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ORIGINAL ARTICLE

Impact of the bacterial nasopharyngeal microbiota on the severity of genus enterovirus lower respiratory tract infection in children: A case–control study

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Abstract

Introduction: Rhinoviruses (RV) and enteroviruses (EV) are among the main causative etiologies of lower respiratory tract infection (LRTI) in children. The clinical spectrum of RV/EV infection is wide, which could be explained by diverse environmental, pathogen-, and host-related factors. Little is known about the nasopharyngeal microbiota as a risk factor or disease modifier for RV/EV infection in pediatric patients. This study describes distinct nasopharyngeal microbiota profiles according to RV/EV LRTI status in children.

Methods: Cross-sectional case–control study, conducted at Hospital Sant de Déu (Barcelona, Spain) from 2017 to 2020. Three groups of children <5 years were included: healthy controls without viral detection (Group A), mild or asymptomatic controls with RV/EV infection (Group B), and cases with severe RV/EV infection admitted to the pediatric intensive care unit (PICU) (Group C). Nasopharyngeal

Abbreviations: ANCOM-BC, analysis of compositions of microbiomes with bias correction; ASV, amplicon sequence variants; DNA, deoxyribonucleic acid; EV, enterovirus; FDR, false discovery rate; IMV, invasive mechanical ventilation; LRTI, lower respiratory tract infection; NMDS, nonmetric multidimensional scaling; NPA, nasopharyngeal aspirate; PBS, phosphate-buffered saline; PCoA, principal coordinates analysis; PERMANOVA, permutational analysis of variance; PICU, pediatric intensive care unit; RDP, ribosomal data project; RNA, ribonucleic acid; RSV, respiratory syncytial virus; RV, rhinovirus.

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samples were collected from participants for viral DNA/RNA detection by multiplex-polymerase chain reaction and bacterial microbiota characterization by 16S rRNA gene sequencing.

Results: A total of 104 subjects were recruited (A = 17, B = 34, C = 53). Children's nasopharyngeal microbiota composition varied according to their RV/EV infection status. Richness and diversity were decreased among children with severe infection. Nasopharyngeal microbiota profiles enriched in genus *Dolosigranulum* were related to respiratory health, while genus *Haemophilus* was specifically predominant in children with severe RV/EV LRTI. Children with mild or asymptomatic RV/EV infection showed an intermediate profile.

Conclusions: These results suggest a close relationship between the nasopharyngeal microbiota and different clinical presentations of RV/EV infection.

KEYWORDS

enterovirus, intensive care units, lower respiratory tract infection, microbiota, rhinovirus

1 | INTRODUCTION

Lower respiratory tract infection (LRTI) is one of the major causes of morbidity and mortality among children under 5 years old, representing the second cause of death worldwide in 2019.[1] Rhinoviruses (RV) and enteroviruses (EV) are among the main causative etiologies of LRTI in children,[2] even during the COVID-19 pandemic.[3] They are RNA viruses belonging to the genus *Enterovirus* of the family *Picornaviridae*. More than 200 types of RV and EV known to infect humans are currently classified into three species of RV (RV-A to C) and four of EV (EV-A to D).

The clinical spectrum of RV/EV infection ranges from asymptomatic or mild symptomatic presentation to severe disease requiring respiratory support in pediatric intensive care units (PICU).[2] This variability could be explained by several factors. Viral coinfection has been suggested to influence the severity of RV/EV infection, although there is no consensus in the literature about its role as a single factor for worse clinical outcomes.[4] Nonetheless, most of the published literature does not focus on specific viral coinfections as a factor of severity, and this could be different depending on the viral etiology.[4] In this sense, a recent study from our group that focused on the role of viral coinfection in the specific scenario of RV/EV infection showed that virus codetection appears not to be decisive to cause severe LRTI in young children in a PICU setting.[5]

Regarding bacterial coinfection, studies have shown that RV/EV infection in children is associated with increased detection of potential pathogenic bacteria in the respiratory tract (*Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*), causing increased risk of LRTI and asthma symptoms.[6] However, most of this research focused on bacterial-viral has been centered on single pathogenic organisms due to their microbiological culture-based nature.

Nowadays, increased recognition is being paid to the bacterial nasopharyngeal microbiota as an important factor that could determine the severity of viral LRTI.[7] As it could either prevent the host to viral infection through a barrier effect or, on the contrary, predispose to infection by facilitating viral invasion.[8] Conversely, the viral infection could facilitate colonization and pathogen bacterial overgrowth in the respiratory tract, by modulating the immune responses and susceptibility to bacterial invasion.[9] In this regard, it has been described that RV/EV infection in adult patients diagnosed with chronic obstructive pulmonary disease alters the nasopharyngeal microbiota and could precipitate bacterial infections.[10] Although RV/EV has often been described as a colonizer in asymptomatic children by some authors,[11] there are very few studies elucidating if there are changes in nasopharyngeal microbiota associated with RV/EV infection severity in pediatric patients. Microbiota analysis could help to distinguish if RV/EV infection in those asymptomatic or mild symptomatic cases has implied some pathogenic changes with respect to healthy noninfected children.

The primary objective of this study was to characterize and compare the nasopharyngeal microbiota profiles in children with severe RV/EV LRTI, children with asymptomatic or mild RV/EV infection, and healthy children without viral detections.

Our underlying hypothesis was that there was a close relationship between the nasopharyngeal microbiota composition and different clinical presentations of RV/EV infection.

2 | MATERIALS AND METHODS

2.1 | Study design and population

This cross-sectional case–control study was conducted at Sant Joan de Deu Barcelona Children's Hospital (HSJD), a reference university medical center located in Barcelona, Spain. This medical center provides health care services to a pediatric population of ≈400,000

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and has a 28-bed PICU. Cases were prospectively recruited from December 2017 to March 2020. Controls were enrolled from routine well-child check-ups and from the Emergency Department. Cases were frequency-paired by age range (<3, 3–11, >11–23, and >23 months) and gender with the controls.

