follow-up, and demonstrate that early recurrence of respiratory symptoms is related to the nasopharyngeal microbial community composition at times of LRTI as well as to impaired microbiota recovery following LRTI.

METHODS

We refer to our previous publication on this cohort (13) for details on study design and microbiota analysis, and to the online data supplement for details on statistical analysis. Data are available from the NCBI Sequence Read Archive database (BioProject ID PRJNA428382).

Study design

We enrolled 154 cases aged 4 weeks to 5 years old, hospitalized for a LRTI, and 307 age-, time- and gender-matched, healthy controls from the community (1:2 ratio except for 1 case). Age, season and gender are known to influence microbiota composition (15–17), and were therefore used as matching criteria to avoid confounding. Nasopharyngeal swabs were obtained from cases at hospital admission and 4-8 weeks after discharge during a follow-up visit to the outpatient clinic or at home. Nasopharyngeal swabs from controls were obtained once during a home visit within 2 weeks from case admission (Figure S1). Extensive information on medical history, lifestyle and environment was collected from all participants using questionnaires. From cases, we also collected clinical data during admission and at follow-up. Two expert paediatricians independently grouped cases into 3 major LRTI phenotypes (pneumonia, bronchiolitis, and wheezing illness) based on medical records. Cases with an unclear or overlapping phenotype were classified as mixed infection. Recurrence of respiratory symptoms was defined as a parent-reported new episode of one or more respiratory symptoms (including rhinorrhoea, wheezing, earache, sore throat, coughing, hoarseness, and 'other' respiratory symptoms) between hospital discharge and the follow-up visit 4-8 weeks later. The study was approved by the Dutch National Ethics Committee (NL42019.094.12). Written informed parental consent was obtained from all participants.

Microbiota analysis

Bacterial DNA was isolated and quantified as previously described (17–19), with the following minor modifications: 16S Real Time PCR was conducted using TAMRA probe 16S-P1 (FAM-ATT AGA TAC CCT GGT AGT CCA-TAMRA) (Life Technologies, Carlsbad, CA) and a PCR mixture containing 12.5 µl 2x

Taqman universal master mix (Life Technologies, Carlsbad, CA), 1 µl of each primer (10 µM), 1 µl of the probe (5 µM), 6.5 µl HPLC graded water (Instruchemie, Delfzijl, Netherlands) and 3 µl of template DNA, on the StepOnePlus System (Applied Biosystems, Foster City, CA). Almost all samples (>99%) contained sufficient DNA for reliable analysis (Figure S1). Following, amplicon libraries of the 16S-rRNA gene (V4 region) were generated, and sequencing was executed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA). Bioinformatic processing was performed as previously described (20, 21). Contaminating sequences were identified using the *decontam* R-package, using their relation to bacterial biomass (frequency method) and their presence/absence in samples versus DNA isolation controls and PCR blanks (prevalence method) (22). In total, 21 OTUs were identified as contaminants, and were removed prior to downstream analyses (Table S1). Each operational taxonomic unit (OTU) was assigned taxonomy and a rank number based on its abundance. Double annotations were assigned to OTUs that could be annotated to both species. Presence of *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* and *Moraxella catarrhalis* was confirmed by quantitative PCR. Viral presence was detected by qualitative multiplex Real Time PCR (RespiFinder SMARTfast 22, Maastricht, Netherlands).

Statistical analysis

Data analysis was performed in R version 3.4.3. Case-control comparisons accounted for matching. P-values or Benjamini-Hochberg adjusted q-values below 0.050 were considered statistically significant. Chi-square and Wilcoxon tests were used to compare host characteristics between cases with and without recurrence of respiratory symptoms during follow-up. Independent relationships between antibiotic treatment, LRTI phenotype, age, and recurrence of respiratory symptoms during follow-up were assessed using multivariable logistic regression, including pairwise interactions and correcting for follow-up time. Conditional logistic regression was used to compare viral presence between cases and controls. Alpha diversity was assessed by the Chao1 index for microbial richness and the Shannon index for diversity (*phyloseq* (23)), and significance of differences between cases and controls was evaluated using linear mixed-effect models. Microbiota

recovery was considered complete when the overall microbial composition was comparable between cases after 4-8 weeks follow-up and matched controls, which was evaluated by permutational analysis of variance (PERMANOVA) on the Bray-Curtis dissimilarity matrix (*vegan* (24)). We similarly analysed differences in microbial diversity at time of admission between cases with and without subsequent recurrence of respiratory symptoms, adjusting for age, gender and month of hospital admission. Discriminant OTUs between cases and controls were identified by combining significant results from *metagenomeSeq* analysis (25) and cross-validated *VSURF* analysis (26), which were then filtered at a fold change (FC) of above 1.5 or below 0.5 (i.e. a 50% change). Stratified analyses were performed of microbiota recovery in relation to recurrence of respiratory symptoms during follow-up, LRTI phenotype, antibiotic treatment, and viral presence. In these stratified analyses, differential abundance testing was limited to the top 100 highest-ranked OTUs, because false positive results in low abundant OTUs are a known risk of *metagenomeSeq* analysis with smaller group sizes (27).

RESULTS

Clinical and microbial factors during LRTI were associated with early recurrence of respiratory symptoms

Cohort characteristics were detailed previously (13). Follow-up data were available for 149 (97%) cases, with a median follow-up time of 39 days (IQR 35-46) (Figure S1). In the 4-8 weeks following hospital discharge, 98 (66%) cases experienced recurrence of respiratory symptoms (from here on called recurrence of symptoms) (Table S2). Of these cases, 57 (58%) specified at least 2 different respiratory symptoms, and 47 (48%) also reported fever (>38°C). Furthermore, 41 cases (42%) consulted a physician, and 8 cases (8%) received antibiotics for these symptoms. Follow-up time was significantly longer in cases with recurrence of respiratory symptoms (p=0.015). Cases with and without recurrence of symptoms during follow-up were not significantly different in terms of baseline characteristics including age, lifestyle and environmental factors, medical history, and clinical findings such as LRTI phenotype and antibiotic treatment (Table 1). However, when age, LRTI phenotype and antibiotic treatment were included in a multivariable model

as predictors of recurrence, we found a borderline significant, independent association between a diagnosis of wheezing illness and an increased rate of subsequent recurrence of respiratory symptoms (β=1.19 compared to pneumonia, 95% CI -0.097-2.54, p=0.074). Furthermore, an independent positive association was found between antibiotic treatment during admission and subsequent recurrence of respiratory symptoms (β=2.47, 95% CI 0.52-4.86, p=0.023), but this effect diminished with increasing age (interaction age and antibiotics: β=-0.078, 95% CI -0.15- -0.013, p=0.028) (Table S3). Viral presence, number of viruses and detection of respiratory syncytial virus (RSV) or Human rhinovirus (HRV) was not significantly different between cases with and without recurrence (Table 1).

Table 1 | Characteristics of cases with and without recurrence of respiratory symptoms during 4-8 weeks follow-up.

	Recurrence	No recurrence	P-value
n	**98**	**51**	
Basics			
Age (months)	12.7 (5.5, 21.6)	16.1 (3.5, 32.1)	0.481
Girl	39 (39.8)	21 (41.2)	1.000
Season of sampling			0.679
Spring	13 (13.3)	7 (13.7)	
Summer	20 (20.4)	14 (27.5)	
Autumn	10 (10.2)	3 (5.9)	
Winter	55 (56.1)	27 (52.9)	
Born at term	90 (91.8)	47 (92.2)	1.000
Mode of delivery			0.190
vaginal	76 (77.6)	43 (84.3)	
elective caesarean section	9 (9.2)	6 (11.8)	
emergency caesarean section	13 (13.3)	2 (3.9)	
Lifestyle and Environmental Factors			
Breastfeeding >3 months	38 (38.8)	18 (35.3)	0.812
Day care attendance	65 (66.3)	28 (54.9)	0.235
Tobacco smoke exposure	19 (19.4)	14 (27.5)	0.359
Number of siblings	1.0 (0.2, 2.0)	1.0 (0.0, 2.0)	0.654
Medical History			
Previous LRTI*	29 (29.6)	11 (21.6)	0.393
Previous hospitalization for RTI†	29 (29.6)	8 (15.7)	0.096
Prior wheezing	25 (25.5)	11 (21.6)	0.740

Table 1 | Continued

	Recurrence	No recurrence	P-value
n	**98**	**51**	
Clinical Data			
Main discharge diagnosis			0.408
bronchiolitis	37 (37.8)	19 (37.3)	
indeterminate	18 (18.4)	8 (15.7)	
pneumonia	18 (18.4)	15 (29.4)	
wheezing	25 (25.5)	9 (17.6)	
Antibiotic treatment during admission	25 (25.5)	14 (27.5)	0.953
Prednison during admission	16 (16.3)	9 (17.6)	1.000
Follow-up time (days after admission)	42.0 (36.0, 49.0)	39.0 (34.5, 44.0)	**0.015**
Viral detection at admission			
Any virus (%)	94 (98.9)	47 (94.0)	0.232
Respiratory syncytial virus (%)	42 (44.2)	28 (56.0)	0.240
Human rhinovirus (%)	51 (53.7)	21 (42.0)	0.245
Number of viruses	1.0 (1.0, 2.0)	1.0 (1.0, 2.0)	0.299

Data are n (%) or median (IQR). Data were acquired from parent questionnaires and medical records. Viral presence was detected by multiplex PCR in nasopharyngeal samples obtained at admission. P-values were calculated by chi-square tests or Wilcoxon rank-sum tests. ***** lower respiratory tract infection; † respiratory tract infection.

In this cohort, significant differences in the microbial community composition between cases at admission and controls were previously shown, with especially increased abundance of pathobionts *Haemophilus influenzae/haemolyticus* and *S. pneumoniae,* and decreased abundance of presumed beneficial bacteria like *Moraxella catarrhalis/nonliquefaciens, Dolosigranulum pigrum* and *Corynebacterium pseudodiphtheriticum/propinquum* (13). Here, a modestly lower Shannon diversity at time of admission was related to a higher rate of recurrence of symptoms, even after adjusting for age, gender and month of hospital admission (p=0.049; Figure 1A). Furthermore, the overall microbial community composition at time of admission was significantly different between cases with versus cases without subsequent recurrence of symptoms, independent of age, gender and month of hospital admission (R^2: 0.020, p=0.012) (Figure 1B), though not correlated with the severity of recurrence of symptoms (i.e. number of symptoms (1 or >1), presence of

fever, or the parents' decision to consult a physician during follow-up (data not shown)). Cases with recurrence of symptoms had higher abundances of gram-negatives like *H. influenzae/haemolyticus, Prevotella oris, Actinomyces* and *Fusobacterium* species and lower abundances of health-associated *C. pseudodiphtheriticum/propinquum* and *D. pigrum* at time of admission compared to controls. On the other hand, cases without recurrence had higher abundances of amongst others gram-positives *Staphylococcus aureus/epidermidis* and *S. pneumoniae* at admission compared to controls (Table 2 and Figure S2).

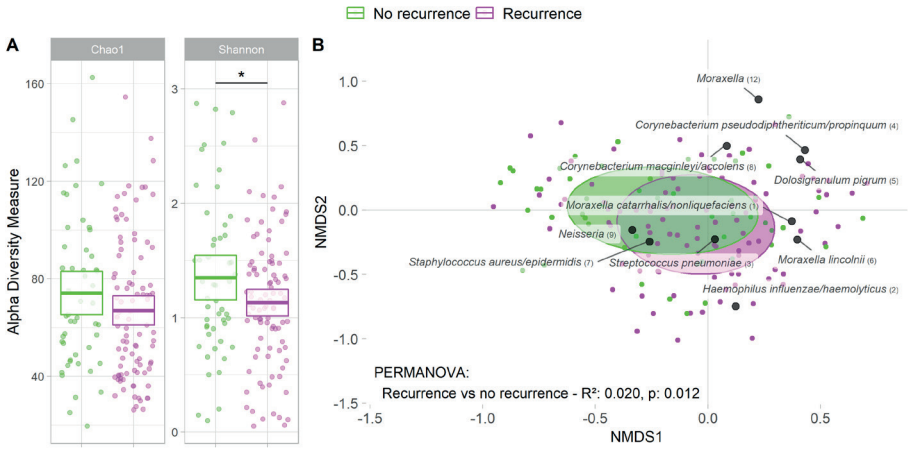

Figure 1 | Nasopharyngeal microbiota during LRTI was associated with early recurrence of respiratory symptoms.
(A) Alpha diversity measures Chao1 index and Shannon diversity index estimated at time of admission for cases with compared to without recurrence of respiratory symptoms during follow-up. Boxes denote means with 95% confidence intervals. Significance was tested by linear models adjusting for age, gender and month of admission, and is indicated by *: p<0.05. (B) Nonmetric multidimensional scaling biplot based on Bray-Curtis dissimilarity depicts nasopharyngeal microbiota composition at time of admission for cases with compared to without recurrence of respiratory symptoms during follow-up, combined with the top 10 operational taxonomic units (OTUs) with highest relative abundance in the entire cohort. Ellipses represent the standard deviation of all points within a sub-cohort. Significance was tested using permutational analysis of variance (PERMANOVA), adjusting for age, gender and month of hospital admission.

Table 2 | Biomarker species during acute LRTI for subsequent recurrence of respiratory symptoms.

Differentially abundant at admission	OTU*	FC†	Significant in
In cases with subsequent recurrence of respiratory symptoms vs. controls	*Haemophilus influenzae/haemolyticus (2)*	5.97	ms‡ + V§
	Fusobacterium (83)	2.57	ms
	Prevotella oris (45)	2.31	ms
	Actinomyces graevenitzii (68)	1.88	ms + V
	Fusobacterium (74)	1.76	ms
	Actinomyces johnsonii (75)	1.76	ms + V
	Actinomyces odontolyticus (48)	1.66	ms + V
	Dolosigranulum pigrum (5)	0.41	ms
	Corynebacterium pseudodiphtheriticum/ propinquum (4)	0.41	ms
In cases without subsequent recurrence of respiratory symptoms vs. controls	*Neisseria lactamica (19)*	2.97	ms + V
	Staphylococcus aureus/epidermidis (7)	2.38	ms + V
	Streptococcus pneumoniae (3)	2.31	V
	Atopobium (100)	1.99	ms
	Klebsiella (11)	1.75	ms + V
	Halomonas (14)	1.57	ms
	Prevotella melaninogenica (16)	1.54	V
	Prevotella nanceiensis (25)	0.49	ms
	Neisseria (9)	0.43	ms + V

* operational taxonomic unit; † fold change; ‡ metagenomeSeq analysis; § VSURF analysis.

Nasopharyngeal microbiota and viral profiles after recovery from LRTI

We also aimed to study remaining differences in microbial community diversity and composition between recovered cases and controls. In general, samples obtained 4-8 weeks after hospital discharge showed significantly higher microbial richness in (former) cases when compared to controls (p=0.042), while Shannon diversity and biomass were comparable (Figure S3). Furthermore, despite the observed major differences in the overall microbial community composition at time of infection, after 4-8 weeks the microbiota of recovered cases had become more similar to controls, and only a small, non-significant difference remained (R^2: 0.004, p=0.080; Figure 2A-B).

On the OTU level, we observed 15 OTUs that were significantly differentially abundant between recovered cases and controls (Table 3 and Figure S4). Of these, *Moraxella* species and *Helcococcus* were already underrepresented at admission, and remained underrepresented in recovered cases, whereas various gram-negative species including *H. influenzae/haemolyticus, P. oris,* and *Neisseria lactamica* remained overrepresented in recovered cases compared to controls.

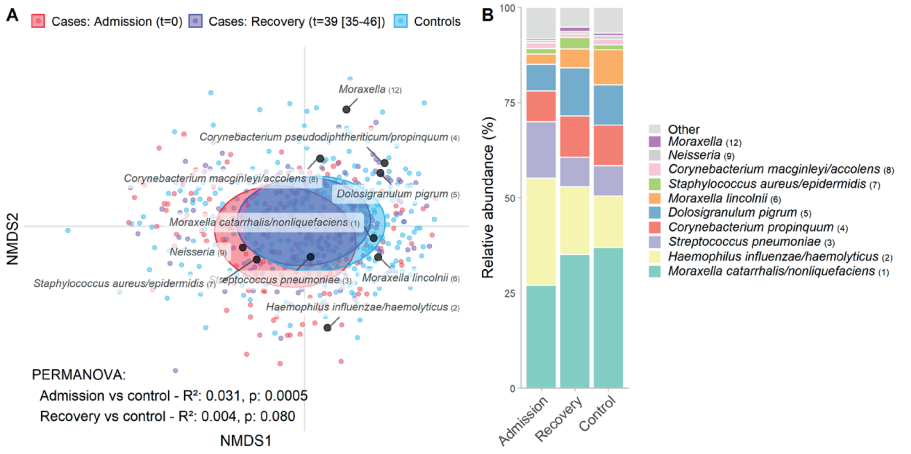

Figure 2 | Nasopharyngeal microbiota recovery following LRTI. (A) Nonmetric multidimensional scaling biplot based on Bray-Curtis dissimilarity depicts nasopharyngeal microbiota composition for cases at admission, cases at follow-up (recovery), and controls, combined with the top 10 operational taxonomic units (OTUs) with highest relative abundance in the entire cohort. Time (t) in days between admission and the follow-up visit was reported as median (IQR). Ellipses represent the standard deviation of all points within a sub-cohort. Significance was tested using permutational analysis of variance (PERMANOVA). (B) Mean relative abundances of the 10 OTUs with highest relative abundance.

Assessment of microbiota recovery in relation to recurrence of respiratory symptoms, showed that when compared to matched controls, cases with recurrence of symptoms during follow-up had a significantly higher microbial richness at the end of follow-up (p=0.034; Figure S5A). This difference in microbial richness was not present between cases without recurrence of symptoms and their matched controls. Also, on microbial community composition level, the microbiota composition had failed to normalize in cases with recurrence of respiratory symptoms during follow-

up (R^2: 0.008, p=0.028), while in cases without recurrence the microbiota were comparable to controls (R^2: 0.005, p=0.50). Especially the abundances of *H. influenzae/haemolyticus, Neisseria, P. oris,* and *Porphyromonas* were persistently increased after recovery in cases with recurrence of symptoms during follow-up compared to controls, while in cases without recurrence, abundance of *S. aureus/epidermidis* and *N. lactamica* were increased after recovery compared to controls (Figure S5B).

Table 3 | Discriminant OTUs for cases during acute LRTI and after 4-8 weeks follow-up compared to controls.

		Cases at admission vs. controls		Cases at recovery vs. controls	
Association	**OTU***	**FC†**	**Significant in**	**FC**	**Significant in**
Admission	*Lactococcus lactis (67)*	2.06	ms‡ + V§	*ns* ‖	*ns*
	Corynebacteriaceae (161)	1.83	ms + V	*ns*	*ns*
	Abiotrophia (111)	1.66	ms + V	*ns*	*ns*
	Corynebacterium (62)	1.64	ms	*ns*	*ns*
	Megasphaera (137)	1.62	ms	*ns*	*ns*
	Fusobacterium (74)	1.61	ms	*ns*	*ns*
	Actinomyces (48)	1.56	ms	*ns*	*ns*
	Fusobacterium (83)	1.53	ms	*ns*	*ns*
	Actinomyces graevenitzii (68)	1.52	ms + V	*ns*	*ns*
	Acinetobacter soli (125)	1.51	ms	*ns*	*ns*
	Actinomyces johnsonii (75)	1.51	ms	*ns*	*ns*
	Dolosigranulum pigrum (5)	0.43	ms	*ns*	*ns*
	Dolosigranulum (122)	0.4	ms + V	*ns*	*ns*
Admission and Recovery	*Haemophilus influenzae/ haemolyticus (2)*	3.85	ms + V	1.69	V
	Neisseria lactamica (19)	2.22	ms + V	1.96	ms + V
	Prevotella oris (45)	2.03	ms	1.72	ms + V
	Moraxella (54)	0.32	ms + V	0.45	ms + V
	Moraxella (58)	0.19	ms + V	0.45	ms + V
	Moraxella lincolnii (6)	0.18	ms + V	0.3	ms + V
	Moraxella (84)	0.17	ms + V	0.34	ms
	Moraxella (163)	0.15	ms + V	0.39	ms + V
	Moraxella (112)	0.08	ms	1.68	ms
	Helcococcus (43)	0.07	ms	0.07	ms + V

Table 3 | Continued

		Cases at admission vs. controls		Cases at recovery vs. controls	
Association	**OTU***	**FC†**	**Significant in**	**FC**	**Significant in**
Recovery	*Neisseriaceae (15)*	*ns*	*ns*	2.26	ms + V
	Moraxella (12)	*ns*	*ns*	1.83	ms
	Porphyromonas (39)	*ns*	*ns*	1.62	ms
	Bradyrhizobium (17)	*ns*	*ns*	1.54	ms + V
	Janthinobacterium lividum (23)	*ns*	*ns*	0.09	ms + V

* operational taxonomic unit; † fold change; ‡ metagenomeSeq analysis; § VSURF analysis; ll not significantly different.

