



Temporal airway microbiome changes related to ventilator-associated pneumonia in children

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In mechanically ventilated children, microbial factors were subtly different at intubation between those who did and did not develop VAP, and changes over time were marginally associated with VAP risk, suggesting other factors may contribute to VAP <https://bit.ly/3ijsaTO>

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ABSTRACT We sought to determine whether temporal changes in the lower airway microbiome are associated with ventilator-associated pneumonia (VAP) in children.

Using a multicentre prospective study of children aged 31 days to 18 years requiring mechanical ventilation support for >72 h, daily tracheal aspirates were collected and analysed by sequencing of the 16S rRNA gene. VAP was assessed using 2008 Centers for Disease Control and Prevention paediatric criteria. The association between microbial factors and VAP was evaluated using joint longitudinal time-to-event modelling, matched case-control comparisons and unsupervised clustering.

Out of 366 eligible subjects, 66 (15%) developed VAP at a median of 5 (interquartile range 3–5) days post intubation. At intubation, there was no difference in total bacterial load (TBL), but Shannon diversity and the relative abundance of *Streptococcus*, Lactobacillales and *Prevotella* were lower for VAP subjects *versus* non-VAP subjects. However, higher TBL on each sequential day was associated with a lower hazard (hazard ratio 0.39, 95% CI 0.23–0.64) for developing VAP, but sequential values of diversity were not associated with VAP. Similar findings were observed from the matched analysis and unsupervised clustering. The most common dominant VAP pathogens included *Prevotella* species (19%), *Pseudomonas aeruginosa* (14%) and *Streptococcus mitis/pneumoniae* (10%). *Mycoplasma* and *Ureaplasma* were also identified as dominant organisms in several subjects.

In mechanically ventilated children, changes over time in microbial factors were marginally associated with VAP risk, although these changes were not suitable for predicting VAP in individual patients. These findings suggest that focusing exclusively on pathogen burden may not adequately inform VAP diagnosis.

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Introduction

Mechanically ventilated children are at high risk of ventilator-associated pneumonia (VAP). Children who develop VAP have an increased risk of mortality [1] and morbidities such as prolonged intubation and paediatric intensive care unit (PICU) stays, and the need for extensive rehabilitation [2]. Suspected VAP is the most common indication for antibiotic use in the PICU, accounting for almost half of all antibiotic days [3]. Limited understanding of the microbial and host factors associated with VAP has precluded the development of effective prevention, diagnostic and treatment strategies.

The prevailing theory behind the pathogenesis of pneumonia, including VAP, is that a pathogen enters the respiratory tract and multiplies until it overwhelms endogenous microbiota and the host defence. Endogenous bacteria [4, 5] are likely critical regulators of both pathogen behaviour and host responses in the airways [6–12]. As such, factors that impact airway microbiota or the host response are key risk factors for development of VAP [11, 13–18]. Yet, the typical culture methodology employed in the clinical environment lacks the sensitivity to assess changes in the microbiota over time.

Culture-independent molecular techniques, using nucleic acid isolated from respiratory samples, can provide sensitive quantification of the bacterial constituents of the lower airway microbiome [19–23], enabling analysis of longitudinal changes in bacterial communities in relation to development of VAP. Intestinal conditions have been associated with changes in bacterial communities over time [24–26]; specifically, lower α -diversity and relative absence of commensal organisms are associated with increased inflammation, barrier permeability and disease status [27, 28]. Early evaluations of the respiratory tract microbiota in mechanically ventilated adults suggest similar shifts in bacterial composition occur among those who develop VAP compared to those who do not [21, 22, 29], but these studies were performed in small numbers of patients and lacked daily molecular assessments to derive conclusive evidence of these associations.

The objectives of this prospective multicentre cohort study of mechanically ventilated children were to determine whether 1) compositional differences at the time of intubation, and 2) decreasing lower airway bacterial α -diversity, increasing bacterial burden and compositional change of the microbiome (increasing pathogen abundance) over time are associated with development of VAP. Furthermore, we sought to determine whether these patterns are evident prior to the clinical determination of VAP, allowing for earlier detection and more effective treatment strategies. Some of the results have been reported previously in abstract form [30].

Material and methods

Study design and subjects

We conducted a prospective cohort study of mechanically ventilated children admitted to the eight PICUs in the National Institute of Child Health and Human Development's Collaborative Pediatric Critical Care Research Network from February 2015 to December 2017. Children aged 31 days to 18 years who were expected to require mechanical ventilation *via* endotracheal tube (ETT) >72 h were eligible. Exclusion criteria included children in whom an ETT aspirate was not obtained within 24 h of intubation; those with a tracheostomy tube or with plans to place one; conditions in which deep tracheal suctioning was contraindicated; a previous episode of mechanical ventilation during the hospitalisation; previous enrolment into this study; and limitations of care.

Eligible patients and their legal guardians were approached for consent within 96 h of intubation. Delayed consent was granted, allowing for tracheal aspirate samples collected from standard-of-care suctioning of

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the ETT *via* sterile specimen trap and stored at -80°C until informed consent could be obtained. Specimens from nonconsenting patients were destroyed. The study was approved by the University of Utah central institutional review board.

Initial specimens were collected within 24 h of intubation, and subsequent samples were collected daily until the first attempted extubation or for up to 14 days. Specimens were frozen at -80°C until analysis. Clinical data were collected prospectively, as detailed in the supplementary material. Subjects were screened daily to identify VAP defined by the paediatric 2008 Centers for Disease Control and Prevention (CDC) criteria [31] in blinded fashion to the caregivers. In addition, separately, physicians were surveyed daily to determine whether they initiated antibiotics for suspected or diagnosed VAP. Details on the method for applying the CDC criteria and physician diagnosis are provided in the supplementary material.