Three groups of children less than 5-year-old were thus defined for the study: (I) A control group (Group A) of healthy children without viral detection; (II) a control group (Group B) of asymptomatic children or with mild symptomatic–upper respiratory tract infection (URTI) (respiratory symptoms with/without fever, but not requiring hospital admission) and microbiological RV/EV detection in the nasopharyngeal aspirate (NPA); (III) and a case group (Group C) of patients with LRTI (bronchiolitis, bronchospasm/viral wheezing, and/or pneumonia) admitted to the PICU with microbiological RV/EV detection in NPA. Admission criteria for the PICU were respiratory failure requiring invasive (IMV) or noninvasive mechanical ventilation (NIV); high flow oxygen therapy with FiO_2 greater than or equal 0.6; or hemodynamic instability.

Inclusion criteria for cases and controls were (I) meeting the case/control definition described above; (II) having signed informed consent by the parents or legal guardians; and (III) not presenting comorbidities (prematurity, immunodeficiency, congenital heart disease, and chronic lung disease). Exclusion criteria were (I) exposure to antibiotics for more than 24 h either just at the moment of sample collection or in the last month before hospital admission.

Relevant epidemiological and microbiological data were recorded from each participant.

2.2 | Laboratory and bioinformatic procedures

NPA samples were collected from all children according to the standard operating procedure established at the study site.[5] Nasopharyngeal secretions were aspirated using a catheter connected to a vacuum source. The mucus collected within the catheter was then eluted by the aspiration of 1.0 mL sterile phosphate-buffered saline (PBS). The sample was aliquoted and conserved at −80°C until further microbial analyses. Microbiological data included respiratory viral species detection, determination of species of RV/EV for RV/EV-positive sample, and nasopharyngeal microbiota characterization.

Total DNA was extracted from NPAs by the automated system NucliSENSeasyMag (BioMérieux) following the manufacturer's instructions.

The presence of viral respiratory infections in NPAs was tested by Allplex™ Respiratory Panels Assays 1, 2, and 3 (Seegene Inc.) targeting 16 viruses (rhinovirus/enterovirus, adenovirus, bocavirus, coronavirus, metapneumovirus, respiratory syncytial virus (RSV) types A and B, Influenza virus types A and B, and Parainfluenza virus types 1, 2, 3 and 4).

Nasopharyngeal microbiota was characterized by 16S rRNA gene sequencing. Specifically, the V3–V4 hypervariable region were amplified and sequenced using Illumina MiSeq. 2 × 300 bp (Illumina) as previously described in the 16S Metagenomic Sequencing Library Preparation protocol (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16-metagenomic-library-prep-guide-15044223-b.pdf). A total number of 15 negative controls were sequenced along with the samples included in the study for further contamination control.

Quality of sequencing data was assessed with the FASTQC toolkit. All further bioinformatic data analyses were conducted using R programming language. Amplicon sequence variants (ASVs) were binned and quantified using the DADA2 package[12] in R using the ribosomal data project set 16 taxonomic reference database.[13] As part of the DADA2 pipeline, before paired-end read merging, forward and reserve reads were trimmed at positions 10/10 and 280/230 starting and ending positions, respectively, to improve read quality.

Decontam package[14] was used to identify potential kit reagents and environmental contaminants in the 15 sequenced negative controls (13 extraction and 2 polymerase chain reaction controls) so they could be further removed from nasopharyngeal participant samples. Following the default Decontam prevalence approach, we only considered real contaminants those ASVs whose mean relative abundance among negative controls was higher than in nasopharyngeal samples. Additionally, reads that were not assigned to kingdom bacteria or were classified as such, but no further taxonomic resolution was reached were excluded. Finally, only nasopharyngeal samples with a minimum sequencing depth of 10,000 reads were considered.

Alpha diversity metrics (Observed and Chao1 for richness, and Shannon index for diversity) were calculated at the ASV level after rarefying samples at 10,000 reads using the Phyloseq package.[15] Nonrarified samples were used for further analyses at higher taxonomic ranks collapsing reads either at the genus or species level using the Phyloseq package.[16] Beta diversity analyses included ordination analyses using nonmetric multidimensional scaling (NMDS) and Bray–Curtis ecological distance over nonfiltered bacterial genera abundance matrix. Differences in overall composition were tested with permutational analysis of variance using *adonis2* function from Vegan package[16] in R. Before differential abundance testing, bacterial genera and/or species were filtered by having at least 0.5% relative abundance in five samples. Differential abundance of bacterial taxa between pairs of groups was tested using analysis of compositions of microbiomes with bias correction[17] on raw reads matrix. The significant threshold for differential abundance was set to 0.05 and $\log_2$ fold change value higher or equal to 2. Pairwise correlations between bacterial genera were tested using Spearman's correlation over the relative abundance matrix. *p* Values were adjusted using false discovery rate method and significance threshold was set to 0.05.

Categorical variables were assessed with the χ^2 or Fisher test if expected values were below 5. Continuous variables were described as median and interquartile range (IQR) values and were further analyzed using Kruskal–Wallis test. Statistical analysis was performed with SPSS v22.0 software (IBM Corp). A $p < 0.05$ was considered statistically significant.

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2.3 | Ethical considerations

The institutional ethics board approved the study (id PIC 146-17) and informed consent was obtained before obtaining samples from parents and/or legal guardians. All information collected has been treated confidentially and in accordance with applicable laws on personal data.

3 | RESULTS

3.1 | Study population

A total number of 114 participants (57 cases and 57 controls) were initially recruited. Four cases and six controls were excluded because there was previous exposure to antibiotics. Therefore, the final study included 104 participants: 17 healthy controls (Group A), 34 mild/asymptomatic controls (Group B), and 53 PICU RV/EV cases (Group C). Main epidemiological, demographic, and microbiological variables were described in Table 1. For reference, there were 38 patients with chest-X-ray opacities in Group C, and in 9 of them antibiotics were started because bacterial superinfection was suspected (fever > 38.5°C, chest-X-ray opacities and either C-reactive protein > 60 mg/L or procalcitonin > 1 ng/mL) but none of them had a culture-confirmed invasive bacterial infection. Overall, Group C showed higher proportion of delivery by C-section ($p = 0.027$), higher RSV detection ($p < 0.001$), higher percentage of subject's recruitment during winter season ($p = 0.043$), and lower percentage of subjects with at least one dose of mandatory vaccinations ($p < 0.001$). Regarding the specific RV/EV species detected, no differences were found between groups.

