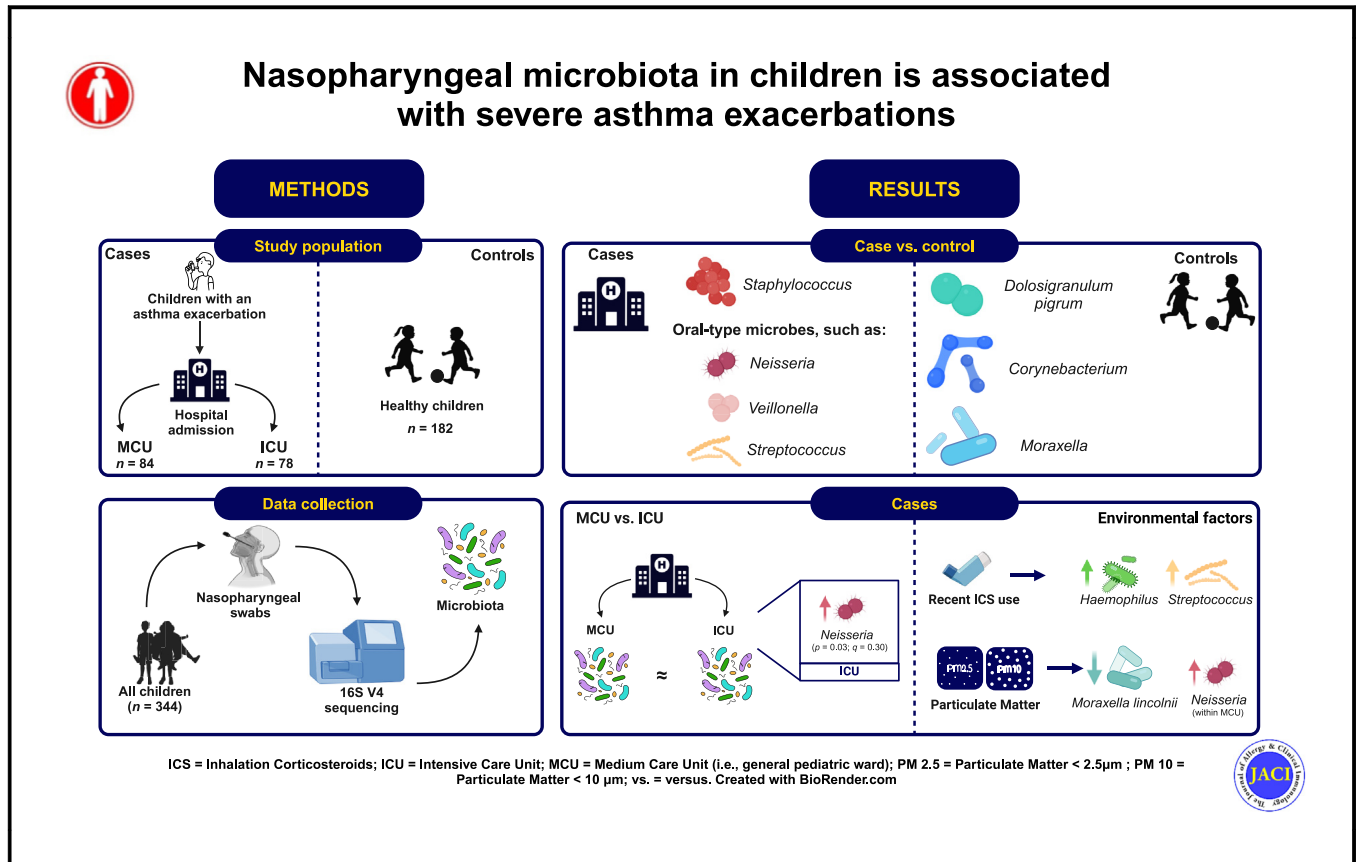


Nasopharyngeal microbiota in children is associated with severe asthma exacerbations



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GRAPHICAL ABSTRACT



Capsule summary: The nasopharyngeal bacterial community in severe pediatric asthma exacerbations is characterized by distinct microbes, including *Staphylococcus* and “oral” microbes, and lacks beneficial niche-appropriate bacteria.

Nasopharyngeal microbiota in children is associated with severe asthma exacerbations



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Background: The respiratory microbiome has been associated with the etiology and disease course of asthma.

Objective: We sought to assess the nasopharyngeal microbiota in children with a severe asthma exacerbation and their associations with medication, air quality, and viral infection.

Methods: A cross-sectional study was performed among children aged 2 to 18 years admitted to the medium care unit (MCU; n = 84) or intensive care unit (ICU; n = 78) with an asthma exacerbation. For case-control analyses, we matched all cases aged 2 to 6 years (n = 87) to controls in a 1:2 ratio. Controls were participants of either a prospective case-control study or a longitudinal birth cohort (n = 182). The nasopharyngeal microbiota was characterized by 16S-rRNA-gene sequencing.

Results: Cases showed higher Shannon diversity index (ICU and MCU combined; $P = .002$) and a distinct microbial community composition when compared with controls (permutational multivariate ANOVA $R^2 = 1.9\%$; $P < .001$). We observed significantly higher abundance of *Staphylococcus* and “oral” taxa, including *Neisseria*, *Veillonella*, and *Streptococcus* spp. and a lower abundance of *Dolosigranulum pigrum*, *Corynebacterium*, and *Moraxella* spp. (MaAsLin2; $q < 0.25$) in cases versus controls. Furthermore, *Neisseria* abundance was associated with more severe disease (ICU vs MCU MaAsLin2, $P = .03$; $q = 0.30$). *Neisseria* spp. abundance was also related with fine particulate matter exposure, whereas *Haemophilus* and *Streptococcus*

abundances were related with recent inhaled corticosteroid use. We observed no correlations with viral infection.

Conclusions: Our results demonstrate that children admitted with asthma exacerbations harbor a microbiome characterized by overgrowth of *Staphylococcus* and “oral” microbes and an underrepresentation of beneficial niche-appropriate commensals. Several of these associations may be explained by (environmental or medical) exposures, although cause-consequence relationships remain unclear and require further investigations. (J Allergy Clin Immunol 2024;153:1574-85.)

Key words: Asthma, exacerbation, respiratory microbiome

Asthma, one of the most common noncommunicable childhood diseases, is characterized by a (sub)acute worsening of symptoms called exacerbation. Exacerbations can be triggered by exposure to allergens, tobacco smoke, air pollution, or viral infections.¹ The severity of these exacerbations ranges from mild and manageable at home to severe exacerbations requiring hospitalization and potentially admission to the intensive care unit (ICU).¹ Risk factors for ICU admission in children with an asthma exacerbation include older age,^{2,3} tobacco smoke exposure, and symptoms for over a week before presentation.³

Recent studies have increasingly focused on the role of the respiratory microbiome in the etiology of asthma. First, early-life colonization with *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae* and lower respiratory tract infections in infancy have been related to the development of wheeze and/or asthma later in childhood.⁴⁻⁶ Second, patients with asthma have shown different airway microbiomes compared with healthy controls,⁷⁻¹¹ with higher relative abundances of *Streptococcus*,⁹ *Moraxella*,⁸ *Haemophilus*,^{7,10,12} and *Staphylococcus*^{7,11} in patients with asthma, whereas *Lactobacillus*,¹³ *Dolosigranulum*,^{14,15} and *Corynebacterium*¹⁵ have been associated with health. Lastly, certain respiratory microbiota profiles have been associated with asthma severity and increased risk of exacerbations in both children^{16,17} and adults.¹⁸⁻²⁰ Particularly, children with increased *Moraxella* and reduced *Dolosigranulum*,^{15,17} *Corynebacterium*,^{16,17} and *Staphylococcus*^{16,21} levels during stable disease are more likely to experience asthma exacerbations.

Currently, studies on the nasopharyngeal microbiota during severe exacerbations are limited and lack differentiation between levels of exacerbation severity. To better understand the nasopharyngeal microbiome composition and its involvement in severe asthma, we (1) investigated differences in nasopharyngeal microbiota between children admitted with an asthma exacerbation and healthy controls and (2) examined the microbiota

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Abbreviations used

ASV:	Amplicon sequence variant
ICS:	Inhaled corticosteroid
ICU:	Intensive care unit
MCU:	Medium care unit
MOL:	Microbiome Onderste Luchtweginfectie (microbiome lower airway infection)
MUIS:	Microbiome Utrecht Infant Study
PDC:	Proportion of days covered
PERMANOVA:	Permutational multivariate ANOVA
PM _{2.5} :	Particulate matter with diameter of less than 2.5 μm
PM ₁₀ :	Particulate matter with diameter of less than 10 μm
SDI:	Shannon diversity index
STATIC-PRO:	Status Asthmaticus on the Intensive Care Prospective

difference between children admitted to the pediatric general ward (medium care unit [MCU]) and ICU as a proxy for exacerbation severity levels. Secondary aims were to explore patient-related (inhaled corticosteroid [ICS] use) and environment-related (viruses and air quality) factors affecting upper airway microbial communities.

METHODS**Study population**

We used samples and data obtained from 3 cohorts: (1) patients participating in the Status Asthmaticus on the Intensive Care Prospective (STATIC-PRO) cohort,³ (2) controls participating in the Microbiome Onderste Luchtweginfectie (MOL; microbiome lower airway infection) cohort,²² and (3) controls participating in the Microbiome Utrecht Infant Study (MUIS) cohort.²³

STATIC-PRO is a cross-sectional study conducted between 2016 and 2018 in the Netherlands. Details on study design were previously described³ and can be found in the Online Repository at www.jacionline.org. Briefly, children (aged 2–18 years) with a doctor-diagnosed asthma exacerbation, requiring systemic corticosteroid treatment and admission to one of the participating pediatric ICUs or pediatric general wards (MCU), were enrolled as cases. Nasopharyngeal samples were collected within 24 hours of admission to hospital and immediately stored at -80°C . Information on environmental triggers, medication use, and medical history was collected from doctors, patients, and caregivers through online questionnaires and from medical and pharmacy records. Asthma severity was determined using the Global Initiative for Asthma definition of mild, moderate, and severe persistent asthma, which is based on treatment¹ and was (re)evaluated before and 3, 6, and 9 months after admission to ensure robustness of the diagnosis. The proportion of days covered (PDC) with ICSs in the year up to admission was calculated using pharmacy records. Respiratory viral pathogens were detected using real-time reverse transcriptase PCR, the details of which were described previously²⁴ and can be found in the Online Repository. Air quality data, including nitrogen dioxide, particulate matter with diameter of less than 2.5 μm (PM_{2.5}), and particulate matter with diameter less than 10 μm (PM₁₀), were collected as the annual average exposure in the sampling year on the basis of a 100 \times 100 m area around the home address postal code.

To maximize statistical power and to minimize potential confounding, healthy controls aged 2 to 6 years were selected from 2 independent studies, the MOL cohort and the MUIS cohort. The MOL cohort (study conducted between 2013 and 2016) was a prospective matched case-control cohort that enrolled children aged 0 to 5 years hospitalized for acute lower respiratory tract infections and their age-, time-, and sex-matched controls. The MUIS study was a longitudinal birth cohort study that followed healthy term-born children from birth to age 6 years. To avoid repeated measures, we selected 1 sample per subject from the MUIS cohort. Details on study design, sample collection, and inclusion criteria for the MOL and MUIS studies have been described previously (MOL²² and MUIS²³).

Data sets for microbiota analyses

Four data sets were constructed from these 3 studies to limit confounding by age and answer different research questions. Data set 1 included all microbiota samples. Data set 2 included cases and healthy controls aged 2 to 6 years to investigate nasopharyngeal microbiota differences between children with asthma exacerbations and healthy controls. Because we did not have access to healthy controls older than 6 years, case-control analyses were limited to children younger than 7 years. To ensure appropriate matching with the case cohort, we age-matched cases to controls, leading to a nearly 1:2 ratio (data set 2). Data set 3 was constructed to compare MCU- and ICU-admitted children. Because age was unevenly distributed between MCU- and ICU-admitted children 10 years and older (see Fig E1, A, in this article's Online Repository at www.jacionline.org), we stratified this analysis for children younger than 10 years (data set 3) to ensure a more even age distribution. Data set 4 included all cases and was used to investigate patient-related (ICS use) and environment-related (viruses and air pollution) factors affecting upper airway microbial communities. For an overview of the data sets and the analyses they were used for, see Table I.

Sample processing

Bacterial DNA was extracted, using a modified bead-based Agowa protocol, as previously described.^{23,25} DNA was quantified through 16S quantitative PCR, and samples with bacterial density greater than 0.1 pg/ μL were amplified using the 515F/806R-primer pairs targeting the V4 region of the 16S-ribosomal RNA gene. Positive and negative control samples were included in each run for quality control. Amplicon libraries were sequenced on an Illumina MiSeq machine (MiSeq reagent Kit v2; Illumina, San Diego, Calif).^{23,25} The same laboratory team performed DNA isolation and amplification using the same protocol for all 3 studies. Bioinformatic processing of the sequence data was performed using DADA2²⁶ to filter, trim (maxEE = 2; truncLen = 200/150 bp), denoise, and merge paired-end reads, as previously described.^{26,27} Chimeras were removed and sequences were combined into amplicon sequence variants (ASVs). Taxonomy was assigned using the naive Bayesian classifier and the Silva v138.2 database.²⁸ Contaminating ASVs were identified and removed using the “decontam” R package (version 1.14.0; see the Online Repository for details). Samples with more than 9500 reads remaining after quality control were included for further analyses. We inspected read counts before and after removing contaminating reads (*decontam*; see Table E1 in this

TABLE I. Overview of the constructed data sets

Dataset	n	Analyses performed on data set
Data set 1: All cases and controls	<ul style="list-style-type: none"> ● n (MCU) = 84 ● n (ICU) = 78 ● n (control) = 182 ● n (total) = 344 	<ul style="list-style-type: none"> ● Hierarchical clustering
Data set 2: Case vs control (age 2-6 y)	<ul style="list-style-type: none"> ● n (MCU) = 63 ● n (ICU) = 24 ● n (control) = 182 ● n (total) = 269 	<ul style="list-style-type: none"> ● Alpha diversity ● Beta diversity (PERMANOVA) ● MaAsLin2 and ALDEx2 ● Multivariable logistic regression
Data set 3: MCU vs ICU (age < 10 y)	<ul style="list-style-type: none"> ● n (MCU) = 80 ● n (ICU) = 46 ● n (total) = 126 	<ul style="list-style-type: none"> ● χ^2/Kruskal-Wallis/Fisher exact/Fisher-Freeman-Halton exact test ● Alpha diversity ● Beta diversity (PERMANOVA) ● MaAsLin2 and ALDEx2
Data set 4: Host/environmental factors in cases (age 2-18 y)	<ul style="list-style-type: none"> ● n (MCU) = 84 ● n (ICU) = 78 ● n (total) = 162 	<ul style="list-style-type: none"> ● Beta diversity (PERMANOVA) ● MaAsLin2

Description of the 4 data sets that were constructed from the 3 study cohorts. The description of the data set includes the number of samples included from each study group (ie, MCU-admitted, ICU-admitted, or healthy control), the age range, and what analyses were performed on the data sets.

article's Online Repository at www.jacionline.org) and evaluated the read depth per study cohort (Fig E1, B). In addition, we confirmed adequate sequence depth through rarefaction curves (Fig E1, C). Comparing the controls of the 2 cohorts regarding alpha and beta diversity and differentially abundant ASVs confirmed the homogeneity of the control group (2-year-olds: see Fig E2 in this article's Online Repository at www.jacionline.org; 3-year-olds: data not shown). See the Online Repository for details. Microbiota data were normalized using total sum scaling and filtered for ASVs with more than 0.1% abundance in at least 1 sample across the 3 studies.

