ORIGINAL ARTICLE

Maturation of the Infant Respiratory Microbiota, Environmental Drivers, and Health Consequences

A Prospective Cohort Study

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Abstract

Rationale: Perinatal and postnatal influences are presumed important drivers of the early-life respiratory microbiota composition. We hypothesized that the respiratory microbiota composition and development in infancy is affecting microbiota stability and thereby resistance against respiratory tract infections (RTIs) over time.

Objectives: To investigate common environmental drivers, including birth mode, feeding type, antibiotic exposure, and crowding conditions, in relation to respiratory tract microbiota maturation and stability, and consecutive risk of RTIs over the first year of life.

Methods: In a prospectively followed cohort of 112 infants, we characterized the nasopharyngeal microbiota longitudinally from birth on (11 consecutive sample moments and the maximum three RTI samples per subject; in total, n = 1,121 samples) by 16S-rRNA gene amplicon sequencing.

Measurements and Main Results: Using a microbiota-based machine-learning algorithm, we found that children experiencing a higher number of RTIs in the first year of life already demonstrate an aberrant microbial developmental trajectory from the first month of life on as compared with the reference group (0–2 RTIs/yr). The altered microbiota maturation process coincided with decreased microbial community stability, prolonged reduction of *Corynebacterium* and *Dolosigranulum*, enrichment of *Moraxella* very early in life, followed by later enrichment of *Neisseria* and *Prevotella* spp. Independent drivers of these aberrant developmental trajectories of respiratory microbiota members were mode of delivery, infant feeding, crowding, and recent antibiotic use.

Conclusions: Our results suggest that environmental drivers impact microbiota development and, consequently, resistance against development of RTIs. This supports the idea that microbiota form the mediator between early-life environmental risk factors for and susceptibility to RTIs over the first year of life.

Keywords: respiratory microbiota; nasopharynx; respiratory tract infections; development; risk factors

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At a Glance Commentary

Scientific Knowledge on the Subject: Factors affecting the risk of respiratory tract infections (RTIs) have been well characterized; however, it is unknown how these factors might impact respiratory microbiota development and thereby susceptibility to RTIs. Studies in mice suggest that timely microbial cues contribute to healthy immune development, in turn enforcing the defense against invading respiratory pathogens.

What This Study Adds to the Field: Using a longitudinal study design and high sampling resolution, we characterized the nasopharyngeal microbiota maturation over the first year of life in 112 infants both during health (11 sampling moments) and at the moment of RTIs. We observed differences in the microbial community maturation in children who ultimately became more susceptible to infections compared to children who were more resistant to infections. These changed dynamics were related to environmental factors that are known to impact susceptibility to RTIs, such as mode of delivery, mode of feeding, early antibiotic use, and crowding. Altered microbiota maturation was evident from the first month of life on and preceded the occurrence of RTIs, strongly suggesting that early-life microbiota development impacts long-term respiratory health.

Acute respiratory tract infections (RTIs) are a leading cause of childhood mortality, being responsible for approximately 0.9 million yearly deaths (15.5% of all deaths) worldwide in children under 5 years of age (1). In addition, these infections are associated with significant morbidity (2) and are a major reason for antibiotic prescription (3), especially in young children. Although it is still unclear why one individual is more vulnerable to respiratory infections compared with another, it was previously hypothesized that—besides environmental and host-related influences—the respiratory microbiota may modulate susceptibility to disease.

Directly after birth, the mucosal surfaces of the respiratory tract of neonates are rapidly colonized with a variety of microbiota that are swiftly molded into niche-specific bacterial communities (4, 5). Over the first months to years of life, these communities are highly dynamic and heavily influenced by environmental factors, including mode of delivery (4, 6), season (7), feeding type (8), and antibiotic treatment (9). In previous studies, we found that the microbial composition at the age of 6 weeks was indicative of microbiota stability and RTI susceptibility over the first 2 years of life (10, 11). This finding underscores the importance of direct postnatal environmental influences and early microbiota maturation on future respiratory health.

The healthy human respiratory microbiome is assumed to stimulate immune maturation (12, 13), promote epithelial integrity (14), and provide colonization resistance (15), thereby preventing overgrowth and invasion of potential pathogenic bacteria (16). In contrast, deviations from a healthy bacterial respiratory community composition have been associated with susceptibility to and/or severity of childhood respiratory diseases, including acute otitis media (17, 18), respiratory syncytial virus disease (19), and asthma development (20) in various retrospective and cross-sectional studies (21).

We here postulate that alterations in the respiratory microbiota development early in life are a consequence of changes in the abundance of specific bacterial biomarker species. We hypothesize that these alterations are controlled by known host-related and environmental influences, and can ultimately lead to altered microbiota stability, in turn affecting RTI susceptibility. Therefore, we prospectively investigated the nasopharyngeal microbiota maturation of 112 unselected, healthy children with frequent, short-interval sampling during the first year of life, as well as during RTI episodes. Hereby, we aimed to study respiratory microbiota development early in life, and investigate its role as potential mediator between early-life drivers and susceptibility to respiratory infectious disease.

Methods

Details on the study design, sample and data collection, and bioinformatics/statistical methods can be found in the Methods in the online supplement. Data have been deposited in the National Center for Biotechnology Information GenBank database (accession no. SRP093519).

Study Population

We enrolled a total of 128 healthy children in an ongoing prospective birth cohort study aiming to investigate the development of the infant microbiota during health and disease. Of 128 infants, 12 children were lost to follow-up (*see* Figure E1 in the online supplement). Details on the trial methods have been described elsewhere (4). Written informed consent was obtained from both parents. The study was approved by the Ethics Committee of Noord Holland, the Netherlands (M012-015, NH012.394, NTR3986). Sequence data of part of the samples (≤6 mo; n = 743 samples of 101 children) were used for a study on the role of mode of delivery on respiratory microbiota acquisition (4).