3.2 | Nasopharyngeal microbiota and RV/EV infection

Alpha-diversity analysis revealed differences in terms of bacterial richness and diversity between groups. Group C showed significantly lower bacterial richness (Observed—$p = 0.009$ and Chao1—$p = 0.009$) and diversity (Shannon—$p = 0.036$) compared to Group A (Figure 1). Although not statistically significant, Group C also showed trends for lower richness (Observed—$p = 0.057$, Chao1—$p = 0.058$) and diversity (Shannon—$p = 0.070$) compared to Group B (Figure 1).

Beta-diversity analysis revealed differences in the nasopharyngeal microbiota composition of children according to their RV/EV infection status. An ordination analysis at the bacterial genus level revealed significant differences in overall composition between groups (ANOVA $R^2 = 6.8\%/p = 0.0009$) (Figure 2A, top) with a gradient from Groups A to C along the X-axis of the ordination plot (Figure 2A, bottom). A more detailed analysis on the taxa contribution of the axes showed a remarkable gradient of genus *Haemophilus* abundance increasing from left to right (from Group A to C) (Figure 2B). Other abundant bacterial genera with a more even distribution along the X-axis included *Moraxella*, *Streptococcus*, among others (Figure 2B).

Mean relative abundance (±SD) of bacterial genera between study groups (Figure 2C) showed that the nasopharyngeal microbiota of Group A was dominated primarily by genus *Moraxella* (35.0 ± 34.8), followed by *Streptococcus* (18.1 ± 24.1), *Dolosigranulum* (mean 17.6 ± 28.7) and *Haemophilus* (15.3 ± 28.2). Group B was dominated by *Haemophilus* (33.6 ± 37.8), *Moraxella* (31.6 ± 35.0), and *Streptococcus* (19.5 ± 27.1), followed far behind by *Staphylococcus* (5.8 ± 23.1) and others. Group C was even more dominated by *Haemophilus* (50.9 ± 38.8), *Moraxella* (24.0 ± 30.4), and *Streptococcus* (14.5 ± 27.1). A more detailed profiling per sample can be found in Supporting Information: e-Figure 1.

A differential abundant test showed that among all pairwise comparisons (Group A–C), genus *Dolosigranulum* was consistently more abundant in those subjects from Group A compared to any of the RV/EV + groups, irrespective of their symptomatology/disease severity (Figure 3A). On the other hand, genus *Haemophilus* was consistently found to increase in those subjects from Group C both compared to Groups A and B (Figure 3A). Similar findings were found at the species level (Figure 3B), with increased *Dolosigranulum pigrum* and *Dolosigranulum unclassified* in Group A, and *H. influenzae* and *Haemophilus* unclassified in Group C. Of note, an unclassified species within genus *Staphylococcus* (identified as *Staphylococcus aureus* by a blast search against the NCBI 16S rRNA/ITS database) was also found increased in Group A compared to Group C.

Haemophilus, *Moraxella*, and *Streptococcus* not only compromised the topmost abundant bacterial genera in our study population, but the latest two showed a negative correlation to the former (Supporting Information: e-Figure 2). Positive correlations were found between *Corynebacterium* and *Dolosigranulum*, as well as amongst other bacterial genera usually found in the oral cavity including *Acinomyces*, *Rothia*, *Granulicatella*, *Porphyromonas*, *Fusobacterium*, *Alloprevotella*, *Gemella*, *Prevotella*, and *Veillonella* (Supporting Information: e-Figure 2).

A specific analysis was performed to identify changes in microbiota richness, diversity, and bacterial genera composition related to the potential confounders unequally distributed between the study groups (RSV coinfection, type of delivery, seasonality, and having received at least 1one dose of mandatory vaccinations). RSV-infected children did not show differences in either alpha diversity or bacterial genera composition compared to RSV noninfected children, yet this comparison was restricted to Group C. Differences were neither observed between children delivered by c-section and those who had a vaginal delivery, nor between those recruited at winter and those included from other seasons. On the other hand, mandatory vaccination was associated with higher bacterial richness and diversity; however, no significant changes were observed in the main bacterial genera abundance.

4 | DISCUSSION

During the last years, several publications have raised the importance of the bacterial nasopharyngeal microbiota as a factor that could determine the severity of viral LRTI. However, scarce evidence has

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TABLE 1 Main epidemiological and microbiological characteristics of study groups.

	Total (*N* = 104)	Group A (*N* = 17)	Group B (*N* = 34)	Group C (*N* = 53)	*p* Value
Epidemiological characteristics					
Age range (months old)					0.111
<3	53 (51%)	5 (30%)	17 (50%)	31 (58%)	
3–11	25 (24%)	6 (35%)	6 (18%)	13 (24%)	
>11–23	7 (7%)	0 (0%)	4 (12%)	3 (6%)	
>23	19 (18%)	6 (35%)	7 (20%)	6 (12%)	
Gender, male (%)	63 (61%)	9 (53%)	20 (59%)	34 (64%)	0.690
Birth weight, kg, median (IQR)	3.2 (2.9–3.6)	3.2 (2.9–3.7)	3.2 (2.9–3.6)	3.2 (2.9–3.5)	0.720
House surface per inhabitant, m^2, median (IQR)	22.5 (16.9–27.1)	22.9 (18.9–28.7)	20.0 (17.5–25.0)	22.5 (16.0–27.5)	0.597
Seasonality, samples collected during winter season[a] (%)	47 (46%)	7 (41%)	10 (32%)	30 (57%)	**0.043**
Race, Caucasian (%)	70 (76%)	13 (76%)	16 (73%)	41 (77%)	0.912
Delivery mode, C-section (%)	33 (32%)	3 (18%)	7 (21%)	23 (44%)	**0.027**
Breastfeeding (%)	82 (79%)	13 (76%)	27 (79%)	42 (79%)	0.966
Breastfeeding duration, months, median (IQR)	2 (1-6)	3 (0.5-6)	2.5 (0.8-10.7)	1 (1-4.7)	0.601
Schooled (%)	21 (20%)	5 (29%)	10 (30%)	6 (11%)	0.063
Parental smoking (%)	34 (33%)	7 (41%)	10 (29%)	17 (33%)	0.700
Superior educational level (%)	68 (76%)	13 (81%)	17 (81%)	38 (73%)	0.681
Mandatory vaccinations (at least one dose)[b] (%)	51/56 (91%)	11/12 (92%)	23/23 (100%)	17/21 (81%)	0.086
LRTI in the last month (%)	11 (11%)	1 (6%)	2 (6%)	8 (15%)	0.312
Microbiological characteristics					
DNA/RNA other viral detections by multiplex PCR (%)	41 (47%)		11 (32%)	30 (57%)	**0.027**
Human respiratory syncitial virus (A and B) (%)	23 (26%)		1 (3%)	22 (41%)	**<0.001**
Human metapneumovirus (%)	6 (7%)		1 (3%)	5 (9%)	0.397
Human coronaviruses (OC43/229E/NL63) (%)	2 (2%)		2 (6%)	0 (0%)	0.150
Human parainfluenza viruses (1, 2, 3, 4) (%)	4 (5%)		1 (3%)	3 (6%)	1
Human influenza viruses (A and B) (%)	1 (1%)		1 (3%)	0 (0%)	0.391
Human adenovirus (%)	10 (12%)		6 (18%)	4 (7%)	0.179
Human bocavirus(%)	2 (2%)		2 (6%)	0 (0%)	0.150
RV/EV species (%)					0.492
EV-A	1 (1%)		1 (3%)	0 (0%)	
EV-B	1 (1%)		0 (0%)	1 (2%)	
RV-A	37 (42%)		15 (44%)	22 (41%)	
RV-B	9 (10%)		2 (6%)	7 (13%)	
RV-C	30 (35%)		11 (32%)	19 (36%)	
Noncharacterized	9 (10%)		5 (15%)	4 (7%)	