Only subjects undergoing mechanical ventilation for >72 h were included in the final analyses (figure 1). Given the limitations of the CDC VAP definition, with significant false-positive and -negative cases [32], we removed subjects with a physician diagnosis or suspected VAP who did not meet CDC VAP criteria ($n=88$) who may represent false-negative CDC cases. The remaining subjects ($n=366$) represent the “supervised analytic cohort”.

Laboratory assays

The supplementary material provides details of laboratory assays. Briefly, DNA extraction was performed using the Qiagen EZ1 advanced extraction platform (Valencia, CA, USA). Total bacterial load (TBL) was estimated using quantitative PCR [33, 34]. Bacterial community composition was assessed by amplification of the V1/V2 region of the 16S ribosomal RNA gene (16S) [34–36]. The relative abundance of each taxon was calculated (number of sequences for specific taxon/total number of sequences $\times 100$). Shannon diversity and evenness indices characterised α -diversity. Morisita–Horn index characterised β -diversity between longitudinally collected samples within subjects.

Statistical analyses

A complete description of statistical methods is included in the supplementary material. Briefly, the association between changes in microbial factor measures over time and development of VAP was estimated using a joint longitudinal time-to-event model (JointModel package in R; R Foundation, Vienna, Austria) that included all subjects in the supervised analytical cohort ($n=366$); covariates included age at intubation, Pediatric Risk of Mortality (PRISM) III score [37], antibiotic exposure (detailed in the supplementary material) and subject-specific random intercepts and slopes. Due to anticipated differences in the baseline characteristics between VAP and non-VAP subjects, an *a priori* sub-analysis was performed employing a group-matching scheme based on age at intubation, PRISM III score, infectious admitting diagnosis and duration of mechanical ventilation. For this analysis, the day of VAP diagnosis in VAP subjects was designated as “day 0” and a corresponding day of mechanical ventilation was assigned in the non-VAP subjects. Mixed-effects models were used to evaluate the changes in microbial factors and β -diversity measures over time. A sensitivity analysis using the full cohort ($n=454$) was also performed.

Given the marked heterogeneity of the cohort, an unsupervised random forest clustering algorithm in the full cohort was used to evaluate the association between select clinical and microbial factors at intubation to identify subpopulations of patients at high risk for VAP.

Results

Cohort description

Out of 1542 subjects screened, 514 were enrolled, 454 were ventilated >72 h and had tracheal aspirate samples available for analysis and 366 subjects met criteria to be included in the supervised analytic cohort (figure 1). Table 1 and supplementary table S1 describe the cohort characteristics, and subject specific reports can be found at https://wkayla.shinyapps.io/subject_specific/ (supplementary figure S1). For the supervised analytic cohort, median (interquartile range (IQR)) age was 17 (5–66) months and 58% were male. Infection was the presenting diagnosis for 284 (78%) patients, of which 203 (55%) were lower respiratory tract infection (LRTI) and 45 (12%) were sepsis. Clinically performed viral PCR testing within 48 h of admission was positive in 127 (35%) subjects. Severity of illness (median (IQR) PRISM III score 5 (1–10)) and antibiotic exposure (128 (35%) within 7 days prior to intubation, and 317 (87%) on the day of intubation) were similar between groups. 66 (18%) subjects developed VAP at a median (IQR) of 5 (3–5) days post-intubation (supplementary figure S2). VAP subjects were more likely to be placed on extracorporeal membrane oxygen support; have longer duration of mechanical ventilation and oxygen support; longer PICU and hospital stays; and be discharged from the hospital on oxygen therapy (table 1). Mortality was higher in VAP subjects (11%, $n=7$) compared to non-VAP subjects (4%, $n=11$; $p=0.027$).