Viral presence at time of admission and after recovery was available for 70 cases and for 139 corresponding controls. As described previously (13), 97% of cases tested positive for any virus at time of admission versus 85% of controls (p=0.019). At follow-up, 91% of (former) cases were virus-positive, which was not significantly different from controls. RSV detection was higher in cases at time of admission compared to controls (40% vs. 4%; $p<0.001$), but had normalized after recovery (3%). Interestingly, in recovered cases, HRV detection was more common (80%) than at time of admission (57%, p=0.006), though comparable to controls (70%; Figure S6). Detection of other respiratory viruses was low and not significantly different between cases and controls following recovery.

Microbiota recovery depends on LRTI phenotype, but not on antibiotic treatment or type of virus

Next, we used stratified analyses to investigate whether microbiota recovery was related to antibiotic treatment, LRTI phenotype or viral presence at time of admission. In total, 43 (28%) cases were treated with antibiotics (33 beta-lactam, 10 macrolide) during admission. Interestingly, only cases not treated with antibiotics showed significantly higher microbial richness after follow-up compared to controls (p=0.001), while no differences in Shannon diversity were observed in either group compared to their respective controls (Figure S7A). Microbiota composition at follow-up also showed little difference between both antibiotic-treated and not antibiotic-treated cases and their

respective controls (treated cases versus controls R^2: 0.007, p=0.47; non-treated cases versus controls R^2: 0.005, p=0.17).

Regarding LRTI phenotype, 57 (37%) cases were classified as bronchiolitis, 37 (24%) as pneumonia, 34 (22%) as wheezing illness, and 26 (17%) as mixed infection. Only (former) bronchiolitis cases had significantly increased microbial richness after follow-up compared to matched controls (p=0.013), though a similar trend was observed for former wheezing illness cases (p=0.088; Figure S7B). Furthermore, only (former) wheezing illness cases still showed a trend towards a different overall microbial community composition at follow-up compared to their respective controls (R^2: 0.023, p=0.072; Figure 3). As aforementioned, cases with wheezing illness had the highest incidence of recurrence of respiratory symptoms, while cases with pneumonia had the lowest incidence (Table 1). On OTU level, we observed that cases recovered from wheezing illness had increased levels of *Streptococcus anginosus,* and gram-negatives like *P. oris, Porphyromonas* and *Neisseria*, which were all also associated with recurrence (Figure 4). By contrast, cases recovered from pneumonia had increased levels of *Klebsiella*, *Neisseriaceae,* and gram-positives *S. aureus/epidermidis* and *Kocuria,* possibly related to antibiotic selection (Figure S8).

Finally, we stratified the analysis based on the most prevalent viruses at time of LRTI, i.e. RSV and HRV, which were detected in 72 (47%) and 73 (47%) cases, respectively, to rule out virus-mediated effects on microbiota recovery. Cases recovered from a RSV-associated LRTI had no significant differences in microbial richness, diversity or the overall microbial composition compared to their matched controls (R^2=0.003, p=0.624). Cases recovered from a HRV-associated LRTI also showed no significant differences in overall microbial composition compared to controls (R^2=0.008, p=0.165), though they had a slightly higher microbial richness upon recovery, which tended towards significance (p=0.070; Figure S7C).

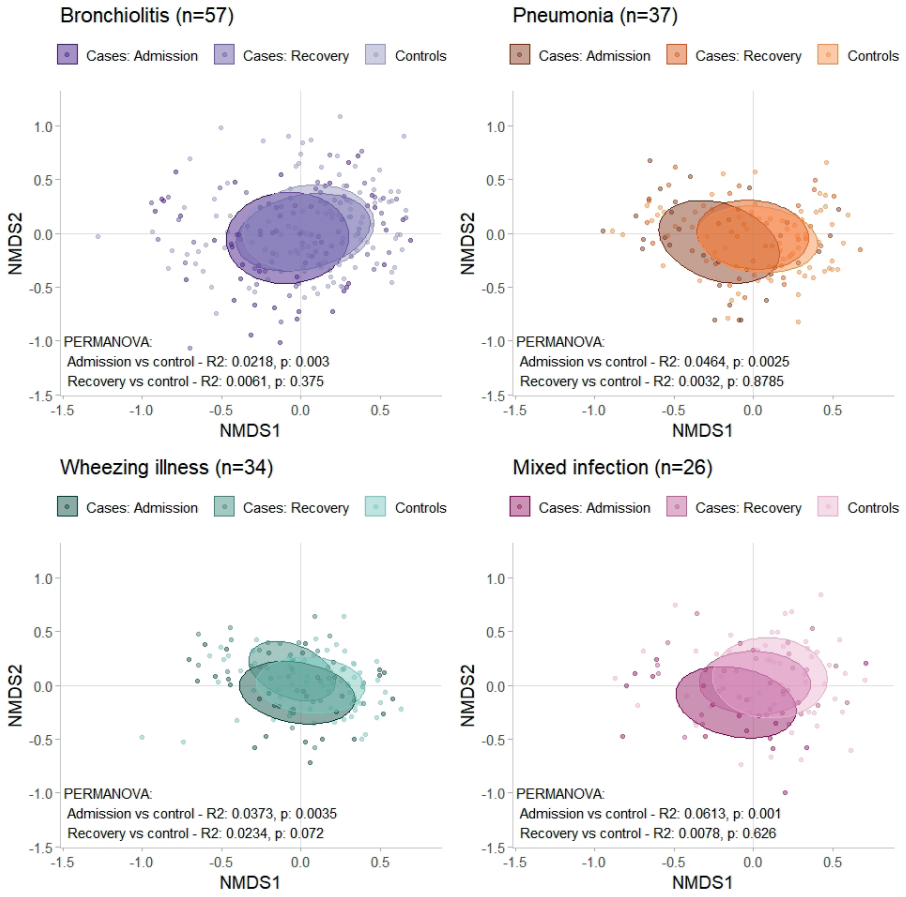

Figure 3 | Microbiota recovery depended on infection phenotype. Nonmetric multidimensional scaling plots based on Bray-Curtis dissimilarity depict nasopharyngeal microbiota composition for cases at admission, cases at follow-up (recovery), and controls, for each of the 4 phenotypes. Ellipses represent the standard deviation of all points within a sub-cohort. Significance was tested using permutational analysis of variance (PERMANOVA).

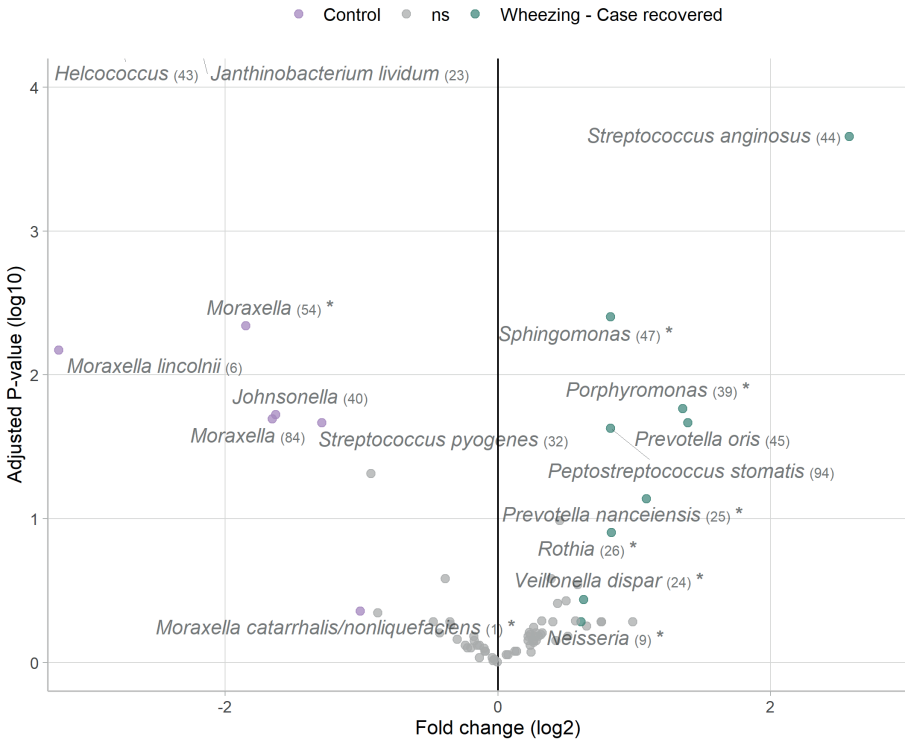

Figure 4 | Discriminant OTUs between cases recovered from wheezing illness and matched controls. Volcano plot of differentially abundant OTUs between cases recovered from wheezing illness and controls. Significance was assessed by metagenomeSeq analysis and cross-validated VSURF analysis limited to the top 100 most highly ranked OTUs, and combined results were filtered at a fold change of at least 1.5 or below 0.5. OTUs marked by an asterisk were identified by cross-validated VSURF analysis. Results of data points falling beyond the limits of the plot: *Helcococcus* log2FC -5.93, adjusted p-value (log10) 13.48; *Janthinobacterium lividum* log2FC -3.67, adjusted p-value (log10) 10.01.

DISCUSSION

LRTI is strongly associated with dysbiosis of the nasopharyngeal microbiota (13). Here, we found in children hospitalized for acute LRTI that lower microbial diversity and the overall microbial community composition in the nasopharynx were modestly associated with subsequent recurrence of even very mild respiratory symptoms within 1-2 months. Specifically, we identified gram-negatives like *H. influenzae/haemolyticus*, *P. oris* and *Actinomyces* species

as potential biomarkers of an increased risk of recurrence of respiratory symptoms, and gram-positives like *S. aureus/epidermidis* and *S. pneumoniae* as potential biomarkers of a reduced risk.

These findings add to a small but growing body of literature suggesting that host-microbial interactions during and following acute (L)RTI may contribute to short- and long-term respiratory outcomes. Previously, Neumann et al. found in noses of infants with their first RTI that lower microbial diversity and increased levels of bacterial families Moraxellaceae or Streptococcaceae were associated with persistent respiratory symptoms (28). Also in the nasal niche, Mansbach et al. related persistently increased levels of *Moraxella* and *Streptococcus* in the weeks following hospitalization for bronchiolitis in infancy to persistent wheeze at the age of 3 years (8). In case of RSV infection, increased nasopharyngeal levels of gram-negatives including *H. influenzae* have been associated with a pro-inflammatory systemic immune response with enhanced neutrophil recruitment and activation, and increased disease severity (29, 30). Correlates with clinical outcomes during recovery remain unknown, but a severity-dependent relationship between RSV bronchiolitis and the risk of recurrent wheezing and asthma has been described (31). Moreover, *Haemophilus*-dominated nasopharyngeal microbiota during RSV infection has also been related to increased viral load (11) and delayed clearance (32), which might also contribute to persistent inflammation, slower recovery and more respiratory morbidity. Finally, in healthy infants, increased influx into the nasopharynx of gram-negatives typically found in the mouth, like *Prevotella, Neisseria* and *Fusobacterium*, has also been associated with higher susceptibility to RTIs in general (20, 33). Alternatively, gram-positive commensals might dampen inflammatory responses. For instance, *S. epidermidis* was shown to enhance mucosal innate immune responses in the nose and to thereby confer resistance to viral infection (34). Moreover, dominance of *Corynebacterium* and *Dolosigranulum* in the infant nasopharyngeal microbiota has been related to decreased incidence of RTIs (35). Future studies should therefore investigate whether antibiotic treatment targeted at gram-negatives and/or preservation or supplementation of gram-positive commensals may prevent recurrence of respiratory symptoms after LRTI and improve long-term respiratory outcomes.

In general, we observed that the nasopharyngeal microbiota had recovered 4-8 weeks after hospitalization for LRTI, though subtle differences remained including a persistently lower abundance of *Moraxella* than seen in healthy controls. Remarkably, this is opposite to observations by Teo and colleagues (14). This study had an unmatched design, later timing of post-LRTI sampling and a different geographic location, but the discrepancy with our findings might also reflect biological differences between their cohort at high risk for atopy and our unselected cohort. In line with our findings, Kaul and colleagues recently demonstrated that in some adult patients with acute influenza infection, microbial communities returned to a healthy state within 22 days from hospital admission (36). It thus appears that the URT microbiota are resilient, but the speed of recovery differs between individuals. In addition, we observed that children with early recurrence of respiratory symptoms following LRTI also had diminished microbiota recovery, despite a longer follow-up duration and more time for the microbiota to recover. The association between longer follow-up duration and recurrence of respiratory symptoms may be directly linked, though also explained by the fact that children had to be asymptomatic at the time of sampling, and as a consequence the follow-up visit was postponed when symptoms were present at that moment. Irrespectively, given the high incidence of symptom recurrence in our cohort, we theorize that while the microbiota gradually recover following LRTI, resistance to viral infection or bacterial pathobiont acquisition and overgrowth might remain diminished for some time, resulting in a (temporarily) elevated risk of new infections upon pathobiont exposure. Aberrant airway immune profiles in asymptomatic neonates and during RSV infection were previously associated with presence and abundance of gram-negatives colonizing the respiratory tract (29, 37, 38), and might mediate this association, but this remains to be investigated. An alternative hypothesis is, however, that children may be genetically predisposed to both microbiota shifts and development of RTIs. For example, genetic variants were previously shown to increase susceptibility to otitis media by modifying the middle ear microbiome (39). This would mean our findings are correlative in nature more than causally linked. Future studies should therefore take genetic factors into consideration to understand the potential mechanisms underpinning our findings.

Importantly, we observed early recurrence of respiratory symptoms followed by diminished microbiota recovery especially in children with wheezing illness, whereas microbiota changes during LRTI were previously shown to be phenotype-independent (13). Together, our findings suggest that especially in children with inflammation-driven illness, the nasopharyngeal microbiota may have more limited property to recover to a state comparable to healthy, matched controls. This is in line with other observational studies where LRTIs accompanied by wheezing symptoms were particularly associated with aberrant respiratory microbiota development (14) and with later-life persistent wheeze and development of asthma (2, 3). We speculate that wheezing illness patients, who were older than patients with a different LRTI phenotype, may have had a more elaborate medical history with LRTIs and atopic symptoms, which may have resulted in reduced resilience of the microbiota. Alternatively, wheezing illness patients often received treatment with inhaled corticosteroids upon hospital discharge, which might also have affected microbiota recovery (40, 41). Unfortunately, limited power hampered us to study the effect of inhaled corticosteroids on the respiratory microbiota and its recovery. Antibiotic-treated patients also had an increased risk of subsequent recurrence, particularly the younger ones. Dutch physicians tend to reserve antibiotic treatment for the more severely ill patients. Therefore, we cannot rule out that this correlation between antibiotic treatment and recurrence might be influenced by more severe disease and accompanying inflammation. Following, the restorative capacity of the nasopharyngeal microbiota appeared in general not related to antibiotic treatment, though we had insufficient power to test if younger antibiotic-treated children may be more prone to prolonged microbiota disturbance, as has been suggested in previous reports (42, 43). Finally, the type of virus detected at time of admission seemed unrelated to recurrence of respiratory symptoms and microbiota recovery in this cohort, despite known relationships between LRTIs associated with RSV or HRV and chronic respiratory morbidity (44).

Strengths of our study include the strictly matched case-control design that allowed us to preclude bias from age, gender and seasonality. Furthermore, inclusion of children up to 5 years old, hospitalized for all LRTI phenotypes allowed us to perform in-depth analyses. However, several limitations need to be acknowledged. We were unable to directly study the lung microbiota

during recovery, but the high concordance between the microbiota in nasopharyngeal and endotracheal samples at time of LRTI in young children implies that the nasopharyngeal microbiota provides a valid proxy (13). Furthermore, follow-up of cases entailed only one timepoint, and therefore, we could not distinguish at what pace microbial recovery occurred, and whether microbial recovery was still ongoing. Follow-up duration also varied and was different between cases with and without recurrence. However, since the microbiota of cases with recurrence had even more time to recover, this strengthens the likelihood that recurrence of respiratory symptoms following LRTI is associated with diminished microbiota recovery. Lastly, for our analyses of recurrence we relied on parental report of respiratory symptoms since hospital discharge, which may have introduced recall bias.

In conclusion, our results suggest that the composition of the nasopharyngeal microbiota during acute LRTI in children may increase susceptibility to new respiratory symptoms in the months following, while the microbiota gradually recover. Future prospective studies with higher resolution and longer follow-up duration, especially focused on recovery from LRTI in high-risk groups of recurrent or chronic respiratory morbidity, are required to confirm and nuance our findings, and should strive to combine host and microbial factors into prediction models of (long-term) clinical outcomes. The current work may be a stepping stone to improved understanding of respiratory outcomes after childhood LRTI and potentially provide input for clinical studies on methods to alleviate recurrent respiratory problems.

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SUPPLEMENTARY METHODS

Statistical analysis

Data analysis was performed in R version 3.4.3 within RStudio version 1.1.383. All analyses comparing cases to controls accounted for the matched nature of the samples. A p-value of less than 0.050 or a Benjamini-Hochberg adjusted q-value of less than 0.050 were considered statistically significant. Chi-square and Wilcoxon tests were used to compare host characteristics between cases with and without recurrence of respiratory symptoms during follow-up. Independent relationships between host characteristics and recurrence of respiratory symptoms during follow-up were assessed in multivariable logistic regression models, with treatment (antibiotics vs. no antibiotics), lower respiratory tract infection (LRTI) phenotype (wheezing illness, bronchiolitis and mixed infection vs. pneumonia) and age in months both as individual explanatory variables and in pairwise interactions, and also correcting for follow-up time in days. The final model was based on backward selection of variables using a p-value of 0.10 as cut-off, until the optimal strength of the model based on the Akaike information criterion was reached. To compare viral presence between cases and controls, conditional logistic regression was used. To assess alpha diversity, we calculated the Chao1 index for microbial richness and the Shannon index for diversity (*phyloseq* (1)), and significance of differences between cases and controls was evaluated using linear mixed-effect models. We also compared alpha diversity measures between cases at time of hospital admission with and without subsequent recurrence of respiratory symptoms using linear models adjusted for age, sex and month of hospital admission. Nonmetric multidimensional scaling (NMDS) biplots based on the Bray-Curtis dissimilarity matrix (*ordinate*-function, *phyloseq* (1), 2 dimensions, maximum 10.000 iterations) were used to visualize differences in the overall microbial community between groups. Statistical significance of differences in the overall microbial community was assessed by permutational analysis of variance (PERMANOVA) using the *adonis*-function or, when comparing cases with and without recurrence of respiratory symptoms during follow-up, the *adonis2*-function adjusting for age, sex and month of hospital admission (*vegan* (2), 1999 permutations). We considered microbiota recovery to be 'complete' if there was no remaining

significant difference in the overall microbial community between cases at recovery and matched controls. Relative abundances of the top 10 most highly abundant operational taxonomic units (OTUs) were visualized in a stacked bar chart. We used *metagenomeSeq* analysis (3) (*fitZig*-function) to identify differentially abundant OTUs between cases and controls (filtered on OTUs present in >10% of the samples, maximum 100 iterations, mixed model design). Next to that, we identified OTUs with highest discriminative abilities between cases and controls with random forest classifier analysis using a 10-fold cross-validated *VSURF* procedure (4). In this analysis, OTUs were considered discriminant when they were selected at least twice in the interpretation step. Log2 fold changes of discriminant OTUs as calculated by *metagenomeSeq* were converted to fold changes using the formula fold change = 2^log2 fold change. Combined results from *metagenomeSeq* and *VSURF* were then additionally filtered at a fold change of at least 1.5 or below 0.5 (i.e. a 50% change) to retain only discriminant OTUs with relevant changes. Above analyses were repeated to assess microbiome recovery in relation to clinical outcome (recurrence vs. no recurrence of respiratory symptoms during follow-up), LRTI phenotype, antibiotic treatment, and presence of respiratory syncytial virus or human rhinovirus. In these sub-analyses, we limited differential abundance testing to the top 100 highest-ranked OTUs, because of limited power, and to avoid false positive results in low abundant OTUs which is a known risk using *metagenomeSeq* analyses in smaller group sizes (5).

SUPPLEMENTARY TABLES

Table S1 | OTUs identified as contaminants.