Note: Statistically significant p values are marked with bold.

Abbreviations: DNA, deoxyribonucleic acid; EV, enterovirus; IQR, interquartile range; LRTI, lower respiratory tract infection; PCR, polymerase chain reaction; RNA, ribonucleic acid; RV, rhinovirus.

[a]Winter season: samples collected between January 1 and March 31.

[b]Official vaccination calendar in Catalonia, Spain includes: diphtheria, tetanus, pertussis (DTaP]), poliovirus, hepatitis B virus, and *Haemophilus influenzae* type b, meningococcal group B and pneumococcal conjugate vaccines.

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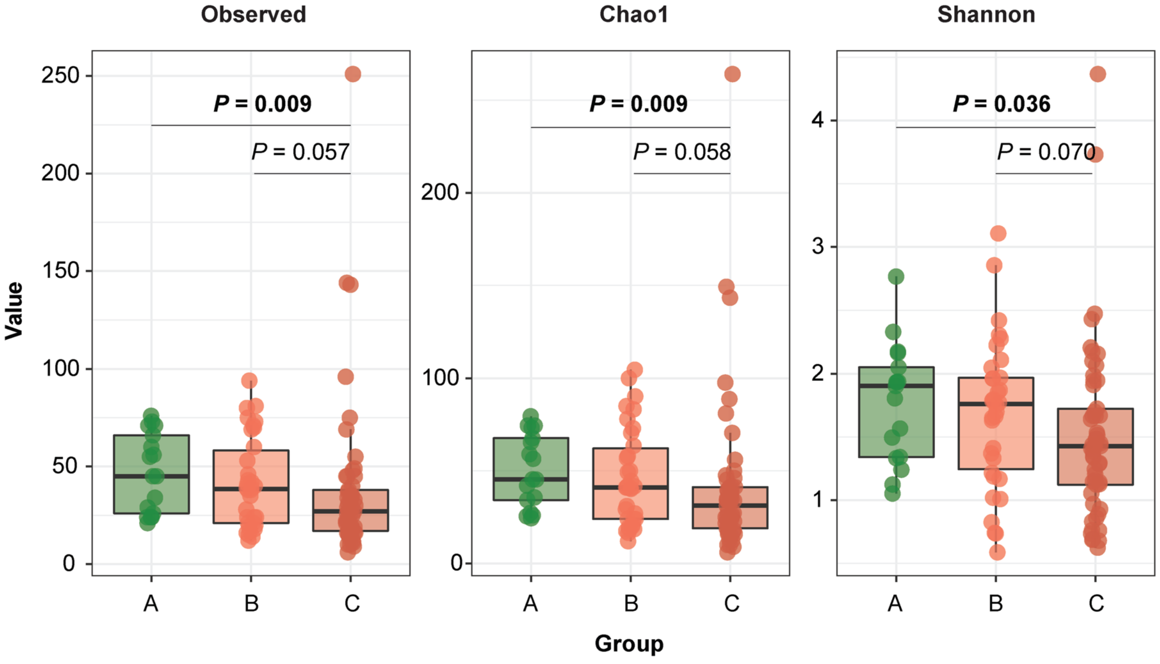

FIGURE 1 Alpha diversity metrics (observed richness, Chao 1 estimator and Shannon diversity index) between study groups. Boxplots showing richness (observed and Chao 1) diversity (Shannon) metrics by study groups (Group A [green], Group B [orange], and Group C (dark red). Nonadjusted *p* values for pairwise comparisons are shown as regular lines if <0.05 or dashed lines if 0.1 > *x* > 0.05.

been published in the specific scenario of RV/EV infection. This study addresses the characterization of different nasopharyngeal microbiota profiles in children according to this infection and its severity.

In terms of bacterial genera composition, in the nasopharynx of our study population topmost abundant players were *Moraxella*, *Haemophilus*, *Streptococcus*, and *Dolosigranulum*. This finding is in line with previous publications showing that during the first year of life, when the nasopharyngeal microbiota is most unstable, initial colonizers such as *Staphylococcus*, *Dolosigranulum*, and *Corynebacterium* are gradually replaced by common pathobionts including *Moraxella*, *Streptococcus*, and *Haemophilus*.[18] Also, in line with previous literature, other bacterial genera found in our data set, yet with lower abundance, included commonly bacterial genera found in the oral cavity of healthy children.[19]

Regarding differences according to RV/EV infection status and its severity, children with severe RV/EV LRTI had a nasopharyngeal microbiota significantly less rich and diverse than healthy controls. Moreover, severe RV/EV infection was also significantly associated to a lower abundance of *Dolosigranulum* and a higher abundance of *Haemophilus*. Interestingly, children with mild or asymptomatic RV/EV infection showed an intermediate profile between the other two groups with less *Dolosigranulum* abundance than the healthy control group, but also trends for higher richness and significantly less *Haemophilus* abundance than the severe RV/EV group. This result is of special interest because RV/EV infection, even in patients with asymptomatic infection, in whom it could be considered a coincidental detection, is associated with changes in bacterial nasopharyngeal composition. This fact would cast doubt on defining as healthy patients those asymptomatic children carrying the virus, but further longitudinal studies focused on this population are needed to elucidate if these changes are long-standing and have clinical consequences.