Microbiota analyses

All analyses were performed in R (version 4.1.2) and RStudio (version 2021.09.1+372). *P* values less than .05 were considered significant, unless otherwise specified. Multiple comparisons were adjusted when necessary using the Benjamini-Hochberg method, resulting in adjusted *P* values (*q* values).

Correlation between baseline characteristics and overall microbiota was inspected via permutational multivariate ANOVA (PERMANOVA) within cases (data set 4).

Variables with a *P* value less than or equal to .2 in univariable analyses were included in a multivariable model to assess their independent effects. All analyses were corrected for covariables that remained significant in the multivariable model. In addition, we corrected all analyses for age, because this is known to affect the microbiome composition and differed significantly among case groups.

Within-sample diversity (alpha diversity) was assessed using observed ASVs (richness), the Shannon diversity index (SDI), and Pielou evenness (data sets 2 and 3) on both rarefied and nonrarefied read counts. Statistical significance between groups was assessed using ANOVA and Dunnett *post hoc* tests. Differences in overall microbiome composition were assessed using the *adonis2* function (vegan package)²⁹ and visualized through principal-coordinate analysis on the basis of the Bray-Curtis dissimilarity matrices (data sets 2-4).

Microbiota clusters were identified through unsupervised hierarchical clustering with complete linkage including all samples. The number of clusters was determined on the basis of average Silhouette and Calinski-Harabasz scores (data set 1).

Chi-square tests were used to examine differences in microbiota clusters between cases and controls. Clusters with at least 10 observations were included in these analyses (data set 2).

Differentially abundant ASVs between groups (ie, cases vs controls, stratified MCU vs controls, and ICU vs controls) were identified using the MaAsLin2 package (input data: relative abundance, normalization = "NONE," min_abundance = 0, min_prevalence = 0, otherwise default settings were used)³⁰ and the ALDEx2 algorithm (input data: raw counts, normalization: center log ratio; test method: *aldex.glm()*).³¹ Given our sample size, we aimed to test a maximum of 30 ASVs with a prevalence of at least 5% in all samples, setting a relative abundance threshold of 0.4% (on the basis of samples in data sets 2 and 3). In total, 29 ASVs fulfilled these criteria. The same ASVs were tested for all comparisons between study groups (data sets 2 and 3). To investigate the contribution of multiple taxa simultaneously, we used a *post hoc* multivariable logistic regression model on the microbial biomarkers that were identified with the MaAsLin2 analysis (data set 2). Using backward selection, we identified the most optimal model. Relative abundance of ASVs plus age were predictors and being a case or control was the outcome.

We explored patient- and environment-related factors associated with the nasopharyngeal microbiota within cases (data set 4). Using MaAsLin2, we assessed the correlation between ICS, virus presence, air pollution exposure, and microbiota for the 15 most abundant ASVs in multivariable models (data set 4). We used the default MaAsLin2 false-detection rate-corrected *q* value (<0.25) to determine significant ASVs in both MaAsLin2 and ALDEx2 to ensure comparability of the results.

Ethics

Informed consent was obtained from all subjects (caregivers) and studies were approved by ethics committees (STATIC-PRO:

Erasmus Medical Center Rotterdam Ethics Committee, MEC 2015-709, MOL; Dutch National Ethics Committee, NL42019-094-12, NTR5132, MUIS; and Dutch National Ethics Committee METC Noord-Holland, M012-015, NH012.394, NTR3986).

Data availability

Scripts are available at <https://gitlab.com/fvanbeveren/STATIC-PRO>. Microbiota data, including minimal patient metadata, have been uploaded in the National Centre for Biotechnology Information GenBank database (accession no. PRJNA911564).

RESULTS

Study population

We studied the nasopharyngeal microbiota of 344 children, including 182 healthy controls and 162 cases (78 ICU cases and 84 MCU cases).

No differences were detected between cases and controls in terms of sex, sampling season, or tobacco smoke exposure (Table II). Significant differences were observed between ICU- and MCU-admitted cases regarding age (mean, 9.0 vs 4.5 years; $P < .001$), ethnicity, duration of symptoms before admission, multiple sensitization, asthma severity posthospitalization, and antibiotic use before sampling (Table II).

Microbiota characteristics

We investigated the correlation between baseline characteristics and the overall nasopharyngeal microbiota composition using PERMANOVA tests, followed by a multivariable PERMANOVA including covariables with a P value less than or equal to .2 in univariable analyses (ie, age, viral detection, (passive) smoke exposure, ICS use ≤ 7 days before admission, multiple sensitization, and duration of symptoms > 7 days before admission) (data set 4). Only age remained significant in this model ($R^2 = 3.9\%$; $P < .001$). Consequently, we stratified and adjusted all downstream analyses for age (data sets 2-4). Given that ICU patients more commonly received antibiotics before sampling and antibiotic treatment is known to affect the nasopharyngeal microbiota, we performed a sensitivity analysis excluding cases who received antibiotics before sampling (data set 1, $n = 25$; data set 2, $n = 9$; and data set 3, $n = 17$). This analysis did not change our findings (data not shown). We first tested alpha and beta diversity between cases and controls (data set 2). Cases had higher numbers of observed ASVs and SDI compared with controls (mean observed species, 63.5 and 33.0; mean SDI, 1.65 and 1.39, for cases and controls, respectively; ANOVA $P < .0001$ and .0023, respectively), but comparable evenness scores. Comparison of rarefied alpha-diversity measures showed similar results (Fig E1, D). Stratified analysis for MCU and ICU cases versus controls demonstrated higher numbers of observed ASVs in both MCU and ICU cases (Dunnnett *post hoc* test, $q < 0.0001$ and $q < 0.0002$, respectively), although SDI was only significantly higher for MCU-admitted children compared with controls (Dunnnett *post hoc* test, $q = 0.003$ and $q = 0.324$, respectively). Overall, children hospitalized with an asthma exacerbation showed higher bacterial diversity compared with controls (Fig 1, A-C). The overall microbial community composition (beta diversity) also differed between cases and controls ($R^2 = 1.89\%$; PERMANOVA

$P < .001$), observing the largest difference between MCU and controls ($R^2 = 1.92\%$; $P < .001$) versus ICU and controls ($R^2 = 0.96\%$; $P = .045$; Fig 1, D) in stratified analysis. We then used unsupervised hierarchical clustering and identified 11 distinct microbiota clusters (data set 1). These clusters were characterized by *Moraxella* (MOR and MOR9), *Corynebacterium* (COR), *Dolosigranulum pigrum* (DOLO), *Moraxella lincolnii* (MORLI), *Haemophilus* (HAE5 and HAE6), *Staphylococcus* (STA), and *Streptococcus* (STREP7). Two clusters lacked a predominant bacterium, and instead were represented by a mixture of different ASVs (MIX and STREP8/MIX; see Fig E3 in this article's Online Repository at www.jacionline.org). Using chi-square tests, we found STREP8/MIX more often in cases compared with controls ($P < .05$; data set 2).

Biomarker analysis

We performed differential abundance testing to identify specific bacterial biomarkers associated with asthma exacerbations and severity (data set 2). The relative abundance of the 29 tested ASVs stratified per group is provided in Table E2 (in the Online Repository available at www.jacionline.org). Of these ASVs, 16 were significantly enriched in cases (MaAsLin2, $q < 0.25$, adjusted for age), including *Staphylococcus*, various *Neisseriaceae*, *Streptococcus*, *Granulicatella*, *Veillonella*, *Actinomyces*, *Haemophilus*, *Prevotella melaninogenica*, *Rothia mucilaginosa*, and a single *Corynebacterium* (10) ASV. Conversely, we identified 8 ASVs enriched in healthy children, including *Moraxella*, *Corynebacterium* (3), *D pigrum*, a different *Haemophilus*, *Helcococcus*, and *Streptococcus* ASVs (Fig 2; see Table E3 in this article's Online Repository at www.jacionline.org). Complementary differential abundance testing using the ALDEx2 algorithm confirmed enrichment of *Staphylococcus*, *Neisseriaceae*, and *Veillonella* in cases and of *Moraxella* spp., *Streptococcus*, and *D pigrum* in healthy controls (Table E3). A sensitivity analysis excluding children who received positive airway pressure or invasive mechanical ventilation ($n = 13$) did not affect these findings. Stratified analysis confirmed most of these findings in both MCU and ICU cases versus controls. Several *Neisseria* ASVs, *Actinomyces*, *Streptococcus* (20), *R mucilaginosa*, and *Corynebacterium* (10) were differentially abundant in MCU children, whereas *Neisseria* (19) was differentially abundant in ICU patients when compared with healthy controls (see Fig E4, B in this article's Online Repository at www.jacionline.org). An additional analysis on genus level showed similar patterns (Fig E4, A). ALDEx2 analysis confirmed enrichment of *Staphylococcus* and *Neisseria* spp. in both MCU- and ICU-admitted children and of *Moraxella*, *Corynebacterium*, and *D pigrum* in healthy controls (to the latter however only in MCU-admitted children; see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). To ensure these analyses were not confounded by age-related differences in disease phenotypes, we performed *post hoc* stratified MaAsLin2 analyses on the subset 2 to 4 years and older than 4 years (data set 2), which showed highly similar differences between cases and controls, irrespective of age group. Next, to identify the joint and independent contributions of the microbial biomarkers identified individually by MaAsLin2 analyses (Fig 2; Table E3), we performed a multivariable backward selection logistic regression analysis (data set 2). The most optimal model included *Moraxella*, *D pigrum*, *Streptococcus*,

TABLE II. Baseline characteristics

Characteristics	All patients			Children aged 2-6 y		
	MCU (n = 84)	ICU (n = 78)	P value*	Case (n = 87)	Control (n = 182)	P value*
Age (y) (mean ± SD)	4.50 (3.00 ± 6.25)	9.00 (5.25 ± 12.00)	<.001	3.84 (1.43)	3.71 (1.44)	.505
Age category, 2-4 y, n (%)	42 (50.0)	16 (20.5)	<.001	58 (66.7)	126 (69.2)	.777
Sex: female, n (%)	33 (39.3)	30 (38.5)	1.000	39 (44.8)	86 (47.3)	.808
Season, n (%)						
Autumn	28 (33.3)	24 (30.8)	.249	25 (28.7)	35 (19.2)	.314
Spring	29 (34.5)	22 (28.2)		28 (32.2)	66 (36.3)	
Summer	15 (17.9)	11 (14.1)		18 (20.7)	37 (20.3)	
Winter	12 (14.3)	21 (26.9)		16 (18.4)	44 (24.2)	
Smoking, yes, n (%)†	7 (10.4)	13 (25.0)		12 (17.4)	23 (13.8)	.610
Cotinine levels, n (%)						
Nonsmoker (<20 µg/L)	60 (89.6)	39 (75.0)	.053	57 (82.6)	—	
Passive smoker (20-100 µg/L)	7 (10.4)	11 (21.2)		12 (17.4)	—	
Active smoker (>100 µg/L)	0 (0.0)	2 (3.8)		0 (0.0)	—	
Any virus (%), positive (CT < 40)	53 (63.9)	40 (52.6)	.203	62 (71.3)	—	
Ethnicity, non-White (%)	25 (29.8)	38 (48.7)	.021	28 (32.2)	—	
Symptoms						
Symptoms > 7 d, n (%), No	64 (78.0)	32 (41.6)	<.001	62 (72.1)	—	
Asthma severity, n (%)						
No medication	19 (22.9)	15 (19.2)	.408	18 (20.9)	—	
Mild asthma	37 (44.6)	30 (38.5)		40 (46.5)	—	
Moderate asthma	17 (20.5)	16 (20.5)		15 (17.4)	—	
Severe asthma	10 (12.0)	17 (21.8)		13 (15.1)	—	
Allergic sensitization, n (%), yes	55 (66.3)	57 (77.0)	.190	47 (56.0)	—	
Multiple sensitization, n (%), yes	8 (9.6)	20 (27.4)	.007	7 (8.3)	—	
Asthma diagnosis before admission, n (%), yes	58 (69.0)	60 (76.9)	.342	63 (72.4)	—	
Asthma severity follow-up, n (%)						
No medication	1 (1.3)	0 (0)	.049	0 (0.0)	—	
Mild asthma	9 (11.5)	11 (15.9)		10 (12.2)	—	
Moderate asthma	54 (69.2)	34 (49.3)		52 (63.4)	—	
Severe asthma	14 (17.9)	24 (34.8)		20 (24.4)	—	
Medication use in cases before admission						
AB prophylaxis < 1 y, n (%), yes	1 (1.3)	2 (2.8)	.610	2 (2.5)	—	
AB treatment < 1 y, n (%), yes	24 (31.6)	20 (28.2)	.786	31 (38.3)	—	
No. of AB treatment courses <1 y, n (%)						
0	52 (68.4)	51 (71.8)	.142	50 (61.7)	—	
1	14 (18.4)	17 (23.9)		21 (25.9)	—	
≥2	10 (13.2)	3 (4.2)		10 (12.3)	—	
AB before sampling, n (%), yes	6 (7.2)	19 (25.0)	.004	9 (10.2)	—	
Prednisone last year, n (%), yes	17 (22.4)	21 (29.6)	.418	18 (22.2)	—	
PDC (mean ± SD)	64.90 (39.30 ± 95.20)	86.60 (46.45 ± 100.00)	.277	66.31 (28.49)	—	
ICS use ≤ 7 d before admission, n (%), yes	42 (63.6)	41 (66.1)	.912	46 (63.0)	—	
Medication use in controls						
AB use last 6 mo, n (%), yes	—	—		—	8 (4.4)	
AB use last month, n (%), yes	—	—		—	1 (0.5)	
ICS use last 3 mo, n (%), yes	—	—		—	2 (1.1)	

AB, Antibiotic; CT, circulation time.

*P values were calculated using the Kruskal-Wallis test for continuous variables and the χ^2 , Fisher exact, or Fisher-Freeman-Halton exact test for categorical variables where appropriate. Significant results are represented in boldface.

†Either passive or active smoking.

Neisseriaceae, and *Gemella* as predictors of disease. Relative abundances of *Moraxella* ASVs, *D pigrum*, and *Gemella* were associated with controls; conversely, relative abundances of *Streptococcus* and *Neisseriaceae* were associated with cases (Table III).