OTU	Method
Tepidimonas (28)	Both
Schlegelella (10)	Both
Acidovorax (66)	Both
Vogesella (69)	Both
Acinetobacter (31)	Both
Acinetobacter seohaensis (64)	Both
Phyllobacteriaceae (52)	Both
Pseudomonas stutzeri (95)	Both
Tardiphaga robiniae (106)	Both
Mesorhizobium (81)	Both
Shewanella (30)	Both
Massilia (88)	Frequency
Pseudomonas aeruginosa (79)	Frequency
Rhizobiales (169)	Prevalence
Xanthomonadales (114)	Prevalence
Cyanobacteria (126)	Prevalence
Hydrotalea (205)	Prevalence
Cyanobacteria (143)	Prevalence
Acinetobacter (139)	Prevalence
Modestobacter (167)	Prevalence
Cupriavidus metallidurans (156)	Prevalence

OTUs were identified as contaminants using their relation with bacterial biomass (frequency method) or their presence in samples compared to negative controls (prevalence method) or both.

Table S2 | Respiratory symptoms in cases during 4-8 weeks follow-up.

n	98
Respiratory symptoms	
Rhinorrhea	73 (74.5)
Cough	61 (62.2)
Wheezing	20 (20.4)
Earache	11 (11.2)
Sore throat	7 (7.1)
Hoarseness	4 (4.1)
Severity measures	
Number of respiratory symptoms	2.00 (1.00, 2.00)
>1 respiratory symptoms	57 (58.2)
Fever (>38°C)	47 (48.0)
Physician visit	41 (41.8)
Antibiotic treatment	8 (8.2)

Data are presented as n (%) or median (IQR). Data were acquired from parent questionnaires.

Table S3 | Independent relationships between clinical characteristics during acute lower respiratory tract infection and early recurrence of respiratory symptoms.

	Coefficient	Std. Error	Z-value	P-value
Intercept	-2.588	1.237	-2.093	0.036
Age (months)	0.002	0.020	0.079	0.937
Antibiotic treatment	2.473	1.088	2.274	0.023
Diagnosis bronchiolitis*	0.751	0.702	1.069	0.285
Diagnosis mixed*	0.479	0.703	0.681	0.496
Diagnosis wheezing illness*	1.190	0.666	1.787	0.074
Follow-up time (days)	0.061	0.024	2.550	0.011
Age (months):Antibiotic treatment	-0.078	0.035	-2.202	0.028

* with pneumonia as a reference

SUPPLEMENTARY FIGURES

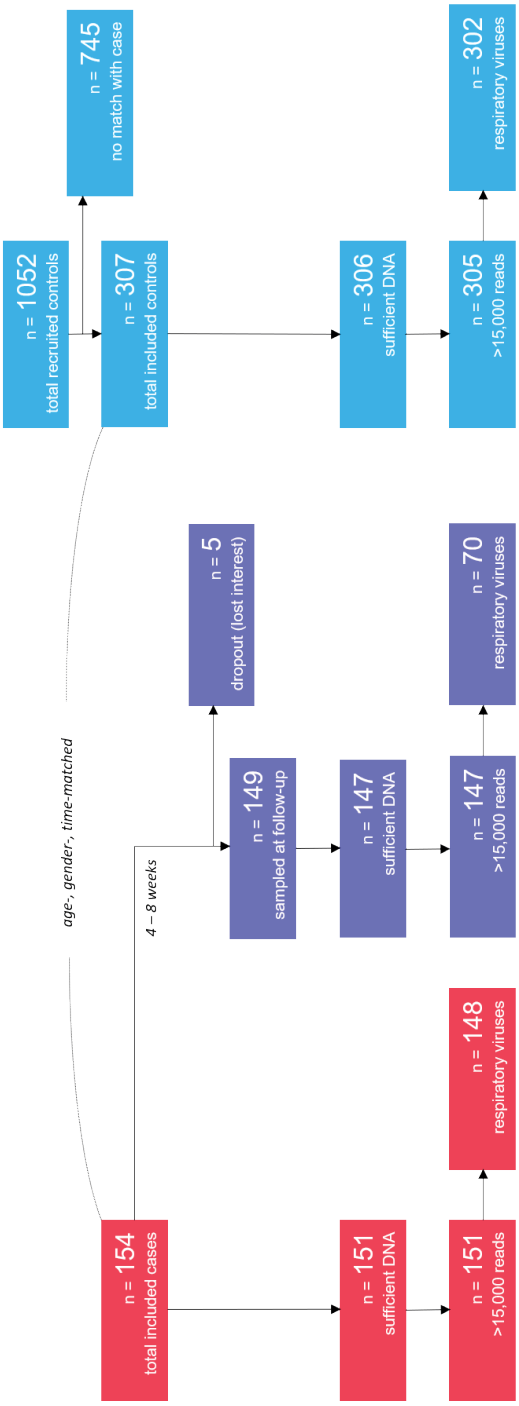

Figure S1 | Study design

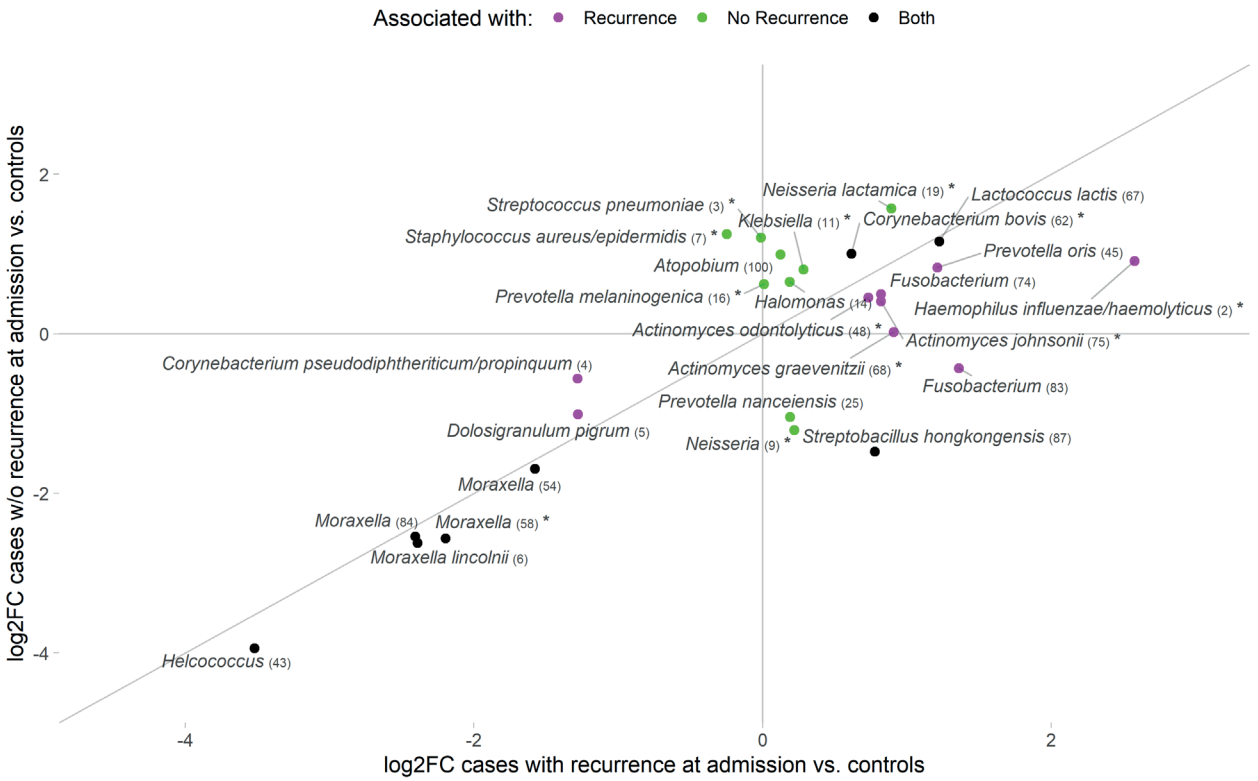

Figure S2 | Discriminant OTUs between cases at admission and controls, stratified by recurrence of respiratory symptoms during follow-up. Log2 fold changes (log2FC) of differentially abundant OTUs between cases at time of admission with subsequent recurrence of respiratory symptoms during follow-up and controls (purple, x-axis), or between cases at time of admission without subsequent recurrence of respiratory symptoms during follow-up and controls (green, y-axis), or both (black). Significance was assessed by metagenomeSeq analysis and cross-validated VSURF analysis limited to the top 100 most highly ranked OTUs, and results were combined and filtered at a fold change of at least 1.5 or below 0.5. OTUs marked by an asterisk were identified by cross-validated VSURF analysis.

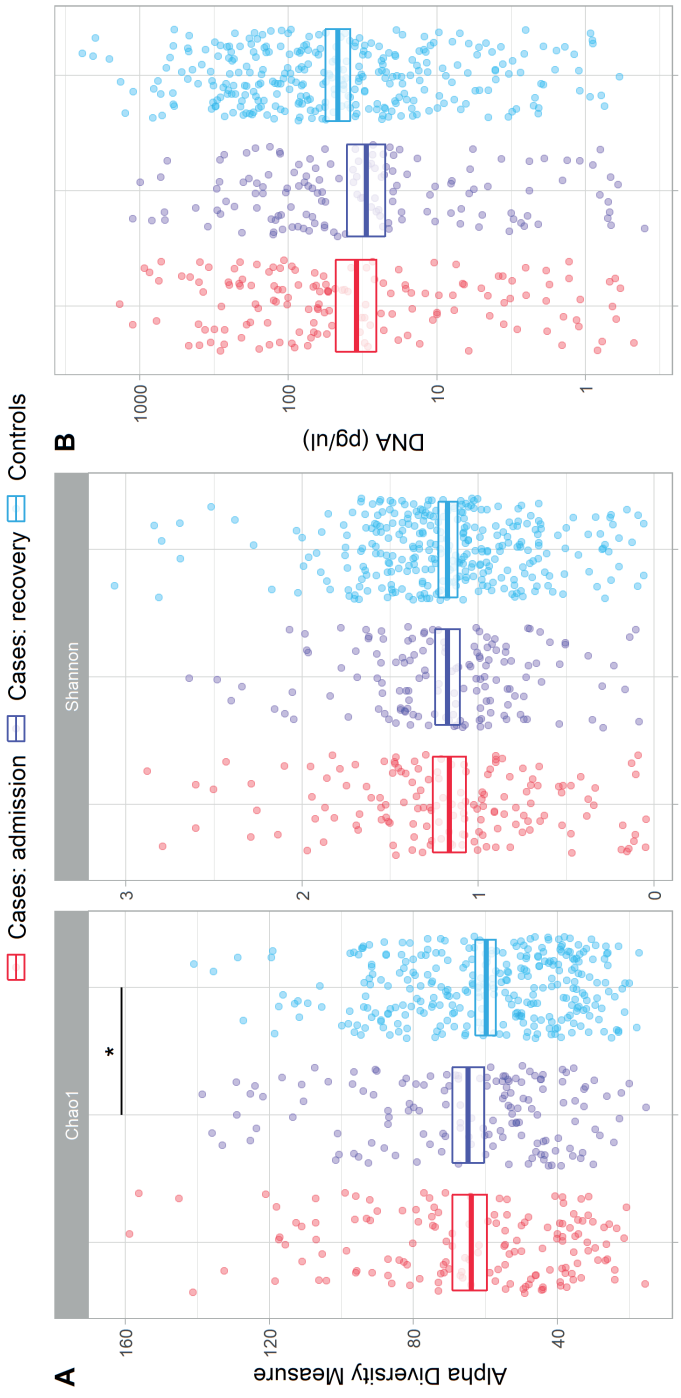

Figure S3 | Microbial richness, diversity and biomass. (A) Alpha diversity measures Chao1 index and Shannon diversity index and (B) microbial biomass estimated by quantitative PCR of the 16S rRNA gene are shown for cases at admission, at recovery and controls. Boxes denote means with 95% confidence intervals. Significance was tested by linear mixed-effect models and indicated by *: p<0.05.

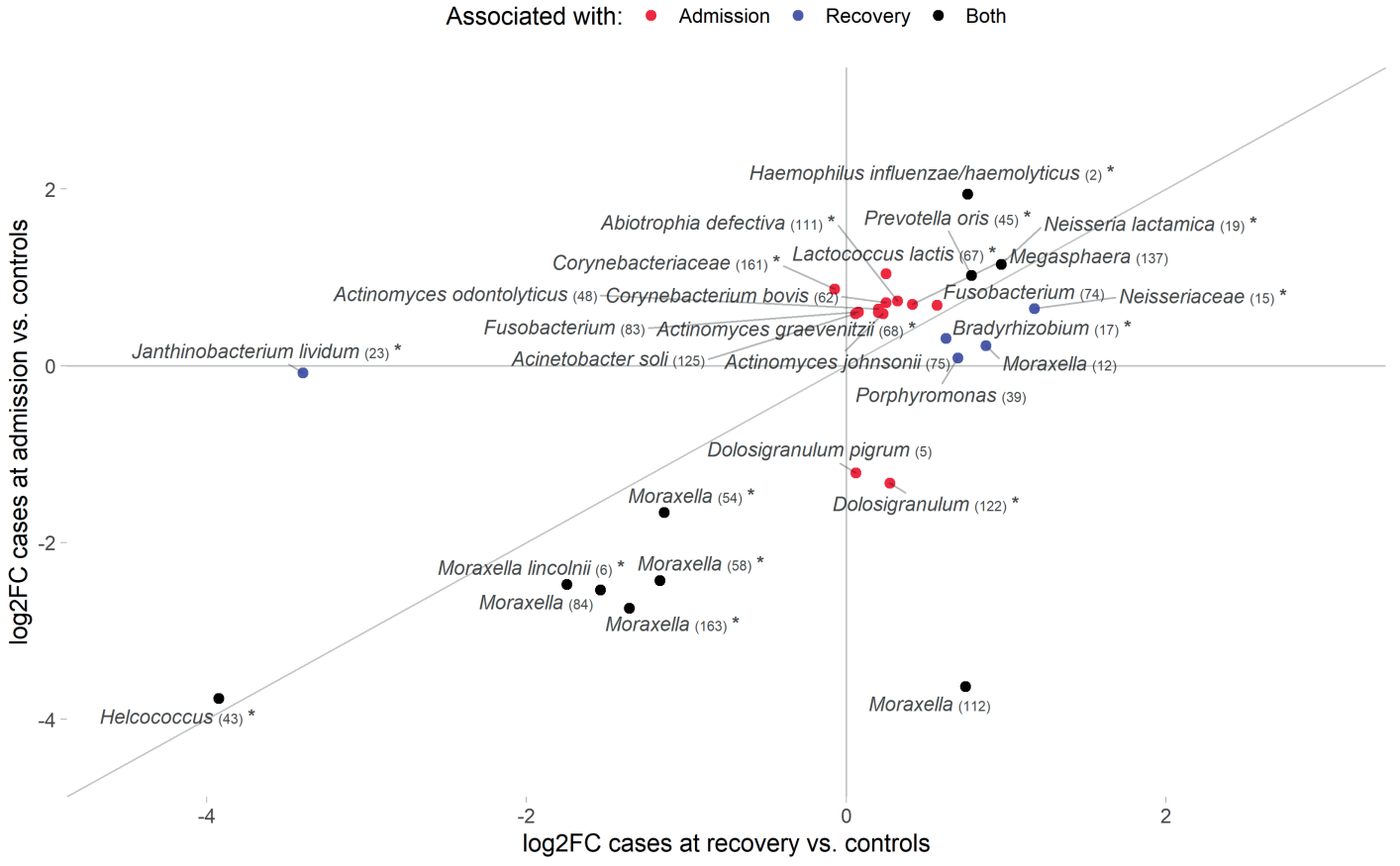

Figure S4 | Discriminant OTUs between cases and controls. Log2 fold changes (log2FC) of differentially abundant OTUs between cases at admission and controls (red, y-axis), or cases at recovery and controls (blue, x-axis), or both (black). Significance was assessed by metagenomeSeq analysis and cross-validated VSURF analysis, and results were combined and filtered at a fold change of at least 1.5 or below 0.5. OTUs marked by an asterisk were identified by cross-validated VSURF analysis.

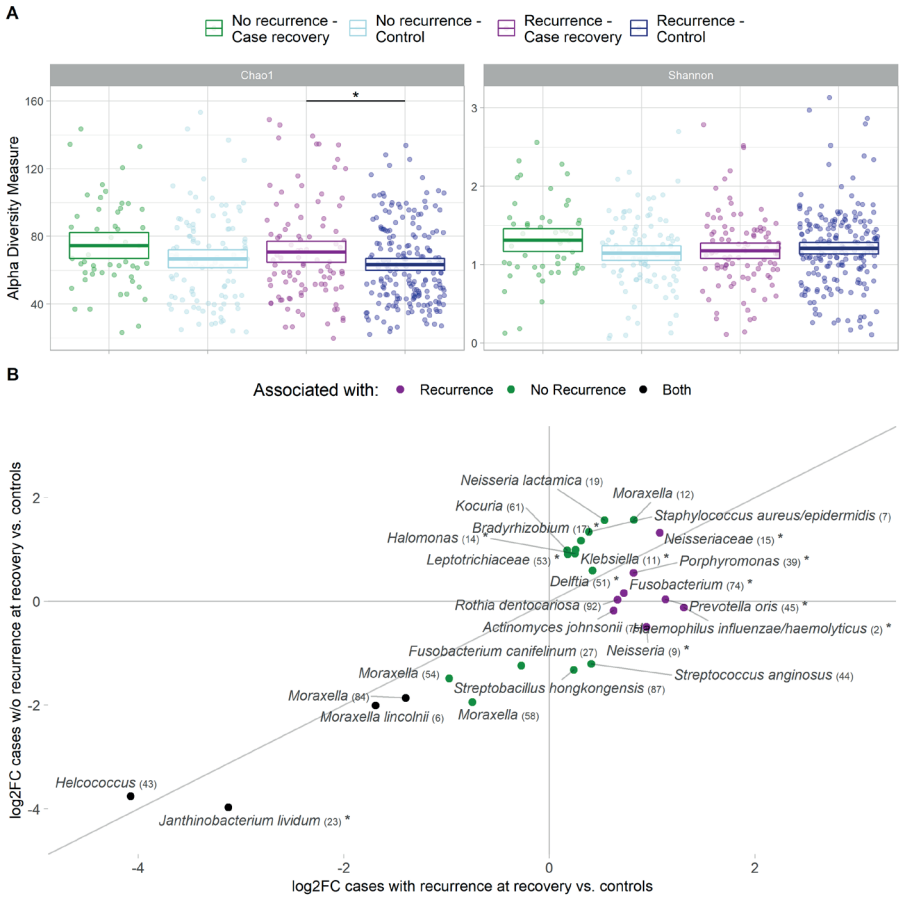

Figure S5 | Microbial recovery in cases with and without recurrence of respiratory symptoms during follow-up. (A) Alpha diversity measures Chao1 index and Shannon diversity index are shown for cases after recovery with and without recurrence of respiratory symptoms and matched controls. Boxes denote means with 95% confidence intervals. Significance was tested by linear mixed-effect models and indicated by *: p<0.05. (B) Log2 fold changes (log2FC) of differentially abundant OTUs between cases at time of recovery who had had recurrence of respiratory symptoms during follow-up and controls (purple, x-axis), or between cases at time of recovery who had not had recurrence of respiratory symptoms during follow-up and controls (green, y-axis), or both (black). Significance was assessed by metagenomeSeq analysis and cross-validated VSURF analysis limited to the top 100 most highly ranked OTUs, and results were combined and filtered at a fold change of at least 1.5 or below 0.5. OTUs marked by an asterisk were identified by cross-validated VSURF analysis.

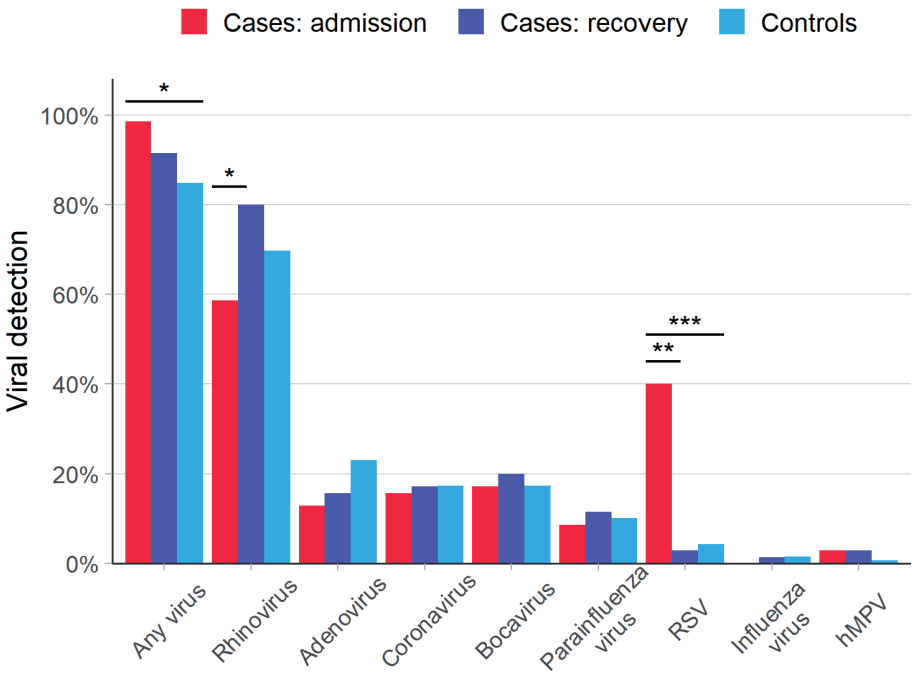

Figure S6 | Viral carriage in cases and controls as detected by quantitative PCR. Bars denote percentages of samples positive for any virus and for each individual respiratory virus for cases at admission, cases at recovery, and controls. Significance was assessed by conditional logistic regression and is indicated by ***: p<0.001, **: p<0.005, or *: p<0.05.