These findings are in line with previous reports by our group, describing significant reductions in bacterial richness and diversity in the microbiota of respiratory diseases such as invasive pneumococcal disease (IPD),[20] *Bordetella* infection,[21] and similar to the ones found by other authors regarding URTI[22] or asthma,[23] among others. Lower *Dolosigranulum* abundance associated to disease states in children has also been previously reported.[24,25] *Dolosigranulum* is a genus with only one specie known so far, *D. pigrum*.[26] *D. pigrum* belongs to the group of lactic acid bacteria, a heterogeneous group of bacteria that produce lactate as the main metabolite from glucose.[26] These bacteria have been detected mainly in the gastrointestinal tract, where they exert numerous beneficial functions for human health.[26] The colonization of the upper respiratory tract by these bacteria has been defined as a protective factor against respiratory infections, asthma, and exacerbations of chronic respiratory diseases.[20,27,28] Of note, in this study, we did not only describe reductions in the abundance of *Dolosigranulum* with RV/EV infection (regardless of disease severity) but also its positive correlation to genus *Corynebacterium*, a known commensal of the respiratory microbiota,[29,30] and their negative correlation to genus *Streptococcus*[31,32] which includes *S. pneumoniae*, a common opportunistic pathobiont in children. These negative correlations with *S. pneumoniae* were also observed in previous studies by our group,[20,24] and in the literature.[33] This could allude to the existence of antagonistic interactions between both

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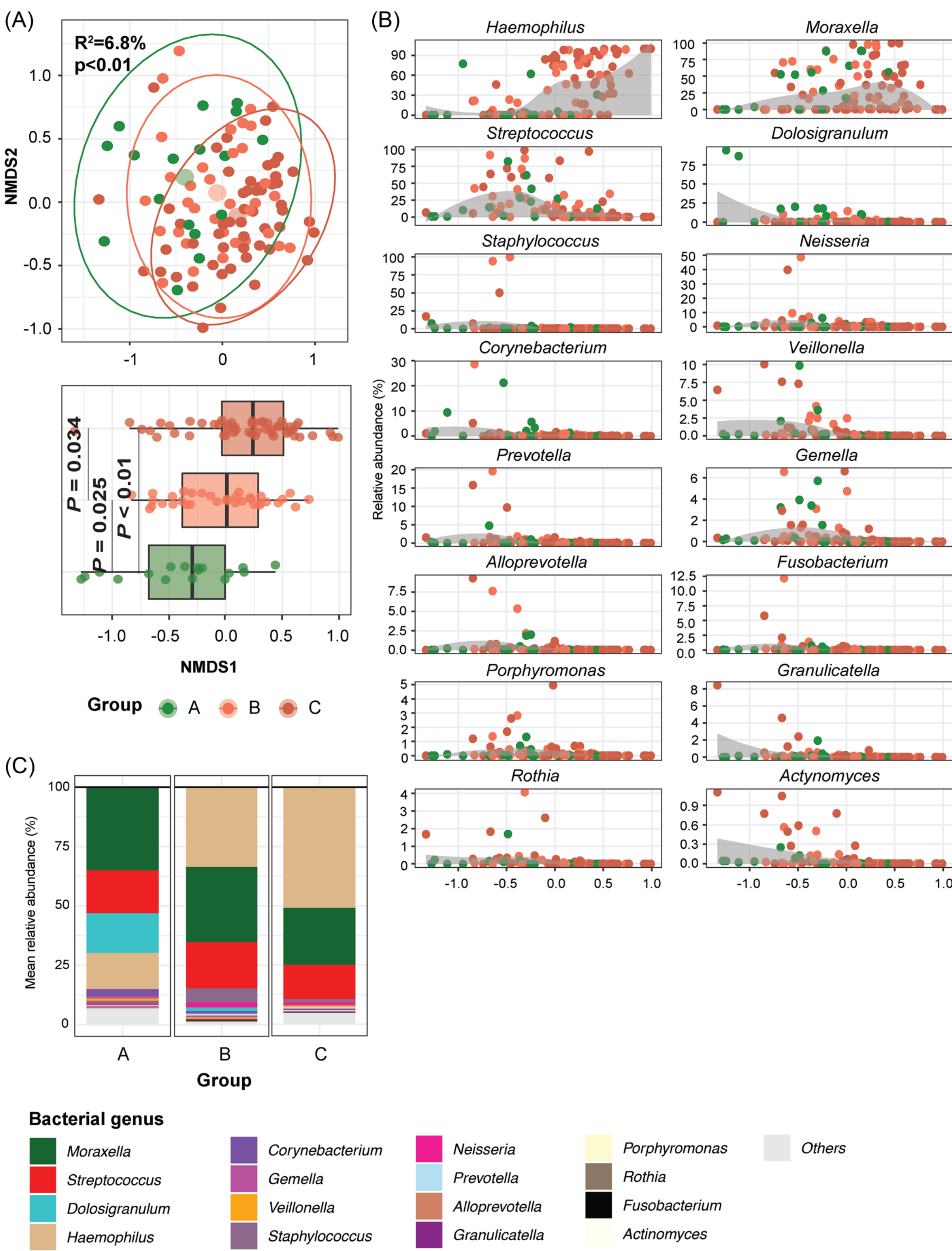

FIGURE 2 Overall nasopharyngeal bacterial genera composition between study groups. (A) Principal coordinates (PCoA) ordination analysis on Bray–Curtis ecological distance matrix calculated on complete bacterial genera abundance taxa (top). Each dot represents a sample and it is colored based on study group (Group A in green, Group B in orange, and Group C in the dark red). Boxplot below shows coordinates from axis 1 (NMDS1) from the ordination plot above. Nonadjusted *p* values for pairwise comparisons are shown as regular lines if <0.05 or dashed lines if 0.1 > *x* > 0.05. (B) Bacterial genera abundance filtered by a minimum of 0.5% relative abundance in at least five samples and ordered from most to least mean abundant. Each dot represents a sample colored by its study group (Group A in green, Group B in orange, and Group C in the dark red). A local regression (gray) shows the relation between the bacterial families' relative abundance and the samples' distribution on Axis 1. (C) Bacterial genera filtered by a minimum of 0.5% relative abundance in at least five samples. Mean relative abundance per group is calculated and shown in each barplot. Bacterial genera not passing the abundance filter are grouped into the "Others" category.