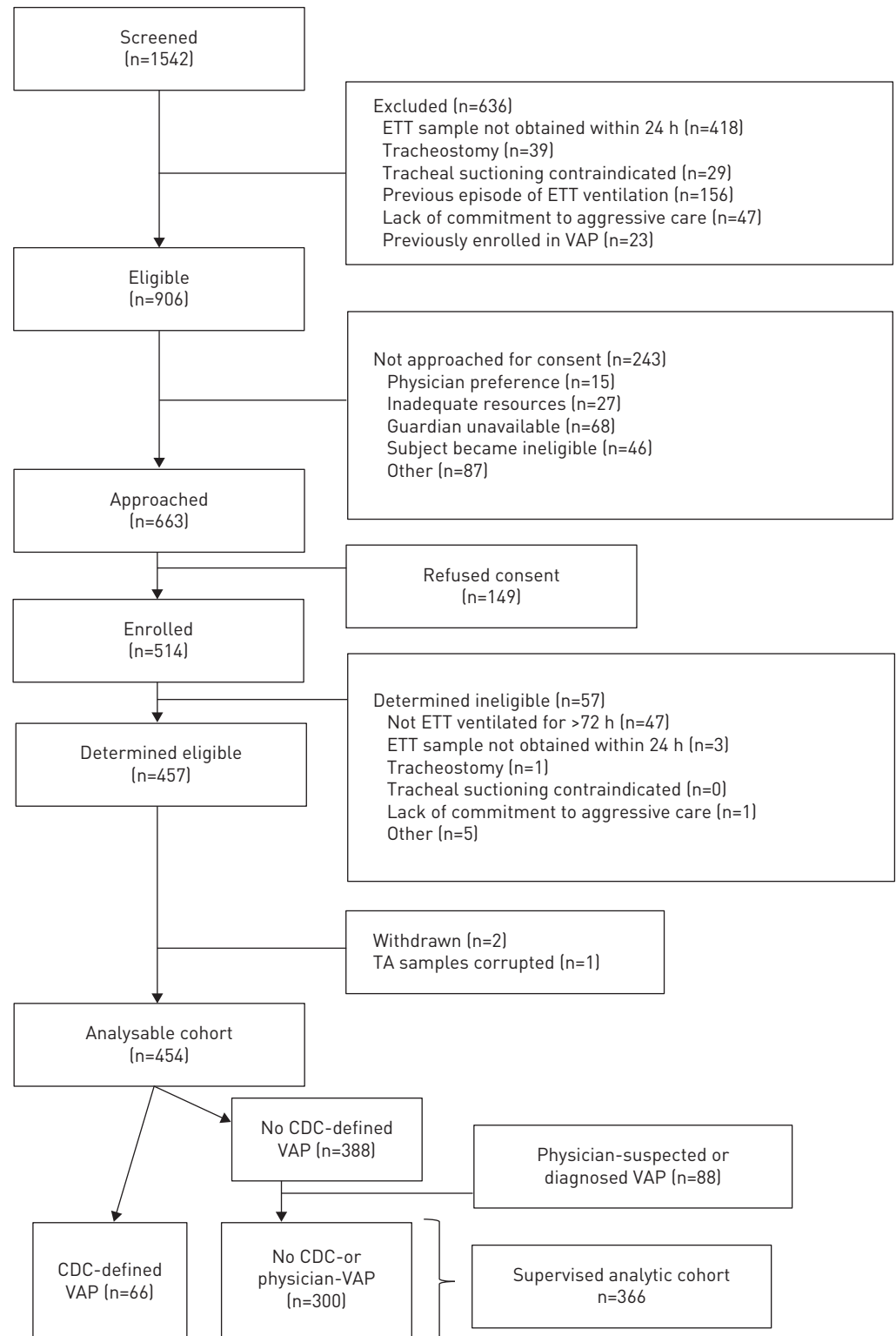


FIGURE 1 Consolidated Standards of Reporting Trials diagram. Reasons listed for exclusion are not mutually exclusive. ETT: endotracheal tube; VAP: ventilator-associated pneumonia; TA: tracheal aspirates; CDC: Centers for Disease Control and Prevention.

Sample collection

For the whole cohort ($n=454$), there was a total of 4031 ventilator days, and 2987 (74%) tracheal aspirate samples were collected. Of those, 2202 (74%) samples had sufficient bacterial DNA present to obtain

TABLE 1 Baseline characteristics and outcomes

	All subjects	Supervised analytic cohort		p-value [#]	Subjects with physician-diagnosed or -suspected VAP not meeting CDC criteria
		No VAP	VAP		
Subjects	454	300	66		88
Male	257 [57]	166 [55]	45 [68]	0.076	46 [52]
Age, in months, at intubation	16.7 [5.0–71.6]	17.5 [4.9–76.5]	14.0 [3.7–38.0]	0.085	17.3 [6.6–89.7]
Admission category[¶]				0.001	
Medical	393 [87]	257 [86]	66 [100]		70 [79]
Surgical	34 [7]	30 [10]	0 [0]		4 [4]
Trauma	27 [6]	13 [4]	0 [0]		14 [16]
Infectious admitting diagnosis	332 [73]	224 [75]	60 [91]	0.007	48 [54]
Primary admitting diagnosis				0.005	
Lower respiratory tract infection	236 [52]	154 [51]	49 [74]		33 [37]
Sepsis	48 [11]	39 [13]	6 [9.1]		3 [3]
Trauma	25 [5.5]	13 [4]	0 [0]		12 [14]
Other	145 [32]	94 [31]	11 [17]		40 [45]
Comorbidity[*]	219 [48]	143 [48]	35 [53]	0.514	41 [47]
Baseline functional status score	6 [6–8]	6 [6–8]	6 [6–9]	0.428	6 [6–8.3]
Received antibiotics within 7 days prior to intubation	152 [33]	104 [34]	24 [36]	0.62	24 [27]
Received antibiotics on day of intubation	386 [85]	259 [86]	58 [88]	0.15	69 [78]
Proportion of intubated days on antibiotics	0.29 [0.14–0.6]	0.32 [0.18–0.68]	0.25 [0.12–0.39]	0.003	0.18 [0.07–0.43]
PRISM III score	5 [2–10]	5 [1.75–10]	5.5 [1.25–10]	0.644	6.5 [2–11]
Patient outcomes					
Length of first intubation days [§]	6 [5–8]	6 [4–7]	10 [7–14]	<0.0001	8 [6–11]
Ventilator-free days in first 28 days	21 [19–23]	22 [20–24]	18 [12–21]	<0.0001	19 [14–22]
PICU LOS [§]	10 [7–15]	9 [7–12]	14 [10–26]	<0.0001	12 [9–19]
Hospital LOS [§]	17 [11–29]	16 [11–24]	20 [14–37]	0.001	21 [14–34]
Hospital mortality [¶]	27 [6]	11 [4]	7 [11]	0.027	79 [10]
ECMO during PICU stay [¶]	14 [3]	6 [2]	7 [11]	0.003	1 [1]
New morbidity	183 [42]	121 [41]	24 [39]	0.873	38 [47]
Discharged from hospital on oxygen [¶]	66 [14]	36 [12]	14 [21]	0.006	16 [18]
Total number of days on oxygen [§]	10 [7–14]	9 [7–12]	14 [9–26]	<0.0001	11 [8–18]

Data are presented as n, n (%) or median (interquartile range), unless otherwise stated. VAP: ventilator-associated pneumonia; CDC: Centers for Disease Control and Prevention; PRISM: Pediatric Risk of Mortality; PICU: paediatric intensive care unit; LOS: length of stay; ECMO: extracorporeal membrane oxygenation. [#]: p-value for comparison between no VAP and VAP subjects in the supervised analytic cohort; [¶]: Fisher's exact test was used to compare groups; ^{*}: specific comorbidities are listed in supplementary table S1; [§]: Wilcoxon rank sum test was used to compare groups.

robust sequence data (supplementary methods). Similar values were observed for the supervised analytic cohort (supplementary table S2, supplementary figures S3 and S4).