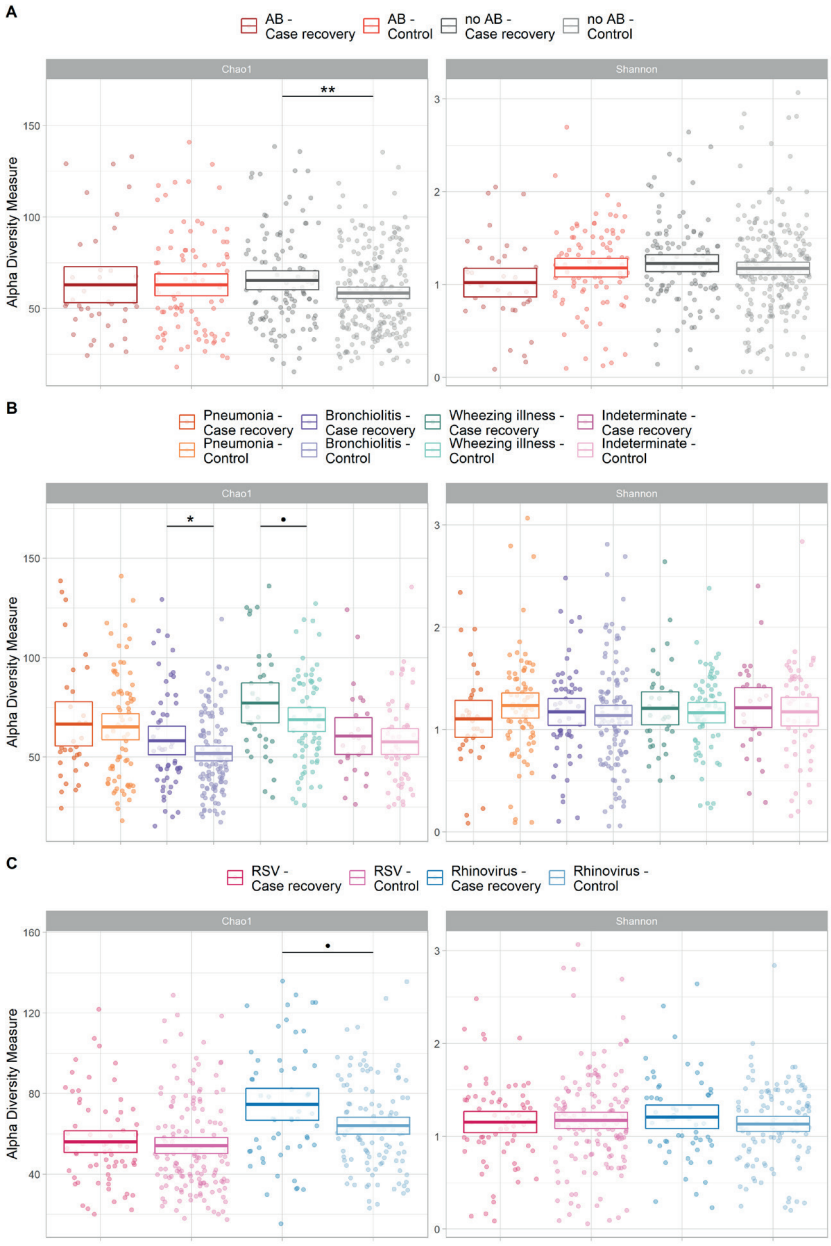

Figure S7 | Microbial recovery according to antibiotic treatment, LRTI phenotype and viral presence. Alpha diversity measures Chao1 index and Shannon diversity index are shown for cases at recovery and matched controls, stratified by (A) antibiotic treatment or no antibiotic treatment, (B) LRTI phenotype and (C) type of virus present at admission. Boxes denote means with 95% confidence intervals. Significance was tested by linear mixed-effect models and indicated by **: p<0.005; *: p<0.05; •: p<0.10.

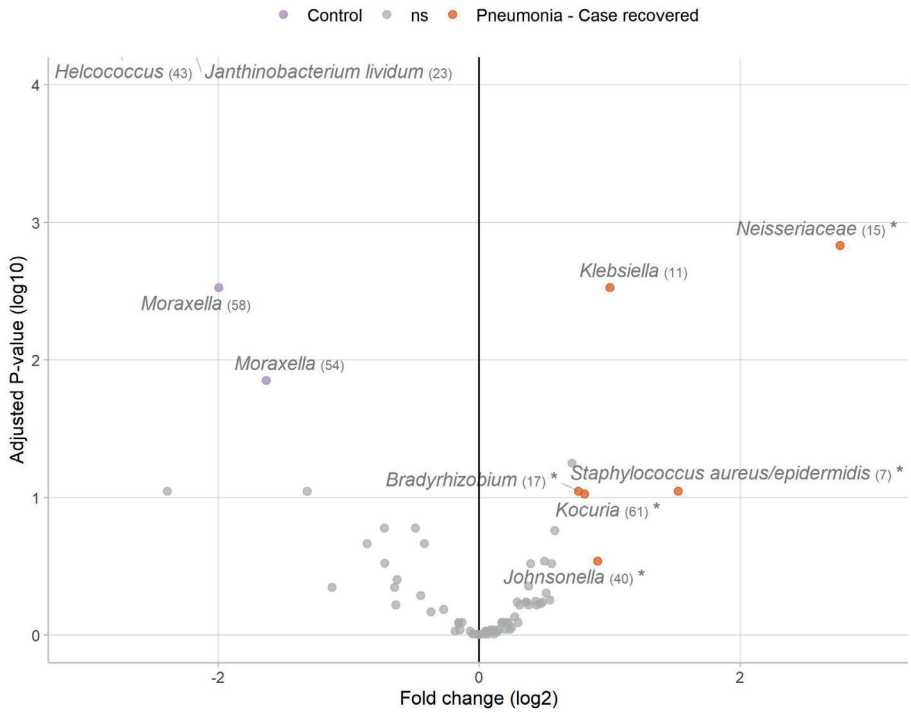

Figure S8 | Discriminant OTUs between cases after recovery from pneumonia and controls. Volcano plot of differentially abundant OTUs between cases with pneumonia at recovery and controls. Significance was assessed by metagenomeSeq analysis and cross-validated VSURF analysis limited to the top 100 most highly ranked OTUs, and combined results were filtered at a fold change of at least 1.5 or below 0.5. OTUs marked by an asterisk were identified by cross-validated VSURF analysis. Results of data points falling beyond the limits of the plot: *Helcococcus* (43) log2FC -4.21, adjusted p-value (log10) 8.30; *Janthinobacterium lividum* (23) log2FC -3.08, adjusted p-value (log10) 6.82.

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CHAPTER 8

The respiratory microbiota during and following mechanical ventilation for respiratory infections in children

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MAIN TEXT

The lower respiratory tract (LRT) harbours distinct, dynamic low-density microbial communities, established through micro-aspiration from the upper respiratory tract (URT) (1–3). However, during intubation and mechanical ventilation, the endotracheal tube temporarily alters the anatomical continuity between URT and LRT, and may provide a bridge for airborne microbes and a barrier for micro-aspiration. Shortly after intubation for a severe LRT infection (LRTI) in children, the microbiota of the nasopharynx and LRT were shown to be very similar (4). However, it remains unknown how the respiratory microbial community develops while the child recovers from the infection under treatment with mechanical ventilation and antibiotics. We therefore analysed respiratory microbiota changes in children participating in our study on acute LRTIs and who were admitted to the paediatric intensive care unit (PICU) for mechanical ventilation (4).

The subset of 29 infants with community-acquired LRTI who required intubation and ventilation, was recruited between September 2013 and September 2016. The mean age of the cohort was 3.4 months (range 1.0-12.8) with 48% being female. All children were diagnosed with bronchiolitis. Conventional microbiological findings were available for 21 of the children. Antibiotics were administered to 28/29 children (25 co-amoxiclav, 2 cephalosporins, 1 azithromycin), 5 of whom were already started on treatment shortly before PICU admission. We obtained nasopharyngeal (NP) swabs, saliva and endotracheal aspirates (ETA) upon intubation (29 NP, 27 saliva, 25 ETA) and shortly before extubation (16 NP, 15 saliva, 14 ETA), which was on average 5.9 days (SD 2.6) after intubation. Saliva was collected by placing an absorbent sponge in the cheek pouches and under the tongue until it became saturated with saliva, which was immediately transferred into glycerol DEPC medium using a sterile syringe. ETA was collected during routine suctioning of the endotracheal tube without instilling saline. We also obtained 20 NP swabs and 19 saliva samples during a follow-up visit, on average 51.9 days (SD 13.5) after PICU discharge.

Microbiota profiles were generated by sequencing of the 16S rRNA gene V4 hypervariable region. Sequence data was deposited in the NCBI Sequence Read Archive database (BioProject ID PRJNA669463). Methodological details

were previously published (4). Overall, 29 NP, 27 saliva, and 24 ETA samples at intubation, 12 NP, 14 saliva, and 11 ETA samples at extubation, and 20 NP and 19 saliva samples at follow-up passed quality control (94.5% of available samples) and were eligible for further analysis. Infants with missing extubation samples were not significantly different from those with available samples in terms of baseline microbiota composition, age or sex (data not shown). Bacterial load was estimated by quantitative (q)PCR targeting the 16S rRNA gene (5, 6). Pneumococcal presence and abundance was tested by *lytA* qPCR.

Alpha diversity was assessed using the Chao1 and Shannon indices for richness and diversity, respectively. Bacterial load and alpha diversity are summarised as median (IQR), and differences by timepoint were evaluated using linear mixed-effect models including subject as a random effect. Differences in overall microbial composition were evaluated by permutational multivariate analysis of variance on the Bray-Curtis dissimilarity matrix with permutations constrained within subject. Microbiota clusters were assigned to each sample using unsupervised hierarchical clustering. Biomarker species of each cluster were identified using random forest classifier analysis as previously described (7). Associations between clusters and timepoints were tested with fisher's exact tests. To assess microbiota concordance between niches, we calculated within-subject Bray-Curtis similarity (1–Bray-Curtis dissimilarity), and Spearman's correlations between individual operational taxonomic unit (OTU) abundances.

Our results show that bacterial load dropped dramatically between intubation and extubation in all niches, though for saliva this difference was not significant (NP: from 92.2 pg/µl (43.9-309.6) to 4.0 pg/µl (1.6-24.0), p=0.024; saliva: from 270.3 pg/µl (80.5-771.8) to 113.0 (20.1-290.6), p=0.158; ETA: from 126.8 pg/µl (31.6-708.2) to 3.9 pg/µl (2.8-13.4), p=0.039). After recovery, the bacterial load had increased only moderately in the NP (to 39.6 pg/µl (13.9-144.6), p=0.459), and more strongly in saliva (to 364.8 pg/µl (200.5-775.5), p=0.014). At the same time, richness and diversity remained comparable in the NP between intubation and extubation (Chao1: from 47.5 (34.6-62.3) to 46.8 (34.3-54.2), p=0.840; Shannon: from 1.17 (0.81-1.87) to 1.53 (0.81-1.75), p=0.945). In saliva, richness and diversity decreased

between intubation and extubation, though the difference was only significant for diversity (Chao1: from 55.0 (43.1-62.6) to 46.8 (39.4-50.9), p=0.443; Shannon: from 2.1 (1.8-2.6) to 1.5 (0.9-1.8), p<0.001), which had also significantly increased again after recovery (to 2.3 (1.8-2.6), p<0.001). In ETA, we observed a modest non-significant increase in richness and diversity between intubation and extubation, which seemed mostly driven by an increase in evenness rather than species richness (Shannon: from 0.14 (0.07-0.76) to 0.99 (0.45-1.63), p=0.112, Chao1: from 32.8 (29.4-42.8) to 51.0 (40.5-62.3), p=0.065). Furthermore, the overall microbial community composition changed significantly between intubation and extubation in both NP (R^2=5.8%, p<0.001) and saliva (R^2=7.6%, p<0.001) and even more in ETA samples (R^2=11.2%, p=0.002; Figure 1A-C). Consequently, when compared to recovery samples, the NP and saliva microbiota composition were even more different from the pre-extubation (NP: R^2=12.8%, p=0.020; saliva: R^2=10.2%, p=0.012) than from the intubation timepoint (NP: R^2=7.0%, p=0.001; saliva: R^2=3.5%, p=0.038), implying marked ecological impact and deviation from healthy microbiota as a consequence of antibiotic treatment and/or mechanical ventilation within a narrow timeframe.

We then performed clustering of NP, saliva and ETA microbiota profiles and distinguished 7 clusters, characterized by either *Streptococcus (1)* (STREP 1), *Moraxella catarrhalis/nonliquefaciens* (MOR), *Haemophilus influenzae/ haemolyticus* (HAEMO), *Corynebacterium propinquum/pseudodiphtheriticum* with *Dolosigranulum pigrum* (COR/DOL), *Streptococcus salivarius (7)* (STREP 2), *Enterobacter/Klebsiella* (ENTERO), *or Staphylococcus aureus/epidermidis* (STAPH) (Figure 1D-F). In NP and ETA, the MOR- and HAEMO-clusters predominated at intubation, and diminished following ventilation and antibiotic treatment. The COR/DOL-profile was exclusively found in NP samples and mostly observed after recovery (p<0.05). At extubation, the MOR-cluster was only observed in the single infant who did not receive antibiotic treatment. By contrast, in saliva, both STREP 1 and STREP 2-clusters predominated at intubation, with the STREP 2-cluster diminishing at extubation, and being completely absent after recovery (not significant).

Overall, at extubation, the STREP 1-, STAPH- and ENTERO-clusters were most prevalent, in line with expected changes following antibiotic exposure.

Within the STREP 1-cluster, a shift from pneumococcal dominance at intubation to non-pneumococcal streptococci pre-extubation was observed (Spearman's correlation *lytA* Ct-values with *Streptococcus (1)* abundance at intubation: ρ=-0.68, p<0.001; at extubation: ρ=-0.08, p=0.883). Interestingly, the STAPH-profile was only present in 2 NP samples at intubation, but predominated in ETA at extubation (p=0.006). The ENTERO-cluster was uniquely found at extubation (p<0.05). *Enterobacter/Klebsiella* became the most predominant OTU in 4 children following (2-8 days of) intubation and ventilation (mean abundance NP: 50.8%, range 0.03-99.4%; saliva: 11.9%, range 0.0-45.1%; ETA: 64.5%, range 30.0-99.7%), even though this OTU was mostly absent at intubation, except for one child with a very low abundance in the NP of 0.008%. To identify this OTU at the species level, we attempted to re-culture the corresponding samples, and identified in 3 of those gram-negative strains that were identified as *Enterobacter cloacae* by MALDI-TOF mass spectrometry. Together, these findings imply that the typically hospital-acquired and antibiotic-resistant pathobiont *E. cloacae* colonized and/or became dominant in the respiratory tract of these children during PICU stay. Similarly, we observed dominance of a *Stenotrophomonas* species (77.7% of ETA microbiota) in 1 case pre-extubation, despite it being nearly absent at intubation (0.002%), again suggesting selection or outgrowth during ventilation. In general, conventional culture performed at admission confirmed the predominant pathogens observed in the NP and/or ETA profile of 12/21 children. Culture results were negative in 5/21 children, and confirmation of non-predominant gram positives but lack of detection of the predominant (gram negative) pathogen was observed in 4 children. These findings underline that culture results, especially in children treated with antibiotics, often lack to provide insight in presence and/or predominance of respiratory pathogens.

We previously demonstrated highly concordant NP and ETA microbiota at intubation in this cohort, suggesting the NP is the source community of the LRTI in young children (4). However, interestingly, NP-ETA concordance at intubation (within-subject median Bray-Curtis similarity 0.66 (IQR 0.44-0.81)), had dropped pre-extubation (0.53 (IQR 0.31-0.63)), although this difference was not significant (Wilcoxon rank-sum test, p=0.188).

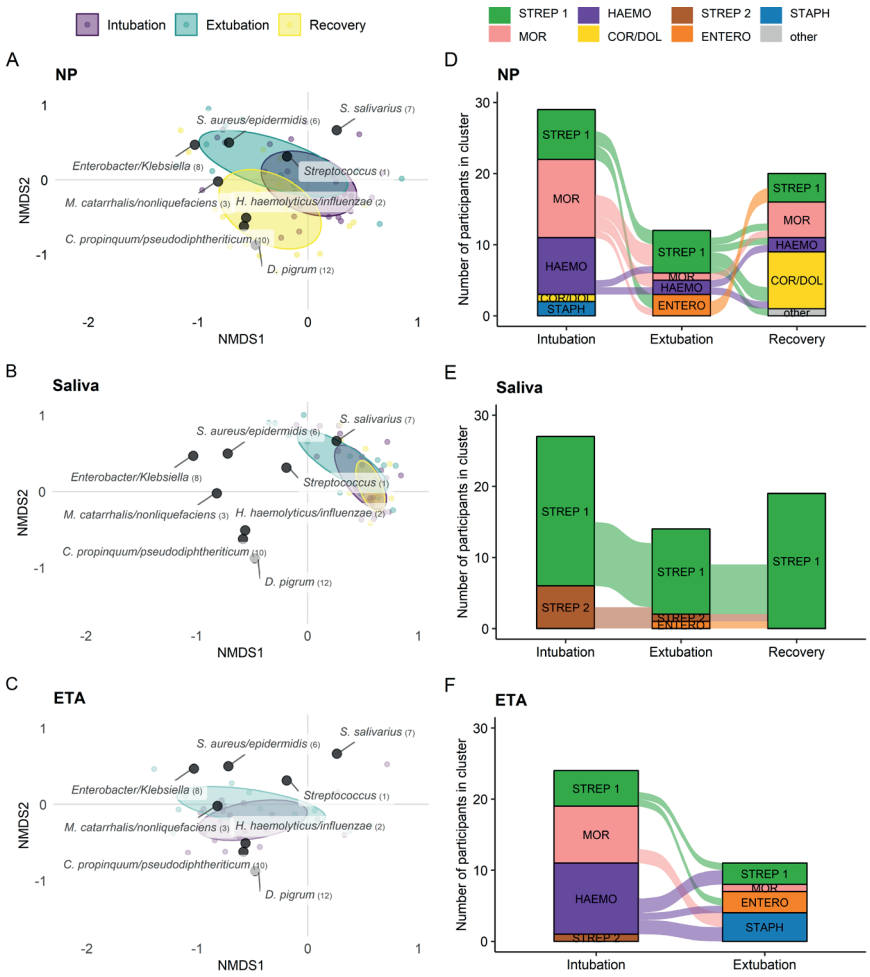

Figure 1 | Respiratory microbiota dynamics during and following mechanical ventilation for severe lower respiratory tract infection. (A-C) Nonmetric multidimensional scaling (NMDS) biplots based on the Bray-Curtis dissimilarity matrix visualizing the overall microbiota composition in the nasopharynx (NP, panel A), saliva (panel B) and endotracheal aspirate (ETA, panel C) at time of intubation, extubation and after 2 months recovery time, along with 8 biomarker operational taxonomic units (OTUs). Ellipses represent the standard deviation of the data points per subgroup. (D-F) Alluvial plots of cluster transitions in the NP (panel D), saliva (panel E) and ETA (panel F) between time of intubation, extubation and after 2 months recovery time. Hierarchical clustering of all samples based on the Bray-Curtis dissimilarity matrix identified 7 distinct clusters, characterized by either *Streptococcus* (1) (STREP 1), *Moraxella catarrhalis/nonliquefaciens* (MOR), *Haemophilus influenzae/haemolyticus* (HAEMO), *Corynebacterium propinquum/pseudodiphtheriticum* with *Dolosigranulum pigrum* (COR/DOL), *Streptococcus salivarius* (7) (STREP 2), *Enterobacter/Klebsiella* (ENTERO), or *Staphylococcus aureus/epidermidis* (STAPH). Stacked bars represent the number of samples in each cluster per timepoint, and connections between bars represent transitions of participants with 2 consecutive samples available between timepoints.