Severity biomarkers

To identify biomarkers of severity, we compared the nasopharyngeal microbiota of the MCU- with the ICU-admitted children (data set 3). We found no differences in both bacterial diversity and overall microbiota composition (Fig 3). Similarly, differential

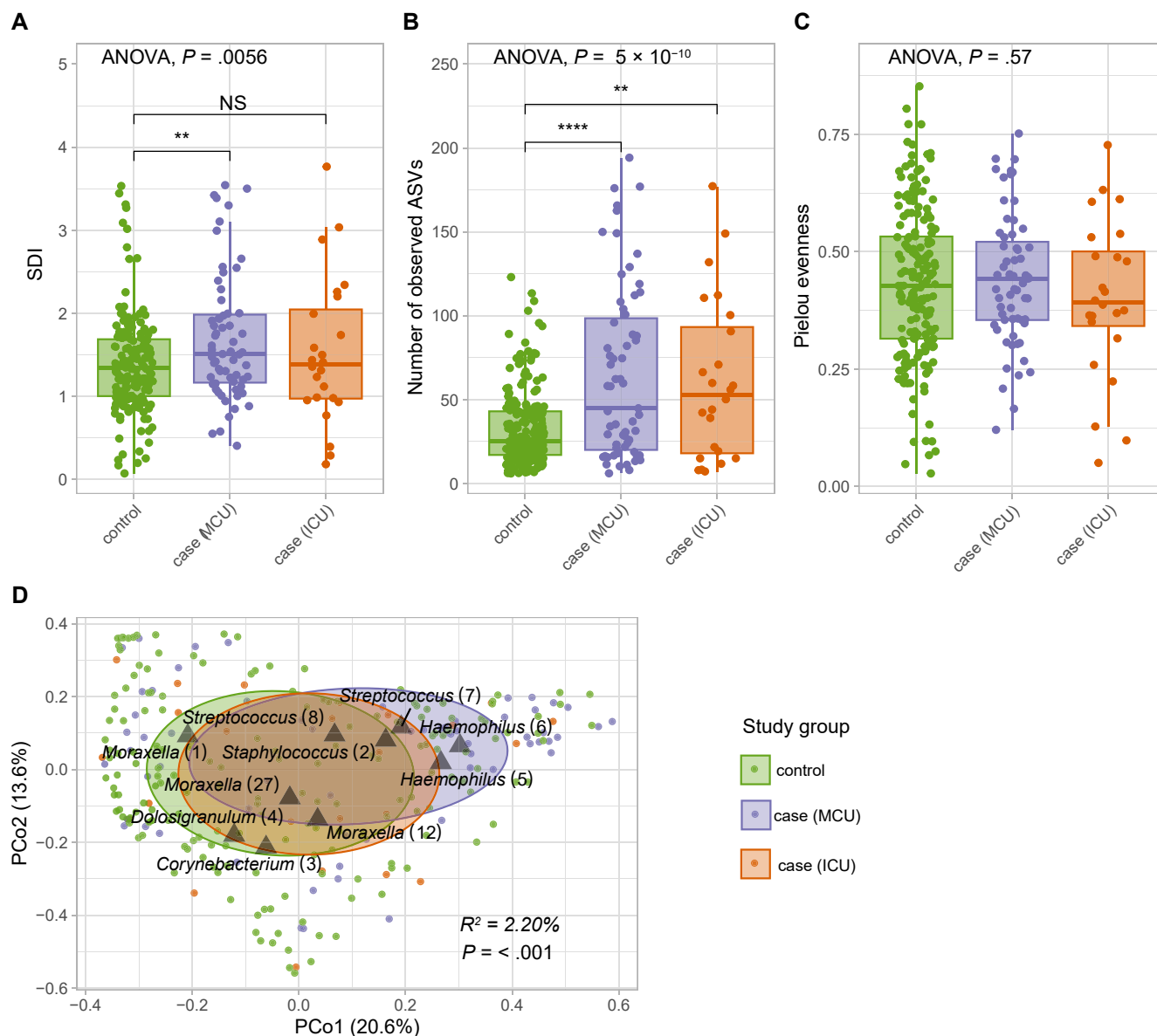


FIG 1. Alpha- and beta-diversity measures in controls and MCU- and ICU-admitted children. Alpha- and beta-diversity measures for children aged 2 to 6 years only (data set 2). **A-C**, Boxplots representing median and interquartile ranges with 25th and 75th percentiles as the upper and lower limit, respectively, stratified per study group. *Post hoc* analyses were performed using the Dunnett test, with healthy controls as reference group, and have been corrected for age: SDI scores (Fig 1, A); observed richness (Fig 1, B); Pielou evenness score (SDI divided by the natural log of the observed number of ASVs) (Fig 1, C). **D**, Principal-coordinate analysis based on Bray-Curtis dissimilarity indices showing the nasopharyngeal microbiota composition for controls and MCU- and ICU-admitted children. The percentages in the brackets on x- and y-axes denote the total variance explained by the first 2 principal coordinates. Each point represents a sample, colored by study group. Ellipses represent the SD of data points per study group. The top 10 highest ranking ASVs are visualized (triangles) and PERMANOVA results are represented and have been corrected for age. NS, Nonsignificant.

abundance testing found no significantly enriched ASVs in either group (using MaAsLin2 and ALDEx2), although a trend toward higher levels of *Neisseria* (19) was found in children admitted to the ICU ($P = .03$; $q = 0.30$; see Table E6 in this article's Online Repository at www.jacionline.org). To ensure these analyses were not confounded by age-related differences in disease phenotypes, we performed a *post hoc* stratified analysis on 2- to 4-year-olds and those older than 4 years for the analyses (data set 3), which confirmed these findings in both age groups.

Patient- and environment-related factors and severe asthma exacerbation

Lastly, we explored the impact of patient- and environment-related factors on the nasopharyngeal microbiota composition in children experiencing asthma exacerbations (data set 4). Because viral infections have been shown to trigger asthma exacerbations, we inspected the microbiota of cases with respiratory viruses more closely. Younger children were more often virus-positive, with rhinovirus being most commonly detected (87.1% in

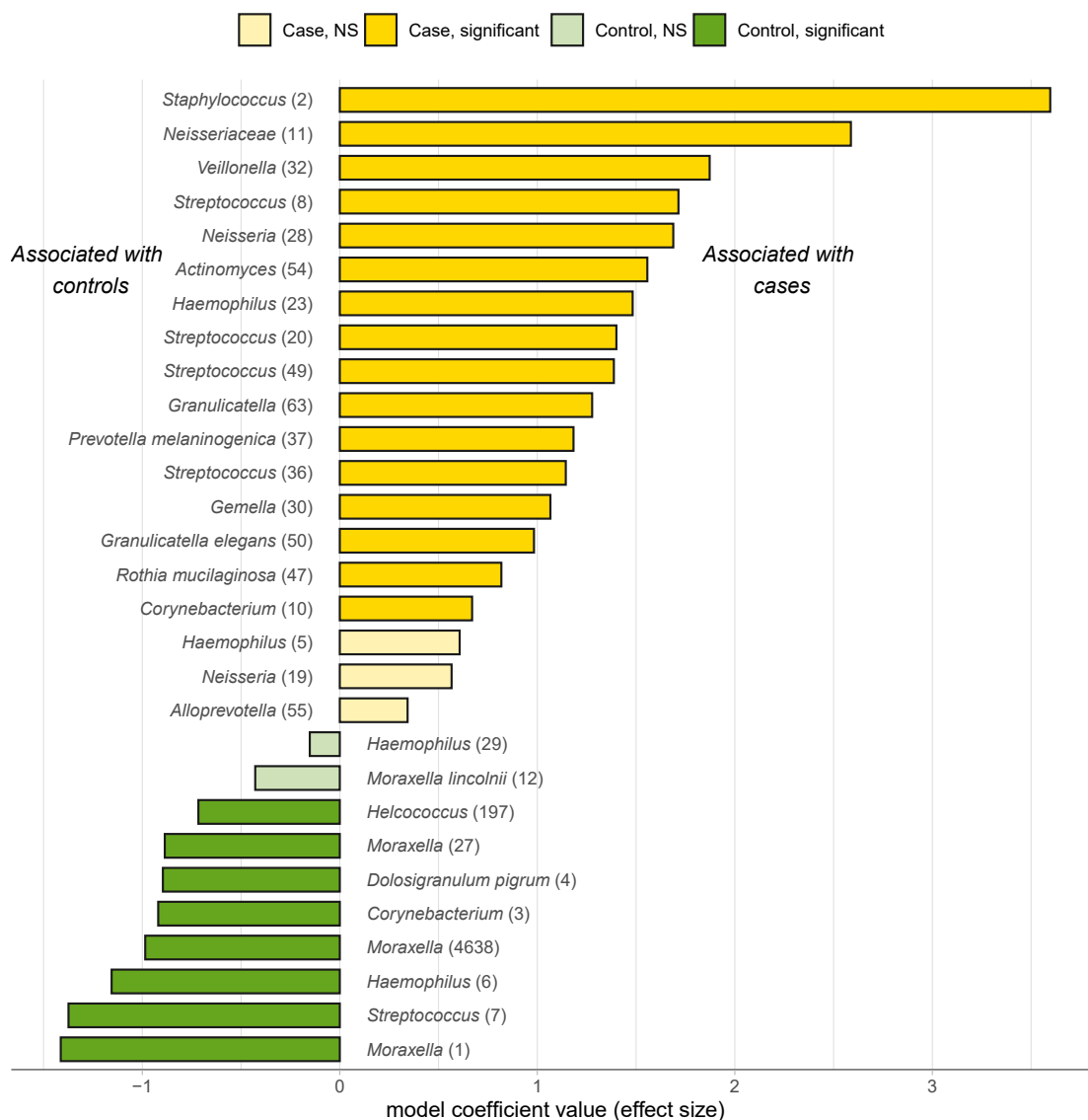


FIG 2. Differential abundance between controls and MCU- and ICU-admitted children. Differential abundance testing between controls and cases (both MCU- and ICU-admitted children; data set 2) using MaAsLin2, for 29 ASVs. We used the default settings except for normalization, prevalence, and abundance. Input data were the relative abundance of ASVs, and therefore no normalization was performed within MaAsLin2. We preselected ASVs, and therefore abundance and prevalence thresholds were set to 0. Results were corrected for multiple testing and age was included as a covariate. The model coefficient indicates the directionality and effect size. Positive results correspond with higher abundance in cases, and negative results correspond with higher abundance in controls. Significant results ($q < 0.25$) are represented in darker shades, and nonsignificant (NS) results by light shades.

virus-positive samples). Consistent with findings from the main cohort,³ viral detection was not associated with severity of asthma exacerbations in this subcohort (data not shown). Multivariable analysis on ASV level (including age, PDC, ICS use in the week before admission, and nitrogen dioxide) showed no ASVs being significantly associated with viral detection (see Fig E5 in this article's Online Repository at www.jacionline.org). ICS use, assessed through (1) PDC and (2) whether ICS was used in the week before admission, was not associated with severity of asthma exacerbations. Multivariable analysis on ASV level showed that higher *Staphylococcus* abundance was associated with lower PDC, whereas higher *Moraxella* and *Neisseriaceae*

abundances were associated with higher PDC (Fig E5). ICS use in the week before admission was associated with higher *Streptococcus* spp. and *Haemophilus* abundances (Fig E5).

Lastly, because air quality is known to contribute to asthma exacerbations, we investigated the relationship between air quality and the nasopharyngeal microbiota within cases. When correcting for age, we found no associations between overall microbiota composition and nitrogen dioxide, PM_{2.5}, or PM₁₀. Multivariable analysis on ASV level, however, demonstrated that *M. lincolnii* was associated with lower average PM_{2.5} and PM₁₀ exposure (see Fig E6 in this article's Online Repository at www.jacionline.org). Stratified analysis for ward of admission

TABLE III. Multivariable logistic regression on microbial biomarkers

Covariable	OR (95% CI)	P value
<i>Moraxella</i> (1)	0.28 (0.09 to 0.81)	.022
<i>D pigrum</i> (4)	0.15 (0.02 to 1.15)	.077
<i>Streptococcus</i> (8)	4.68×10^{10} (7.64×10^3 to 4.49×10^{20})	.013
<i>Neisseriaceae</i> (11)	2.19×10^{34} (1.59×10^{12} to 5.66×10^{67})	.016
<i>Moraxella</i> (27)	0.01 (0 to 3.52)	.234
<i>Gemella</i> (30)	3.02×10^{-44} (0 to 4.46×10^{-9})	.040

OR, Odds ratio.

(to minimize confounding by location of home residence) confirmed the relationship between *M lincolnii* and lower levels of PM_{2.5} and PM₁₀, although this did not reach statistical significance (see Fig E7 in this article's Online Repository at www.jacionline.org). Within the MCU group, we observed positive associations between PM_{2.5} exposure and *Neisseriaceae* abundance and between nitrogen dioxide exposure and *Corynebacterium* and lower *Streptococcus* (7) abundances (Fig E7).

DISCUSSION

We found differences in the nasopharyngeal microbiota between hospitalized children with asthma exacerbations and healthy controls. Children with exacerbations showed increased abundance of *Staphylococcus* and “oral” taxa, including *Neisseria* and (facultative) anaerobes.^{32,33} Interestingly, we found minimal differences in microbiota composition between MCU- and ICU-admitted children, suggesting limited association between microbiota and exacerbation severity. Lastly, we described the relationship between risk factors for exacerbation, including viruses, ICS use, air quality, and microbiota composition at time of severe exacerbation.

Previous literature has shown contrasting findings regarding upper airway bacterial diversity in asthma exacerbations. Studies reported lower bacterial diversity in asthmatic children compared with healthy controls,^{8,15,34} whereas some found no differences,^{8,9,35} and other studies associated higher alpha diversity with longer wheezing episodes,³⁶ and the loss of asthma control.¹⁷ These contrasting alpha-diversity findings may be partially attributed to factors such as (1) differing anatomical locations in the (upper) respiratory tract that were studied (ie, hypopharynx, oropharynx, nasopharynx, and nasal cavity); (2) differences in timing, patients selected, or asthma exacerbation definition; (3) age differences; and most importantly (4) differences in received treatment, such as antibiotic treatment, which will dramatically reduce diversity temporarily (in our study antibiotic treatment preadmission was very low).

We further found that the lactic acid producing commensal *D pigrum* is low abundant at time of severe exacerbation, which is consistent with previous findings during exacerbation¹⁴ and during stable asthma.^{15,17} Children with *D pigrum*-dominated microbiota have also shown better asthma control^{15,17} and developed fewer respiratory infections in early life.²³ The observed absence of *Dolosigranulum*, not only in asthma but also in other respiratory diseases, supports the relationship between *D pigrum* and respiratory health. Biomechanistic studies in mouse models of respiratory syncytial virus, severe acute respiratory syndrome coronavirus 2, and *S pneumoniae* infection confirmed that these bacteria elicit beneficial immunomodulatory

properties.^{37,38} In addition, cooccurrence with *Corynebacterium* has been shown to inhibit *S pneumoniae* growth *in vitro*.³⁹ Further research is therefore warranted to understand the potential role of this bacterium in asthma (exacerbation) prevention.

We also showed that, consistent with previous findings in asthma and asthma exacerbations,^{7,40} in our cohort high *Staphylococcus* levels were associated with asthma exacerbations when compared with healthy controls. In addition, early-life oropharyngeal and nasopharyngeal *Staphylococcus*-dominated microbiota have been associated with asthma development later in childhood.^{13,41} Conversely, 2 other studies reported a lower risk of developing exacerbations in asthmatic children with a *Staphylococcus*-dominated nasal microbiota.^{16,21} Taken together, these findings suggest that *Staphylococcus* abundance is higher in asthma compared with health, although *Staphylococcus* may not be related to exacerbation risk. Alternatively, these contrasting findings indicate that different *Staphylococcus* spp. could have distinct roles in asthma and health. Despite clinical differences between MCU- and ICU-admitted children, we found minimal associations between the microbiota and asthma exacerbation severity (MCU vs ICU). Although there are limited data on microbiota and exacerbation severity, a study comparing the respiratory microbiota between mild/moderate and severe asthmatic children also found minimal differences between the 2 severity groups.³⁵ The modest microbiota differences may be a result of other (clinical) factors that add to the decision whether a child is admitted to ICU or MCU, such as previous severe exacerbations requiring ICU admission or age. Alternatively, the limited differences in nasopharyngeal microbiota during disease exacerbation in MCU- or ICU-admitted children may be driven by acute respiratory symptoms and accompanying inflammation that all children experienced at time of sampling. In that case, their acute inflammatory state may overshadow differences in nasopharyngeal microbial communities preceding exacerbations and potentially contributing to severity of consecutive symptoms. This is especially relevant for cumulative effects of treatment, such as steroids and antibiotics, in the phase leading up to an exacerbation. Our study is not designed to identify these pathways; instead, prospective longitudinal studies should be executed to identify these pathways.