Association between microbial factors and development of VAP

On the day of intubation, there were statistically lower Shannon diversity and evenness indices for VAP subjects *versus* non-VAP subjects, but no differences in TBL. The relative abundance was lower at intubation in the VAP subjects for the following taxa: *Streptococcus*, Lactobacillales, *Prevotella* and *Prevotella* taxon JF146818 (supplementary table S3). The first sample with sequencing data within 48 h of intubation revealed a dominant organism (taxon with relative abundance >50%) in 187 (51%) subjects, of whom 120 (64%) were admitted with a LRTI. The most common dominant taxa included *Haemophilus*, *Moraxella*, *Streptococcus mitis/pneumoniae*, *Staphylococcus aureus* and *Prevotella melaninogenica*. At the time of VAP diagnosis, *Pseudomonas aeruginosa* represented the most common dominant taxon (n=6, 14%), followed by *Prevotella melaninogenica* (n=4, 10%) and *Streptococcus mitis/pneumoniae* (n=4, 10%) (table 2). Overall, *Prevotella* species represented eight (19%) cases. Of the VAP subjects with a dominant organism, 45% had the dominant organism present in the initial tracheal aspirate sample. For 24% of VAP subjects, the most abundant taxon represented <50% relative abundance.

β -diversity measures quantify the divergence in communities within individual subjects over time. Evaluation of trends in β -diversity indicated that VAP subjects had a bimodal pattern of higher divergence

TABLE 2 Dominant taxa[#] in ventilator-associated pneumonia (VAP) patients on day±1 of diagnosis

	VAP cases [¶]
Subjects	42
<i>Pseudomonas aeruginosa</i>	6
<i>Prevotella melaninogenica</i>	4
<i>Streptococcus mitis/pneumoniae</i>	4
<i>Enterobacteriaceae</i> spp.	3
<i>Mycoplasma</i> spp.	3
<i>Haemophilus</i> spp.	3
<i>Streptococcaceae</i> spp.	2
<i>Moraxella</i> spp.	2
<i>Ureaplasma</i> spp.	2
<i>Staphylococcus epidermidis</i>	2
<i>Stenotrophomonas</i> spp.	2
<i>Escherichia/Shigella</i> spp.	1
<i>Staphylococcus aureus</i>	1
<i>Bacillus</i> spp.	1
<i>Acinetobacter</i> spp.	1
<i>Neisseria mucosa</i>	1
<i>Prevotella</i> oral taxon 299 strF0039 ⁺	1
<i>Prevotella</i> oral taxon 306 strF0472 [§]	1
<i>Prevotella</i> taxon JF146818	1
<i>Prevotella</i> spp.	1

[#]: defined as those with >50% relative abundance; [¶]: 55 VAP cases had samples/sequence data available with±1 day of diagnosis, 13 had top ranked taxa <50%; ⁺: accession number CP003666; [§]: Accession number AJIN01000000.

in their bacterial communities early (days 3–4) and late (days 10–11) compared to non-VAP subjects (figure 2a and b). When sequential samples were compared to their intubation sample, VAP subjects appeared to have more divergence over time than non-VAP subjects (figure 2c and d). However, these overall differences were not statistically significant when evaluated with the mixed-effects models.

Joint models time-to-event analysis

Three joint models were constructed, each of which included time to VAP as one outcome and a microbial factor (TBL, Shannon diversity or Shannon evenness) modelled over time during mechanical ventilation as a second longitudinal outcome. The joint model assumes a linear trend over time for the longitudinal outcomes, and each sequential day's microbial factor value is included into the time to VAP diagnosis component of the model. On average, TBL increased over time; no significant changes were observed for Shannon diversity or evenness (supplementary table S4). Multiple measures of antibiotic exposure were associated with diversity and evenness, but not with TBL (supplementary table S4, figure 3a). In the subset of subjects with sequencing data, younger age was associated with shorter time to VAP after adjusting for PRISM III and microbial factors. After adjustment for days on mechanical ventilation, measures of antibiotic exposure, age, and PRISM III score, only TBL was associated with development of VAP (supplementary table S4, figure 3b). Surprisingly, and counterintuitively, higher TBL on each sequential day of mechanical ventilation was associated with a lower hazard (hazard ratio 0.39; 95% CI 0.23–0.64) for developing VAP (supplementary table S5). There was no association between each sequential day's diversity or evenness values and the hazard for VAP. Employing different approaches to incorporate the longitudinal outcome, the association between TBL and development of VAP was consistent for lagged values (values from the days preceding the current day's value in the model) and slopes of the sequential values (supplementary table S5). However, for diversity and evenness there was an association only with the slopes of those measures over time and the development of VAP. Because sequence data were missing on day of diagnosis for some VAP patients (n=18, 27%), a sensitivity analysis was performed which included VAP subjects with at least three samples, one of which had to be collected within a day of diagnosis. The results were consistent with the findings of the initial analysis (data not shown). We also performed these analyses in the entire cohort (n=454) including all subjects not meeting CDC VAP criteria (n=388) with similar results (data not shown).