Data Collection

For the current analyses, we included samples and data of 112/116 children who completed the 1-year follow-up and for whom we had eight or more samples available for further analyses after laboratory work-up (Figure E1). Home visits were conducted within 2 hours after birth, at 24 hours, at 7 and 14 days, and at 1, 2, 3, 4, 6, 9, and 12 months of age. During each home visit, a trained doctor or research nurse obtained a nasopharyngeal swab according to World Health Organization protocol (22) and completed an extensive survey on the health status of the child, as well as on the presence or absence of environmental factors and potential risk factors related to respiratory disease (4). Aside from these regular visits, parents were asked to contact the study team in case of an active RTI, defined as fever 38°C or higher for longer than 6 hours combined with malaise and presence of RTI symptoms. Subsequently, an RTI visit was planned within 48 hours after the start of the fever to collect additional samples and to obtain more detailed medical information.

16S-rRNA Gene Amplicon Sequencing

Bacterial DNA of the nasopharyngeal samples was isolated, amplicon libraries of the 16S-rRNA gene (V4 region) were generated, and sequencing was executed as previously described (4, 23). Amplicon

pools were paired-end sequenced in eight runs using an Illuminia MiSeq instrument (Illumina Inc., San Diego, CA). Bioinformatic processing included trimming, error correction, assembly, and 97%-identity clustering of reads into operational taxonomic units (OTUs). After removal of chimeric reads, OTUs were taxonomically annotated using SILVA and BLASTN (Table E1). We refer to OTUs using maximum genus-level annotations, combined with a rank number based on the abundance of each given OTU. Details on processing and quality control, including the use of negative controls, are described in the METHODS in the online supplement. After abundance filtering, a rarefied dataset was generated and used for downstream analyses (24). α-diversity measures were averaged over 100 rarefactions. β-diversity was assessed using the Bray-Curtis dissimilarity metric.

Statistical Analysis

All analyses were performed in R version 3.3.0 within R studio version 0.99.902 (Boston, MA).

Random Forest Analysis

We hypothesized that the nasopharyngeal microbial succession patterns would be altered in children who experienced more RTIs during their first year of life. Therefore, we stratified our population into three groups based on the normal distribution of RTIs over the first year of life (Figure E2): 39 children with 0–2 RTIs (reference group; n = 372 samples); 52 children with 3–4 RTIs (n = 496 samples); and 21 children with 5–7 RTIs (n = 197 samples). To identify OTUs characteristic of a healthy microbiota maturation, we regressed the relative abundance of all 576 OTUs against chronological age in the reference group using a random forest model (*randomForest* package), and selected age-discriminatory OTUs using a step-wise backward 10-fold cross-validation procedure (*see* METHODS in the online supplement and Figures E3A and E3B) (24). This selection of OTUs was subsequently used as input to a new, reduced random forest model in which we regressed the relative abundance of these OTUs versus chronological age in the reference group. The resulting final model was then used to predict chronological age, referred to as "microbiota age," in samples from individuals who experienced 3–4 and 5–7 RTIs, and on the group of samples collected during RTIs (n = 56 samples). To generate accurate microbiota age estimates for the reference group, we used a 10-fold cross-validation procedure. Relative microbiota age (RMA) was calculated as follows: RMA = microbiota age of a given child minus microbiota age of the reference group at similar age, as determined by a spline fit (24). As a *post hoc* analysis, we studied the effect of the *Moraxella* genus on the performance of the microbiota age model by excluding the OTUs belonging to the *Moraxella* genus from the model while monitoring the amount of variance explained by this reduced model.

Associations between Environmental Factors and Microbiota Parameters

"Environmental factors" used in the descriptions of the various models comprises birth mode, breast feeding until 3 months of age, day care attendance, presence of siblings under 5 years of age, antibiotic treatment in the previous 4 weeks, and season of birth, if not specified otherwise. If applicable, correction for multiple testing was performed using the Benjamini-Hochberg procedure.

Microbial succession patterns were visualized using nonmetric multidimensional scaling (*vegan* package) based on the Bray-Curtis dissimilarity matrix. We performed two separate analyses based on permutational multivariate ANOVA (PERMANOVA) tests and the Bray-Curtis dissimilarity matrix, to study the effect of: (*1*) environmental factors, age, and subject; and (*2*) the number of RTIs experienced in the first year of life on the overall bacterial community structure. Permutations were constrained within subjects to account for repeated measures. This analysis was repeated over 100 rarefactions to assess the robustness of our results based on one rarefied set.

To complement the group-based analyses, we also assessed the microbial development at the individual level using an unsupervised clustering approach. The proportion of samples within each cluster at each time point was visualized using an alluvial diagram, stratified by the number of RTIs that children experienced over the first year of life.

We used separate linear mixed models to assess the associations between (relative) microbiota age and stability (α-/β-diversity measures) as dependent variables and: (*1*) environmental factors; and (*2*) the number of RTIs (fixed effects), while adjusting for age and with the subject variable included as a random intercept (*lme4* package). In addition, the relationships between (*1*) bacterial density and (*2*) relative abundance (dependent) and sampling moment (fixed) were assessed using linear mixed models.

We used smoothing spline ANOVA (SS-ANOVA; *metagenomeSeq* package) for the analyses of: (*1*) the differences in abundance of age-discriminatory OTUs between RTI-groups; and (*2*) the effects of birth mode and breastfeeding on the nasopharyngeal microbiota, as it simultaneously tests for the existence and timing of differences in OTU abundance. To confirm associations between environmental factors and relative abundance of microbiota in a multivariable manner, we used Multivariate Association with Linear Models (MaAsLin; *MaAsLin* package), adjusting for age and with subject as a random effect.