Interestingly, although strictly sampling the nasopharynx, we detected taxa associated with the oropharyngeal niche³³ (oral flora), including *Neisseria*, *Streptococcus* spp., *Veillonella*, *Actinomyces*, *P melaninogenica*, *Granulicatella*, and *Gemella* spp. Microbial community composition is in part governed by ecological factors, such as oxygen tension, pH, and epithelium.⁴² Therefore, the increased overlap in bacterial communities between nasopharynx and oropharynx during acute asthma exacerbation may indicate that the physiological boundaries between these

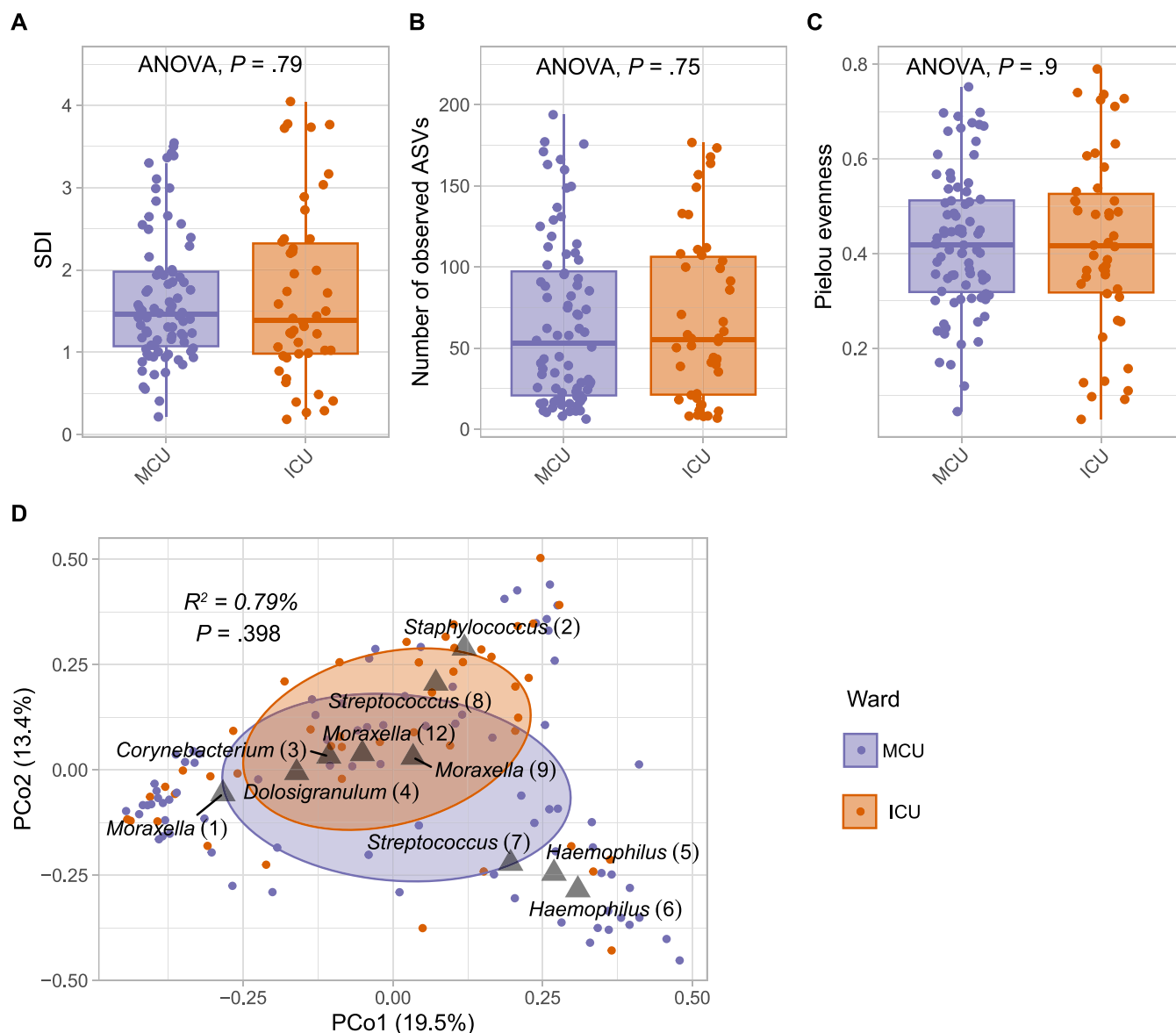


FIG 3. Alpha- and beta-diversity measures in MCU- and ICU-admitted children. Alpha- and beta-diversity measures for children admitted with an asthma exacerbation who are younger than 10 years only (data set 3). **A-C**, Boxplots representing median and interquartile ranges with 25th and 75th percentiles as the upper and lower limit, respectively, stratified per study group. Differences for ward of admission were calculated using ANOVA and have been corrected for age: SDI scores stratified per ward of admission (Fig 3, A); observed richness stratified per ward of admission (Fig 3, B); Pielou evenness score (SDI divided by the natural log of the observed number of ASVs) (Fig 3, C). **D**, Principal-coordinate analysis based on Bray-Curtis dissimilarity indices, showing nasopharyngeal microbiota composition for MCU- and ICU-admitted children. The percentages in brackets on x- and y-axes denote the total variance explained by the first 2 principal coordinates. Each point represents a nasopharyngeal sample, colored by ward of admission. Ellipses represent the SD of data points per ward. The top 10 highest ranking ASVs are visualized (triangles) and PERMANOVA results are represented in the plot and have been corrected for age. NS, Nonsignificant.

niches have become obscured and that their ecological properties became more alike. To rule out that these results were confounded by treatment effects, we performed a sensitivity analysis excluding children who received a form of positive airway pressure or invasive mechanical ventilation, which did not change our results. The observed collapse of niche-specific bacterial communities, which we previously termed “loss of bacterial topography,” was also observed preceding and during intercurrent infections in infants.⁴³ This phenomenon was illustrated by the

appearance of oral-type taxa, including *Neisseria lactamica*, *Streptococcus* spp., *Prevotella nanceiensis*, and *Fusobacterium*, into the nasopharyngeal niche.⁴³ We speculate that a similar ecological phenomenon may occur before and during asthma exacerbations. Previously, in hospitalized children with respiratory syncytial virus bronchiolitis, a nasopharyngeal microbial profile enriched with *Neisseria*, *Prevotella*, and *Veillonella* was also associated with atopy and inflammation,⁴⁴ suggesting a potential link between mucosal inflammation, nasopharyngeal

colonization, and influx of oral-type taxa in asthma. Although the topographical collapse did precede respiratory infection in one of these studies,⁴³ more evidence is needed to establish that mucosal inflammation promotes enrichment with oropharyngeal-type taxa and subsequent (severe) asthma exacerbations.

Among the enrichment of oral-type taxa, we particularly found a higher abundance of *Neisseria* in the nasopharynx of children hospitalized with an asthma exacerbation compared with healthy controls, with ICU-admitted children showing a trend toward higher levels compared with MCU-admitted children. *Neisseria* spp., including *N. flavescens/subflava/meningitidis*,^{32,33} are regarded respiratory tract commensals and are often found in oral niches (ie, tongue, gingiva, and hard palatum). Although generally considered commensals, recent research in patients with bronchiectasis found that *Neisseria* abundance was related to poor clinical outcomes.⁴³ In asthma, higher *Neisseria* levels were also found in the bronchial microbiota of patients with severe asthma compared with healthy controls.²⁰ *In vivo* mouse models and *ex vivo* human cell experiments showed that *N. subflava* weakened cell barrier integrity and increased IL-6 and IL-8 expression,⁴⁵ indicating that certain *Neisseria* spp. hold immunopathologic potential in chronic respiratory diseases. In addition, we describe increased *Neisseria* levels in MCU-admitted children exposed to higher fine particulate matter levels, suggesting a potential relationship between air pollution, *Neisseria* spp. abundance, and (severe) asthma exacerbations. Previous research described a relationship between microbiota, air pollution, and immune responses, noting an inverse relationship between extracellular vesicles with immunomodulatory properties and PM_{2.5} exposure in subjects lacking *Moraxella*.⁴⁶ These findings suggest that the microbiome may modulate the immune response to PM_{2.5} exposure, and as such mediate asthma exacerbations. However, because our study was not specifically designed to investigate the relationship between air quality and asthma exacerbation, future studies are needed to confirm our findings.

The main limitation of our study is use of samples collected in 3 separate cohorts, which could introduce cohort-specific bias to the microbiota data. To minimize potential bias, we executed sample collection, transport, and storage according to the same standard operating procedures, benchmarked our laboratory processes for these studies,²⁵ and performed thorough quality control including the comparison of microbiota between control cohorts and a robust decontamination procedure. Lastly, to limit age-related confounding, we created separate data sets, maximizing the sample size for all microbiota analyses while minimizing age-related bias. This approach did result in a limited number of ICU-admitted children in our analyses, potentially reducing the power to detect differences in asthma exacerbation severity (ie, MCU or ICU admission), the ability to extrapolate the findings to older children, and to detect differences between ICU and controls. We observed larger differences between MCU and controls than between ICU and controls; however, this trend may be influenced by the limited number of ICU-admitted children.

Nevertheless, few studies have investigated variations in airway microbiota across different levels of severe asthma exacerbations, and we were still able to compare a substantial subset of admitted children to age-matched healthy controls. Because we studied the nasopharyngeal microbiota at time of severe asthma exacerbation, we cannot distinguish whether our

findings result from or contribute to the susceptibility of developing a (severe) asthma exacerbation. Therefore, although we found associations between recent ICS use and *Haemophilus* and *Staphylococcus* abundances, as well as high levels of PDC in the year before admission and *Moraxella* and *Neisseriaceae* abundances, it remains unclear whether these exposures initiate microbiome shifts or contribute to disease. Further longitudinal studies are therefore needed to understand the role of these factors in host-microbial community interactions in the upper airways in relation to the pathophysiology of asthma exacerbations.

DISCLOSURE STATEMENT

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Key messages

- Nasopharyngeal microbiota at time of severe asthma exacerbation is characterized by an overrepresentation of *Staphylococcus* and oral-type microbes and an underrepresentation of, among others, *Moraxella*, *D. pigrum*, and *Corynebacterium* spp.
- There are limited differences in nasopharyngeal microbiota between children with a severe asthma exacerbation admitted to either the pediatric general ward or the ICU.

REFERENCES

1. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Available at: <https://ginasthma.org/wp-content/uploads/2022/04/GINA-Main-Report-2022-V2-WMS.pdf>. Accessed April 3, 2023.
2. Lyell PJ, Villanueva E, Burton D, Freezer NJ, Bardin PG. Risk factors for intensive care in children with acute asthma. *Respirology* 2005;10:436-41.
3. Boeschoten SA, Boehmer AL, Merkus PJ, van Rosmalen J, de Jongste JC, Fraaij PLA, et al. Risk factors for intensive care admission in children with severe acute asthma in the Netherlands: a prospective multicentre study. *ERJ Open Res* 2020;6:00126-2020.
4. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bønnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;357:1487-95.
5. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 2015;17:704-15.