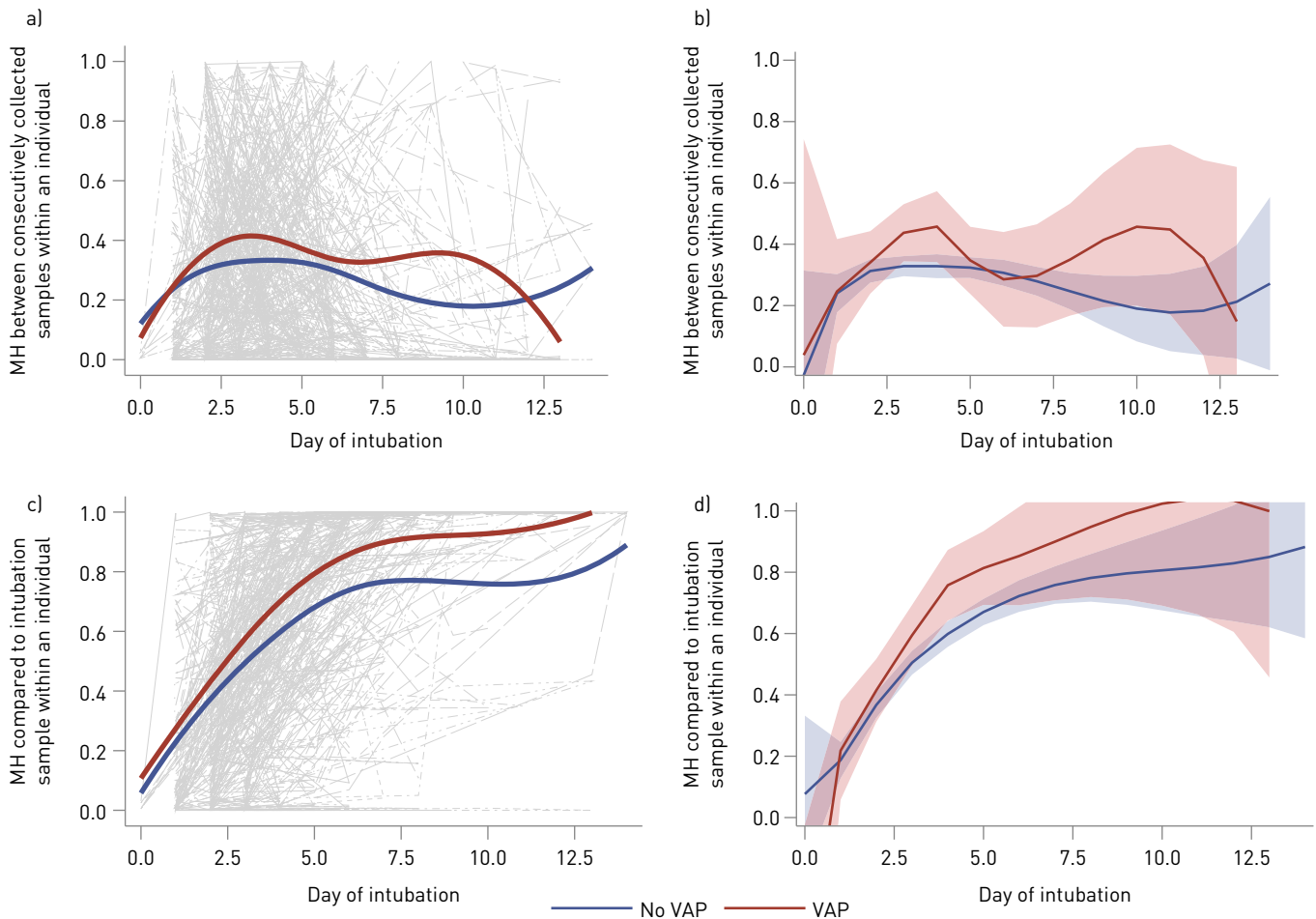


FIGURE 2 Comparison of β -diversity between ventilator-associated pneumonia (VAP) and non-VAP subjects in the supervised analytic cohort. a and c) Plots present the raw Morisita-Horn (MH) values for each individual (grey lines) and the average trends for the VAP groups using smoothing splines (coloured curves). b and d) Plots include the estimates and the 95% confidence intervals from the mixed model. The solid lines are included in the confidence interval for the other group, indicating there is no significant difference between groups. a and b) Plots correspond to the MH between consecutively collected samples within an individual; c and d) plots correspond to the MH between each sample and the intubation sample for each subject. All plots exclude samples collected after VAP diagnosis for VAP subjects.

Matched case-control analysis

The 66 VAP patients were group-matched to 227 patients who did not develop VAP either by the CDC diagnostic criteria or by physician diagnosis or suspicion of VAP. The VAP and non-VAP groups were balanced based on age at intubation, PRISM III score, infectious admitting diagnoses and length of mechanical ventilation (match quality is reported in supplementary table S6). Comparisons of the lower airway bacterial communities at intubation and at day 0 (VAP group: day of VAP diagnosis, non-VAP group: corresponding mechanical ventilation day) are provided in supplementary figure S5. Applying the β -diversity metrics to the matched cohort, we found that the degree of divergence was greatest 3 days prior to day 0 and was more divergent in the VAP subjects, although not statistically higher compared to non-VAP subjects (difference of 0.15, $p=0.06$; supplementary figure S6). We observed lower diversity and evenness in the VAP cases at day -2, but not at days -3, -1 or 0 (supplementary table S7, figure 4). TBL was lower in the VAP group at day -3 only (figure 4), but there were no differences in the day-to-day changes from intubation in any of the measures between the VAP and non-VAP groups from days -3 to 0 (supplementary table S7, supplementary figure S7).