Results

Baseline Characteristics of the Study Population

Baseline characteristics of the study population stratified by number of RTIs experienced in the first year of life can be found in Table E2.

Nasopharyngeal Microbiota Composition in the First Year of Life

A median of 20,670 reads was generated per sample (range = 3,911–97,870 reads), which were binned into a total 576 OTUs (after filtering), representing a total of 14 bacterial phyla. Firmicutes was the most abundant phylum, with a maximum abundance of 65.4% at Day 1 (mainly *Staphylococcus* [3], *Dolosigranulum* [4], and *Streptococcus* [5]). Later, Proteobacteria emerged and became predominant, with a maximum abundance of 71.7% at 12 months of life (mostly *Moraxella* [1], *Haemophilus* [6], and *Moraxella* [7]; Figures 1 and 2, Figure E4). We observed major shifts in nasopharyngeal microbiota composition between Day 0 and Day 1 and between Day 1 and Week 1 (Figure E5). The difference in microbiota composition between Day 1 and Week 1 coincided with a strong increase in absolute bacterial abundance, which then increased up to the age of approximately

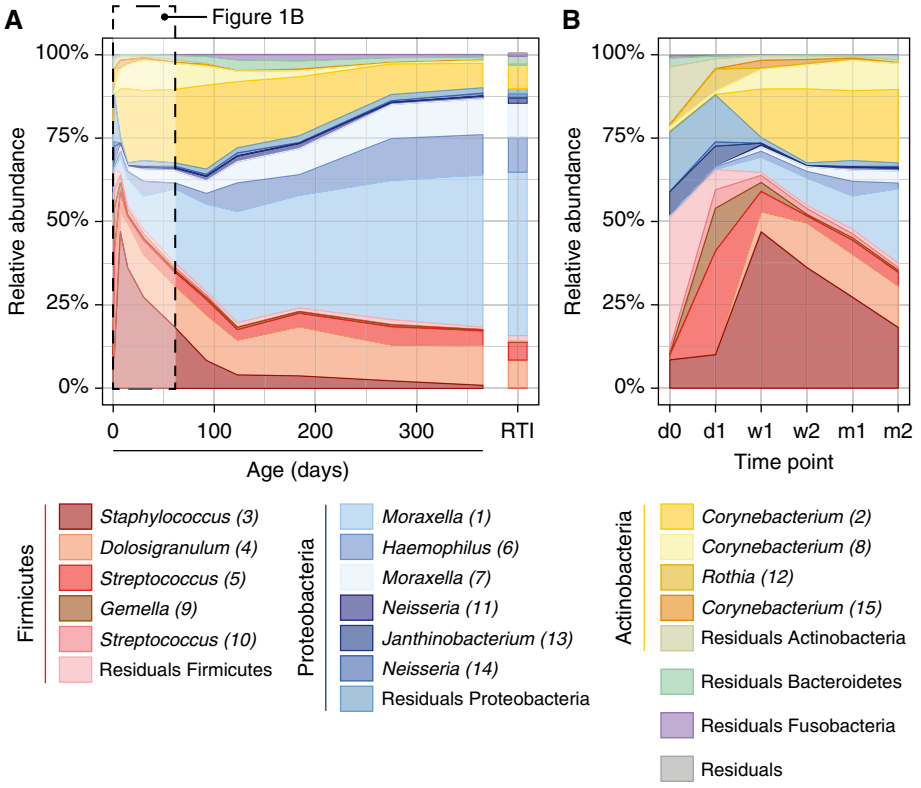

Figure 1. Microbiota development over the first year of life. (*A*) Relative abundance of the 15 highest-ranking operational taxonomic units (OTUs) over the first year of life (age in days) and during respiratory tract infections (RTIs). OTUs are color coded, as indicated, which was based on their phylum-level taxonomic annotation: *red*, Firmicutes; *yellow*, Actinobacteria; and *blue*, Proteobacteria. We observed a high abundance of Firmicutes (*Staphylococcus* [3] and *Dolosigranulum* [4]) and Actinobacteria (*Corynebacterium* spp.) early in life, which was gradually replaced by *Proteobacteria* (*Moraxella* [1], *Moraxella* [7], *Haemophilus* [6], and *Neisseria* spp.). OTUs that were not among the 15 highest ranking were collapsed and referred to as "residuals," stratified by phylum for the five most abundant phyla. (*B*) Relative abundance of the 15 highest-ranking OTUs over the first 2 months of life. Visualization of microbiota profiles per time point allows for a more detailed assessment of microbial dynamics at early time points. Over the first week of life, a relatively high abundance of *Streptococcus* (5), *Janthinobacterium* (13), *Neisseria* spp., and *Rothia* (12) was observed, apart from other OTUs belonging mainly to the Firmicutes, Proteobacteria, and Actinobacteria phyla (*see* Figure E5). d = day; m = month; w = week.

1 month, after which it stabilized (linear mixed model, $q < 0.001$; Figure 3).