Also, only 36 OTUs (combined relative abundance 36.6%) were still significantly correlated between NP and ETA samples pre-extubation, compared to 74 OTUs (combined relative abundance 84.2%) at intubation, suggesting non-NP microbes may have settled in the LRT community. We therefore investigated whether micro-aspiration could explain these findings, and studied the concordance between saliva and ETA samples both at intubation and pre-extubation. We observed that the concordance in microbial community composition between saliva and ETA was low at both intubation (within-subject median Bray-Curtis similarity 0.13 (IQR 0.03-0.33)), and pre-extubation (0.17 (IQR 0.04-0.61)). Moreover, the number of OTUs that correlated between both niches dropped from 70 OTUs at intubation (combined relative abundance 57.0%), to 52 OTUs at extubation (combined relative abundance 14.7%). Collectively, our data suggest that the NP is a more important source community for the LRT compared to the oral microbiota in children, and that NP, saliva and ETA microbiota evolve relatively independently during mechanical ventilation, which resulted in increased segregation between the URT and LRT microbial communities.

In summary, we observed that during intubation and ventilation, combined with antibiotic treatment in critically ill children suffering from a community-acquired LRTI, the respiratory microbiota composition clearly changed, even deviating further from 'healthy' profiles. The bacterial load dropped and the relative abundance of predominant pathogens decreased, simultaneously allowing antibiotic-resistant bacteria including *Staphylococcus* species, non-pneumococcal streptococci, and *Enterobacter/Klebsiella* species, to colonize and/or overgrow the respective niches. Furthermore, our data suggest differential effects of intubation/ventilation and/or antibiotic use on microbial communities in the respective niches. Our findings are in line with previous results in adults (8). However, unlike findings in adults, the LRT microbiota of young children with a severe LRTI reflected the NP more than the oral microbiota before intubation, which did not change during ventilation. The main limitation of this study is its small sample size. Future, larger studies are required to disentangle independent effects of (different) antibiotic therapies and intubation for mechanical ventilation on respiratory microbiota dynamics. Further study is especially important because the respiratory microbiota composition during intubation has recently been

related to clinical outcomes in adults. For instance, Dickson and colleagues reported that detection of species of the Enterobacteriaceae family in the lungs of critically ill adults was associated with acute respiratory distress syndrome and prolonged duration of mechanical ventilation (9). Similarly, in a large cohort of mechanically ventilated patients, worse clinical outcomes were related to low alpha diversity combined with pathogen overgrowth in the LRT, in contrast to high alpha diversity with dominance of typically oral taxa (10). In line with these findings, Woo and colleagues observed that increased abundance of oral taxa including *Streptococcus* during intubation and ventilation were related to successful extubation (11). Findings presented here thus warrant similar studies of respiratory microbiota changes during intubation and ventilation in relation to recovery in critically ill paediatric patients, and exploration of methods to prevent rapid in-hospital acquisition and/or enrichment of antibiotic-resistant pathobionts in this already vulnerable patient population.

ACKNOWLEDGEMENTS

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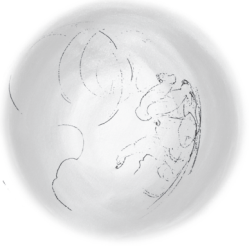

CHAPTER 9

Respiratory pathogen detection in children: saliva as a diagnostic specimen

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ABSTRACT

We compared respiratory pathogen detection in saliva with nasopharyngeal (NPS) and/or oropharyngeal swabs (OPS) in 29/57 children with respiratory symptoms. The sensitivity in NPS was 93% (95% CI 78-98%), in OPS 79% (95% CI 60-90%), in saliva overall 76% (95% CI 58-88%), and in 18 saliva samples collected with drooling or sponges, 94% (95% CI 74-99%). Saliva offers attractive practical advantages, and could thus be a relevant specimen alternative in the pediatric population.

INTRODUCTION

Respiratory infections are the most common cause of illness in childhood, and may be caused by various viral and bacterial pathogens (1). Accurate and timely identification of pathogens causing infectious diseases is important to guide treatment and infection control precautions, which is highlighted by the current SARS-CoV-2 pandemic. Multiplex PCR of nasopharyngeal swabs (NPS) and oropharyngeal swabs (OPS) is widely applied for respiratory pathogen detection. However, particularly for children, NPS and OPS collection holds disadvantages, as the procedure is uncomfortable and may be experienced as painful, induces sneezing or coughing, and requires a trained health care worker to obtain a high quality sample. Saliva collection causes little discomfort, is safer, and can be done by self-sampling or by parents, which may reduce anxiety and increase the willingness of children to undergo sampling for microbiological testing. To date, only one study in children demonstrated a 74% sensitivity of respiratory pathogen detection in saliva compared to NPS (2). Therefore, we evaluated the sensitivity of respiratory pathogen detection in saliva in children using multiplex PCR.

MATERIALS AND METHODS

This study prospectively enrolled children (<18 years old) who presented to the emergency department of a general hospital in The Netherlands between April 14, 2020 and August 9, 2020, within 14 days of onset of symptoms of a potential SARS-CoV-2 infection (3). NPS and OPS were routinely obtained using flexible swabs (Copan eSwabs® 490CE and 484CE, Brescia, Italy), which were placed in Amies medium. Upon oral (parental) consent, saliva was obtained at the same time using at least one of three methods, depending on the age of the child: stroking both cheek pouches and the tongue with a swab (DNA-col, Labonovum, Limmen, the Netherlands), which was placed in RNA protect Cell Reagent (Qiagen, Hilden, Germany) (all ages); drooling 0.5 ml into a tube with 10% glycerol in DEPC water (≥5 years); or consecutively rubbing two absorbent sponges (Oracol Saliva Collection Device, Malvern Medical Developments Ltd., Worcester, United Kingdom) on the gums, cheek pouches and tongue for 60 seconds each (<5 years). From 5 children, 2 extra saliva sponges were collected for volume measurements. Additionally, patient characteristics were extracted from medical records.

NPS, OPS and saliva were transported to the laboratory at room temperature. Sponges were centrifuged at 800g for 5 minutes to extract saliva. All samples were tested within 72 hours for the presence of respiratory pathogens using qualitative multiplex ligation-dependent probe amplification (MLPA, RespiFinder® 2Smart kit 22 FAST, PathoFinder, Maastricht, Netherlands). MLPA is designed to detect 21 pathogens, including rhinovirus, influenza viruses, respiratory syncytial viruses, human metapneumovirus, adenovirus, bocavirus, coronaviruses, parainfluenza viruses, *Bordetella pertussis* and *Mycoplasma pneumoniae*. In addition, all samples were tested for presence of SARS-CoV-2 using quantitative RT-PCR based on the presence of the E-gene (4).

Data analysis was performed in R version 3.6.1. The sensitivity of saliva (overall and per collection method), NPS and OPS for respiratory pathogen detection was compared with a combined reference standard, defined as detection of any pathogen in at least one sample (NPS, OPS or saliva). Wilson 95% confidence intervals (CI) were calculated using the *DescTools* package. The number of pathogens per sample type was compared using Mann-Whitney U tests. Clinical characteristics (i.e. number of symptoms, duration of hospitalization, and medical treatment with bronchodilators and corticosteroids) were compared between children with the same pathogens detected in saliva (collected with drooling or sponges) as in NPS and OPS and children with fewer/different pathogens detected in saliva (collected with drooling or sponges) than in NPS and OPS, using chi-square tests and Mann-Whitney U tests.

RESULTS

In total, 57 children with symptoms indicative of a potential SARS-CoV-2 infection participated in the study and had saliva collected alongside NPS and OPS, of whom 29 children (51%) had one or more pathogens detected in at least one sample and were included in the analysis. Saliva was collected with swabs and drooling from 6 children (21%), with swabs only from 11 children (38%), by drooling only from 3 children (10%), and with sponges from 9 children (31%). The median age was 2.1 years [interquartile range (IQR), 1.1-3.5 years], and 11 (38%) children were female. Symptoms had been present for a median of 2 days [IQR, 1-3 days] at time of emergency department presentation, and most commonly included fever (18 children,

62%), rhinitis (16, 55%), shortness of breath (15, 52%), coughing (15, 52%), decreased appetite (12, 41%), and vomiting (6, 21%). Most children required hospital admission (24, 83%) for a median duration of 1 day [IQR, 0.5-2 days].

Rhinovirus was the most commonly detected pathogen, followed by adenovirus and bocavirus, coronaviruses (types 229E and NL63/HKU1) and *B. pertussis* (Table 1). No SARS-CoV-2 was detected. Two and 3 pathogens were simultaneously detected in nine children and one child, respectively. Overall, 22/29 (76%, 95% CI 58-88%) children had at least one pathogen detected in saliva, compared to 27/29 children having at least one pathogen detected in NPS (93%, 95% CI 78-98%), and 22/28 children in OPS (79%, 95% CI 60-90%). Rhinovirus, coronavirus, and *B. pertussis* were more often detected in saliva than adenovirus and bocavirus.

Among the different saliva collection methods, swabs appeared to perform poorly compared to saliva by drooling or sponges, which, when taken together, tested positive for at least one pathogen in 17/18 children (94%, 95% CI 74-99%; Table 1). The swabs were not intended for microbiological testing and yielded very low saliva volumes, which may both contribute to their poor performance.

Table 1 | Respiratory pathogen detection by multiplex PCR according to sample type, compared to the combined reference standard.

Pathogen	Total*	NPS	OPS	Saliva, any	Saliva, swab	Saliva, drooling	Saliva, sponge
Any	29	27/29 (93%)	22/28 (79%)	22/29 (76%)	6/17 (35%)	8/9 (89%)	9/9 (100%)
Rhinovirus	24	24/24 (100%)	20/23 (87%)	18/24 (75%)	4/12 (33%)	7/8 (88%)	8/9 (89%)
Adenovirus	6	5/6 (83%)	4/6 (67%)	2/6 (33%)	0/2 (0%)	0/1 (0%)	2/3 (67%)
Bocavirus	6	3/6 (50%)	3/6 (50%)	1/6 (17%)	1/3 (33%)	0/1 (0%)	0/2 (0%)
Coronavirus	3	3/3 (100%)	1/3 (33%)	2/3 (67%)	1/2 (50%)	1/1 (100%)	0/1 (0%)
Bordetella pertussis	1	0/1 (0%)	0/1 (0%)	1/1 (100%)	0/1 (0%)	1/1 (100%)	0/0 (NA)

*Total = number of children with pathogen in any of the NPS, OPS or saliva specimens. Pathogens that were tested, but were not detected in the study participants: influenza viruses (A and B), respiratory syncytial virus (A and B), human metapneumovirus, parainfluenza viruses (1, 2, 3 and 4), Mycoplasma pneumoniae, Legionella pneumophila, Chlamydophila pneumoniae, severe acute respiratory syndrome coronavirus 2. Abbreviations: NPS = nasopharyngeal swab; OPS = oropharyngeal swab.

By contrast, the volume obtained by drooling was fixed at 0.5 ml. We then measured the volume of saliva obtained with 2 consecutive sponges from 5 children (5 months-4 years old), which exceeded 0.5 ml in four of them. Both of these methods thus yielded sufficient volumes of saliva for laboratory analyses. Eleven children who had only swabs collected were excluded from further analyses.

The average number of pathogens detected per specimen type was 1.0 (range 0-2) in saliva by drooling and 1.1 (range 1-2) in saliva from sponges, which was not significantly different from the average number of pathogens detected in NPS (1.2, range 0-2; $p>0.323$) and OPS (1.0, range 0-2; $p>0.661$) from the same children. Eleven children had the same pathogen(s) detected in saliva as in NPS and OPS. However, 5 children had some but not all pathogens in NPS and OPS detected in saliva, and 1 child had none of the pathogens in NPS and OPS detected in saliva. Children for whom all pathogens in NPS and OPS were also detected in saliva, reported more symptoms (median 4 [IQR 2.5-4] versus 2 [IQR 2-2.75]), were hospitalized for a longer period (median 2 days [IQR 2-2] versus 1 day [IQR 0.62-2.12], $p=0.306$), and more often required medical treatment with bronchodilators (82% versus 33%, $p=0.142$) and systemic corticosteroids (46% versus 17%, $p=0.512$) compared to children for whom pathogen detection in saliva was unsuccessful, but these differences were not significant. By contrast, 1 child had *B. pertussis* detected in saliva, which was not found in NPS or OPS.

DISCUSSION

We found a 94% sensitivity of saliva for respiratory pathogen detection in children, provided that saliva was collected by drooling or sponges, which is comparable with results from studies in adults (5). Our results add to the only previous study in children which found a 74% sensitivity of respiratory pathogen detection in saliva collected with a single absorbent sponge (2). Concordance between pathogens detected in saliva, NPS and OPS seemed to be higher in more severely ill children, which may be explained by higher viral loads increasing the likelihood of pathogen detection in saliva. Our results also suggest that some pathogens, like adenovirus and bocavirus, are less likely to be detected in saliva than others, which may be due to post-

infectious shedding at low viral loads, but our numbers are too low to draw definite conclusions on pathogen-specific differences in saliva detection. Furthermore, detection of *B. pertussis* in saliva in one case directly altered decisions on antimicrobial treatment and isolation measures. Testing saliva alongside NPS and OPS may thus have an additional benefit, which was previously demonstrated in adults (6,7), but is novel for children.

Our work is mainly limited by the small sample size, which did not allow us to evaluate significance of differences in sensitivity between different sample types. Furthermore, the more clinically relevant respiratory syncytial virus, parainfluenza virus or influenza virus were not circulating at the time of inclusion. Future studies to validate our findings should, therefore, take place in winter, when prevalence of these viruses will normally be higher, and should evaluate viral loads, which we were not able to assess. SARS-CoV-2 was also not detected, reflecting its low prevalence in (symptomatic) children in the first epidemic wave in The Netherlands (8). Finally, we did not quantify practical aspects of using saliva, such as collection time and costs, so the implementation of microbiological testing of saliva in medical practice remains to be investigated.

In summary, we have shown that saliva can be used with high sensitivity for respiratory pathogen detection in children, and can provide additional results compared to NPS and OPS with direct consequences for clinical management. Collection should be by drooling or with sponges to ensure adequate volumes. However, sponges take more time to collect and require some additional steps during laboratory processing, so this method may be reserved for cases when drooling is not possible. Collecting saliva is simpler, less uncomfortable, and better tolerated by children than NPS or OPS and therefore, saliva could be a relevant specimen alternative in the pediatric population.

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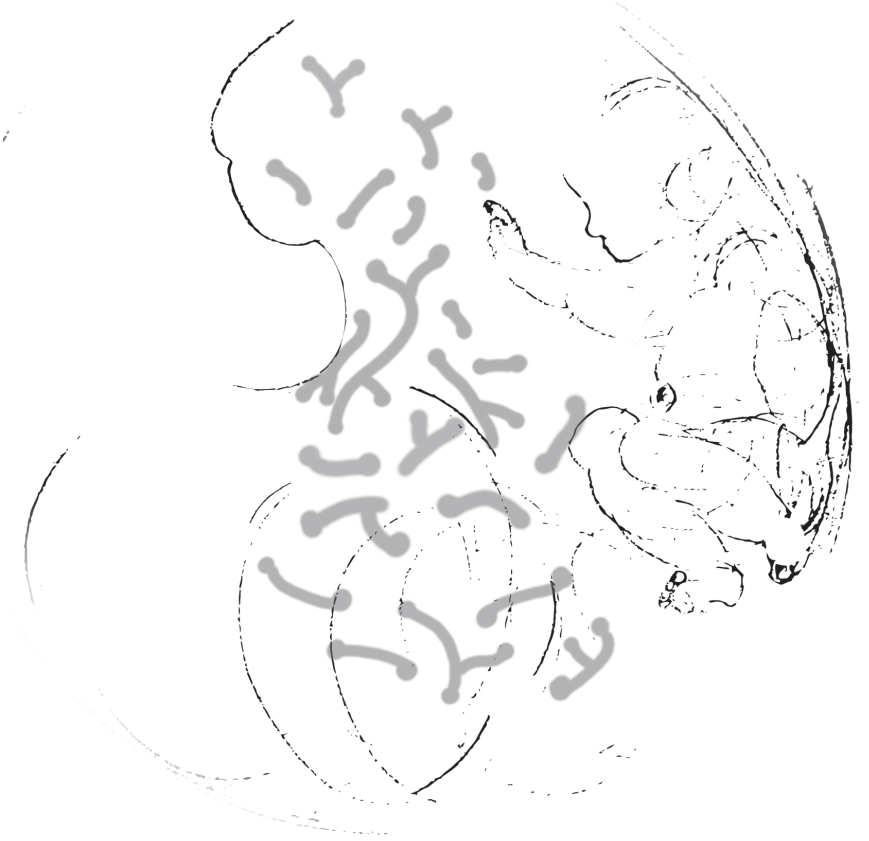

CHAPTER 10

Summarizing discussion and future perspectives

INTRODUCTION

Human evolution in symbiosis with bacteria and other micro-organisms inhabiting all bodily surfaces including the respiratory and gastro-intestinal tract (i.e., the microbiota) has selected for mutualistic functions of the microbiome that support an equilibrium with the host (1). As a result, the human microbiome plays a pivotal role for general health. For example, microbes have immune-modulating properties, provide colonization resistance to prevent pathogen invasion and infection and aid in the digestion and production of important nutrients (2). The immune-modulating functions of the human microbiome may be particularly important in the earliest phase of life. In infancy, both the microbiome and the immune system undergo rapid developmental changes at the same time. Accumulating evidence suggests that these two processes are strongly intertwined: microbial cues provide important input for the maturing immune system, and *vice versa* (3). It is even believed that in the period spanning approximately the first 100 days of life, the so-called 'window of opportunity', the microbiome leaves a permanent mark on the immune system, and thereby may thus already pave the way for future health or disease development. The primary motivation for studying the composition and development of the microbiome in infancy, early childhood and also later in life, is to shed light on the pathogenesis of infectious and immune-mediated childhood diseases, and open up new avenues of research into (preventative) microbiota-based interventions.

The infant's first microbiota are seeded at birth. Some studies identified bacterial DNA in the placenta or amniotic fluid and proposed the existence of a prenatal microbiota (4, 5), but a thorough investigation convincingly showed that these findings are most likely based on contamination and perinatal acquisition of bacteria (6). During birth, the mother provides her child with a rather uniform bacterial "starter kit". Consequently, the microbial composition of different niches including the nasopharynx, oral cavity and gut, is strikingly similar directly after birth, but niche-specific microbial communities begin to develop soon after (1, 7). In contrast, variation in microbiota composition between individual neonates can be large, for instance due to mode of delivery: the initial composition of the microbiota of vaginally born infants, who came into contact with the maternal genital

and fecal flora while passing through the birth canal, strongly differs from the more skin- and environment-transmitted microbiota of Caesarean (C-) section born infants (8–10). Infants born by C-section show, among other differences, later colonization with and lower relative abundances of beneficial lactic acid-producing bacteria in both the upper respiratory tract and the gut microbiota compared with infants born by vaginal delivery, and these differences persist for months (11, 12).

Subsequent microbiota maturation follows dynamic, niche-specific trajectories in healthy infants, although the individual course is highly sensitive to exogenous exposure to, for instance, breastmilk and solid foods, antibiotics and other drugs, other children and animals (13). The nasopharyngeal microbiota rapidly diversify in the first weeks of life: typically, initial *Staphylococcus* predominance is first replaced by Gram-positive commensals *Dolosigranulum* and *Corynebacterium* that become predominant, and later on by *Moraxella*, which stably predominates the niche from around the age of three months (14–16). This microbial colonization pattern is more often observed in vaginally delivered, breastfed infants as opposed to C-section born, formula fed infants (12, 16). In contrast to the nasopharynx, the gut microbiota have a more complex composition and develop over a longer time period, in parallel with (required adaptation to) dietary changes. An initial phase of breastmilk-stimulated *Bifidobacterium* predominance may be followed by a transitional phase with higher abundances of members of the family Lachnospiraceae upon the introduction of solid foods, reaching a stable state at around three years of age (17, 18). For both niches, early-life deviations from these natural microbial maturation trajectories have been associated with disadvantageous effects on respiratory health later on, such as a higher susceptibility to respiratory tract infections (RTIs) (11, 16) and atopic diseases like asthma (19, 20).