Cluster analysis: temporal changes in community composition within endotypes

Given the high degree of heterogeneity of subjects in the cohort (table 1 and supplementary table S1), an unbiased clustering algorithm based on clinical and microbiological characteristics at the time of intubation was used to create subgroups of subjects with similar presentation. The dendrogram, a graphical description of the hierarchical clustering (supplementary figure S8), indicated several tight, small clusters of patients with similar microbial composition and clinical characteristics. The contribution of

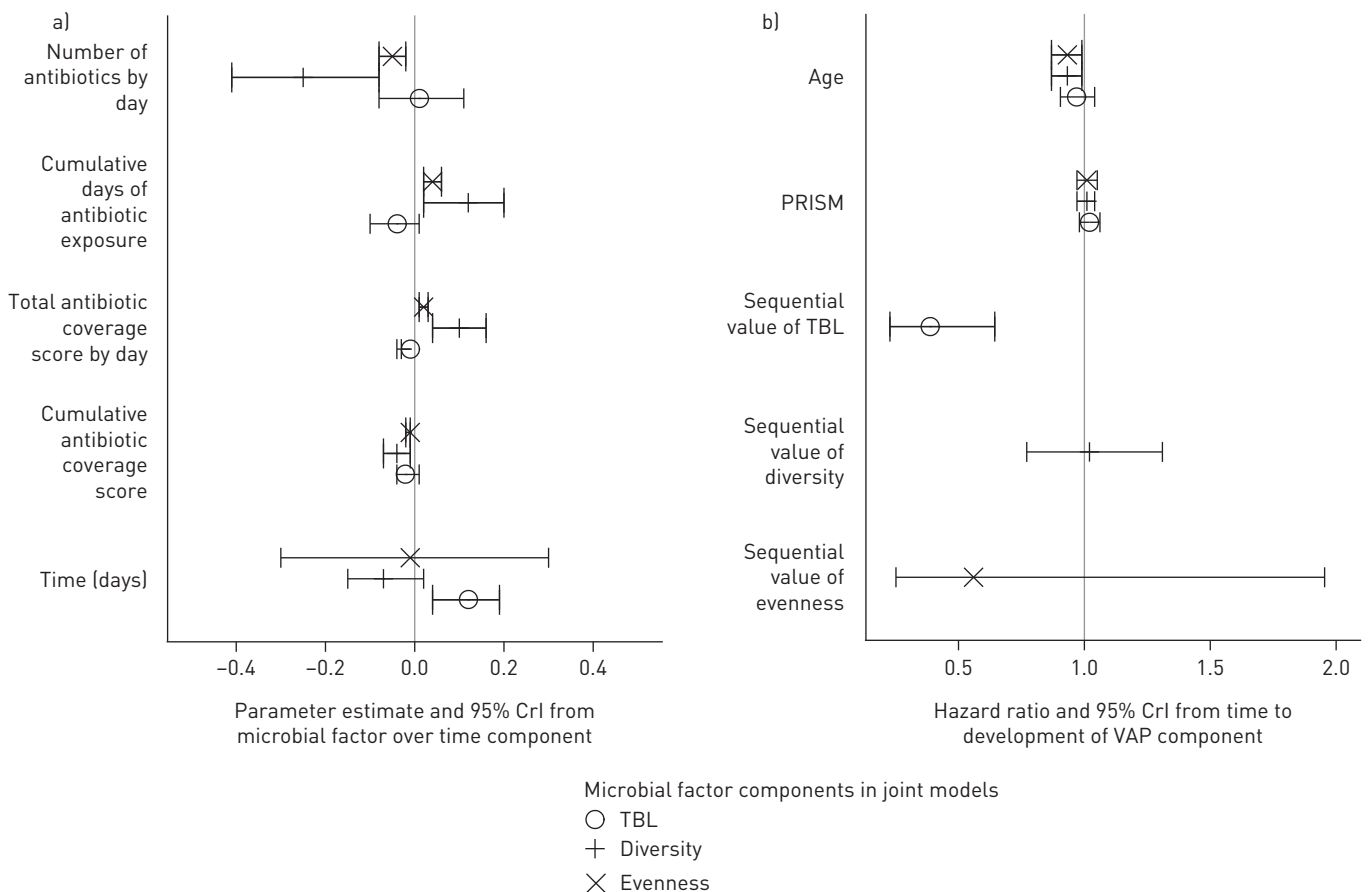


FIGURE 3 Parameter estimates from the three joint models, each of which includes time to ventilator-associated pneumonia (VAP) and a microbial factor modelled over time as outcomes. The joint model assumes a linear trend over time for the longitudinal outcomes and the current or sequential values for the microbial factor is included into the time to VAP diagnosis component of the model. Antibiotic exposure was measured in four ways: cumulative antibiotic coverage score indicates the overall broadest spectrum antibiotic coverage the patient received throughout the period of intubation up until the time the sample was collected; total antibiotic coverage score by day indicates the broadest spectrum coverage of antibiotic the patient received on the day the sample was collected; cumulative days of antibiotic exposure indicates number of antibiotics given during the time of intubation up until the sample was collected; and number of antibiotics by day indicates number of drugs given on the day of sample collection. A forest plot displaying the a) parameter estimates from the longitudinal outcome component of the joint model for each of the microbial factors and b) the hazard ratios from the time to VAP component of the joint model. These model estimates indicate that microbial factors change over time and with antibiotic exposure. Time to VAP diagnosis is associated with total bacterial load (TBL). In the subset of subjects with sequencing data, younger age is associated with shorter time to VAP after adjusting for Pediatric Risk of Mortality (PRISM) III and microbial factors. After adjustment for time, antibiotic exposure, age and PRISM III score, only TBL was associated with development of VAP. Error bars correspond to 95% credible intervals [CrI] from the joint model, intervals that exclude values of 0 in a) or 1 in b) are significantly associated with the variables listed on the y-axes.

changes in microbial composition or antibiotic treatment to the development of VAP was then evaluated within each cluster (figure 5). The clusters were not clearly associated with VAP diagnosis. For example, the leftmost cluster of subjects contains 33 subjects with tracheal aspirate samples dominated by *Haemophilus*, the majority of whom had an infectious diagnosis and low PRISM III scores upon intubation. Of this subset, four subjects were diagnosed with VAP with variation in the dominant organism at or near the time of VAP (n=2 *Streptococcus mitis/pneumoniae*, n=1 *Bacilli* and n=1 *Prevotella*). There were no consistent differences in the temporal changes in microbial factors, site effects or administered antibiotics that might explain an association with VAP in this more homogeneous group of subjects. Similar heterogeneity was noted among the other clusters.