Trajectories of Microbial Development

We aimed to study whether nasopharyngeal microbiota development is different in infants experiencing more RTIs in the first year of life compared with the low-burden infants. First, we demonstrated that the microbial community composition was significantly associated with the number of RTIs experienced in the first year of life (i.e., 0–7 RTIs; categorical), after adjusting for age, using a PERMANOVA test (Table E3A; 1.7% of the variance explained, $P = 0.001$). Subsequently, we stratified the study participants over three groups based on the number of RTIs they experienced within the first 12 months of life (i.e., 0–2, 3–4, and 5–7 RTIs; Figure E2 and Table E2). To explore the microbial succession patterns at the individual level, we clustered samples using an unsupervised clustering approach. The proportion of individuals in each cluster at each time point was then visualized using an alluvial diagram stratified by the number of RTIs experienced over the first year of life (Figure E6). We identified eight clusters over all time points, of which the largest four were enriched for: *Moraxella* (1) (38.5% of samples), *Corynebacterium* (2) and *Dolosigranulum* (4) (CDG; 19.7%), *Staphylococcus* (3) (19.4%), and *Streptococcus* (5) (8.4%). In concordance with our previous observations, we found that the CDG cluster has a much more prominent and prolonged role in the reference group compared with children who suffered from 5–7 RTIs. Instead, these children appear to "skip" the CDG cluster altogether, transitioning directly from the early-life *Staphylococcus* cluster to the *Moraxella* cluster (Figure E6C), the latter of which is typically observed more often at later time points in the reference cohort (Figure E6A). In the children who experience 3–4 RTIs, the cluster distributions at each time point do resemble those of the reference group, although an early rise of the *Haemophilus* (6) cluster was noted (Figure E6B).

Nasopharyngeal Microbiota Maturation in Relation to Susceptibility to RTI and Identification of Age-Discriminatory Taxa

To further assess these differences in microbiota dynamics, we used a random forest regression model. First, we identified age-discriminatory OTUs in the reference group (i.e., 0–2 RTIs; Figures E3A and E3B) and regressed their relative abundance against chronological age, enabling us to model healthy microbiota development (65.9% of variance explained, based on 10-fold cross-validation, 100 repetitions). Then, the model was used to calculate predicted chronological age or "microbiota age" in children with 3–4 and 5–7 RTIs and in samples taken during RTIs (58.1% variance explained), subsequently comparing these estimates to chronological age. We first observed that children with 5–7 RTIs showed an accelerated microbiota maturation when compared with the reference group from very early in life on (linear mixed model, $P = 0.007$). A similar, although nonsignificant, trend was observed in children with 3–4 RTIs versus the reference group (linear mixed model, $P = 0.13$; Figure 4A). The accelerated microbiota developmental patterns in children with more than two RTIs were related to an early enrichment of *Moraxella* (1) from just after birth on (SS-ANOVA, $q = 0.007$), enrichment of *Neisseria*, *Prevotella*, and *Alloprevotella* spp. from Month 2 onward (SS-ANOVA, $q \leq 0.021$) and (prolonged) absence of *Corynebacterium* (2) and *Corynebacterium* (80), *Dolosigranulum* (4), and *Streptococcus* (10) (SS-ANOVA, $q \leq 0.039$; Figure 4B, Figure E7, and Table E4A). Subgroup analyses comparing either the 3–4 or 5–7 RTI groups to the reference group yielded highly similar results (Tables E4B and E4C).

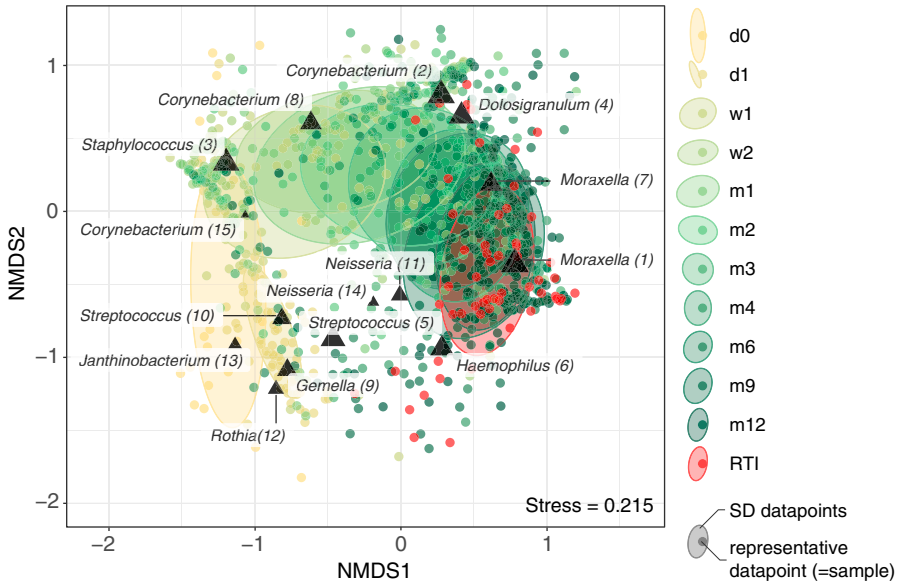

Figure 2. Nonmetric multidimensional scaling (NMDS) plot visualizing the microbiota succession patterns in the first year of life. Each point represents the microbial community composition of one sample. Samples taken during health (n = 1,065) are colored based on the age at which they were taken (colors ranging from *yellow* [Day 0] to *dark green* [Year 1]). In addition, samples taken during respiratory tract infection (RTI) are depicted (*dark red*; n = 56). The SD of data points within time point/RTI strata is shown by ellipses. The 15 highest-ranked operational taxonomic units (OTUs) were simultaneously visualized (*triangles*). The size of the *triangles* is relative to the mean relative abundance of the OTU it represents. The stress value indicates how well the high-dimensional data are captured in the two-dimensional space of this NMDS; a value of approximately 0.2 indicates that the representation of some points is potentially misleading, and that a representation in a higher dimensional space might be more appropriate (*see* Figure E4 for detailed assessment) (32). d = day; m = month; w = week.