6. Teo SM, Tang HHF, Mok D, Judd LM, Watts SC, Pham K, et al. Airway microbiota dynamics uncover a critical window for interplay of pathogenic bacteria and allergy in childhood respiratory disease. *Cell Host Microbe* 2018;24:341-52.e5.
7. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. *PLoS One* 2010;5:e8578.
8. Depner M, Ege MJ, Cox MJ, Dwyer S, Walker AW, Birzele LT, et al. Bacterial microbiota of the upper respiratory tract and childhood asthma. *J Allergy Clin Immunol* 2017;139:826-34.e13.
9. Chun Y, Do A, Grishina G, Grishin A, Fang G, Rose S, et al. Integrative study of the upper and lower airway microbiome and transcriptome in asthma. *JCI Insight* 2020;5:e133707.
10. Boutin S, Depner M, Stahl M, Graeber SY, Dittrich SA, Legatzki A, et al. Comparison of oropharyngeal microbiota from children with asthma and cystic fibrosis. *Mediators Inflamm* 2017;2017:5047403.
11. Kim BS, Lee E, Lee MJ, Kang MJ, Yoon J, Cho HJ, et al. Different functional genes of upper airway microbiome associated with natural course of childhood asthma. *Allergy* 2018;73:644-52.
12. Bisgaard H, Hermansen MN, Bonnelykke K, Stokholm J, Batty F, Skjott NL, et al. Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. *BMJ* 2010;341:c4978.
13. Dzidic M, Abrahamsson TR, Artacho A, Collado MC, Mira A, Jenmalm MC. Oral microbiota maturation during the first 7 years of life in relation to allergy development. *Allergy* 2018;73:2000-11.
14. Song Y, Hou J, Kwok JSL, Weng H, Tang MF, Wang MH, et al. Whole-genome shotgun sequencing for nasopharyngeal microbiome in pre-school children with recurrent wheezing. *Front Microbiol* 2022;12:792556.
15. Hou J, Song Y, Leung ASY, Tang MF, Shi M, Wang EY, et al. Temporal dynamics of the nasopharyngeal microbiome and its relationship with childhood asthma exacerbation. *Microbiol Spectr* 2022;10:e0012922.
16. McCauley K, Durack J, Valladares R, Fadrosch DW, Lin DL, Calatroni A, et al. Distinct nasal airway bacterial microbiotas differentially relate to exacerbation in pediatric patients with asthma. *J Allergy Clin Immunol* 2019;144:1187-97.
17. Zhou Y, Jackson D, Bacharier LB, Mauger D, Boushey H, Castro M, et al. The upper-airway microbiota and loss of asthma control among asthmatic children. *Nat Commun* 2019;10:5714.
18. Taylor SL, Leong LEX, Choo JM, Wesselingh S, Yang IA, Upham JW, et al. Inflammatory phenotypes in patients with severe asthma are associated with distinct airway microbiology. *J Allergy Clin Immunol* 2018;141:94-103.e15.
19. Zhang X, Zhang X, Zhang N, Wang X, Sun L, Chen N, et al. Airway microbiome, host immune response and recurrent wheezing in infants with severe respiratory syncytial virus bronchiolitis. *Pediatr Allergy Immunol* 2020;31:281-9.
20. Huang YJ, Nariya S, Harris JM, Lynch SV, Choy DF, Arron JR, et al. The airway microbiome in patients with severe asthma: associations with disease features and severity. *J Allergy Clin Immunol* 2015;136:874-84.
21. McCauley KE, Flynn K, DiMassa V, LaMere B, Fadrosch DW, Lynch KV, et al. Seasonal airway microbiome and transcriptome interactions promote childhood asthma exacerbations. *J Allergy Clin Immunol* 2022;150:204-13.
22. Man WH, van Houten MA, Mérelle ME, Vlieger AM, Chu MLJN, Jansen NJG, et al. Bacterial and viral respiratory tract microbiota and host characteristics in children with lower respiratory tract infections: a matched case-control study. *Lancet Respir Med* 2019;7:417-26.
23. Bosch AATM, de Steenhuijsen Piters WAA, van Houten MA, Chu MLJN, Biesbroek G, Kool J, et al. Maturation of the infant respiratory microbiota, environmental drivers, and health consequences. A prospective cohort study. *Am J Respir Crit Care Med* 2017;196:1582-90.
24. Hoek RAS, Paats MS, Pas SD, Bakker M, Hoogsteden HC, Boucher CAB, et al. Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients. *Scand J Infect Dis* 2013;35:65-9.
25. Hasrat R, Kool J, de Steenhuijsen Piters WAA, Chu MLJN, Kuiling S, Groot J, et al. 2021 Benchmarking laboratory processes to characterise low-biomass respiratory microbiota. 2021;11:17148.
26. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13:581-3.
27. de Steenhuijsen Piters WAA, Watson RL, de Koff EM, Hasrat R, Arp K, Chu MLJN, et al. Early-life viral infections are associated with disadvantageous immune and microbiota profiles and recurrent respiratory infections. *Nat Microbiol* 2022;7:224-37.
28. Quast C, Priesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2012;41:D590-6.
29. Oksanen J, Simpson GL, Blanchet GF, Kindt R, Legendre P, Minchin PR, et al. vegan: community ecology package. 2022. Available at: <https://CRAN.R-project.org/package=vegan>. Accessed May 19, 2023.
30. Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, et al. Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput Biol* 2021;17:e1009442.
31. Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DE, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2014;2:15.
32. Segata N, Haake S, Mannon P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol* 2012;13:R42.
33. Eren AM, Borisy GG, Huse SM, Mark Welch JL. Oligotyping analysis of the human oral microbiome. *Proc Natl Acad Sci U S A* 2014;111:E2875-84.
34. Perez-Garcia J, González-Carracedo M, Espuela-Ortiz A, Hernández-Pérez JM, González-Pérez R, Sardón-Prado O, et al. The upper-airway microbiome as a biomarker of asthma exacerbations despite inhaled corticosteroid treatment. *J Allergy Clin Immunol* 2023;151:706-15.
35. Thorsen J, Stokholm J, Rasmussen MA, Roggenbuck-Wedemeyer M, Vissing NH, Mortensen MS, et al. Asthma and wheeze severity and the oropharyngeal microbiota in children and adolescents. *Ann Am Thorac Soc* 2022;19:2031-43.
36. Thorsen J, Stokholm J, Rasmussen MA, Mortensen MS, Brejnrod AD, Hjelmsø M, et al. The airway microbiota modulates effect of azithromycin treatment for episodes of recurrent asthma-like symptoms in preschool children: a randomized clinical trial. *Am J Respir Crit Care Med* 2021;204:149-58.
37. Ortiz Moyano R, Raya Tonetti F, Tomokiyo M, Kanmani P, Vizoso-Pinto MG, Kim H, et al. The ability of respiratory commensal bacteria to beneficially modulate the lung innate immune response is a strain dependent characteristic. *Microorganisms* 2020;8:727.
38. Islam MdA, Albarracín L, Melnikov V, Andrade BGN, Cuadrat RRC, Kitazawa H, et al. *Dolosigranulum pigrum* modulates immunity against SARS-CoV-2 in respiratory epithelial cells. *Pathogens* 2021;10:634.
39. Brugger SD, Eslami SM, Pettigrew MM, Escapa IF, Henke MT, Kong Y, et al. *Dolosigranulum pigrum* cooperation and competition in human nasal microbiota. *mSphere* 2020;5:e00852-20.
40. Aydin M, Weisser C, Rué O, Mariadassou M, Maaß S, Behrendt AK, et al. The rhinobiome of exacerbated wheezers and asthmatics: insights from a German pediatric exacerbation network. *Front Allergy* 2021;2:667562.
41. Tang HHF, Lang A, Teo SM, Judd LM, Gangnon R, Evans MD, et al. Developmental patterns in the nasopharyngeal microbiome during infancy are associated with asthma risk. *J Allergy Clin Immunol* 2021;147:1683-91.
42. Man WH, de Steenhuijsen Piters WAA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* 2017;15:259-70.
43. Man WH, Clerc M, de Steenhuijsen Piters WAA, van Houten MA, Chu MLJN, Kool J, et al. Loss of microbial topography between oral and nasopharyngeal microbiota and development of respiratory infections early in life. *Am J Respir Crit Care Med* 2019;200:760-70.
44. Raita Y, Pérez-Losada M, Freishtat RJ, Harmon B, Mansbach JM, Piedra PA, et al. Integrated omics endotyping of infants with respiratory syncytial virus bronchiolitis and risk of childhood asthma. *Nat Commun* 2021;12:3601.
45. Li L, Mac Aogáin M, Xu T, Jaggi TK, Chan LLY, Qu J, et al. Neisseria species as pathobionts in bronchiectasis. *Cell Host Microbe* 2022;30:1311-27.e8.
46. Mariani J, Favero C, Carugno M, Pergoli L, Ferrari L, Bonzini M, et al. Nasal microbiota modifies the effects of particulate air pollution on plasma extracellular vesicles. *Int J Environ Res Public Health* 2020;17:611.

METHODS

Study population and data collection

Cases were recruited through the STATIC-PRO cohort.^{E1} In this study, children were eligible for inclusion on admission to hospital with an asthma exacerbation requiring systemic corticosteroid treatment and admission to any of the 7 Dutch pediatric ICUs or to 1 of the 4 participating pediatric general wards (MCUs). The 4 MCUs were included on the basis of their geographical distribution across the Netherlands. Criteria for ICU admission were, in line with Dutch guidelines,^{E2} as follows: (1) respiratory failure and impending exhaustion, (2) need for intravenous salbutamol, and/or (3) mechanical ventilation. Guidelines did not change during the study period. Details on study design were previously described.^{E1} Respiratory viral pathogens (ie, adenovirus, human coronaviruses-NL63, -OC43, -229E, bocavirus, human metapneumovirus, influenza A and B, parainfluenza 1-4, respiratory syncytial virus type A and B, and human rhinovirus) were detected with real-time reverse transcriptase PCR and classified as pathogen if the cycle threshold (Ct value) was less than 40. For subtyping of rhinovirus, a Ct value of less than 30 was required (in-house protocol). Primers, probes, and PCR assay conditions were as in the routine setting of the Molecular Viral Diagnostics Department of the Erasmus Medical Centre and were described previously for these specific viruses.^{E3} Briefly, RNA extraction was performed using MagnaPureLC (Roche Diagnostics, Almere, The Netherlands) total nucleic acid isolation kit. The addition of a known concentration of phocine distemper virus was used to control the extraction.^{E3} Urinary cotinine levels were assessed within 6 hours of admission to determine exposure to environmental smoke and categorized as active (>100 µg/L), passive (20-100 µg/L), or nonsmoking (<20 µg/L).

The MOL study was a prospective matched case-control study, conducted between 2013 and 2016, enrolling children aged 0 to 5 years requiring hospitalization for an acute lower respiratory tract infection and age-, time- and sex-matched controls.^{E4} The MUIS was a longitudinal birth cohort study in which healthy term-born children were followed from birth until age 6 years.^{E5} To avoid repeated measures, we selected 1 sample per subject from the MUIS study. Details on study design, sample collection,

and inclusion criteria for the MOL and MUIS studies have been described previously (MOL^{E4} and MUIS^{E5}).

Quality control

Contaminating ASVs were identified using the “decontam” R package (version 1.14.0; either method for STATIC-PRO/MOL). For MUIS samples, contaminants were removed using the combined method as previously described.^{E6} In addition, we manually inspected suspect ASVs, defined as ASVs not identified by “decontam” but present (>0.1% abundance) in 1 or more negative control samples and in 2 or more nasopharyngeal samples (STATIC-PRO/MOL).

The MUIS and MOL cohorts were compared on alpha diversity, beta diversity, and differentially abundant ASVs. The top 25 most abundant ASVs were included in the MaAsLin2 analysis. The analyses were performed on all available samples of children aged 2 years and 3 years separately to limit confounding by age and increase the power of the quality control analysis.

REFERENCES

- E1. Boeschoten SA, Boehmer AL, Merkus PJ, van Rosmalen J, de Jongste JC, Fraaij PLA, et al. Risk factors for intensive care admission in children with severe acute asthma in the Netherlands: a prospective multicentre study. *ERJ Open Res* 2020; 6:00126-2020.
- E2. Nederlandse Vereniging voor Kindergeneeskunde. Dutch Society of Pediatrics - Guideline Status Asthmaticus in Children.. 2012. Available at: <https://www.nvk.nl/Portals/0/richtlijnen/acuut%astma/Methodenacuutastma.pdf>. Accessed January 2021.
- E3. Hoek RAS, Paats MS, Pas SD, Bakker M, Hoogsteden HC, Boucher CAB, et al. Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients. *Scand J Infect Dis* 2013;45:65-9.
- E4. Man WH, van Houten MA, Mérelle ME, Vlieger AM, Chu MLJN, Jansen NJG, et al. Bacterial and viral respiratory tract microbiota and host characteristics in children with lower respiratory tract infections: a matched case-control study. *Lancet Respir Med* 2019;7:417-26.
- E5. Bosch AATM, de Steenhuijsen Pters WAA, van Houten MA, Chu MLJN, Biesbroek G, Kool J, et al. Maturation of the infant respiratory microbiota, environmental drivers, and health consequences. A prospective cohort study. *Am J Respir Crit Care Med* 2017;196:1582-90.
- E6. de Steenhuijsen Pters WAA, Watson RL, de Koff EM, Hasrat R, Arp K, Chu MLJN, et al. Early-life viral infections are associated with disadvantageous immune and microbiota profiles and recurrent respiratory infections. *Nat Microbiol* 2022;7:224-37.

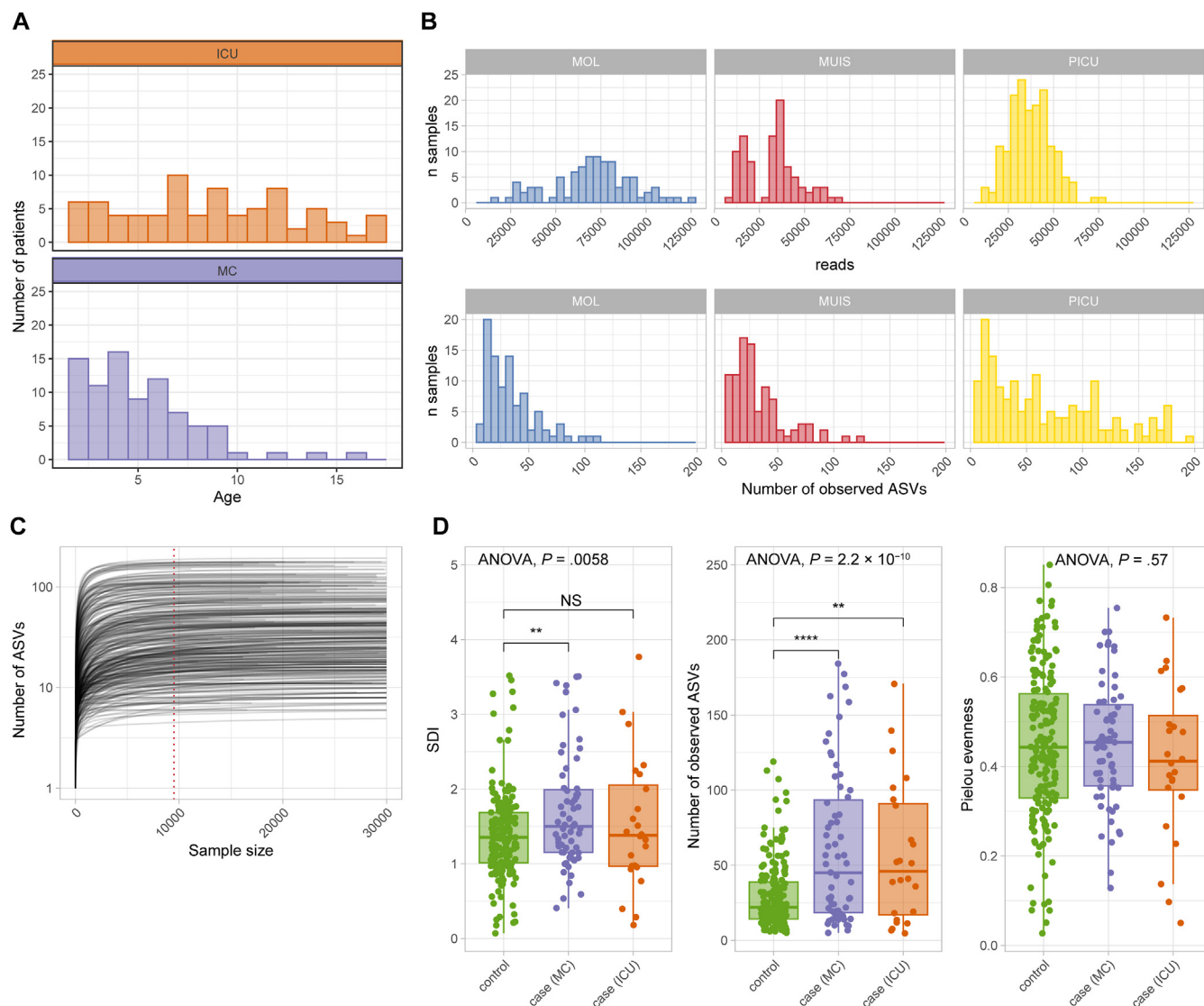


FIG E1. Descriptives and quality control for sequencing depth. **A**, Histograms showing the age distribution of children admitted to the MCU and ICU. **B**, Histograms showing the number of reads and the number of observed ASVs per study cohort. **C**, Rarefaction curve showing the number of reads sequenced on the x-axis and the number of observed ASVs on the y-axis. **D**, Rarefied alpha-diversity measures for children aged 2 to 6 years only (data set 2), stratified per study group. Boxplots represent median and interquartile ranges with 25th and 75th percentiles as the upper and lower limit. *Post hoc* analyses were performed with the Dunnett test, with healthy controls as the reference group. NS, Nonsignificant.