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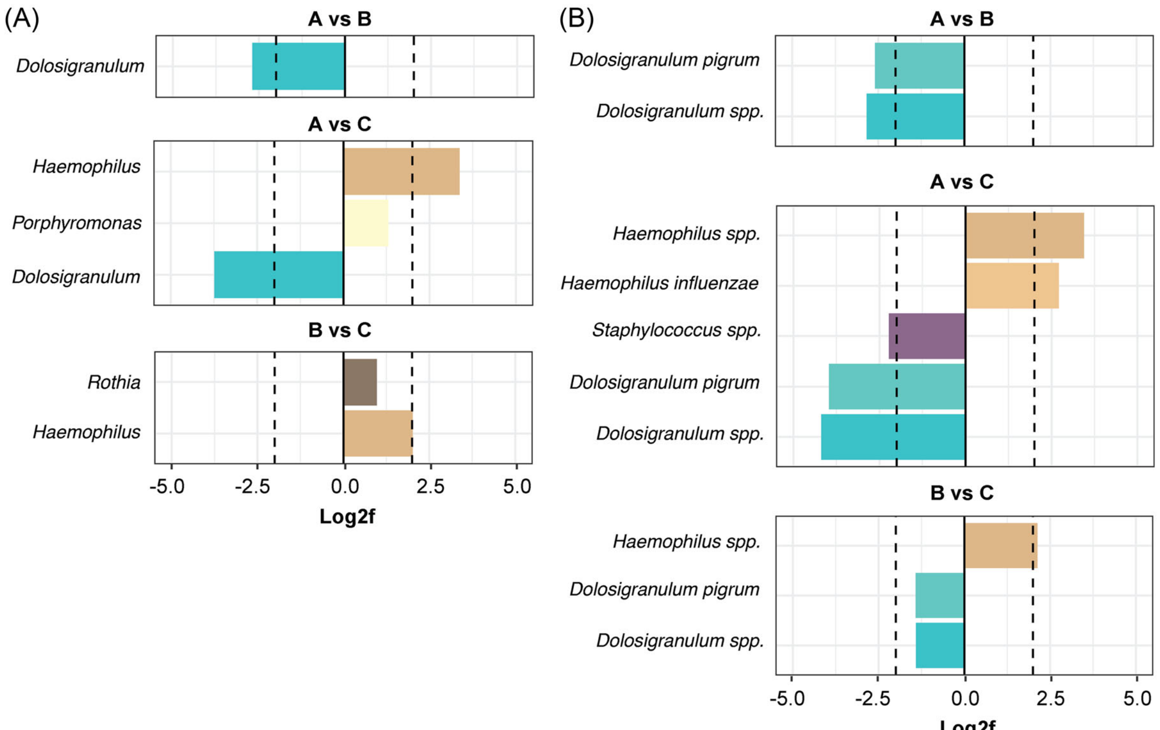

FIGURE 3 Differentially abundant bacterial taxa by RV/EV infection status and severity. Pairwise differential abundance analysis on bacterial taxa per study groups (A, B, and C). Bacterial genera (A) and species (B) filtered by a minimum of 0.5% relative abundance in at least five samples. Log2F is shown along the X-axis and taxa are colored based on their taxonomic classification. EV, enteroviruses; RV, rhinoviruses.

bacteria in the nasopharynx of children as a possible mechanism for the prevention of IPD. Moreover, studies have been carried out in animal models that advance a future clinical application of these observations. In vivo mouse models have shown an increased resistance to pneumococcal infection in those mice in which *D. pigrum* was administered intranasally, as well as differential cytokine production and a reduction in lung damage.[34,35]

In addition to decreased *Dolosigranulum* abundance, severe RV/EV infection in our study showed increased *Haemophilus* abundance. Higher *Proteobacteria* abundance, specifically genus *Haemophilus*, has also been linked to many respiratory diseases such as childhood asthma[36,37] as well as to increased incidence[38] and symptom severity[39] of viral infections. In a small experimental longitudinal series of young adults,[40] RV/EV infection was associated to a very significant increase of *Haemophilus* bacterial load, which is in line with our findings. A similar *Haemophilus* dominant profile has been also observed in children with severe bronchiolitis and RV infection.[41]

Regarding RSV infection, Mansbach et al. showed that clearance of RSV during bronchiolitis was more likely to be reduced in subjects with a *Haemophilus* dominant profile.[42] A more severe RSV disease has been associated with a nasopharyngeal microbiota profile enriched in *H. influenzae* and *Streptococcus*.[42,43] However, it is worth noting that in our study RSV infection, which was only detected in 1 (3%) mild/asymptomatic controls and in 22 (41%) cases, did not relate to significant changes neither on alpha diversity nor bacterial genera differential abundance within our RV/EV case group. Therefore, we have not observed the high dominance of *Streptococcus* reported by others in RSV severe cases. We speculate that this fact could imply that RV/EV + RSV coinfection has differential features in reference to RSV bronchiolitis.

Vaccination strategies have also been reported to have beneficial effects through the modulation of commensal nasopharyngeal microbiota.[44] In our study, the subgroup of children subjected to mandatory vaccination was characterized by increased bacterial richness and diversity. These results are aligned with those of previous studies by our group[20] and in the literature,[45] suggesting that the microorganisms from the surrounding environment (for instance, the oral cavity) might occupy the nasopharyngeal niche left by bacteria serotypes included in vaccines. Of note, cases in our study were less vaccinated than controls and showed lower bacterial richness and diversity, as well as decreased *Dolosigranulum* abundance, reinforcing the potential beneficial effect of vaccination.

This study presents several limitations. First, the study population was small but of similar size to previous studies examining these relationships in respiratory contexts.[46] Second, due to the cross-sectional case–control design, no causality could be established from the outcomes, only associations, and no follow-up could be made on changes in the nasopharyngeal microbiota after PICU discharge.