To assess whether the above differences were predominantly driven by the *Moraxella* genus rather than by the total group of biomarker species, we assessed the impact of *Moraxella* spp. on the performance of the microbiota age model by repeating the analyses, including all biomarker OTUs, except those belonging to the *Moraxella* genus. This model, containing 18 OTUs, showed a confined effect of *Moraxella* spp., with a small reduction of performance in the reference group (60.9% variance explained), and a slightly improved performance in children who experienced 3–4 or 5–7 RTIs over the first year of life and in samples taken during RTI (60.1% variance explained), compared with the model based on 22 OTUs.

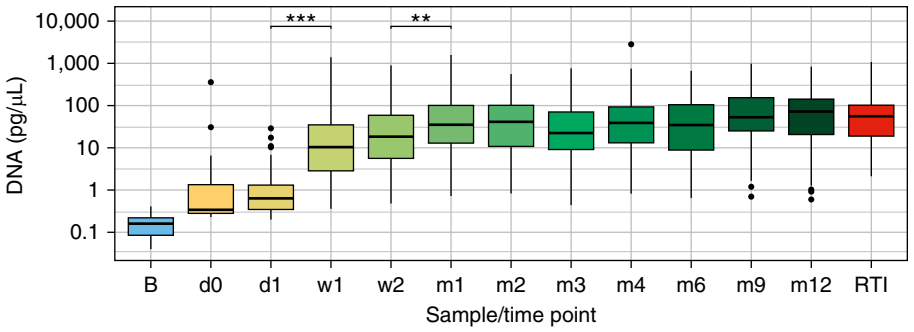

Figure 3. Absolute bacterial density over the first year of life. Boxplots showing the absolute bacterial density (in pg/µl 16S-rRNA gene) in blanks (n = 55; *blue*), in samples taken during health at various time points (n = 1,065; colors ranging from *yellow* [Day 0] to *dark green* [Year 1]), and during respiratory tract infection (RTI) (n = 56; *red*). Bacterial density is particularly low at Days 0 and 1, then gradually increases until the age of approximately 1 month, after which it remained largely stable. Box plots represent the 25th and 75th percentiles (*lower* and *upper boundaries of boxes*, respectively), the median (*middle horizontal line*), and measurements that fall within 1.5 times the interquartile range (IQR; distance between 25th and 75th percentiles; *whiskers*) or outside 1.5 times the IQR (points). *q* values were derived from a linear mixed model with $\log_{10}$-transformed bacterial density as outcome variable, time point as fixed effect, and subject as a random effect. Only samples taken at regular intervals were considered, and each consecutive time point was compared with the previous time point using the *multcomp* package. **$0.001 \leq q < 0.01$; ***$q < 0.001$. d = day; m = month; w = week.

Relative Microbiota Age in Relation to (Susceptibility to) RTI

By calculating the RMA (defined as the difference in microbiota age between susceptible groups and the reference group), we verified that microbiota age was increased in children with 5–7 RTIs compared with the reference group (linear mixed model, adjusted for age, $P = 0.007$; Figure E8), which was already apparent in the first month of life ($P = 0.011$, linear mixed model; *post hoc* analysis in children ≤1 mo of age). This latter finding was substantiated by a PERMANOVA test, demonstrating that the microbiota composition over the first month of life was significantly associated with the number of RTIs over the first year of life (Table E3B; 0.8% of the variance explained, $P = 0.001$). The RMA was not significantly different between the group with 3–4 RTIs and the reference group ($P = 0.12$). Moreover, although the RMA was maximal during RTIs (median RMA = +67.8 d in RTI samples), we already observed an increase in RMA during the period preceding the factual RTI (median RMA = +37.1 d at the first time point preceding RTI [T = −1]; $P = 0.004$), suggesting that the microbiota maturation alterations precede RTIs. After recovery from an RTI, RMA decreased towards the reference group, but did not normalize (median RMA = +29.7 d [T = +1]; $P = 0.04$; Figure 4C). Although these changes in RMA appeared to be related to individual OTUs (Figure E9), these changes were not statistically significant.

Nasopharyngeal Microbiota Stability over Time

We next investigated whether bacterial community stability over time was different for children who experienced 0–2, 3–4, and 5–7 RTIs over the first year of life. Community stability, measured by the Bray-Curtis dissimilarity between