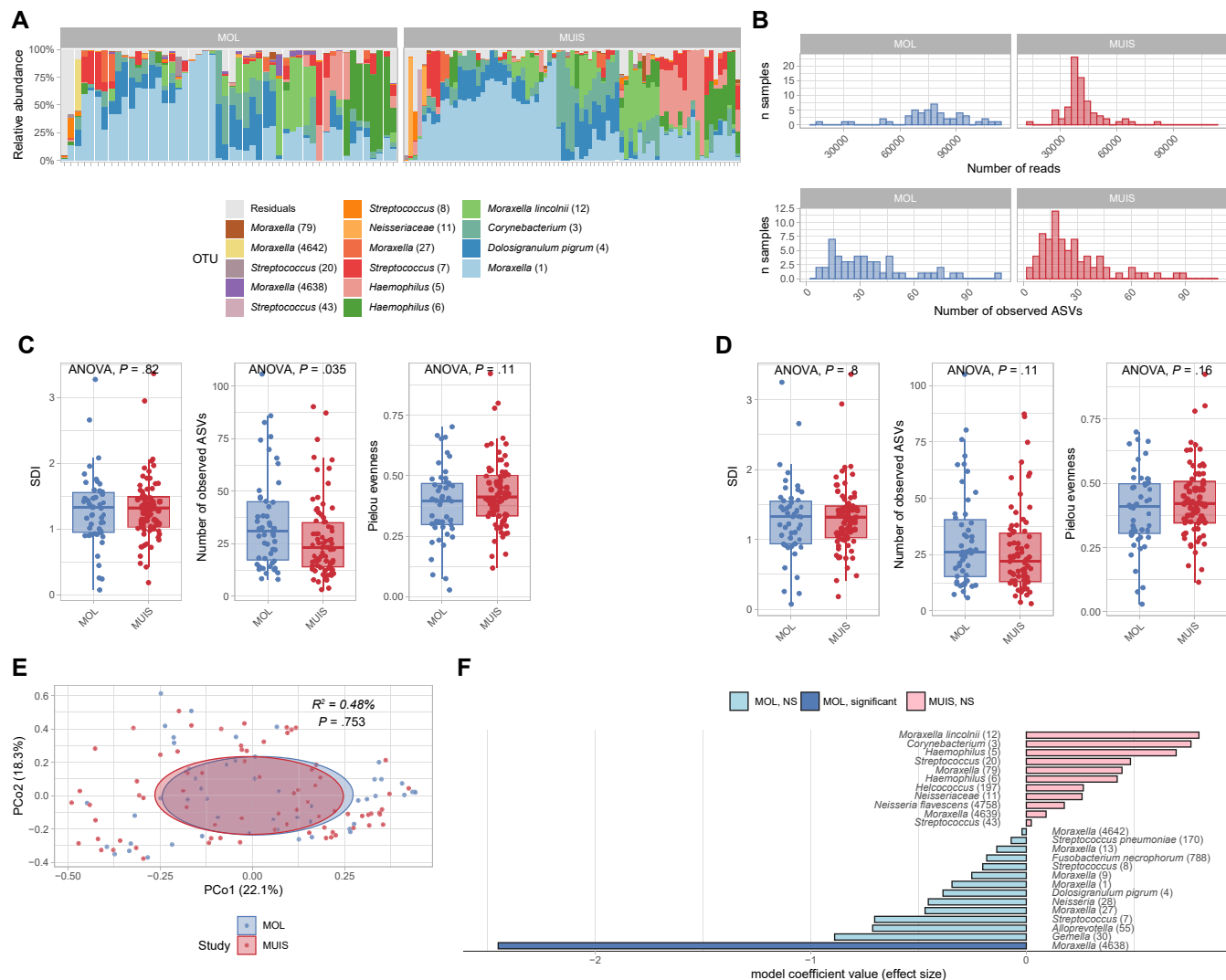


FIG E2. Descriptives and quality control measures for 2-year control samples from MUIS and MOL cohorts. Quality control to ensure homogeneity of MOL and MUIS samples, by comparing the nasopharyngeal microbiota at different levels in all available samples from the 2 cohorts of children aged 2 years (MOL, $n = 50$; MUIS, $n = 75$). **A**, Relative abundance of the top 15 highest abundant ASVs. **B**, Histograms displaying the number of reads and the number of observed ASVs per control study. **C** and **D**, Nonrarefied (**C**) and rarefied (**D**) alpha-diversity measures stratified per control cohort. Boxplots represent median and interquartile ranges with 25th and 75th percentiles as the upper and lower limit. **E**, Principal-coordinate analysis based on Bray-Curtis dissimilarity indices showing the nasopharyngeal microbiota composition for the 2 control cohorts. The percentages in the brackets on x- and y-axes denote the total variance explained by the first 2 principal coordinates. Each point represents a sample, colored by control cohort. Ellipses represent the SD of data points per study group. **F**, Results of the differential abundance testing of the 25 most abundant ASVs in the 2 control cohorts, using MaAsLin2. The model coefficient indicates the directionality and effect size. Positive results correspond with higher abundance in MUIS, and negative results correspond with higher abundance in MOL. Significant results ($q < 0.25$) are represented in darker shades and nonsignificant (NS) results by light shades. We observed only 1 ASV that was significantly different between the cohorts, underlining their comparability.

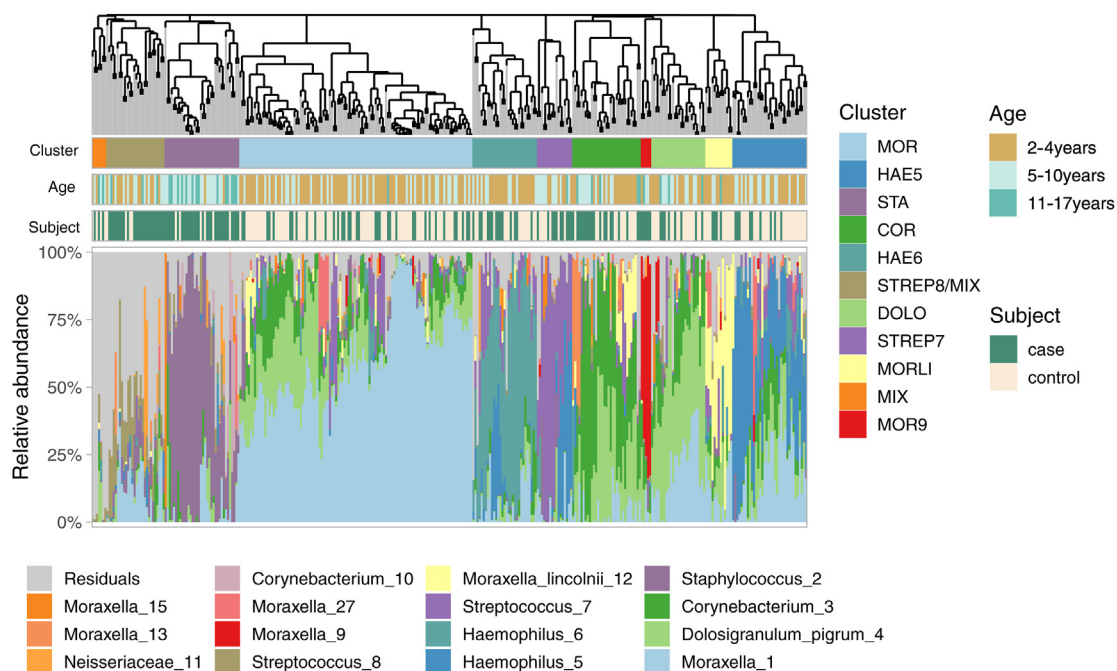


FIG E3. Hierarchical clustering. Clustering was performed on the entire data set ($n = 344$), on the basis of Bray-Curtis dissimilarity indices and using complete linkage. The top bars show information on cluster membership, age, and disease status of the child. The compositional bars represent the relative abundances of the 15 most abundant ASVs in all samples.

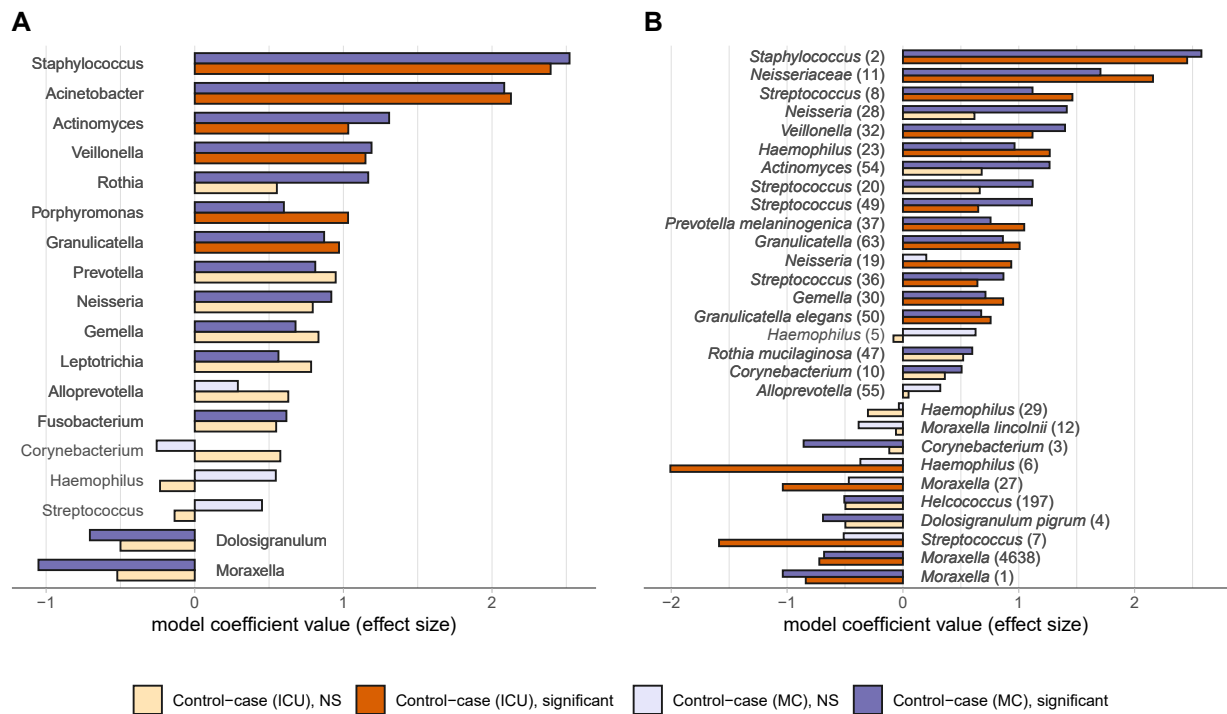


FIG E4. Differential abundance between controls and MCU- and ICU-admitted children. Differential abundance testing between controls and either MCU-admitted (purple) and ICU-admitted (orange) children (data set 2) using MaAsLin2 for (1) genus level and (2) for ASVs. We used the default settings except for normalization, prevalence, and abundance. Input data were the relative abundance of the genus or ASV, and therefore no normalization was performed within MaAsLin2. We preselected genera and ASVs, and therefore abundance and prevalence thresholds were set to 0. Results were corrected for multiple testing. The model coefficient indicates the directionality and effect size. Positive results correspond with higher abundance in cases, and negative results correspond with higher abundance in controls. Significant results ($q < 0.25$) are represented in darker shades and nonsignificant (NS) results by light shades.

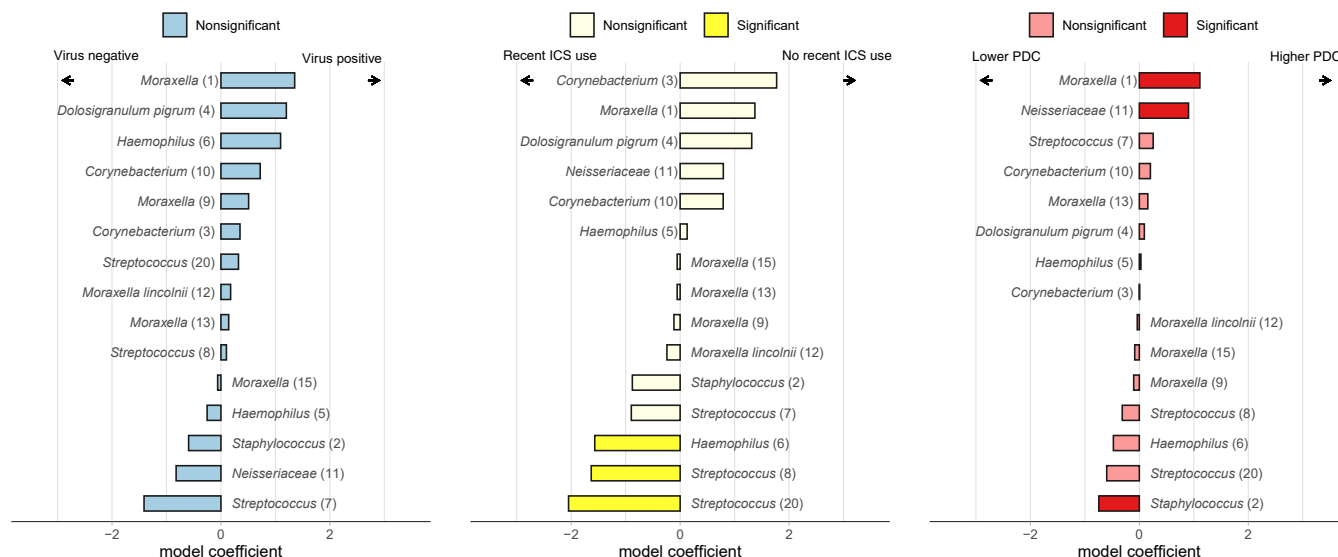


FIG E5. Differential abundance analysis related to viral presence and ICS use. Differential abundance of the 15 most abundant ASVs in relation to viral presence, ICS use in the week before admission (yes/no), and the PDC with ICS in the year before admission in cases. This was using multivariable models (including age, viral carriage, recent ICS use, PDC, and nitrogen dioxide). Darker colors indicate significant results (MaAsLin2, $q < 0.25$).

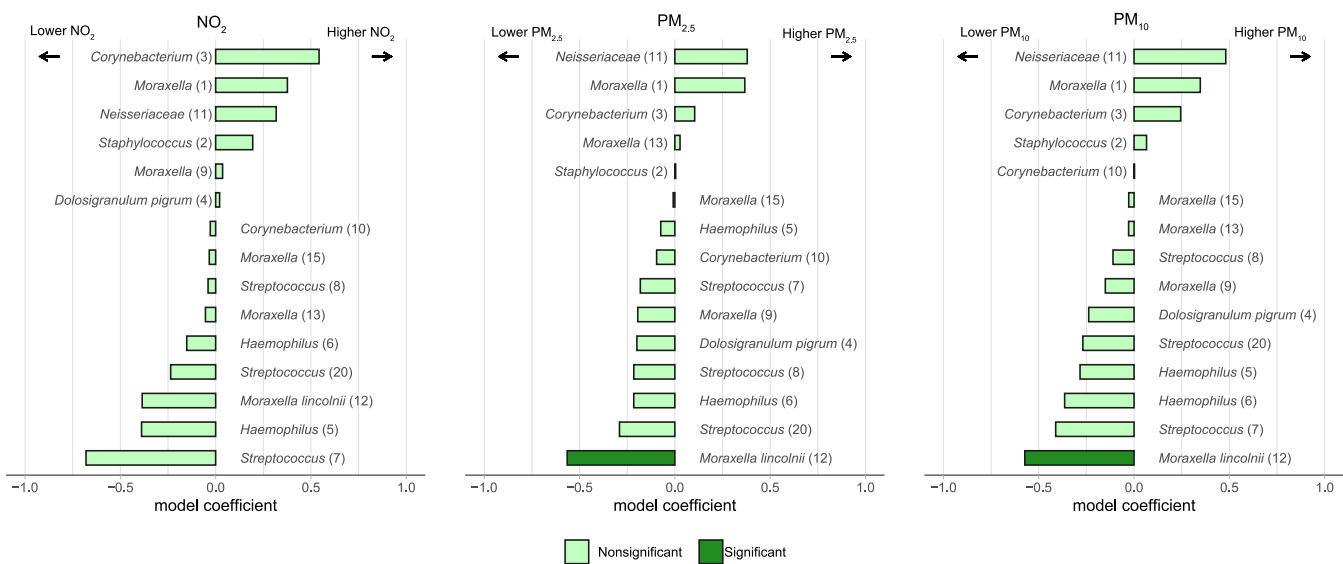


FIG E6. Differential abundance analysis related to air quality. Differential abundance of the 15 most abundant ASVs for yearly average nitrogen dioxide (NO₂), PM_{2.5}, and PM₁₀ exposure. Tested within patient cohort in multivariable models (including age, viral carriage, recent ICS use, PDC, and nitrogen dioxide). Darker colors indicate significant results (MaAsLin2, $q < 0.25$).