The microbial ecosystem is especially sensitive to perturbations in infancy and early childhood (21). The terms “dysbiosis” or “microbial imbalance” are difficult to define, but they are commonly used to describe microbiota aberrations that are related to the manifestation or progression of a disease. The current scientific literature contains countless examples of over- and underrepresentation of specific bacterial taxa either directly or indirectly

associated with disease (see **chapter 2** for a review focused on the respiratory microbiota and respiratory diseases). On the one hand, microbiota shifts may directly affect infection susceptibility. The most well-known example is that antibiotic-induced reduction of gut microbiota diversity may in some patients lead to the selection of (intrinsically resistant) *Clostridium difficile* causing recurrent episodes of severe diarrhea, that can in some cases only be treated by a drastic microbial reset through a fecal microbiota transplant (22). Another example of intervention-related changes to the microbiota with consequences for infection susceptibility is pneumococcal conjugate vaccination, which successfully reduces nasopharyngeal colonization with (pathogenic) pneumococcal vaccine serotypes. However, pneumococcal colonization was shown to be replaced by *Staphylococcus* and *Haemophilus*, upon which, for example, the incidence of otitis media associated with these respiratory pathogens increased (23–26). Microbiota changes may also have indirect effects on the host, for example through microbiota-induced immune modulation. A clear example is the acquisition of *Bifidobacterium* colonization, which is generally delayed in C-section born infants compared with vaginally born infants (11), and appears to be essential for adequate functioning of the immune system in early life. Consequently, the lack of the metabolically active *Bifidobacterium* as well as *Bacteroides* in the first three months of life has been associated with immune dysregulation and systemic inflammation (27) as well as an increased risk of developing several immune-mediated disorders with childhood onset like type 1 diabetes and asthma later on (20, 28). In the respiratory tract, very early *Moraxella* enrichment in the first weeks of life was associated with a stronger activation of the local antiviral immune response, and with a higher frequency of symptomatic RTIs in the first year of life (16, 29).

All in all, current evidence suggests that perturbations to the microbial community composition in early childhood may have long-term effects for the host susceptibility to disease. However, direct associations between the early-life microbiota composition and the development of the immune system, as an intermediate player between the microbiome and health outcomes, are less studied. Furthermore, the capacity of the microbiome to recover by itself following a perturbation (i.e., resilience of the microbiome) has not been studied extensively, while this is likely critical to maintaining

good health (30). The primary aims of this thesis were to a) study associations between the early-life development of the microbiota and humoral adaptive immune function, and b) the resilience of the microbiota in children following perturbation from a severe lower respiratory tract infection (LRTI).

KEY FINDINGS OF THIS THESIS

Early-life microbes leave their mark on mucosal and systemic humoral immunity

At this stage, only few human infant studies investigated a link between microbiota development from birth onward and relevant immune functions. In **chapters 4 and 5** of this thesis, we demonstrated associations between early-life microbiota development in a healthy birth cohort (the MUIS study (16)) and antibody responses, an important effector component of both mucosal and systemic immunity. Demonstrating such temporal associations is an important first step to prove causal relationships between the early-life microbiota, immune maturation and health consequences.

Local host-microbiota crosstalk in the upper respiratory tract and infection susceptibility

First, **chapter 4** focused on the nasopharyngeal microbiota and the dynamics of local mucosal IgG, IgA and IgM concentrations in saliva obtained at 11 timepoints in the first year of life. Our primary aim was to explore correlates between the composition of the nasopharyngeal microbiota and the subsequent intrinsic mucosal antibody response. However, unlike other studies that used animal models (31), our prospective, observational design did not allow us to experimentally disconnect the bidirectional interactions between microbial colonization and antibody responses. This aspect combined with the age-dependent dynamics of both the nasopharyngeal microbiota and antibody concentrations confound the interpretation of direct correlations (32), and to overcome these challenges, we took a stepwise approach in the statistical analysis.

First, we aligned nasal expression of genes involved in antibody production with IgG, IgA and IgM concentrations over time. Twice in the first year of life, sharp increases in gene expression were directly followed by overall

increases in antibody concentrations, namely IgM and IgA in the first two weeks, and IgG between months six and nine. This approach allowed us to estimate the onset of endogenous antibody production, which thus seemed to be much earlier for IgA and IgM than for IgG.

We then focused our analysis on nasopharyngeal microbiota development leading up to the induction of week two IgA and month six IgG production, and showed that early enrichment of *Moraxella* and underrepresentation of *Dolosigranulum* and *Corynebacterium* were consistently associated with higher antibody concentrations at these timepoints. Intriguingly, elevated IgA levels at week two and IgG levels at month six were in turn also associated with a higher total number of parent-reported RTIs in the first year of life. High induction of IgG at six months of age was likely reactive to prior RTIs and other characteristics that were in line with a history of higher microbial exposure (i.e. daycare attendance, viral detection, high bacterial load). This was not the case for IgA. Instead, high IgA induction at two weeks of age was only associated with very early *Moraxella* enrichment, which we have previously also associated with stronger interferon signalling (29) and higher RTI susceptibility (16), and underrepresentation of the health-associated commensal *Dolosigranulum*. We hypothesize that initially, high mucosal IgA induction may be an excessive, perhaps nonspecific response to the microbiota, which may reflect inadequate tuning of the (mucosal) immune system by the local microbial community. This may then result in a higher number of symptomatic RTIs. Indeed, colonization of the infant upper respiratory tract with *Moraxella catarrhalis* and *Haemophilus influenzae* has previously been related to topical inflammation (33). Local baseline inflammation in older, immunosenescent mice has also been linked to an inappropriate mucosal innate immune response and subsequent failure to clear the respiratory pathogen *Streptococcus pneumoniae*, which was suggested contribute to the increased susceptibility to pneumococcal infections at older age (34). Nevertheless, we cannot distinguish whether the number of RTIs in the first year of life would have been even higher if early boosting of the mucosal IgA response had not occurred.

Though cause and consequence cannot be exactly unraveled, we concluded that maintaining stable antibody concentrations may be a hallmark of

respiratory health in the first year of life. All in all, mucosal antibodies might thus be an early and easily accessible biomarker of mucosal immune imbalance in the upper respiratory tract.

Long-term effects of microbiota-mediated immune maturation reflected in vaccine responses

Next, **chapter 5** focused on the gut microbiota and antibody responses to routinely administered PCV-10 (at 2, (3,) 4 and 11 months of age) and meningococcus type C (MenC) vaccination (at 14 months of age). Antigen-specific vaccine responses were measured in saliva, but since salivary IgG responses to vaccination correspond well with serum levels, these were considered to reflect systemic immunity (35, 36). Based on the "window of opportunity"-concept, we hypothesized that we would find the strongest associations between the gut microbiota composition in the first weeks of life and vaccine responses to routine vaccinations of the national immunization program starting at two months of age, even though the latter were measured months later at the age of 1-1.5 years. We identified a chain of events, where vaginally born infants had a gut microbiota profile with high relative abundances of *Bifidobacterium* and *Escherichia coli* at the age of one week and higher antibody responses to pneumococcal and meningococcal vaccination later on compared with C-section born infants. This finding supports the hypothesis that the gut microbiota may be a mediator between delivery by means of C-section and immune effects including the increased risk of childhood immune-mediated diseases (37). Interestingly, we found stronger associations of the early-life microbiota with the pneumococcal vaccine response than with the meningococcal vaccine response. While several explanations are possible, we believe that this is most likely due to the different timing of vaccine administration: PCV-10 was first administered at the age of 2 months in our cohort, while MenC vaccination was not given until the age of 14 months. While the immune system appears to be most sensitive to bacterial cues in the earliest phase of life, accumulating evidence also suggests that with time, the effect of initial lack of exposure can be mitigated by appropriate subsequent microbiota maturation. For example, in a large Danish birth cohort, the risk of asthma was only increased in C-section born infants who still retained

C-section-induced microbiota changes by the age of one year (38). Olin and colleagues unexpectedly observed rapid convergence of initially strongly different immune phenotypes in term and preterm born infants onto shared developmental paths, and proposed a framework where (opposing) adaptive responses to multiple, varied antigens ultimately limit the number of possible immune phenotypes (39). So, full understanding of the role of the gut microbiota in the development of immune-mediated disorders with childhood onset requires high-resolution evaluation of longitudinal developmental trajectories of the microbiome with emphasis on the first weeks of life, but continuing throughout childhood.

Distinguishing drivers and consequences

A major challenge we faced with the studies discussed in **chapters 4 and 5**, was clearly distinguishing early-life environmental drivers, microbial community shifts, and consequences for immune parameters in the statistical analysis. For example, in **chapter 4**, we found that breastfeeding was the primary source of IgA until the induction of endogenous production, and early-life breastmilk-derived IgA was, in turn, associated with nasopharyngeal microbiota assembly. This may suggest that early-life IgA influences the formation of the microbiota, which is in line with findings from other studies (40–42). However, breastmilk also contains microbial communities of its own (43). Feeding type could thus be both a potential confounder as well as an independent driver of the effect of IgA on the nasopharyngeal microbial community composition. Reassuringly, statistical significance of the effects persisted when we considered breastfeeding as a confounding factor and included feeding type as an independent variable in multivariable models. A similar issue arose in **chapter 5**, where microbial community types observed at one week of age were primarily driven by mode of delivery, and both were also associated with the outcome, i.e. the antibody response to vaccination. Given the clear temporal succession, we applied the principles of mediation analysis to unravel whether these triangular associations could reflect a sequence of events (44). Indeed, the association between the week-one microbial community types and the immune response to pneumococcal vaccination persisted, regardless of delivery mode. This finding provided additional evidence for a role of the gut microbiota as a mediator between

delivery mode and vaccine responses, and at the same time demonstrated an added value of mediation analysis for longitudinal human microbiome studies.

Molecular mechanisms underlying microbiota-mediated effects on host immunity

The underlying molecular mechanisms of microbiota-induced immune modulation are likely many and complex. The findings presented in **chapters 4 and 5** of this thesis are merely associative in nature, but they align with mechanistic studies from other research groups, which supports the biological plausibility of our results. First, direct interaction of bacterial constituents with epithelial receptors may trigger immunomodulatory pathways. *E. coli* was shown to promote antibody responses to the trivalent inactivated influenza vaccines through TLR5-mediated flagellin sensing that stimulated plasma cell differentiation (45), and was also found to be associated with a higher antibody response to pneumococcal and meningococcal vaccination in **chapter 5**. Second, some bacteria including *Bifidobacterium* species are well-known for their ability to metabolize human milk oligosaccharides and thereby produce short chain fatty acids (SCFA) (46, 47). SCFAs are considered key metabolites that support symbiotic host-microbiota relationships, and the gut microbiome functional capacity for SCFA biosynthesis was demonstrated to be lower in infants who developed type 1 diabetes than in healthy controls (48). *Bifidobacterium* abundance was also associated with higher vaccine immunogenicity in **chapter 5** and other studies (49, 50), and it remains to be investigated whether SCFAs play a role. A different metabolic pathway affecting host immunity to vaccination was described by Hagan and colleagues, who showed that an antibiotic-induced reduction of bile acid metabolism by the gut microbiome activated inflammatory responses and impaired the antibody response to influenza vaccination in healthy adults without pre-existing immunity (51). Third, in mice, bacterial antigens from the gut have been shown to disseminate systemically and trigger the production of antigen-specific IgG and IgA antibodies elsewhere, which were in turn shown to protect against sepsis caused by bacteria that originated from the gut microbiota (52, 53). This phenomenon could underlie some of the correlations between specific

bacterial taxa and antibody induction described in **chapter 4**, though this mechanism has not yet been demonstrated in humans, nor for bacterial antigens originating from the respiratory tract. All in all, it appears to be possible that the associations we found between the early-life microbiota and clinically relevant immune parameters reflect true biological processes.

Lower respiratory tract infections as part of a vicious cycle of host-microbiota interactions

An important property of a healthy microbiota is its capacity to recover to the baseline state after a perturbation, which is called microbiome resilience (30). In **chapters 7 and 8**, we studied the resilience of the nasopharyngeal microbiota following a severe LRTI. During a severe LRTI (i.e. an LRTI requiring hospitalization), nasopharyngeal microbial communities are characterized by (hyper)dominance with potentially pathogenic bacteria like *H. influenzae* and *S. pneumoniae*, and virtual absence of beneficial commensals like *Corynebacterium* and *Dolosigranulum*, regardless of phenotypic characteristics traditionally linked to 'viral' versus 'bacterial' infections (54). Studies using animal models have also shown that experimental respiratory pathogen introduction disrupted the composition of the local microbiota (55, 56). A LRTI can thus be considered a perturbation of the respiratory microbiome. High nasopharyngeal abundances of *Haemophilus* and *Streptococcus* species have also been associated with increased severity of respiratory syncytial virus bronchiolitis, indicating that the extent of the microbial ecosystem perturbation can contribute to inflammation leading to symptoms as well as to the course of disease of this viral infection (57). However, subsequent recovery of the perturbed microbial ecostate, as well as determinants of this recovery and potential clinical consequences of (lack of) microbiota recovery, remain elusive at this point.

Potential drivers of respiratory microbiota recovery following a LRTI

In **chapter 7**, we described both clinical and nasopharyngeal microbiota recovery in young children hospitalized for a LRTI in a case-control study (the MOL study (54)). With stringent matching of LRTI cases with two healthy controls of the same age and sex and within the same season, we were able to approximate microbiological recovery as the discrepancy of the

nasopharyngeal microbiota profiles of the case approximately six (range: four to eight) weeks after hospital admission compared with its two matched controls, as a measure of normalization to an age-appropriate configuration. Using this measure, we observed interesting associations between clinical factors, like antibiotic treatment, LRTI phenotype and age, and microbiota recovery. Overall, children showed a microbial composition comparable to healthy controls six weeks after hospital discharge, regardless of whether they had received antibiotic treatment or not. By contrast, the microbiota of children with the clinical LRTI phenotype of wheezing illness, who were generally older than two years of age, had not (yet) recovered to a state comparable to healthy controls at the follow-up visit, as opposed to children with a bronchiolitis, pneumonia or 'mixed' phenotype. Children with a wheezing illness LRTI also more often experienced new respiratory symptoms within the period between hospital discharge and the follow-up visit. This is in line with epidemiological data showing that children with a wheezing LRTI remain at an increased risk of developing wheezing illness and asthma during childhood (58–60). The group of children who experienced new respiratory symptoms during follow-up also showed reduced recovery of the microbial composition, with persistently increased relative abundances of *Haemophilus* and typically oral Gram-negatives such as *Prevotella* and *Fusobacterium* species as well as reduced abundances of health-associated commensals, and thereby the circle was complete.

In **chapter 8**, we investigated the microbiota of the nasopharynx, saliva and the lower respiratory tract in a small cohort of even more severely ill children who required pediatric intensive care unit (PICU) admission and mechanical ventilation for a LRTI (the MEREL study (54)). These children also all received antibiotic treatment for the LRTI. During the PICU stay, initial hyperdominance of 'classic' respiratory pathogens like *Haemophilus* and pneumococcus shifted to entirely different profiles. When the ventilatory tube was removed after a few days, microbiota profiles were characterized by high relative abundances but a low bacterial load of *Staphylococcus*, Enterobacteriaceae*, Stenotrophomonas* or non-pneumococcal streptococci, which we attributed to antibiotic selection of resistant species and/or introduction from the intensive care environment through the ventilatory tube. Despite this strong microbiota shift during mechanical ventilation, we

observed quite 'normal' nasopharyngeal and salivary microbiota profiles at the follow-up visit approximately two months after discharge from the intensive care. When we compared the nasopharyngeal microbiota profiles to age-matched healthy controls (taken from a different study, since we lacked a direct control group for the intensive care cohort), no differences in the overall composition were apparent anymore (results not reported in the chapter). This finding might indicate that the upper respiratory tract microbiota also showed a good recovery after intensive care treatment for a LRTI. Taken together, the degree to which the microbiota are perturbed during the acute infection, the clinical severity of a LRTI, and/or antibiotic treatment thus did not appear to restrain the recovery of the upper respiratory microbiota to an age-appropriate composition. In contrast, in the subgroup of (generally older) children hospitalized for a wheezing illness LRTI, microbiota recovery was more limited or slower. This finding suggests impaired resilience of the microbiota in this group, potentially as a consequence of underlying (chronic) immune-mediated/inflammatory processes and/or prior corticosteroid use present in these children.

Reduced microbiota resilience may precede a critical transition to an alternative ecostate

The human microbiota forms a complex, dynamical ecosystem. Variation in the microbiota composition is large between as well as within individuals over time, which hampers the definition of a 'normal' or 'healthy' microbiota (1). At this stage, we are not yet able to use our accumulating knowledge of the microbiota to predict, let alone prevent, disease outcome for the individual patient. For this purpose, we may need to discover early markers of critical bifurcations where the microbial ecosystem passes a threshold and shifts to a contrasting ecostate that is associated with a negative clinical outcome, like asthma. Scheffer and colleagues formulated a theory of generic early-warning signals for such transition points in dynamical systems that can also be applied to the microbiome (61). This theory states, among others, that complex systems (e.g. the microbiome) show "critical slowing down" before reaching a transition point, which is reflected in increasing correlation of the state of the system at consecutive timepoints, increasing variance and slower recovery to baseline after a perturbation (61).

The results from **chapters 7 and 8** support this theory. Impaired microbiota resilience in children recovering from a wheezing illness LRTI, might reflect "critical slowing down" as a sign that the microbiota of these children is nearing a transition point towards an alternate microbial ecostate. Once extrinsic and intrinsic forces push the respiratory microbiota over this point to this alternate ecostate, this may in turn affect the host in various ways (e.g. by producing certain metabolites or inducing inflammation) with potential health effects (62). In this case, wheezing LRTIs in particular are associated with asthma development (63, 64). Hence, we speculate that the observed "critical slowing down" might predict a clinically relevant transition point of the microbiome to an alternate ecostate associated with asthma. Greater fluctuations over time are another characteristic of a complex system nearing a shift to an alternate ecostate, because consecutive perturbations have additive effects when recovery is slow. We may also have observed this phenomenon in **chapter 7**, where children who had again experienced new respiratory symptoms particularly demonstrated reduced microbiota recovery 4-8 weeks after a severe LRTI compared with children who remained asymptomatic in this period. This may reflect a vicious cycle where slower microbiota recovery after a LRTI predisposes to the recurrence of respiratory symptoms upon new pathogen exposure, which further disrupts the microbial ecosystem, and so on (65). Testing this hypothesis requires large, longitudinal studies with frequent clinical phenotyping and microbiota characterization of pediatric (wheezing illness) LRTI patients.

Another example of microbiome perturbation is antibiotic treatment. Nevertheless, in **chapter 7**, the subset of children who received antibiotic treatment overall showed adequate microbiota recovery. The microbiota of these children thus proved to be resilient to the antibiotics, or the antibiotics might even have 'helped' to reconstitute the original microbiota. In other cases, antibiotics may be intended to correct the microbiota to the original ecostate, but without success, due to a resilient dysbiotic state of the microbiota (30). In patients with cystic fibrosis (CF), establishment of chronic *Pseudomonas aeruginosa* colonization in the lung causes frequent pulmonary exacerbations and a steady decline in lung function. Antibiotic treatment targeted at this pathogen often ameliorates clinical symptoms for a short while. However, at the ecological level, antibiotic treatment

was shown to lead to an increase rather than a decrease in *P. aeruginosa* abundance, which was followed by a recovery of the microbiota to the pre-treatment composition within 30 days (66). Presence of *P. aeruginosa* in the CF microbiota may mark an alternative stable ecostate that is associated with worse disease outcome and is difficult to reverse permanently by antibiotic therapy.

In summary, limited ability of the respiratory microbiota to recover to a pre-perturbation state after an infection might be an early warning sign for a future transition to an alternate and possibly undesirable ecostate, which is at least difficult and perhaps even impossible to revert. Hence, we might be better able to predict an individual's risk of certain complications or long-term health problems if we could measure microbiota resilience. However, studying resilience ideally requires frequent sampling of the same individuals in a long time series starting before a perturbation, which may, for example, be feasible for CF patients followed from birth, but not for healthy children who may or may not develop a LRTI. In the latter case, our approach to assess microbiota resilience in post-LRTI patients by comparison with healthy, matched controls may be an option to consider in future studies.

Opportunities for using saliva as a specimen for infection and immunity research

The results presented in this thesis are in part gained from the analysis of saliva specimens: in **chapters 4 and 5** to measure either total or vaccine antigen-specific antibody concentrations with fluorescent bead-based multiplex immunoassays, and in **chapter 8** to characterize the oral microbiota composition with 16S rRNA gene sequencing. Saliva offers attractive practical advantages, especially in children. Collection of saliva is simple, non-invasive and does not require assistance from a health care professional. This reduces fear and discomfort experienced by children, which increases the likelihood that they will cooperate, especially when repeated samples are needed. Here, we take a small sidestep to make a plea for the more widespread use of saliva in pediatric infectious diseases and immunology research based on the findings also presented in **chapters 6 and 9** of this thesis.