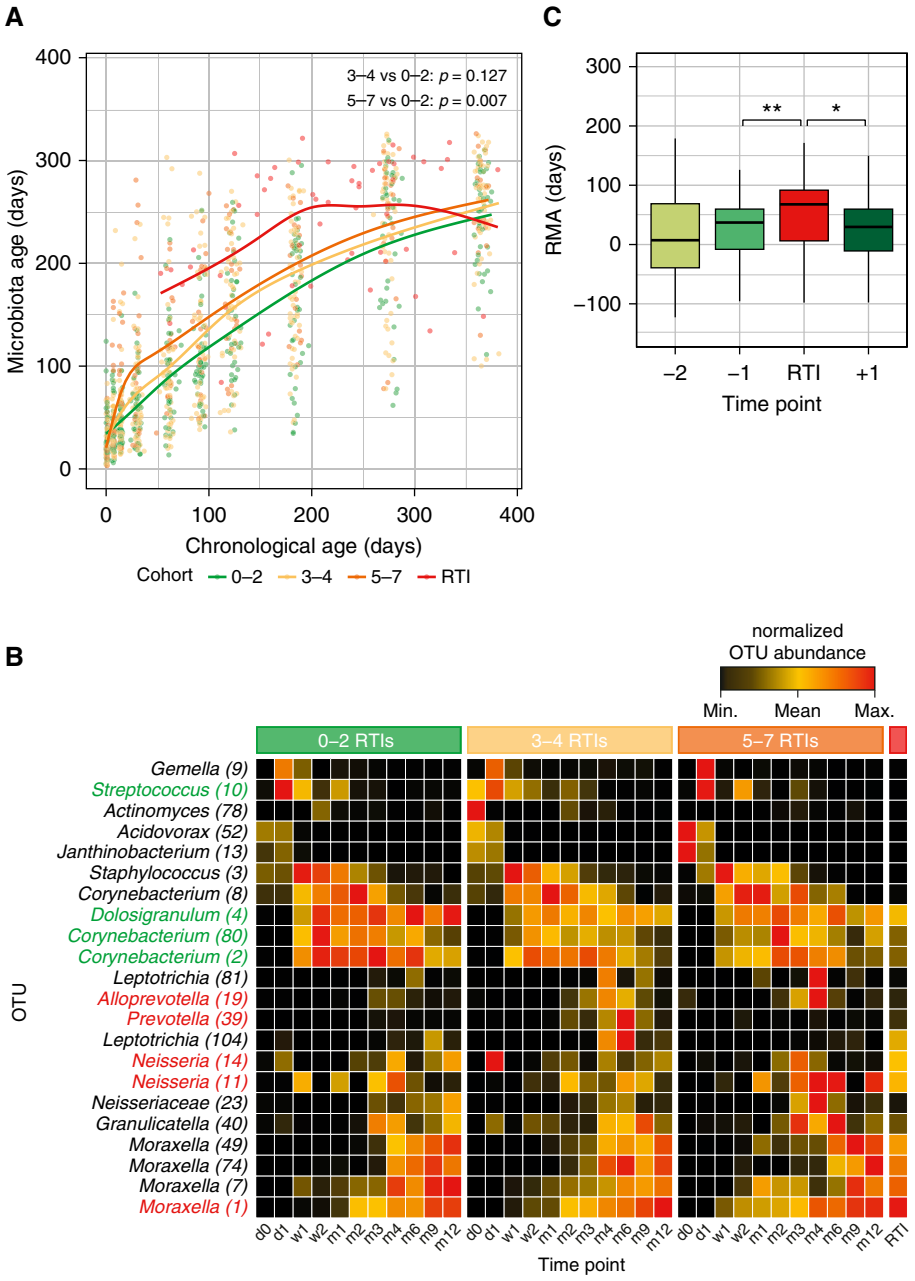

Figure 4. Microbiota maturation and age-discriminatory taxa stratified by respiratory tract infection (RTI) susceptibility. (*A*) Microbiota age estimates plotted against chronological age stratified by number of RTIs experienced during the first year of life. The *curves* represent smooth spline fits for each cohort. *P* values are based on a linear mixed model, including age (spline) and number of RTIs (i.e., 0–2, 3–4, or 5–7 RTIs) as fixed effects and subject as random effect. (*B*) Heatmap of the mean relative abundance of the 22 age-discriminatory operational taxonomic units (OTUs) against moment of sampling in each cohort. OTUs are ordered vertically based on average linkage hierarchical clustering using the Euclidean distance matrix. *Colors* correspond with row-wise normalized relative abundances (i.e., *red* indicates the maximum relative abundance of that OTU over all cohorts; *black* indicates the minimum relative abundance). OTU names are colored *green* if they were significantly enriched in the reference group (0–2 RTIs) compared with children with more than two RTIs. *Red* was used to denote the OTUs that were observed in higher abundance in children with more than two RTIs (based on smoothing spline ANOVA *q* values; *see* Table E4A). d = day; m = month; w = week. (*C*) Relative microbiota age (RMA) before (*light green shades*), during (*red*), and after RTI (*dark green*). The relative microbiota age two time points before RTI (−2; n = 51; on average 104 d to RTI), one time point before RTI (−1; n = 47; 50 d to RTI), at RTI (RTI; n = 56; mean age at consecutive time points, was significantly different between children with 0–2 RTIs and those with 3–4 and 5–7 RTIs (linear mixed model, $P = 0.005$ and $P = 0.02$, respectively). This phenomenon was apparent from the age of 3 months on (Figure 5).

Impact of Environmental Drivers on Bacterial Community Composition

We then aimed to assess the effect of environmental factors on nasopharyngeal microbiota composition and succession. Using PERMANOVA tests, we found that factors with the largest impact comprised subject (unadjusted $R^2 = 18.7\%$), age (10.4%), and environmental drivers, including presence of siblings under 5 years of age (1.6%), day care attendance (0.9%), season of birth (0.7%), breastfeeding for at least 3 months (0.5%), birth mode (0.4%), and antibiotic usage in the previous month (0.3%; all *P* values ≤ 0.016; Tables E3C and E3D).

Environmental Drivers and Their Effects on Microbiota Maturation, Stability, and Individual Bacterial Taxa

After showing that microbiota maturation is accelerated in children more susceptible to RTIs, we next set out to determine the influence of environmental drivers on this process. We modeled the RMA using a linear mixed model, including environmental factors. We observed that particularly the presence of young siblings and day care attendance are associated with an increased RMA early in life (both $P < 0.0005$). Similar associations were found when directly modeling microbiota age instead of RMA versus environmental drivers (data not shown). In contrast, the observed differences in microbiota stability between groups could not be explained by environmental factors (linear mixed model, $P > 0.05$) and did not relate to differences in α-diversity measures between groups (linear mixed model, $P > 0.05$; Figure E10). We also did not detect differences in microbiota stability directly before, during or after an RTI episode.