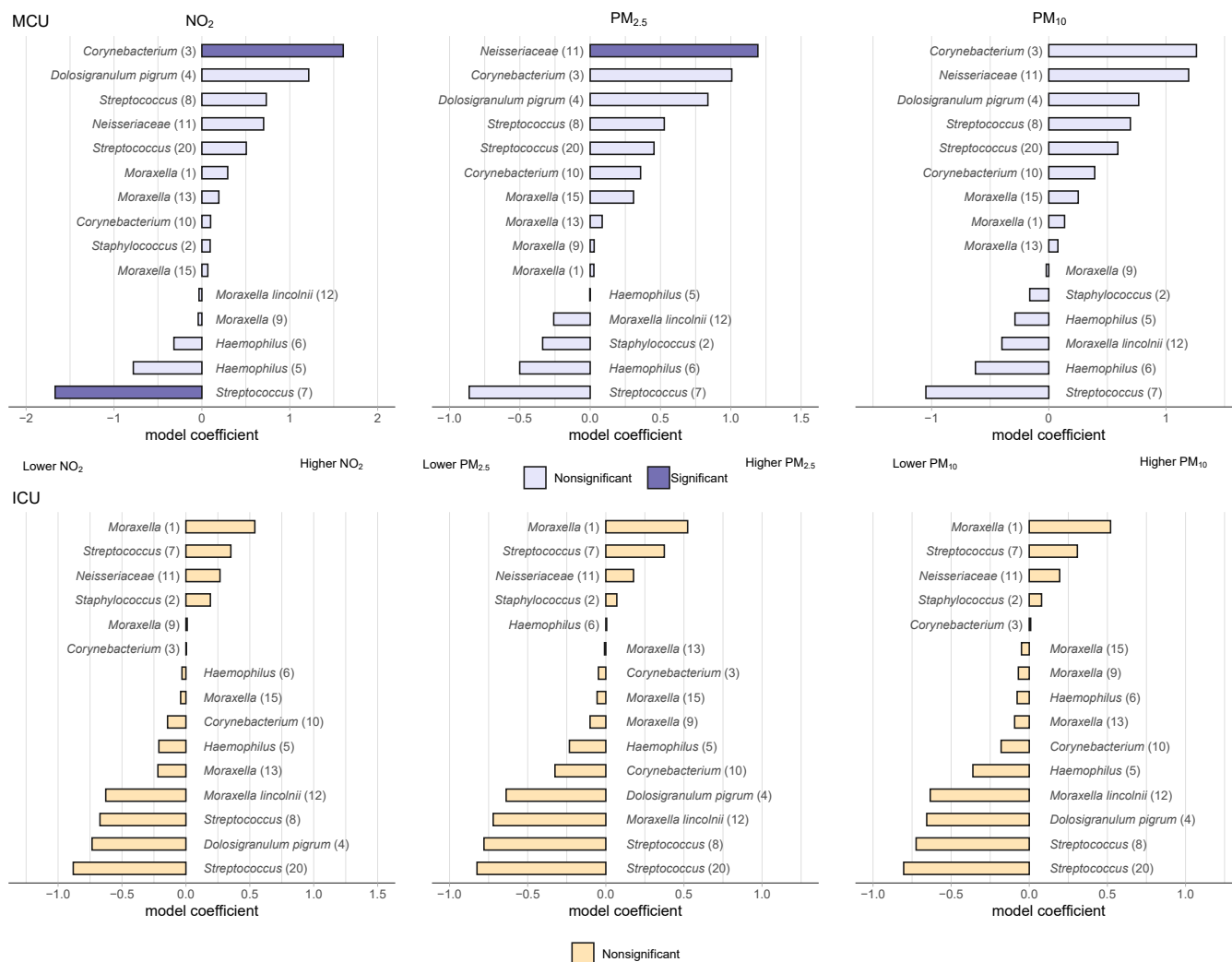


FIG E7. Differential abundance analysis in relation to air quality, stratified per ward of admission. Differential abundance of the 15 most abundant ASVs for yearly average nitrogen dioxide, PM_{2.5}, and PM₁₀ exposure, tested within MCU- and ICU-admitted children, respectively. Darker colors indicate significant results (MaAsLin2, $q < 0.25$).

TABLE E1. Overview of reads before and after *decontam*

Reads	STATIC-PRO			MOL			MUIS		
	Mean	Median	IQR	Mean	Median	IQR	Mean	Median	IQR
Reads before <i>decontam</i>	40,864.6	40,241.5	13,143	71,449	72,325	23,044	33,084	35,848	22,480.5
Reads after <i>decontam</i>	37,098.6	36,571	14,715.8	70,700.5	71,748	23,417	31,964.9	34,250	21,930
Read loss	3,766	811	4,880	748.5	413	765.5	1,119	594	1,379.5
% Read loss	9.7	1.9	12.8	1.3	0.6	1	4.1	1.9	5.5

IQR, Interquartile range.

TABLE E2. Relative abundance of tested ASVs in children aged 2-6 y

ASV	ICU					MCU					Controls				
	Mean	Median	IQR	Min	Max	Mean	Median	IQR	Min	Max	Mean	Median	IQR	Min	Max
Moraxella_1	0.279	0.254	0.452	0	0.942	0.354	0.36	0.572	0.001	0.972	0.304	0.315	0.441	0.005	0.654
Staphylococcus_2	0.051	0.005	0.05	0	0.31	0.07	0.002	0.013	0	0.754	0	0	0	0	0.001
Corynebacterium_3	0.16	0.043	0.181	0	0.815	0.135	0.112	0.187	0	0.349	0.096	0.046	0.15	0	0.282
Dolosigranulum_pigrum_4	0.122	0.062	0.171	0	0.436	0.143	0.118	0.163	0	0.596	0.082	0.012	0.106	0.001	0.284
Haemophilus_5	0.102	0.002	0.015	0	0.965	0.116	0.004	0.082	0	0.969	0.136	0.02	0.076	0	0.578
Haemophilus_6	0.038	0	0	0	0.407	0.086	0	0.011	0	0.632	0.042	0.03	0.031	0	0.146
Streptococcus_7	0.064	0	0.019	0	0.984	0.028	0	0.031	0	0.145	0.005	0	0	0	0.023
Streptococcus_8	0.061	0.01	0.093	0	0.465	0.016	0.003	0.022	0	0.084	0.076	0.03	0.052	0.001	0.289
Corynebacterium_10	0.009	0	0	0	0.206	0.001	0	0	0	0.005	0	0	0	0	0
Neisseriaceae_11	0.006	0	0.006	0	0.07	0.003	0	0.001	0	0.02	0	0	0	0	0.002
Moraxella_lincolnii_12	0.031	0	0.015	0	0.437	0.016	0.001	0.023	0	0.08	0.013	0	0.031	0	0.034
Neisseria_19	0.005	0.001	0.005	0	0.041	0.004	0	0.003	0	0.031	0.02	0.002	0.024	0	0.074
Streptococcus_20	0.023	0.001	0.006	0	0.476	0.003	0.001	0.001	0	0.024	0.004	0	0.008	0	0.01
Haemophilus_23	0.01	0.001	0.005	0	0.099	0.002	0	0.003	0	0.015	0.005	0	0.002	0	0.025
Moraxella_27	0	0	0	0	0	0.003	0	0	0	0.034	0	0	0	0	0
Neisseria_28	0.01	0	0.002	0	0.142	0.002	0	0.003	0	0.009	0.003	0	0.004	0	0.009
Haemophilus_29	0	0	0	0	0.005	0.011	0	0	0	0.147	0.006	0	0	0	0.029
Gemella_30	0.006	0.001	0.011	0	0.027	0.002	0	0.002	0	0.008	0.027	0.003	0.051	0	0.081
Veillonella_32	0.002	0	0.003	0	0.014	0.001	0	0	0	0.008	0.001	0	0	0	0.005
Streptococcus_36	0.002	0	0.001	0	0.015	0.001	0	0	0	0.004	0.001	0	0.001	0	0.002
Prevotella_melaninogenica_37	0.002	0	0.002	0	0.011	0.001	0	0.001	0	0.004	0	0	0	0	0.001
Rothia_mucilaginosa_47	0.004	0	0.001	0	0.058	0.001	0	0	0	0.014	0.005	0	0	0	0.024
Streptococcus_49	0.004	0	0.002	0	0.026	0.001	0	0.002	0	0.005	0.006	0	0.008	0	0.023
Granulicatella_elegans_50	0.002	0	0.002	0	0.01	0.001	0	0.001	0	0.003	0.005	0	0.004	0	0.021
Actinomyces_54	0.002	0	0.002	0	0.008	0.001	0	0.001	0	0.002	0.005	0	0.003	0	0.021
Alloprevotella_55	0.002	0	0.001	0	0.028	0.001	0	0.001	0	0.006	0.156	0.003	0.028	0	0.748
Granulicatella_63	0.002	0	0.001	0	0.023	0.001	0	0.001	0	0.003	0.001	0	0.002	0	0.003
Helcococcus_197	0	0	0	0	0.002	0	0	0	0	0.004	0	0	0	0	0
Moraxella_4638	0	0	0	0	0	0	0	0	0	0	0.002	0	0	0	0.008

Relative abundance of the 29 ASVs tested in MaAsLin2 and ALDEx2 in children aged 2-6 y (Table I, data set 2).

IQR, Interquartile range.

TABLE E3. Control-case

Feature	MaAsLin2 results					ALDEx2 results					
	Estimated coefficient	SE	P value	q value	Association	Estimated coefficient	SE	t statistic	P value	q value	Association
Staphylococcus_2	−3.59693	0.452807	5.57×10^{-14}	3.23×10^{-12}	Case	−3.51055	0.526555	−6.67026	3.48×10^{-10}	1.01×10^{-8}	Case
Neisseriaceae_11	−2.58764	0.406787	8.7×10^{-10}	2.52×10^{-8}	Case	−2.72129	0.634907	−4.29218	.000243	0.005824	Case
Veillonella_32	−1.87257	0.431546	2.03×10^{-5}	0.000236	Case	−1.49722	0.534138	−2.80792	.012952	0.211148	Case
Streptococcus_8	−1.71472	0.4622	.000252	0.001046	Case	−1.25505	0.570992	−2.20094	.038468	0.484469	Case
Neisseria_28	−1.68854	0.43709	.000141	0.000763	Case	−1.42924	0.563665	−2.54086	.028518	0.328533	Case
Actinomyces_54	−1.55709	0.403813	.000145	0.000763	Case	−1.22268	0.536502	−2.28483	.0496	0.435328	Case
Haemophilus_23	−1.48272	0.363347	5.94×10^{-5}	0.000383	Case	−1.27083	0.539452	−2.36249	.036381	0.395466	Case
Streptococcus_20	−1.40046	0.443208	.001761	0.006362	Case	−1.02028	0.532826	−1.91814	.074896	0.704867	Case
Streptococcus_49	−1.38754	0.332685	4.11×10^{-5}	0.000298	Case	−1.21174	0.544883	−2.22923	.058261	0.503958	Case
Granulicatella_63	−1.27761	0.339866	.00021	0.000935	Case	−0.97888	0.506104	−1.93736	.100693	0.642669	Case
Prevotella_melaninogenica_37	−1.18317	0.424304	.005678	0.017331	Case	−0.66929	0.512415	−1.31022	.256797	0.887708	Case
Streptococcus_36	−1.14421	0.302562	.000192	0.00093	Case	−1.17316	0.553906	−2.1217	.070647	0.563823	Case
Gemella_30	−1.06618	0.424046	.012518	0.034572	Case	−0.42665	0.442039	−0.96463	.36954	0.991464	Case
Granulicatella_elegans_50	−0.98318	0.321148	.002428	0.007824	Case	−0.57482	0.482023	−1.19781	.30271	0.923698	Case
Rothia_mucilaginosa_47	−0.81797	0.329308	.013611	0.035883	Case	−0.51186	0.564375	−0.9054	.408803	0.985294	Case
Corynebacterium_10	−0.66995	0.287657	.02061	0.049807	Case	−0.76569	0.625253	−1.22722	.280914	0.916158	Case
Haemophilus_5	−0.60684	0.554801	.275034	0.409025	Case	−0.07739	0.810055	−0.09601	.822867	1	Control
Neisseria_19	−0.56605	0.420286	.179184	0.296933	Case	0.180549	0.586056	0.306755	.653963	0.997653	Control
Alloprevotella_55	−0.34324	0.423753	.41866	0.55187	Case	0.364697	0.519075	0.709111	.478082	0.994025	Control
Haemophilus_29	0.151945	0.258339	.556923	0.633363	Control	0.750925	0.668154	1.125908	.310754	0.951277	Control
Moraxella_lincolnii_12	0.428095	0.578062	.459608	0.592384	Control	1.184079	0.890025	1.331373	.212641	0.955355	Control
Helcococcus_197	0.716013	0.315269	.023939	0.053403	Control	1.576649	0.646357	2.445184	.04049	0.391527	Control
Moraxella_27	0.886027	0.377964	.019804	0.049807	Control	2.144476	0.740187	2.899889	.011313	0.175154	Control
Dolosigranulum_pigrum_4	0.895653	0.541944	.099578	0.192518	Control	1.842869	0.683591	2.696569	.008663	0.171301	Control
Corynebacterium_3	0.919393	0.574295	.110585	0.200538	Control	1.929691	0.779966	2.475818	.016909	0.286461	Control
Moraxella_4638	0.985068	0.233444	3.36×10^{-5}	0.000278	Control	2.777565	0.636064	4.378156	.000215	0.005321	Control
Haemophilus_6	1.155251	0.721739	.110641	0.200538	Control	2.298402	0.983368	2.338411	.026093	0.392379	Control
Streptococcus_7	1.373529	0.668653	.040936	0.084796	Control	2.819382	0.952263	2.962068	.004813	0.101306	Control
Moraxella_1	1.412775	0.435499	.001329	0.005139	Control	2.447338	0.579145	4.227995	4.72×10^{-5}	0.001255	Control

Results differential abundance testing for case or control, when corrected for age, using MaAsLin2 and ALDEx2. ASVs that were considered significant after adjustment for multiple testing (using Benjamini-Hochberg correction; $q < 0.25$) are printed in boldface.