Serum IgG antibody titers and seroconversion remain the golden standard to assess the efficacy of many vaccines and different dosing schedules to evaluate possible changes to national immunization programs (67, 68). In **chapter 6**, we compared anti-pneumococcal antibody concentrations in saliva between infants participating in our healthy birth cohort study, who had received either three (at 2, 3 and 4 months of age) or two (at 2 and 4 months of age) primary doses of the 10-valent pneumococcal conjugate vaccine due to a change in the national immunization program. We found only minor decreases in the salivary antibody response after the booster dose (at 11 months of age for both groups) in the group with the two-dose schedule. The neutralizing IgG antibody response is the primary immune effector of the pneumococcal vaccination (69), which does not only provide protection against invasive infections, but also prevents pneumococcal acquisition in the upper respiratory tract. This suggests that systemic IgG antibodies transported to saliva may play a direct role in protection from pneumococcal infection (70–72). Monitoring salivary antibody responses to pneumococcal vaccination would require knowledge of a surrogate of protection against infection, which has previously been estimated for MenC vaccination (73), but not yet for pneumococcal vaccination. Finding this surrogate could pave the way for a more prominent role of saliva in pneumococcal vaccine trials, in particular when they concern large-scale vaccination campaigns or populations where drawing blood is difficult (e.g. young children).

For respiratory pathogen detection in children with a RTI, the deep nasopharyngeal swab remains the golden standard, which most children experience as uncomfortable. At the onset of the Coronavirus Disease 2019 (COVID-19) pandemic, the sudden need for high volume viral testing attracted attention to more patient- and child-friendly sample collection methods. This led to a heap of studies demonstrating a comparable or even higher sensitivity for detecting severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in saliva compared with nasopharyngeal swabs in both adults and children (74–76). In the same period, we initiated a similar pilot study comparing different sampling methods in the pediatric population, but we took a broader approach testing for a range of

respiratory viral and bacterial pathogens (**chapter 9**). Though we did not detect SARS-CoV-2 in our study population (children were largely unaffected in the first wave of COVID-19 in The Netherlands (77)), we found an overall sensitivity of respiratory pathogen detection in saliva of 94%, comparable to nasopharyngeal and oropharyngeal swabs, provided that we used sponges or drooling collection methods rather than swabs. In our subsequently initiated household SARSLIVA-study, repeated self-collection of saliva at home by family members of a laboratory-confirmed COVID-19 patient provided detailed insight into viral transmission within the household (78). Moreover, the study participants, including children, found the frequent self-collection of saliva manageable and preferable to nasopharyngeal swabs (unpublished data). With the persisting threat of SARS-CoV-2 and perhaps future outbreaks of new respiratory viruses, viral testing may remain crucial for effective infection control and child-friendly sampling methods remain highly relevant. More research in children may enable the ranking of different sample types, alone or in combination, for respiratory pathogen testing in different settings (e.g. hospital vs. community) and populations (e.g. asymptomatic vs. symptomatic, younger vs. older), taking into account the degree of (dis)comfort experienced by the child and their caregivers.

For early-life microbiota studies, **chapter 8** again underlined that nasopharyngeal samples are likely more representative of the (lower) respiratory microbiota than saliva (13). Nonetheless, saliva is a very informative substance that can be used to assess relevant outcomes such as antibody concentrations or viral presence, which is particularly attractive in longitudinal studies with repeated measurements over time at high resolution.

FUTURE PERSPECTIVES

Moving from correlation to causation

Knowledge of the human early-life microbiota continues to expand at a rapid pace, but the translation of this knowledge into practical applications is still in its infancy. Longitudinal studies with prospective follow-up from birth and extensive phenotyping now begin to provide important insight into the intrinsic dynamics of the microbiota in relation to immune maturation

and disease susceptibility, which is the first step to establish causal links in humans. Examples are **chapters 4 and 5** of this thesis, where the design of our cohort of healthy infants with longitudinal follow-up from birth allowed us to carefully disentangle associations between early-life host factors (e.g. mode of delivery), subsequent microbiota maturation, and immune effectors later on (e.g. vaccine response) with a clear temporal succession, which is not definite proof but could help to understand causal links. Similar birth cohorts with longitudinal microbiota follow-up also exist of infants who are genetically predisposed to the development of immune-mediated diseases like asthma (14) and diabetes (17) and of infants who were exposed to a perturbating factor like antibiotics in early life (79). Meta-analysis of different microbiota birth cohort studies would provide an additional layer of evidence, but is complex because of the many confounders introduced by differences in study design and setting (e.g. sampling methods, geographic region) as well as laboratory methods (e.g. DNA isolation and sequencing protocols), as explained in **chapter 3**. Nonetheless, the current drive of the scientific community to publicly share raw sequencing data already makes it much more feasible to harmonize different data sets by using increasingly advanced bioinformatic techniques (80, 81). Such analyses that combine, compare and contrast different cohorts may reveal both generic and cohort-specific associations between key players in the microbiota and health outcomes in childhood.

For mechanistic support of correlative findings linking bacterial taxa to clinical outcomes, researchers tend to turn to animal studies. Antibiotic perturbation studies in infant mice have shown microbiota-mediated effects on host immunity, metabolism and even lifespan that were independent from the antibiotics themselves (82–84). The controlled transplantation of defined consortia resembling the neonatal microbiota in mouse models to assess their dynamics and effects on the host, represents another, new line of investigation which may prove enlightening in the future (85). Although murine models allow for some experimental control of host effects, they also suffer from specific limitations, including cage effects and coprophagy (ingesting feces), and have a profoundly different microbiota composition as well as immune forces acting on the microbiota than humans, hampering the translation of results to the human situation. In an upcoming *in natura*

approach, taxonomic data is aligned with other readouts reflecting microbial activity, such as transcriptomics, metabolomics and proteomics. Such integrated analysis of multi-omic studies requires careful interpretation of the different data layers in light of each other, but can yield multilevel insight into molecular pathways linking microbial shifts via metabolic or host gene expression changes to a disease outcome (29). However, causal pathways can still only be derived if the human study participants are exposed to an intervention, which can be antibiotics, pro- or prebiotics, vaccination or even pathogen exposure using a human challenge model (86). Lastly, when multiple epidemiological microbiota studies point towards bacteria that may be highly beneficial to human health, this warrants in-depth species-specific studies. One such species is *Dolosigranulum pigrum*, which has consistently been associated with respiratory health and is considered a candidate for live biotherapeutic agents (15, 16, 87). Detailed *in vitro* experiments of *D. pigrum* revealed that this lactic acid bacterium can indeed enhance nasal barrier function, dampen inflammatory responses and exert antimicrobial activity against *Staphylococcus aureus*, and, when cooperating with *Corynebacterium*, also against *S. pneumoniae* (88, 89). These and other confirmations of biological underpinnings for correlative observations like those presented in this thesis, strengthen the body of evidence for functional host-microbiota interactions.

Translation to the clinic

Making the move to microbiota modulation for health benefits would not only require a thorough functional understanding of the microbial ecosystem, but also a workable definition of a desired target endpoint. Defining a 'healthy' microbiome has proven exceptionally difficult (1, 62). Some have argued that microbiotas follow the 'Anna Karenina principle' adapted from Leo Tolstoy – while health-associated microbiotas are all alike, microbiotas associated with disease states are all different in their own way (90). We observed this phenomenon in **chapter 5**, where the microbiota community types that were associated with higher vaccine responses had a relatively homogeneous composition (predominated by either *E. coli* or *Bifidobacterium*), while the composition of the microbiota associated with lower vaccine responses strongly varied between infants. Besides specific

microbiota configurations, age-appropriate microbiota maturation may be a hallmark of health, especially in the youngest pediatric population: several studies calculating a microbiota 'age' showed that maturation was delayed in infants with more frequent RTIs (16) or with severe malnutrition (91) compared with their peers. Analogously, we associated very early *Moraxella* predominance in the nasopharynx, which would normally occur around the age of three months, with stronger mucosal IgA induction and more frequent RTIs in **chapter 4.** Constructing a growth curve of normal microbiota development, as is common for other child developmental parameters like weight and height, might be an interesting option to detect abnormalities (early). As outlined above in our discussion of **chapters 7 and 8**, it has also been proposed that the resilience of the microbiota in response to a perturbation like a LRTI is an indicator of health (30). However, assessing this in an individual patient requires repeated sampling both before and after the perturbation, which is often not feasible in clinical practice. Instead, compiling a population-scale public health database that allows for the comparison of an individual's microbiota after a perturbation to a selection of microbiotas from a group of carefully matched peers, might be a solution in the future (92).

Although exact therapeutic microbiologic goals remain to be defined, interventions that have already proven to alter the infant gut microbiota composition range from seeding of C-section born infants with maternal fecal or vaginal microbiota soon after birth (93, 94), via live therapeutic agents for preterm infants (95) to prebiotics added to formula (96). Long-term health benefits remain elusive at this stage and will take time to evaluate, but it is likely that ongoing investigations will shed light on the consequences of early-life microbiota modulation. The findings presented in this thesis have three main implications for microbiota-directed intervention trials. First, **chapter 7** pointed towards nasopharyngeal enrichment with Gram-negatives like *Haemophilus* and less abundant typically oral bacteria combined with decreased abundance of health-associated commensals, as potential mediators of worse respiratory outcomes after a LRTI. Previously, especially higher *Haemophilus* abundance during a LRTI had consistently been associated with inflammation, increased viral load, prolonged viral presence, and increased illness severity (57, 97–99). Furthermore, influx

of typically oral bacteria in the nasopharynx has also been associated with increased RTI susceptibility (100). Future studies should determine whether Gram-negatives like *Haemophilus*, *Prevotella* and *Fusobacterium* might be biomarkers of ongoing respiratory morbidity in pediatric post-LRTI patients, and whether antibiotic or pre/probiotic interventions targeted at the containment of these species and/or the support of beneficial commensals, might alleviate some of the burden. However, the potential of respiratory microbiota manipulation has barely been explored to date and presents specific challenges (101). Second, in **chapters 4 and 5**, microbiota configurations in the first 7 days of life seemed to be the most relevant for the immune effectors we studied, again underlining that the immune tone is already set as early as the first weeks of life. This finding suggests that interventions may be most effective when administered very early in life, in line with the 'window of opportunity'-hypothesis. Third, we have demonstrated associations between the microbial composition and clinically relevant outcomes like (vaccine-induced systemic and natural mucosal) antibody responses or recurrence of respiratory infection symptoms in **chapters 4, 5 and 7**. These outcomes can be easily measured in the short term and are very common in the pediatric population, which offers major advantages like smaller sample sizes and shorter follow-up duration compared with trials with a long-term, rare outcome such as the development of chronic, and/or immune-mediated disorders. The antibody response to vaccination has even been implicated as an early predictor of other future health parameters: two-year-long monitoring of mice who received antibiotics early in life and subsequently followed an aberrant microbial maturation trajectory, revealed that these mice did not only mount lower antibody responses to vaccination, but also had higher insulin resistance, more inflammation, and a reduced lifespan than antibiotic-naïve mice (82). Importantly, antibody levels can even be easily measured in a non-invasive manner if saliva is used.

Take home message for clinicians

Although, as aforementioned, the influence of the early-life microbiota on immune maturation in infancy with health effects that may last a lifetime, still needs to be mechanistically proven in humans, the immense body

of evidence has become difficult to ignore. Therefore, at this point, our main message to clinicians is to make an effort to cherish the early-life microbiota in daily practice. Practices that disrupt the vertical mother-infant transmission as well as the maintenance of early-life microbes are highly abundant nowadays (102). C-sections on demand are increasingly common in certain parts of the world and form an avoidable threat to the microbiota-immune axis (37). Antibiotics are often prescribed to newborns with signs of an infection that prove to be innocent later on, and we encourage the use of risk stratification tools including sensitive biomarkers of early-onset sepsis to reduce unnecessary antibiotic use (103). Breastfeeding has many obvious health benefits including effects on the microbiota and should be stimulated, and should be replaced by infant formula resembling breast milk when breastfeeding is not possible (96). Notwithstanding that practices like emergency C-sections and antibiotics for suspected early-onset sepsis can of course be lifesaving, weighing short- and long-term microbial and immunological effects might still lead to a reduction of their use without medical urgency. Safeguarding critical early-life microbes is likely to prove beneficial to the health of future generations.

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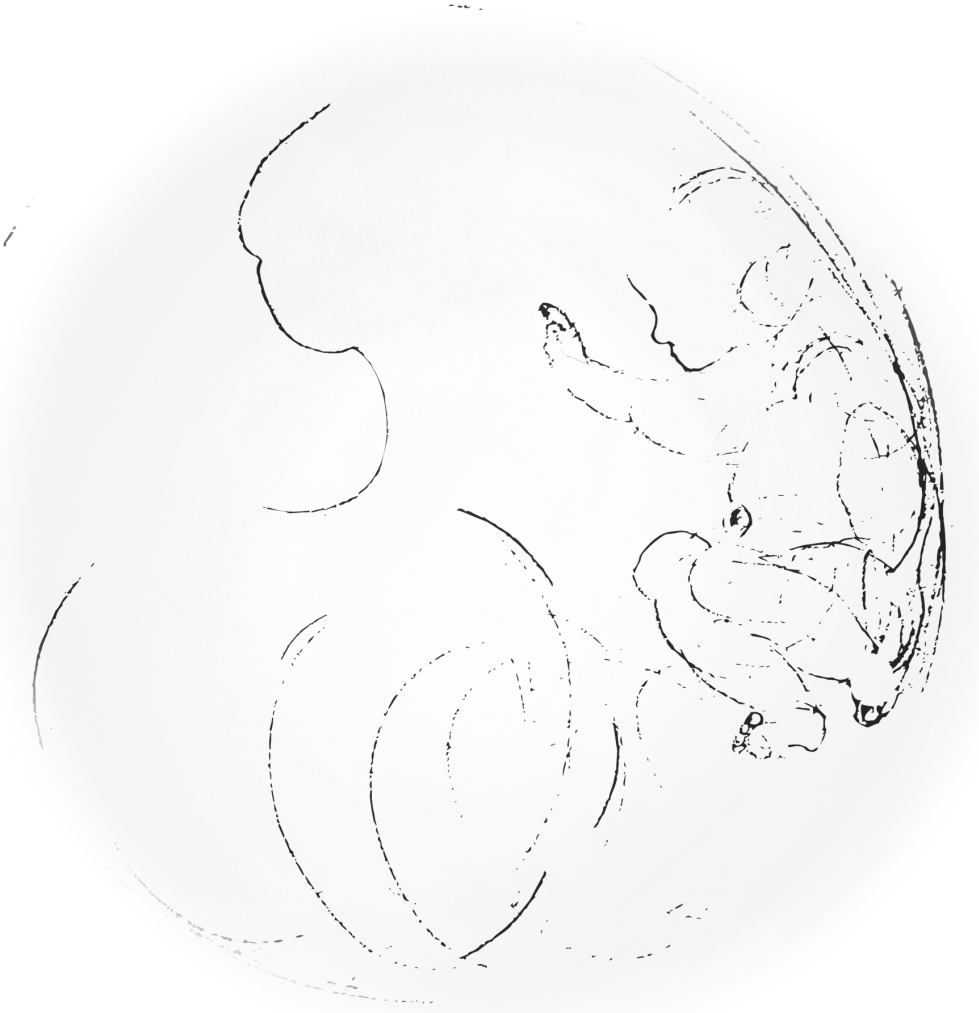

APPENDICES

NEDERLANDSE SAMENVATTING

Introductie

Ongezien leeft de mens samen met een ontelbaar aantal bacteriën, die voorkomen op de slijmvliezen van de darmen en de luchtwegen en op de huid. Samen vormen de koloniserende bacteriën het menselijke (bacteriële) microbioom. Dankzij de komst van nieuwe moleculaire technologie die het mogelijk heeft gemaakt om deze bacteriën nader te bestuderen en te classificeren op basis van hun DNA (zie ook **hoofdstuk 3**), hebben we in de afgelopen 15 jaar veel geleerd over de samenstelling van het microbioom en tal van belangrijke functies die het microbioom vervult voor onze gezondheid op korte en lange termijn. Bacteriën en andere micro-organismen zoals virussen, schimmels en parasieten ontwikkelden zich vele miljoenen jaren eerder dan de mens en daarom is het niet meer dan logisch dat het microbioom de menselijke evolutie heeft beïnvloed en belangrijke functies vervult voor de gezondheid van de mens. Het microbioom speelt bijvoorbeeld een rol bij de vertering en productie van belangrijke voedingsstoffen, beschermt tegen kolonisatie en infectie door pathogenen en beïnvloedt het functioneren van het immuunsysteem.

Een pasgeboren baby verkrijgt diens eerste flora van de moeder tijdens de passage door het geboortekanaal. Dit microbiële “startpakket” is vrij uniform, wat betekent dat vlak na de geboorte de samenstelling van het microbioom op verschillende plekken (niches) van het babylichaam, zoals de darmen, de huid en de luchtwegen, nog tamelijk hetzelfde is. Echter al snel daarna past het microbioom zich aan aan lokale omstandigheden in de verschillende niches, onder invloed van bijvoorbeeld temperatuur, vochtgehalte en de beschikbaarheid van voedingsstoffen. Zo ontwikkelen zich niche-specifieke verschillen in de samenstelling van het microbioom. Bij drie maanden oude baby’s zijn de darmen in grote mate gekoloniseerd met bacteriën van de genera *Bifidobacterium* en *Escherichia*, terwijl bacteriën van de genera *Moraxella, Corynebacterium* en *Dolosigranulum* vooral voorkomen in de (bovenste) luchtwegen.

In de darmen en de luchtwegen is er nauw contact tussen de lokale microbiota en cellen van het immuunsysteem en de samenstelling van het microbioom is geassocieerd met de ontwikkeling van het immuunsysteem

en de gezondheid van een kind op langere termijn. Een afwijkende microbioom samenstelling op jonge leeftijd is bijvoorbeeld geassocieerd met een hogere gevoeligheid voor luchtweginfecties, maar ook met een toegenomen risico op chronische ziektes die gekenmerkt worden door afwijkend functioneren van het immuunsysteem, zoals astma, allergieën of suikerziekte. Steeds meer studies laten zien dat vooral het microbioom in de allereerste levensweken, wanneer ook het immuunsysteem grote ontwikkelingen doormaakt, geassocieerd is met de gezondheid van een kind op latere leeftijd. De samenstelling van het microbioom is, juist in deze eerste levensfase, ook heel gevoelig voor invloeden van buitenaf. Een kind dat middels een keizersnede geboren wordt, mist de eerste blootstelling aan moeders vaginale en darmflora tijdens de passage door het geboortekanaal, en begint daardoor met een andere microbioom samenstelling dan een kind dat geboren wordt middels een vaginale bevalling. Borstvoeding bevat goede bacteriën en voedingsstoffen en stimuleert daarmee een microbioom samenstelling die we associëren met gezondheid, terwijl antibiotica het microbioom juist in ongunstige zin aantasten. Uiteraard zijn een keizersnede en antibiotica vaak medisch noodzakelijk, maar door het effect dat deze blootstellingen hebben op de samenstelling van het microbioom in het vroege leven, beïnvloeden ze mogelijk tevens de gezondheid van het kind op de korte en langere termijn. Ook na de eerste levensweken blijft de samenstelling van het microbioom geassocieerd met gezondheid en ziekte; zo geeft een afwijkende samenstelling van het microbioom van de (bovenste) luchtwegen een verhoogd risico op (ernstige) luchtweginfecties in de opvolgende jaren. In **hoofdstuk 2** hebben we een samenvatting geschreven over de bestaande kennis over het verband tussen het microbioom van de luchtwegen in het vroege leven en ziekte en gezondheid. Dit proefschrift borduurt hierop voort.

Interacties tussen het microbioom van de luchtwegen in het vroege leven en lokale antistoffen

In **hoofdstuk 4** bestudeerden we de samenhang tussen factoren waar een pasgeboren kind aan blootgesteld wordt (zoals borstvoeding), de ontwikkeling van het microbioom van de neus en het verloop van de totale concentratie antistoffen in speeksel in het eerste levensjaar. Hiervoor maakten we gebruik

van gegevens uit de 'Microbiome Utrecht Infant Study', oftewel de MUIS studie. Aan deze studie namen 130 gezonde, pasgeboren baby's deel, van wie op elf vooraf vastgestelde momenten, tussen twee uur na hun geboorte tot hun eerste verjaardag, een diepe neuswat, speeksel, huidwat en ontlasting verzameld werden. Daarnaast werd informatie verzameld over onder andere geboortewijze, borstvoeding, antibioticagebruik en luchtweginfecties. Uitscheiding van antistoffen in speeksel biedt bescherming tegen bacteriën en virussen die een luchtweginfectie kunnen veroorzaken. Er bestaan verschillende typen antistoffen, waaronder immuunglobuline A (IgA), dat vooral voorkomt op slijmvliezen zoals in de mond en de darm, en immuunglobuline G (IgG), dat vooral voorkomt in het bloed, maar ook wordt uitgescheiden in speeksel. Wij verwachtten dat blootstelling van baby's aan veel verschillende, gezondheids-bevorderende bacteriën in de neus zou leiden tot hogere concentraties van IgA en IgG in speeksel en daarmee een betere bescherming tegen luchtweginfecties. Deze hypothese bleek deels juist en deels onjuist.