We further tested the contribution of individual bacterial taxa to the associations between environmental factors and microbiota maturation using MaAsLin. With respect to age-discriminatory taxa, we found that *Moraxella* spp. were positively associated and *Staphylococcus* spp. were negatively associated with day care (both $q < 0.0005$). Furthermore, we found that *Corynebacterium* (2) and *Dolosigranulum* (4) were strongly reduced

after antibiotic usage ($q < 0.03$). In addition, we observed many associations between environmental drivers and bacterial taxa that were not previously assigned as age-discriminatory biomarkers. Notably, the presence of siblings was associated with increased abundance of the family *Pasteurellaceae* ($q = 0.003$), which includes the *Haemophilus* genus (Table E5).

Temporal Effects of Mode of Delivery and Feeding Type on Bacterial Taxa

Because MaAsLin is not suited to identifying temporary effects and the timeframes within which they occur, we additionally studied the impact of early-life drivers, such as mode of delivery and feeding type, on the microbial succession patterns using SS-ANOVA. Of the age-discriminatory taxa, early and/or prolonged predominance of *Corynebacterium* (2), *Corynebacterium* (8), and *Dolosigranulum* (4) ($q \leq 0.03$), and late enrichment of *Moraxella* spp. ($q < 0.05$; from ~Month 3 on), were associated with vaginal birth and/or breastfeeding. In contrast, in formula-fed and/or caesarian-born children, we observed a high abundance of *Gemella* (9) and *Streptococcus* (10) ($q \leq 0.012$) from birth on, and prolonged (4–11 mo) predominance of *Neisseria* spp. and (facultative) anaerobes, including *(Allo)prevotella*, *Granulicatella*, and *Actinomyces* spp. ($q < 0.05$) after the first month of life. Abundance of the age-discriminatory taxum *Staphylococcus* (3) was related to birth by caesarian section in the first month of life only ($q = 0.016$). Besides, although not directly linked to microbiota maturation, we found that the additional early enrichment of *Streptococcus* (5) was associated with caesarian section and/or formula feeding (from birth on; $q \leq 0.026$), which could be confirmed using MaAsLin (Table E5). In addition, we observed temporal enrichment of oral-type bacteria, including streptococci and facultative anaerobic bacteria, such as *Prevotella*, *Porphyromonas*, and *Veillonella* spp., in formula-fed children (from ~Months 1–2 onward) and early abundance of *Dolosigranulum* (4) in breastfed children (Tables E6 and E7, Figures E11 and E12).

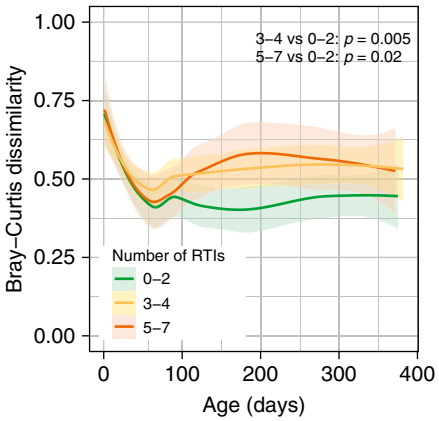

Figure 5. Microbiota stability over time stratified by respiratory tract infection (RTI) susceptibility. Bray-Curtis dissimilarities were calculated within each subject between each pair of consecutive time points. The bacterial community stability was significantly lower in children with 3–4 ($P = 0.005$) or 5–7 RTIs ($P = 0.02$) compared with the reference group of children experiencing 0–2 RTIs within the first year of life. *P* values are based on a linear mixed model, including age (spline) and number of RTIs as fixed effects and subject as random effect. The *shaded area* around each *smoothing spline* represents the 95% confidence interval.

Discussion

Microbial colonization of the upper respiratory tract occurs directly after birth and develops rapidly toward niche-specific profiles during the first weeks of life (4, 5, 10, 25). Several cross-sectional case–control studies have shown differences in respiratory microbial profiles between children with and without acute otitis media (18, 26), and between infants with mild, moderate, and severe respiratory syncytial virus (19). Longitudinal studies, linking respiratory microbiota development and maturation and (risk of) RTIs, however, are sparse, lack detailed information, and are only retrospectively executed (10, 20).

Our results suggest that microbiota maturation in healthy children who experience a limited number of 0–2 RTIs in the first year of life (reference group) is associated with a specific timing of colonization events accompanied by the consecutive appearance and disappearance of specific community members. In general, we observed that, during the first week of life, the microbiota development is typified by a strong increase in absolute bacterial abundance. In the reference group, this coincides with the initial expansion of *Streptococcus* spp. at Day 1, supplanted by rapid niche differentiation at 1 week of life, initially driven by staphylococcal predominance, but quickly followed by the establishment of multiple *Corynebacterium* and *Dolosigranulum* spp., a process which is strongly related to vaginal delivery (4) as well as breastfeeding. Although *Moraxella* spp. become predominant community members over time in most children, in the reference group they only become the main community members from 2–3 months of life on. From that age on, *Moraxella* spp. may still co-occur with *Corynebacterium* and *Dolosigranulum* spp. in a mixed-community profile, or they can truly dominate all other community members in a *Moraxella* spp.–dominated community profile (4). This natural process of consecutive events coincides with normalization of ecological stability from the age of 3 months on, and with fewer infections.