TABLE E4. Control-MCU

Feature	MaAsLin2 results					ALDEx2 results					
	Estimated coefficient	SE	P value	q value	Association	Estimated coefficient	SE	t statistic	P value	q value	Association
Staphylococcus_2	2.576171	0.359207	8.96×10^{-12}	5.2×10^{-10}	Case (MCU)	2.467181	0.424161	5.819828	2.47×10^{-8}	7.15×10^{-7}	Case (MCU)
Neisseriaceae_11	1.704851	0.312656	1.22×10^{-7}	1.77×10^{-6}	Case (MCU)	1.698986	0.495393	3.436206	.002667	0.056893	Case (MCU)
Neisseria_28	1.414252	0.337037	3.81×10^{-5}	0.000316	Case (MCU)	1.340631	0.438412	3.060285	.005219	0.110501	Case (MCU)
Veillonella_32	1.399049	0.33654	4.47×10^{-5}	0.000324	Case (MCU)	1.049211	0.42414	2.482454	.029878	0.385398	Case (MCU)
Actinomyces_54	1.265525	0.316152	8.32×10^{-5}	0.000536	Case (MCU)	1.03261	0.424933	2.43616	.032142	0.403906	Case (MCU)
Streptococcus_20	1.12015	0.347067	.001422	0.005889	Case (MCU)	0.854598§	0.426574	2.008683	.069066	0.668574	Case (MCU)
Streptococcus_8	1.118657	0.364955	.002422	0.00878	Case (MCU)	0.674883	0.455183	1.489588	.171605	0.943875	Case (MCU)
Streptococcus_49	1.113654	0.256147	2.03×10^{-5}	0.000196	Case (MCU)	1.087761	0.425543	2.568905	.02578	0.325504	Case (MCU)
Haemophilus_23	0.963374	0.28351	.000793	0.003835	Case (MCU)	0.676433	0.433356	1.561556	.156838	0.908815	Case (MCU)
Streptococcus_36	0.867612	0.237112	.000311	0.001802	Case (MCU)	0.856439	0.436434	1.966088	.088448	0.668959	Case (MCU)
Granulicatella_63	0.86285	0.265154	.0013	0.005799	Case (MCU)	0.58413	0.400038	1.469535	.210168	0.872537	Case (MCU)
Prevotella_melaninogenica_37	0.756435	0.330455	.022937	0.060397	Case (MCU)	0.304889	0.402098	0.761312	.435145	0.988936	Case (MCU)
Gemella_30	0.712533	0.333918	.03386	0.081829	Case (MCU)	0.202764	0.343451	0.587834	.558435	1	Case (MCU)
Granulicatella_elegans_50	0.675574	0.251378	.007699	0.023502	Case (MCU)	0.377611	0.381783	0.990979	.371895	0.981129	Case (MCU)
Haemophilus_5	0.62699	0.440512	.155931	0.27602	Case (MCU)	0.259048	0.638781	0.404798	.680093	1	Case (MCU)
Rothia_mucilaginoso_47	0.598468	0.256261	.020341	0.056181	Case (MCU)	0.331434	0.448986	0.735626	.479354	0.994368	Case (MCU)
Corynebacterium_10	0.506555	0.222932	.02395	0.060397	Case (MCU)	0.581998	0.494383	1.179856	.296563	0.957246	Case (MCU)
Alloprevotella_55	0.322225	0.331395	.331858	0.50652	Case (MCU)	−0.28012	0.402129	−0.69677	.523311	0.982668	Control
Neisseria_19	0.201523	0.328644	.540322	0.653842	Case (MCU)	−0.46449	0.465767	−1.00231	.375571	0.97268	Control
Haemophilus_29	−0.0351	0.212492	.868934	0.91633	Control	−0.43078	0.53312	−0.81119	.445095	0.982247	Control
Haemophilus_6	−0.36735	0.578389	.525949	0.653842	Control	−1.14047	0.782745	−1.45783	.166483	0.96495	Control
Moraxella_lincolni_12	−0.38232	0.457235	.403895	0.570222	Control	−0.97982	0.706481	−1.38788	.194026	0.966267	Control
Moraxella_27	−0.4671	0.313313	.137308	0.256899	Control	−1.3032	0.602479	−2.16472	.05533	0.572913	Control
Helcococcus_197	−0.5061	0.257639	.050632	0.108765	Control	−1.20133	0.517979	−2.32332	.048212	0.495418	Control
Streptococcus_7	−0.51128	0.450105	.257115	0.426076	Control	−1.79538	0.755848	−2.37621	.022701	0.400068	Control
Moraxella_4638	−0.68058	0.192877	.0005	0.002635	Control	−2.01837	0.516253	−3.91686	.001243	0.027076	Control
Dolosigranulum_pigrum_4	−0.68993	0.430689	.110479	0.220958	Control	−1.40984	0.537961	−2.62282	.011096	0.225452	Control
Corynebacterium_3	−0.85731	0.45335	.05981	0.123893	Control	−1.65646	0.615756	−2.69065	.009321	0.196741	Control
Moraxella_1	−1.03712	0.335565	.002231	0.008625	Control	−1.70035	0.436269	−3.90276	.000185	0.004935	Control

Results differential abundance testing for case or control, when corrected for age, using MaAsLin2 and ALDEx2. ASVs that were considered significant after adjustment for multiple testing (using Benjamini-Hochberg correction; $q < 0.25$) are printed in boldface.

TABLE E5. Control-ICU

Feature	MaAsLin2 results					ALDEx2 results					
	Estimated coefficient	SE	P value	q value	Association	Estimated coefficient	SE	t statistic	P value	q value	Association
Staphylococcus_2	2.453168	0.549203	1.32×10^{-5}	0.000191	Case (ICU)	2.482867	0.665502	3.735459	.000353	0.009979	Case (ICU)
Neisseriaceae_11	2.158347	0.394309	1.29×10^{-7}	3.75×10^{-6}	Case (ICU)	2.594221	0.705261	3.686884	.000915	0.024387	Case (ICU)
Streptococcus_8	1.462947	0.526305	.005953	0.049325	Case (ICU)	1.486528	0.671364	2.213637	.035385	0.620584	Case (ICU)
Haemophilus_23	1.267242	0.398234	.001691	0.016351	Case (ICU)	1.440939	0.632682	2.281021	.040281	0.557862	Case (ICU)
Veillonella_32	1.118572	0.478107	.020277	0.090466	Case (ICU)	0.992918	0.635798	1.565351	.175989	0.891312	Case (ICU)
Prevotella_melaninogenica_37	1.046919	0.467854	.026327	0.102691	Case (ICU)	0.837661	0.595142	1.419014	.232416	0.907144	Case (ICU)
Granulicatella_63	1.007217	0.375924	.007983	0.056393	Case (ICU)	1.005812	0.595502	1.694539	.147442	0.848121	Case (ICU)
Neisseria_19	0.935022	0.475079	.050413	0.146198	Case (ICU)	0.77632	0.68564	1.136391	.308737	0.994768	Case (ICU)
Gemella_30	0.864942	0.498641	.084329	0.208618	Case (ICU)	0.467741	0.530382	0.88459	.418489	0.998927	Case (ICU)
Granulicatella_elegans_50	0.757073	0.363771	.038672	0.135997	Case (ICU)	0.529907	0.577415	0.919413	.401986	0.991126	Case (ICU)
Actinomyces_54	0.679808	0.453954	.13581	0.30296	Case (ICU)	0.510563	0.635362	0.808787	.416485	1	Case (ICU)
Streptococcus_20	0.663228	0.520408	.203965	0.387223	Case (ICU)	0.297393	0.648308	0.458184	.582432	1	Case (ICU)
Streptococcus_49	0.649916	0.365982	.077262	0.208618	Case (ICU)	0.340511	0.636195	0.539808	.539766	1	Case (ICU)
Streptococcus_36	0.642879	0.32659	.050376	0.146198	Case (ICU)	0.779209	0.651063	1.203126	.294342	0.981767	Case (ICU)
Neisseria_28	0.61729	0.500865	.219207	0.387223	Case (ICU)	0.445299	0.675823	0.659158	.504136	1	Case (ICU)
Rothia_mucilaginosa_47	0.51975	0.36626	.157411	0.338142	Case (ICU)	0.445135	0.657996	0.678005	.493036	0.99933	Case (ICU)
Corynebacterium_10	0.361767	0.328168	.271601	0.450081	Case (ICU)	0.449332	0.736339	0.612747	.517895	1	Case (ICU)
Alloprevotella_55	0.048734	0.491555	.921124	0.936843	Case (ICU)	−0.41006	0.614803	−0.66918	.528573	0.996392	Control
Moraxella_lincolni_12	−0.06023	0.696194	.93114	0.936843	Control	−0.38201	1.05448	−0.36131	.702767	1	Control
Haemophilus_5	−0.08195	0.648218	.899527	0.936843	Control	−0.40541	0.939627	−0.433	.668334	1	Control
Corynebacterium_3	−0.1179	0.668722	.860231	0.936843	Control	−0.64495	0.904016	−0.71498	.485032	1	Control
Haemophilus_29	−0.3021	0.306878	.326076	0.511146	Control	−0.78352	0.774252	−1.01566	.383002	0.961176	Control
Helcococcus_197	−0.49638	0.40373	.220316	0.387223	Control	−1.02904	0.779523	−1.32346	.254719	0.939285	Control
Dolosigranulum_pigrum_4	−0.49685	0.641345	.439417	0.606813	Control	−1.06041	0.80845	−1.31291	.200047	1	Control
Moraxella_4638	−0.72185	0.308123	.020112	0.090466	Control	−1.8682	0.791505	−2.36618	.043257	0.525113	Control
Moraxella_1	−0.83858	0.483169	.084154	0.208618	Control	−1.81715	0.670342	−2.71025	.009189	0.22506	Control
Moraxella_27	−1.03673	0.46402	.026558	0.102691	Control	−2.07069	0.898707	−2.30634	.040207	0.566807	Control
Streptococcus_7	−1.58723	0.767338	.039861	0.135997	Control	−2.55351	1.078416	−2.36987	.024944	0.498634	Control
Haemophilus_6	−2.00752	0.831605	.016665	0.087868	Control	−2.85741	1.128535	−2.53295	.01741	0.372748	Control

Results differential abundance testing for case or control, when corrected for age, using MaAsLin2 and ALDEx2. ASVs that were considered significant after adjustment for multiple testing (using Benjamini-Hochberg correction; $q < 0.25$) are printed in boldface.

TABLE E6. ICU-MCU

Feature	MaAsLin2 results					ALDEx2 results					
	Estimated coefficient	SE	P value	q value	Association	Estimated coefficient	SE	t statistic	P value	q value	Association
<i>Neisseria_19</i>	1.19583	0.553145	.032564	0.303933	Case (ICU)	1.462284	0.59745	2.458995	.031713	0.493006	Case (ICU)
<i>Neisseriaceae_11</i>	0.751464	0.770226	.331157	0.786719	Case (ICU)	0.896338	0.729327	1.229745	.250152	1	Case (ICU)
<i>Gemella_30</i>	0.493911	0.60989	.419598	0.826882	Case (ICU)	0.495138	0.379121	1.318154	.233774	0.98237	Case (ICU)
<i>Granulicatella_elegans_50</i>	0.450169	0.512897	.381818	0.822378	Case (ICU)	0.459481	0.471507	0.978268	.367824	0.99773	Case (ICU)
<i>Alloprevotella_55</i>	0.431734	0.607102	.478345	0.826882	Case (ICU)	0.56176	0.537684	1.053663	.334792	0.998938	Case (ICU)
<i>Streptococcus_8</i>	0.400595	0.683037	.558621	0.826882	Case (ICU)	0.523232	0.502334	1.042369	.321974	1	Case (ICU)
<i>Haemophilus_23</i>	0.361765	0.644935	.575864	0.826882	Case (ICU)	0.534912	0.519454	1.027106	.346891	0.996079	Case (ICU)
<i>Staphylococcus_2</i>	0.253609	0.650392	.697261	0.887423	Case (ICU)	0.303187	0.44608	0.683203	.505042	1	Case (ICU)
<i>Corynebacterium_3</i>	0.199114	0.825349	.809764	0.910075	Case (ICU)	0.152522	0.874752	0.175602	.817077	1	Case (ICU)
<i>Prevotella_melaninogenica_37</i>	0.177145	0.566777	.755153	0.910075	Case (ICU)	0.238795	0.537537	0.446233	.610194	1	Case (ICU)
<i>Rothia_mucilaginosa_47</i>	0.176515	0.538756	.743746	0.910075	Case (ICU)	0.21095	0.602594	0.348514	.62018	1	Case (ICU)
<i>Granulicatella_63</i>	0.13781	0.475237	.772319	0.910075	Case (ICU)	0.278026	0.498502	0.563847	.564782	1	Case (ICU)
<i>Helcococcus_197</i>	−0.02653	0.198356	.893824	0.910075	Case (MCU)	0.096198	0.551943	0.174036	.611201	0.995505	Case (ICU)
<i>Streptococcus_36</i>	−0.05475	0.384862	.887113	0.910075	Case (MCU)	−0.03744	0.577812	−0.06145	.641748	1	Case (ICU)
<i>Corynebacterium_10</i>	−0.08286	0.468149	.859807	0.910075	Case (MCU)	0.123666	0.679965	0.17989	.677664	1	Case (ICU)
<i>Moraxella_lincolnii_12</i>	−0.1044	0.544174	.848177	0.910075	Case (MCU)	0.046456	0.817225	0.055839	.72851	1	Case (MCU)
<i>Dolosigranulum_pigrum_4</i>	−0.15682	0.710129	.825594	0.910075	Case (MCU)	−0.0865	0.745192	−0.11745	.849977	1	Case (ICU)
<i>Moraxella_27</i>	−0.18637	0.200489	.354421	0.793902	Case (MCU)	−0.23748	0.606573	−0.39532	.55227	0.998753	Case (MCU)
<i>Veillonella_32</i>	−0.24874	0.601556	.679966	0.887423	Case (MCU)	−0.14555	0.574542	−0.25176	.691091	1	Case (MCU)
<i>Streptococcus_49</i>	−0.25054	0.443153	.572863	0.826882	Case (MCU)	−0.46625	0.578034	−0.80464	.475318	0.993777	Case (MCU)
<i>Haemophilus_29</i>	−0.28573	0.230528	.217537	0.737817	Case (MCU)	−0.32119	0.657916	−0.49049	.600477	1	Case (MCU)
<i>Moraxella_1</i>	−0.40971	0.789334	.604652	0.846513	Case (MCU)	−0.42337	0.783734	−0.53875	.597169	1	Case (MCU)
<i>Neisseria_28</i>	−0.4184	0.74006	.572857	0.826882	Case (MCU)	−0.18402	0.591188	−0.31381	.667895	1	Case (ICU)
<i>Actinomyces_54</i>	−0.43736	0.520616	.402493	0.826882	Case (MCU)	−0.57772	0.543658	−1.06797	.324277	0.991986	Case (MCU)
<i>Streptococcus_20</i>	−0.69929	0.611431	.254969	0.737817	Case (MCU)	−0.82828	0.504575	−1.65153	.139645	0.943493	Case (MCU)
<i>Haemophilus_5</i>	−0.76714	0.796164	.337165	0.786719	Case (MCU)	−0.41453	0.793571	−0.52395	.610594	1	Case (MCU)
<i>Haemophilus_6</i>	−1.34769	0.738832	.070568	0.439088	Case (MCU)	−1.25112	0.98378	−1.27297	.239321	0.995936	Case (MCU)
<i>Streptococcus_7</i>	−1.89762	0.956485	.049487	0.346412	Case (MCU)	−1.40949	1.012771	−1.39396	.189908	0.9975	Case (MCU)

Results differential abundance testing for case or control, when corrected for age, using MaAsLin2 and ALDEx2.