In conclusion, different nasopharyngeal microbiota profiles according to RV/EV LRTI status in children were characterized in

our study. Richness and diversity were decreased among children with severe infection by viruses from the genus *Enterovirus*. Nasopharyngeal microbiota profiles enriched in genus *Dolosigranulum* were related to respiratory health, while genus *Haemophilus* was specifically predominant in children with severe RV/EV LRTI. Children with mild or asymptomatic RV/EV infection showed an intermediate profile. These findings suggest a close relationship between the nasopharyngeal microbiota and different clinical presentations of RV/EV infection.

AUTHOR CONTRIBUTIONS

Daniel Penela-Sánchez, Muntsa Rocafort, Desirée Henares, Iolanda Jordan, Maria Cabrerizo, and Cristian Launes performed case–control recruitment and data collection. Daniel Penela-Sánchez, Muntsa Rocafort, Desirée Henares, and Pedro Brotons did the bioinformatic and statistical analysis. Cristian Launes and Carmen Muñoz-Almagro conceptualized the study and obtained funding for its realization. All authors contributed to the manuscript preparation, revision and edition, and have provided approval for submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Daniel Penela-Sánchez, Cristian Launes and Muntsa Rocafort have access to all study data and takes responsibility for the data integrity and accuracy of the data analysis. The datasets generated during and/or analyzed during the current study are available in the European Nucleotide Archive (ENA) repository (https://www.ebi.ac.uk/ena/browser/home) under Bioproject PRJEB52956. Code used for the bioinformatic and statistical analysis and plotting the data (10.6084/m9.figshare.19826008) as well as the input data file (10.6084/m9.figshare.19825837) and color dictionary (10.6084/m9.figshare.19825249; 10.6084/m9.figshare.19825450) have been uploaded to FigShare.

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REFERENCES

1. GBD 2019 Under-5 Mortality Collaborators. Global, regional, and national progress towards Sustainable Development Goal 3.2 for neonatal and child health: all-cause and cause-specific mortality findings from the Global Burden of Disease Study 2019. *Lancet*. 2021;398(10303):870-905. doi:10.1016/S0140-6736(21)01207-1
2. Lauinger IL, Bible JM, Halligan EP, et al. Patient characteristics and severity of human rhinovirus infections in children. *J Clin Virol*. 2013;58(1):216-220. doi:10.1016/j.jcv.2013.06.042
3. Brotons P, Jordan I, Bassat Q, et al. The positive rhinovirus/enterovirus detection and SARS-CoV-2 persistence beyond the acute infection phase: an intra-household surveillance study. *Viruses*. 2021;13(8):1598. doi:10.3390/v13081598
4. Meskill SD, O'Bryant SC. Respiratory virus co-infection in acute respiratory infections in children. *Curr Infect Dis Rep*. 2020;22(1):3. doi:10.1007/s11908-020-0711-8
5. Penela-Sánchez D, González-de-Audicana J, Armero G, et al. Lower respiratory tract infection and genus enterovirus in children requiring intensive care: clinical manifestations and impact of viral co-Infections. *Viruses*. 2021;13(10):2059. doi:10.3390/v13102059
6. Kloepfer KM, Lee WM, Pappas TE, et al. Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. *J Allergy Clin Immunol*. 2014;133(5):1301-1307.e3. doi:10.1016/j.jaci.2014.02.030
7. de Steenhuijsen Piters WAA, Sanders EAM, Bogaert D. The role of the local microbial ecosystem in respiratory health and disease. *Philos Trans R Soc Lond B Biol Sci*. 2015;370(1675):20140294. doi:10.1098/rstb.2014.0294
8. Di Pierro F, Colombo M, Zanvit A, Risso P, Rottoli A. Use of *Streptococcus salivarius* K12 in the prevention of streptococcal and viral pharyngotonsillitis in children. *Drug Healthc Patient Saf*. 2014;6:15-20. doi:10.2147/DHPS.S59665
9. Allen EK, Koeppel AF, Hendley JO, Turner SD, Winther B, Sale MM. Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge. *Microbiome*. 2014;2:22. doi:10.1186/2049-2618-2-22
10. Molyneaux PL, Mallia P, Cox MJ, et al. Outgrowth of the bacterial airway microbiome after rhinovirus exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2013;188(10):1224-1231. doi:10.1164/rccm.201302-0341OC
11. Jartti T, Jartti L, Peltola V, Waris M, Ruuskanen O. Identification of respiratory viruses in asymptomatic subjects: asymptomatic respiratory viral infections. *Pediatr Infect Dis J*. 2008;27(12):1103-1107. doi:10.1097/INF.0b013e31817e695d
12. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from illumina amplicon data. *Nature Methods*. 2016;13(7):581-583. doi:10.1038/nmeth.3869
13. Cole JR, Wang Q, Fish JA, et al. Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res*. 2014;42(Database issue):D633-D642. doi:10.1093/nar/gkt1244
14. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*. 2018;6(1):226. doi:10.1186/s40168-018-0605-2
15. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013;8(4):e61217. doi:10.1371/journal.pone.0061217
16. Oksanen J, Blanchet FG, Kindt R, et al. *Package 'vegan.' R Package Version 2.0–8*. 2013;2:254. https://github.com/vegandevs/vegan
17. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. *Nat Commun*. 2020;11(1):3514. doi:10.1038/s41467-020-17041-7
18. Bosch AATM, Piters WAAS, van Houten MA, et al. Maturation of the infant respiratory microbiota, environmental drivers, and health consequences. A prospective cohort study. *Am J Respir Crit Care Med*. 2017;196(12):1582-1590. doi:10.1164/rccm.201703-0554OC