In contrast, children with high susceptibility to RTIs over the first year of life exhibit an accelerated bacterial community maturation from as early as the first month of life on (i.e., before development of their first RTIs). This pattern was characterized by diminished and less-prolonged establishment of *Corynebacterium* and *Dolosigranulum* spp. coinciding with premature predominance of *Moraxella* spp. colonization, and more abundant and prolonged presence of oral types of bacteria in the nasopharyngeal niche, including *Neisseria* and *Prevotella* spp. The observed aberrant microbial succession in children with more RTIs also coincided with decreased bacterial community stability over time, which is in line with previous observations, and supports the ecological theory that more stable microbiota are more resistant to RTIs (10). Interestingly, we could also show that acceleration of microbiota age preceded the occurrence of RTIs, supporting the hypothesis that microbiota changes forego a clinically symptomatic RTI. Conjointly, these findings support our hypothesis that the initial early colonization after birth and subsequent development of URT microbiota over the first months of life impact respiratory health.

Figure 4. (Continued). sampling of 216 d), and after RTI (+1; n = 41; 57 d after RTI) is depicted in boxplots (*see* Figure 2 legend). RMA already increased at time points preceding a factual RTI (median RMA = +7.3 d at T = −2, +37.1 d at T = −1, and +67.8 d at RTI). *P* values are based on a linear mixed model, including timing of sampling (i.e., −2, −1, RTI, or +1) and age (continuous) as fixed effects and subject as random effect. The contrasts −2 versus −1, −1 versus RTI, and RTI versus +1 were tested (*multcomp* package). *$0.01 \leq q < 0.05$; **$0.001 \leq q < 0.01$.

Our data, in line with others, show that prolonged abundance of *Corynebacterium* and *Dolosigranulum* spp. are linked to healthy microbiota development and microbiota stability (10, 17, 20, 26), and are related to breastfeeding and vaginal delivery (4, 8, 27). Their co-occurrence may be explained by the ability of *Dolosigranulum* spp. to produce lactic acid, which plausibly selects for *Corynebacterium* spp. outgrowth (21). Antagonism between *Corynebacterium* spp. and *Streptococcus pneumoniae*, a known respiratory pathogen, may, at least in part, explain their association with respiratory health (17, 26, 28). Because we and others (20, 29) have shown that antibiotic use in infancy is associated with depletion of *Corynebacterium* and *Dolosigranulum* spp., routinely used antibiotics may have more (prolonged) consequences for microbiota-driven resistance against RTIs than is currently thought.

Conversely, accelerated microbial succession patterns in children with more RTIs were characterized by enrichment of *Neisseria* spp. and (facultative) anaerobic, mainly oral species, including *Prevotella* spp., which, in turn, were linked to formula feeding. Similar findings have been reported previously (10, 30), and imply a loss of topography within the upper respiratory tract, suggesting that the host or the local ecosystem is unable to restrain oral microbiota within their niche early in life. As presence of these bacteria is linked to RTI susceptibility, further studies on their role in respiratory health are warranted.

In the literature, conflicting results have been reported regarding the role of *Moraxella* spp. in the pathogenesis of RTIs. Some studies found that *Moraxella* spp. colonization was associated with respiratory infections, including pneumonia and bronchiolitis (11), whereas others reported that the *Moraxella*-dominated profile was associated with bacterial community stability (10, 20) and fewer RTI episodes (10). Although, in our study, development from a *Staphylococcus*- into a *Corynebacterium/Dolosigranulum*-, toward a *Moraxella*-dominated profile, eventually occurs in the great majority of children, we show here that especially lack of *Corynebacterium/Dolosigranulum* spp. establishment coincides with a premature transition from *Staphylococcus*-dominated towards a *Moraxella*-dominated profile, which is associated with influx of oral bacterial species and an increased risk of RTIs (20). In line with this observation, several studies in mice have demonstrated that the neonatal immune system requires cues from the respiratory microbiota for its development within a specific timeframe (12, 13). Indeed, premature *Moraxella* spp. colonization is shown to induce a mixed proinflammatory immune response (31), although data on the effects of *Moraxella* spp. colonization at greater age are lacking. In addition, whether the required microbial triggers might be species and/or strain specific deserves further study.

In our prospective birth cohort study, we collected frequent nasopharyngeal samples of a large number of healthy children at regular intervals over the first year of life as well as during RTIs, allowing us to study microbial development during health and preceding and during RTI episodes. More importantly, it allowed us to explore microbiota dynamics and drivers of susceptibility to RTIs. Strengths of our study include the frequency of sampling and the consistency in data and sample collection by trained doctors and research nurses. We made a rigorous effort to minimize the potential effect of environmental contamination on low-density nasopharyngeal samples collected from children at very early ages. Last, we used nonparametric machine-learning techniques combined with (multivariable) spline-based mixed models to explore specific age-dependent patterns in microbial succession.

Our study also has limitations. First, parents were asked to contact the research team in case of an RTI. Therefore, it is likely that not all RTI episodes were captured for in-depth analyses. Exhaustive efforts were however made to obtain detailed information on all experienced RTIs when questionnaires were filled out during regular home visits to minimize reporting bias in our multivariable analyses (Bosch and colleagues, unpublished data). Second, despite frequent sampling, our samples capture snapshots of a highly dynamic and developing microbiota; therefore, we can only make assumptions about the dynamics in between sampling moments. Third, although we observed that microbiota changes seem to forego RTIs and are associated with RTI susceptibility, our study design precludes any definite statements on causality.

We here provide evidence that accelerated microbiota maturation is associated with microbiota instability and number of RTIs over the first year of life. These changed dynamics could be observed as early as within the first month of age (i.e., before the first RTI experiences). We also were able to link the impact of known important drivers, such as birth mode, feeding type, the presence of siblings, early day care attendance, and recent use of antimicrobial therapy, via altered microbiota development to susceptibility to RTIs. The potential implications of these findings for our understanding of pathogenesis of disease, as well as diagnostic and preventive strategies, deserve further investigation. ■

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