Discussion

In this prospective multicentre cohort study of mechanically ventilated children at high risk of VAP, those who developed VAP exhibited lower Shannon diversity and lower relative abundance of *Streptococcus*, *Lactobacillales* and *Prevotella* on the day of intubation compared with those without VAP. The composition of bacterial communities diverged more over time in VAP subjects compared to non-VAP subjects; these differences were not statistically significant. Subtle differences in microbial factors (TBL,

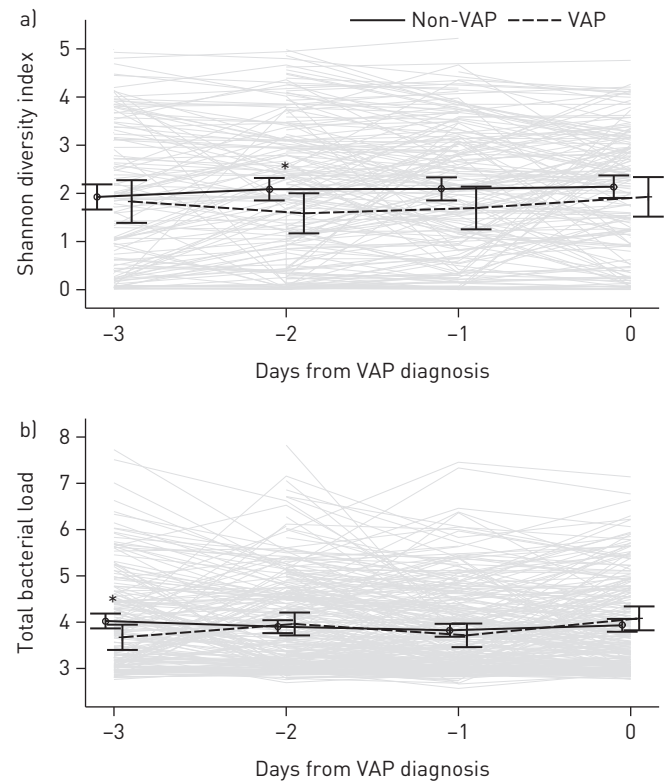


FIGURE 4 Average trajectories of change in microbial factors reveal subtle statistical differences between subjects who developed ventilator-associated pneumonia (VAP) compared to subjects who did not develop VAP in the matched cohort. Day 0 denotes day of diagnosis in VAP cases ($n=66$) and the reference day of mechanical ventilation in controls ($n=227$; see supplementary material for details). Comparisons are displayed for a) Shannon diversity and b) total bacterial load (Shannon evenness not shown), for up to 3 days preceding day 0. Comparison of diversity between VAP cases and controls at each of 3 days prior to day 0 indicated a lower diversity in the VAP cases at day -2 prior to VAP diagnosis compared to controls; however, significant differences were not present on days -3, -1 or 0. *: $p<0.05$.

diversity and evenness) were associated with development of VAP after adjusting for antibiotic exposure in both joint time-to-event and matched case-control analyses.

To our knowledge, this is the first comprehensive evaluation of the lower-airway microbiota in mechanically ventilated children relative to the development of VAP. Our findings are consistent with previous studies performed in adults [20, 21, 29, 38], including one report of an association between VAP and lower abundance of Bacilli, which includes *Streptococcus* and *Lactobacillus*, at intubation [29]. While these associations may provide insight into potential pathogenic mechanisms, given the variability in the longitudinal evolution of the microbiome, it is unlikely they will provide useful clinical prognostication for individual patients. Unsupervised clustering of subjects using baseline characteristics did not identify subgroups of subjects at significant risk of developing VAP. Additionally, within relatively homogeneous clusters of subjects, there were no discernible changes in the bacterial community or antibiotic usage patterns that were associated with VAP, suggesting that other unmeasured factors may contribute to development of VAP.

The hypothesis that VAP, like other infections, is characterised by an increase in bacterial burden and a decrease in community diversity [21, 29, 39] was not clearly evident in our study. The decrease in microbial diversity during mechanical ventilation support is consistent with other studies, and we found that antibiotic exposure is directly associated with diversity and evenness, but change in diversity was not consistently associated with the development of VAP over time (only observed 2 days prior to VAP in the matched cohort), even after adjusting for antibiotic exposure. In general, these changes over time appear subtle in context of the large longitudinal intra- and inter-subject variation. In both analytic approaches, we found that on average, TBL was lower in VAP cases than in non-VAP cases, contrary to our hypothesis. Yet, there were individuals who exhibited increases in TBL with development of VAP. Given the high use of antibiotics, it is possible that the lower TBL signifies microbiome depletion, providing a gap for pathogen colonisation and subsequent infection, but this explanation would require more detailed