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19. Dewhirst FE, Chen T, Izard J, et al. The human oral microbiome. *J Bacteriol*. 2010;192(19):5002-5017. doi:10.1128/JB.00542-10
20. Henares D, Brotons P, de Sevilla MF, et al. Differential nasopharyngeal microbiota composition in children according to respiratory health status. *Microb Genom*. 2021;7(10):000661. doi:10.1099/mgen.0.000661
21. Rocafort M, Henares D, Brotons P, et al. Exploring the nasopharyngeal microbiota composition in infants with whooping cough: a test-negative case-control study. *PLoS One*. 2021;16(10): e0259318. doi:10.1371/journal.pone.0259318
22. Sakwinska O, Bastic Schmid V, Berger B, et al. Nasopharyngeal microbiota in healthy children and pneumonia patients. *J Clin Microbiol*. 2014;52(5):1590-1594. doi:10.1128/JCM.03280-13
23. Chen M, He S, Miles P, et al. Nasal bacterial microbiome differs between healthy controls and those with asthma and allergic rhinitis. *Front Cell Infect Microbiol*. 2022;12:841995. doi:10.3389/fcimb.2022.841995
24. Camelo-Castillo A, Henares D, Brotons P, et al. Nasopharyngeal microbiota in children with invasive pneumococcal disease: identification of bacteria with potential disease-promoting and protective effects. *Front Microbiol*. 2019;10:11. doi:10.3389/fmicb.2019.00011
25. Lappan R, Imbrogno K, Sikazwe C, et al. A microbiome case-control study of recurrent acute otitis media identified potentially protective bacterial genera. *BMC Microbiol*. 2018;18(1):13. doi:10.1186/s12866-018-1154-3
26. Mokoena MP. Lactic acid bacteria and their bacteriocins: classification, biosynthesis and applications against uropathogens: a mini-review. *Molecules*. 2017;22(8):1255. doi:10.3390/molecules22081255
27. Hasegawa K, Linnemann RW, Mansbach JM, et al. Nasal airway microbiota profile and severe bronchiolitis in infants: a case-control study. *Pediatr Infect Dis J*. 2017;36(11):1044-1051. doi:10.1097/INF.0000000000001500
28. Kaul D, Rathnasinghe R, Ferres M, et al. Microbiome disturbance and resilience dynamics of the upper respiratory tract during influenza A virus infection. *Nat Commun*. 2020;11(1):2537. doi:10.1038/s41467-020-16429-9
29. Cremers AJ, Zomer AL, Gritzfeld JF, et al. The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. *Microbiome*. 2014;2:44. doi:10.1186/2049-2618-2-44
30. De Boeck I, Wittouck S, Wuyts S, et al. Comparing the healthy nose and nasopharynx microbiota reveals continuity as well as niche-specificity. *Front Microbiol*. 2017;8:2372. doi:10.3389/fmicb.2017.02372
31. Bomar L, Brugger SD, Yost BH, Davies SS, Lemon KP. Corynebacterium accolens releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. *mBio*. 2016;7(1): e01725-15. doi:10.1128/mBio.01725-15
32. Brugger SD, Eslami SM, Pettigrew MM, et al. Dolosigranulumpigrum cooperation and competition in human nasal microbiota. *mSphere*. 2020;5(5):e00852-20. doi:10.1128/mSphere.00852-20
33. de Steenhuijsen Piters WAA, Jochems SP, Mitsi E, et al. Interaction between the nasal microbiota and S. pneumoniae in the context of live-attenuated influenza vaccine. *Nat Commun*. 2019;10(1):2981. doi:10.1038/s41467-019-10814-9
34. Ortiz Moyano R, Raya Tonetti F, Tomokiyo M, et al. The ability of respiratory commensal bacteria to beneficially modulate the lung innate immune response is a strain dependent characteristic. *Microorganisms*. 2020;8(5):727. doi:10.3390/MICROORGANISMS8050727
35. Raya Tonetti F, Tomokiyo M, Ortiz Moyano R, et al. The respiratory commensal bacterium *Dolosigranulum pigrum* 040417 improves the innate immune response to *Streptococcus pneumoniae*. *Microorganisms*. 2021;9(6):1324. doi:10.3390/MICROORGANISMS9061324
36. Hyde ER, Petrosino JF, Piedra PA, Camargo CA, Jr., Espinola JA, Mansbach JM. Nasopharyngeal proteobacteria are associated with viral etiology and acute wheezing in children with severe bronchiolitis. *J Allergy Clin Immunol*. 2014;133(4):1220-1222.e3. doi:10.1016/j.jaci.2013.10.049
37. Teo SM, Mok D, Pham K, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe*. 2015;17(5):704-715.
38. Rosas-Salazar C, Shilts MH, Tovchigrechko A, et al. Nasopharyngeal microbiome in respiratory syncytial virus resembles profile associated with increased childhood asthma risk. *Am J Respir Crit Care Med*. 2016;193(10):1180-1183. doi:10.1016/j.chom.2015.03.008
39. Langevin S, Pichon M, Smith E, et al. Early nasopharyngeal microbial signature associated with severe influenza in children: a retrospective pilot study. *J Gen Virol*. 2017;98(10):2425-2437. doi:10.1099/jgv.0.000920
40. Hofstra JJ, Matamoros S, van de Pol MA, et al. Changes in microbiota during experimental human rhinovirus infection. *BMC Infect Dis*. 2015;15:336. doi:10.1186/s12879-015-1081-y
41. Toivonen L, Camargo CA, Jr., Gern JE, et al. Association between rhinovirus species and nasopharyngeal microbiota in infants with severe bronchiolitis. *J Allergy Clin Immunol*. 2019;143(5): 1925-1928.e7. doi:10.1016/j.jaci.2018.12.1004
42. Mansbach JM, Hasegawa K, Piedra PA, et al. Haemophilus-dominant nasopharyngeal microbiota is associated with delayed clearance of respiratory syncytial virus in infants hospitalized for bronchiolitis. *J Infect Dis*. 2019;219(11):1804-1808. doi:10.1093/infdis/jiy741
43. de SteenhuijsenPiters WAA, Heinonen S, Hasrat R, et al. Nasopharyngeal microbiota, host transcriptome, and disease severity in children with respiratory syncytial virus infection. *Am J Respir Crit Care Med*. 2016;194(9):1104-1115. doi:10.1164/rccm.201602-0220OC
44. Mika M, Maurer J, Korten I, et al. Influence of the pneumococcal conjugate vaccines on the temporal variation of pneumococcal carriage and the nasal microbiota in healthy infants: a longitudinal analysis of a case–control study. *Microbiome*. 2017;5(1):85. doi:10.1186/s40168-017-0302-6
45. Biesbroek G, Wang X, Keijser BJF, et al. Seven-valent pneumococcal conjugate vaccine and nasopharyngeal microbiota in healthy children. *Emerging Infect Dis*. 2014;20(2):201-210. doi:10.3201/eid2002.131220
46. Marsh RL, Smith-Vaughan HC, Chen ACH, et al. Multiple respiratory microbiota profiles are associated with lower airway inflammation in children with protracted bacterial bronchitis. *Chest*. 2019;155(4): 778-786. doi:10.1016/j.chest.2019.01.002

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