Ten eerste zagen we bij baby's die (deels) gevoed werden met moedermelk in de eerste weken na de geboorte, hogere IgA concentraties in speeksel vergeleken met baby's die alleen flesvoeding kregen. Moedermelk bevat veel IgA, wat de baby beschermt tegen infecties in de eerste levensfase, wanneer de eigen antistofproductie nog op gang moet komen. Het verband tussen voedingswijze en speeksel IgA concentraties verdween na de leeftijd van twee weken, wat erop kan duiden dat de eigen productie van IgA na 14 dagen op gang was gekomen. Dit werd ondersteund door een toename van de expressie van genen die een rol spelen bij antistof productie op de leeftijd van 14 dagen. Ten tweede zagen we dat baby's met hoge speeksel IgA concentraties direct na de geboorte, op jongere leeftijd al een groter gehalte aan gezondheids-bevorderende bacteriën zoals *Corynebacterium* soorten en *Dolosigranulum pigrum* in het microbioom hadden dan baby's met lage speeksel IgA concentraties direct na de geboorte. Uit eerder onderzoek was reeds bekend dat een luchtwegmicrobioom met een hoge aanwezigheid van *Corynebacterium* soorten en *D. pigrum* mogelijk beschermend is tegen het doormaken van veel luchtweginfecties gedurende het eerste levensjaar. Deze bevindingen tezamen suggereren dat de bekende beschermende werking van borstvoeding tegen luchtweginfecties ten dele berust op de aanwezigheid van IgA in de moedermelk direct na de geboorte,

wat mogelijk een vroege ontwikkeling van een luchtwegmicrobioom met veel gezondheids-bevorderende bacteriën stimuleert. Ten derde vonden we hogere speeksel IgA concentraties op de leeftijd van twee weken en hogere IgG concentraties op de leeftijd van zes maanden bij kinderen die betrekkelijk veel luchtweginfecties doormaakten in het eerste levensjaar en die een luchtwegmicrobioom hadden met weinig *Corynebacterium* soorten en *D. pigrum.* Deze associatie tussen hogere concentraties van IgA rond twee weken en later ook IgG en meer luchtweginfecties was tegenovergesteld aan wat we vooraf hadden verwacht. Een mogelijke verklaring is dat een ongunstigere microbioom samenstelling en meer luchtweginfecties de antistofproductie juist in grotere mate stimuleren. Een beperking van dit onderzoek is dat het MUIS geboortecohort een relatief gezond cohort is en er geen kinderen meededen die extreem lage antistofconcentraties hadden en om die reden heel gevoelig zouden kunnen zijn voor het ontwikkelen van luchtweginfecties.

Het darmmicrobioom van de pasgeborene beïnvloedt de werking van vaccinaties

De opkomst van vaccinaties in de 20e eeuw heeft de morbiditeit en mortaliteit als gevolg van diverse ernstige infectieziekten op de kinderleeftijd verder teruggedrongen, nadat betere hygiëne, voeding en behuizing de totale sterfte al belangrijk hadden gereduceerd. Een belangrijk mechanisme waarmee vaccinaties beschermen tegen specifieke ziekteverwekkers is door het opwekken van een IgG antistofrespons in het bloed. Hoewel vaccins vrijwel altijd hoge individuele bescherming bieden tegen infectieziekten, bestaan er aanzienlijke verschillen in de hoogte van de antistofrespons van individuele kinderen. Het is niet geheel duidelijk wat die verschillen veroorzaakt. In **hoofdstuk 5** onderzochten we of het darmmicrobioom hiermee geassocieerd kon zijn, waarbij we opnieuw gebruik maakten van gegevens van de MUIS studie. Specifiek onderzochten we of de samenstelling van het darmmicrobioom in het vroege leven samenhing met de IgG antistofrespons tegen het 10-valente pneumokokkenconjugaatvaccin (die de kinderen kregen op de leeftijd van 2, 3, 4 en 11 maanden) en het meningokokken C conjugaatvaccin (die kinderen kregen op de leeftijd van 14 maanden). Beide vaccins zijn onderdeel van het Rijksvaccinatieprogramma

en bieden bescherming tegen ernstig verlopende infecties veroorzaakt door pneumokokken en meningokokken. Ons onderzoek liet zien dat de antistofrespons gemeten in speeksel op beide vaccins minder hoog was bij kinderen die geboren waren middels een keizersnede dan kinderen die middels een vaginale bevalling ter wereld waren gekomen. Verder was er een verschil in samenstelling van het darmmicrobioom, waarbij een hogere mate van aanwezigheid van *Bifidobacterium* soorten en *Escherichia coli* op de leeftijd van één week geassocieerd was met een hogere antistofrespons, vooral na de pneumokokkenvaccinaties. *Bifidobacterium* soorten en *E. coli* op de leeftijd van één week kwamen op hun beurt meer voor bij kinderen die geboren waren middels een vaginale bevalling. Deze bevindingen tezamen suggereren daarom dat geboortewijze effect heeft op het darmmicrobioom dat vervolgens een afdruk achterlaat op de functie van het immuunsysteem en de respons op vaccinaties. Verrassend was dat het darmmicrobioom op de leeftijd van één week het meest sterke verband toonde met de antistofrespons op vaccinaties later in het leven, vanaf de eerste verjaardag: de laatste dosis van het pneumokokkenvaccin werd gegeven rond 11 maanden waarna we de antistoffen bepaalden op de leeftijd van 12 maanden en het meningokokken C werd gegeven rond 14 maanden waarna we de antistofrespons bepaalden op 18 maanden. Deze bevinding steunt de hypothese dat het darmmicrobioom vlak na de geboorte nog altijd invloed uitoefent op het immuunsysteem later in het leven, met mogelijk langdurige consequenties voor de gezondheid. Onze bevindingen zijn echter alleen associaties en verder onderzoek is nodig om een oorzakelijk verband aan te tonen.

Microbioom herstel na ernstige onderste luchtweginfecties en het risico op terugkerende luchtwegklachten

Infecties van de bronchiën en longen, zogenoemde onderste luchtweginfecties, zijn nog altijd één van de belangrijkste oorzaken van overlijden van jonge kinderen wereldwijd. Klassiek wordt vaak onderscheid gemaakt tussen een virale of een bacteriële oorzaak, maar in de praktijk is het een samenspel. Eerder onderzoek heeft aangetoond dat het luchtwegmicrobioom bij kinderen ten tijde van een onderste luchtweginfectie, ongeacht de veronderstelde verwekker, sterk afwijkt van dat van gezonde kinderen.

Daarnaast is reeds bekend dat een samenstelling van het microbioom van de bovenste luchtwegen (neus en keel) waarbij van jongs af aan een groot gehalte aan bekende bacteriële luchtwegpathogenen, zoals pneumokokken en *Haemophilus influenzae* worden gezien, predisponeert voor frequentere en ernstigere luchtweginfecties. Deze bevindingen impliceren dat de samenstelling van het microbioom van de bovenste luchtwegen een rol speelt in de pathogenese van onderste luchtweginfecties. In **hoofdstuk 7** borduurden wij voort op dit onderzoek en bestudeerden wij het herstel van het microbioom in de neus na het doormaken van een ernstige onderste luchtweginfectie en mogelijke determinanten van dit herstel. Hiervoor maakten we gebruik van gegevens van de 'Microbioom Onderste Luchtweginfecties' (MOL) studie. In deze studie werd het microbioom van de neus van 154 kinderen vanaf de leeftijd van zes weken tot vijf jaar, die opgenomen waren geweest in het ziekenhuis vanwege een ernstige onderste luchtweginfectie (patiënten), vergeleken met dat van twee gezonde kinderen van dezelfde leeftijd en hetzelfde geslacht en op dezelfde tijd van de opname (gezonde controles). Het microbioom van de neus van de controles diende als vergelijking voor een 'normaal', gezond microbioom voor de gepaarde patiënt. Over het algemeen vertoonde de patiënten groep als geheel vier tot zes weken na de ziekenhuisopname een goed herstel van het microbioom, dat wil zeggen dat het microbioom vergelijkbaar was met de gezonde controles, ongeacht of er antibiotische behandeling had plaatsgevonden tijdens de ziekenhuisopname. Wel vertoonde de subgroep van kinderen die waren opgenomen vanwege een onderste luchtweginfectie getypeerd door benauwdheid met piepende uitademing ten gevolge van bronchusobstructie, een minder goed herstel van het microbioom. Ziekenhuisopname vanwege een dergelijke ernstige onderste luchtweginfectie met bronchusobstructie is geassocieerd met de diagnose astma op latere leeftijd. De ontwikkeling van astma is ook geassocieerd met een afwijkend microbioom. Het niet herstellen tot een samenstelling zoals bij gepaarde, gezonde controles na een ernstige onderste luchtweginfectie met bronchusobstructie zou kunnen duiden op betrokkenheid van een afwijkend luchtwegmicrobioom bij astma. In de eerste weken na ontslag uit het ziekenhuis werd gezien dat twee derde van de patiënten opnieuw luchtwegklachten ontwikkelde. Dit percentage lag zelfs nog hoger bij de kinderen die een onderste luchtweginfectie met

bronchusobstructie hadden doorgemaakt. Terugkerende luchtwegklachten gingen op hun beurt samen met een luchtwegmicrobioom met een hogere aanwezigheid van *H. influenzae* alsook anaerobe, gramnegatieve bacteriën zoals die van de genera *Prevotella* en *Fusobacterium*. Dit verschil was al aanwezig tijdens de ziekenhuisopname en persisteerde na de herstelperiode van vier tot zes weken. Samen suggereren deze bevindingen een vicieuze cirkel waarbij een afwijkende samenstelling van het luchtwegmicrobioom tijdens een ernstige onderste luchtweginfectie geassocieerd is met terugkerende luchtwegklachten, waarna afwijkingen in het luchtwegmicrobioom persisteren. Deze afwijkende luchtwegmicrobioom samenstelling en verminderd microbioom herstel na een ernstige luchtweginfectie lijken ook geassocieerd met de diagnose astma op later leeftijd, maar nader onderzoek is nodig om hier een uitspraak over te kunnen doen.

Sterke veranderingen in het luchtwegmicro-bioom tijdens een intensive care opname voor ernstige onderste luchtweginfecties

Een zeer kleine groep kinderen met een ernstige onderste luchtweginfectie behoeft een intensive care opname met mechanische ventilatie. Bij deze kinderen is het mogelijk om via de beademingsbuis materiaal uit de lagere luchtwegen te verkrijgen en om daarin het microbioom van de lagere luchtwegen te bestuderen. In **hoofdstuk 8** onderzochten we veranderingen in het microbioom van de neus, het speeksel en de lagere luchtwegen bij 29 kinderen die aan de beademing lagen vanwege een ernstige onderste luchtweginfectie. Deze kinderen namen deel aan de 'Microbioom Ernstige Luchtweginfecties' (MEREL) studie. We vonden dat ten tijde van de beademing de meer bekende luchtwegpathogenen zoals *H. influenzae* en pneumokokken overheersend aanwezig waren in zowel de bovenste als de lagere luchtwegen. Enkele dagen later, na klinisch herstel en het afkoppelen van de beademing, werd een geheel andere microbioom samenstelling gevonden met een sterk gedaalde totale hoeveelheid bacteriën (waarschijnlijk als gevolg van behandeling met antibiotica) en overheersing van *Staphylococcus* soorten, Enterobacteriaceae, *Stenotrophomonas maltophilia* of streptokokken anders dan pneumokokken. Deze bacteriën zijn vaak resistent voor antibiotica en komen veel voor in een ziekenhuisomgeving, en waren dus waarschijnlijk geïntroduceerd en/of uitgeselecteerd tijdens de ziekenhuisopname.

Desalniettemin observeerden we na een herstelperiode van ongeveer twee maanden, dat de microbioom profielen in de neus van de kinderen weer vergelijkbaar waren met die van gezonde kinderen van dezelfde leeftijd. Dit suggereert dat het microbioom van deze kinderen een grote veerkracht vertoont, ondanks de grote veranderingen die werden geobserveerd tijdens het ernstig ziekzijn.

Mogelijkheden voor een bredere toepassing van speeksel bij onderzoek naar infecties en immuniteit

Een deel van de resultaten uit dit proefschrift zijn verkregen door het gebruik van speeksel voor het meten van antistofconcentraties (**hoofdstukken 4** en **5**) en het karakteriseren van het microbioom (**hoofdstuk 8**). Het gebruik van speeksel heeft veel praktische voordelen: het verzamelen is gemakkelijk, non-invasief en vereist geen assistentie van een zorg- of onderzoeksmedewerker, in tegenstelling tot het verkrijgen van bijvoorbeeld bloed of het afnemen van een diepe neuswat. Desondanks wordt in klinisch onderzoek betrekkelijk weinig gebruik gemaakt van speekselmonsters. Voor het onderzoeken van de antistofrespons na vaccinatie is bijvoorbeeld bloed de gouden standaard. In **hoofdstuk 6** vergeleken wij antistofresponsen tussen twee verschillende toedieningsschema's van het 10-valente pneumokokkenconjugaatvaccin (op de leeftijd van 2, 3, 4 en 11 maanden vergeleken met 2, 4 en 11 maanden) als onderdeel van het Rijksvaccinatieprogramma bij de deelnemers van de MUIS studie. Onze bevindingen waren vergelijkbaar met eerdere bevindingen van studies die de antistofrespons vergeleken in bloed. Een belangrijk obstakel voor wijdverbreid gebruik van speeksel bij onderzoek naar nieuwe vaccins en vaccinatieschema's is het ontbreken van een afkapwaarde voor de speeksel antistofconcentratie waarboven een vaccin goede bescherming biedt tegen infectie. Het bepalen van dergelijke waardes voor antigeen-specifieke antistofresponsen in speeksel zou bij toekomstig vaccinonderzoek invasieve bloedafnames kunnen besparen, wat met name aantrekkelijk is voor studies met kinderen als doelgroep. Voor het aantonen van luchtwegpathogenen bij kinderen met ernstige luchtwegklachten wordt vaak een diepe neuswat gebruikt. Sommige kinderen ervaren ook dit onderzoek echter als te vervelend en weigeren dit. In **hoofdstuk 9** onderzochten wij of speeksel een gelijkwaardig alternatief zou kunnen zijn. De vraag of we speeksel konden

gebruiken in plaats van een diepe neuswat werd erg relevant toen het op grote schaal testen van kinderen op Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) onderdeel vormde van de maatregelen om de COVID-19 pandemie in te dammen. In ons onderzoek bij 57 kinderen met luchtwegklachten, vonden wij dat de gevoeligheid van speeksel voor het aantonen van luchtwegpathogenen (virussen en bacteriën anders dan SARS-CoV-2) nagenoeg vergelijkbaar was met die van de diepe neuswat, op voorwaarde dat voldoende speekselvolume werd verkregen door te spugen of met behulp van een absorberend sponsje. Wij concludeerden dat het gebruik van speeksel overwogen kan worden indien het verkrijgen van een diepe neuswat praktisch niet mogelijk is. Deze beide studies demonstreren de mogelijkheden van speeksel als een meer kindvriendelijk alternatief bij onderzoek naar infecties en immuniteit.

De toekomst van microbioomonderzoek in het vroege leven en gezondheidseffecten

Hoewel het aantal studies naar het microbioom in het vroege leven en gezondheidseffecten op latere leeftijd in hoog tempo groeit, staat de vertaling van deze kennis naar de klinische praktijk nog in de kinderschoenen. Longitudinale studies waarbij blootstellingen op jonge leeftijd (bijvoorbeeld geboortewijze of antibioticagebruik) worden gelinkt aan microbioom veranderingen en vervolgens aan uitkomsten voor de gezondheid (bijvoorbeeld een vaccinrespons of terugkerende luchtwegklachten) zijn een goede stap richting het aantonen van verbanden, maar vormen geen definitief oorzakelijk bewijs. Dierstudies waarbij het microbioom wordt gemanipuleerd en vervolgens gezondheidsuitkomsten worden gemeten verlenen ondersteunend bewijs, maar kunnen niet één op één worden geëxtrapoleerd naar de situatie bij de mens. Verschillende onderzoeksgroepen experimenteren met nieuwe methodes om de samenstelling van het microbioom van pasgeborenen te beïnvloeden, bijvoorbeeld met specifieke probiotica die gezondheids-bevorderende bacteriën bevatten en door de pasgeborene in te smeren met vaginaal vocht of te transplanteren met de fecale flora van hun moeders. Deze methodes lijken in de eerste plaats veilig en hebben inderdaad een effect op de samenstelling van het microbioom in het vroege leven, maar toekomstige studies zullen moeten uitwijzen of

ze vervolgens ook een positief effect op de gezondheid bewerkstelligen. Dit vereist grote en langlopende studies. Onze bevindingen suggereren dat het grootste gezondheidseffect wellicht behaald kan worden wanneer dergelijke “correcties” van een verstoord microbioom (bijvoorbeeld als gevolg van een keizersnede of antibioticagebruik) op een zo jong mogelijke leeftijd worden ingezet. Het bepalen van totale antistofconcentraties of antistofresponsen na vaccinaties als maat voor gezondheidseffecten van dergelijke interventies zou kunnen worden overwogen, met als belangrijk voordeel dat dit bij elk (gevaccineerd) kind al op jonge leeftijd mogelijk is (in tegenstelling tot aandoeningen die zich pas op latere leeftijd ontwikkelen, zoals astma en allergieën). Daarnaast zou onderzoek naar mogelijkheden voor ondersteuning van herstel van een gezond microbioom na een ernstige onderste luchtweginfectie kunnen worden opgezet met het toedienen van antibiotica gericht op reductie van *Haemophilus* en/of anaeroben of door probiotica met gezondheids-bevorderende bacteriën en het bepalen van een eventuele gezondheidswinst (bijvoorbeeld de frequentie van nieuwe luchtweginfecties).

Voor nu blijven preventieve en therapeutische opties gericht op microbioom verbetering nog grotendeels toekomstmuziek. Desondanks kan kennis van interacties tussen het microbioom, infecties en immuniteit reeds van nut zijn in de klinische praktijk. Ingrepen zoals een keizersnede of antibiotische behandeling zijn vaak medisch noodzakelijk, maar bewustzijn van effecten van medische beslissingen op het microbioom van een kind met eventuele lange termijn consequenties voor de gezondheid zou in sommige gevallen kunnen leiden tot een andere afweging. Wanneer we leren essentiële bacteriën in het vroege leven veilig te stellen, dan plukken toekomstige generaties daar hopelijk de vruchten van.

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LIST OF PUBLICATIONS

Publications in this thesis

E.M. de Koff, W.A.A. de Steenhuijsen Piters, M.A. van Houten, M. Kuijer, I. Tcherniaeva, M.L.J.N. Chu, R. Hasrat, G. den Hartog, E.A.M. Sanders, D. Bogaert. Interplay between the early-life respiratory microbiota and mucosal antibody development is associated with infection susceptibility. *Submitted*.

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CURRICULUM VITAE

Emma Margaretha de Koff was born on February 24 in 1992, in the city of Utrecht, where she lived her entire childhood with her parents and younger sister Sarah. She completed her secondary education at the Christelijk Gymnasium Utrecht in 2010. That same year, she left home to live on-campus in Amsterdam and attend the Amsterdam University College, where she obtained her Bachelor degree in the Liberal Arts and Sciences with a major in Biomedical Sciences (pre-med track) in 2013. She subsequently continued her education with the Selective Utrecht Medical Master (SUMMA) at the University Medical Center Utrecht, where she graduated in 2017 as a physician and a researcher. She first gained a couple of months of work experience as a junior doctor at the pediatrics department of the Spaarne Gasthuis in Hoofddorp. However, the research she started as a student in the group of prof. dr. D. Bogaert, prof. dr. E.A.M. Sanders and dr. M.A. van Houten continued to fascinate her, and she was given the opportunity to work as a PhD student from 2018 to 2022 at the Spaarne Gasthuis, UMC Utrecht and RIVM, which ultimately culminated in the publication of this thesis.

Currently, she is working as a resident in training in Clinical Microbiology at the Amsterdam University Medical Center. She lives in Utrecht with her husband Jonathan, and they plan to continue on their parallel medical career paths together.