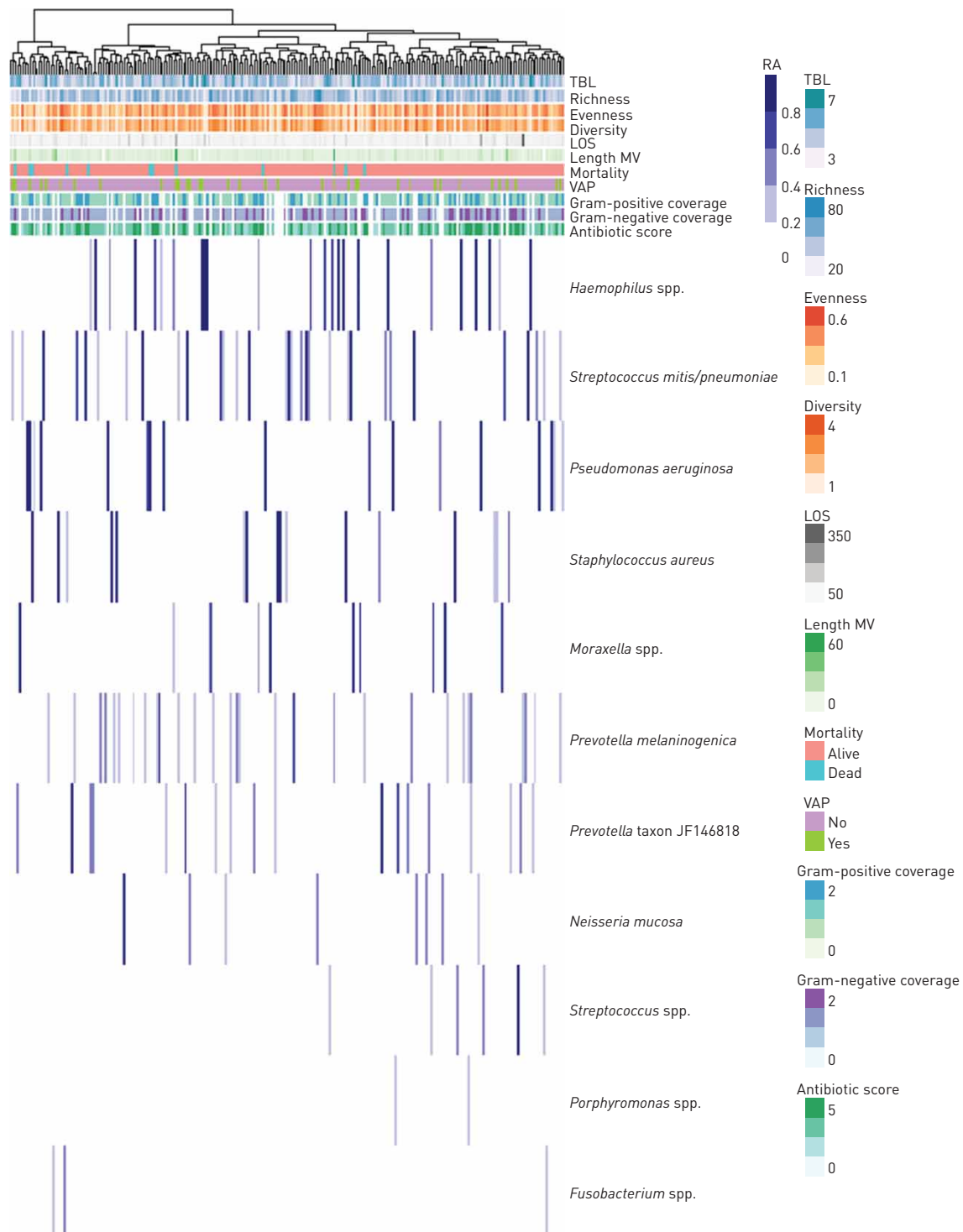


FIGURE 5 Clustering analysis does not reveal high-risk ventilator-associated pneumonia (VAP) phenotypes. The patient clusters represented in supplementary figure S8 are displayed by the dendrogram at the top of the figure. Outcomes (VAP, length of mechanical ventilation, mortality) for each subject are indicated using colour bars underneath the corresponding terminal end of the dendrogram. The heatmap displays the relative abundance for the taxa identified in the sample either at VAP diagnosis for cases or 48 h prior to extubation for non-VAP subjects. Subjects clustered together based on their clinical and microbial factors at intubation are displayed next to each other, the distance between subjects is indicated by the dendrogram at the top. There are no discernable differences in any clinical, treatment (antibiotic score; see supplementary material for details) or microbial factors that might explain why subjects in the same cluster develop VAP while others did not. Total bacterial load, richness, diversity and evenness are presented for day of VAP diagnosis or 48 h prior to extubation in non-VAP subjects. LOS: length of hospital stay; TBL: total bacterial load; length MV: length of mechanical ventilation; mortality: in-hospital mortality; RA: relative abundance.

studies to confirm. Because TBL is a measure of the entire bacterial community burden, it may not directly represent pathogen burden, *e.g.* if the pathogen burden increases while other commensal bacterial burden decrease, the TBL may not change significantly.

To sufficiently characterise the existence of infection, an increase in pathogen burden must be coupled with evidence of tissue injury and host inflammatory response. The latter is a key measure that is missing in our study and suggests that even with the increased sensitivity of molecular microbial detection methodology and a large sample size, simply focusing on the quantitative composition of the bacterial microbiome of tracheal aspirate specimens is not sufficient to fully elucidate the pathogenesis and key risk factors for VAP. The evolution of the airway microbiota as it relates to development of VAP is likely a more dynamic process than daily molecular taxonomic analysis can assess given its inability to measure metabolic activity, replication rates and virulence of the bacteria as they interact with each other and the host. ~20% of VAP cases did not have a dominant organism. These could represent false-positive cases, consistent with known limitations of the CDC definition [32]. We attempted to correct for confounding by antibiotic administration by excluding cases in which physician suspicion for VAP led to antibiotic administration before CDC criteria could be satisfied, but many patients received antibiotics for reasons other than VAP. Alternatively, it is possible that the “increased pathogen burden” hypothesis may not be entirely correct. Emerging evidence suggests that bacterial virulence may change without necessarily changing bacterial abundance [40]. This could explain why VAP patients had worse outcomes without having marked differences in their bacterial constituencies and despite similar initial illness severity. Thus, different approaches centred on gene expression changes in both host and microbial populations (viral, bacterial and fungal) may be required to redefine pneumonia constructively [23, 41]. One recent study measured human DNA content (a surrogate for host inflammatory response) in respiratory samples together with 16S RNA gene sequencing and found that higher human DNA content was more robustly associated with VAP than microbial factors [29].

Molecular detection identified common pathogens in many of the VAP patients in our cohort, including *Pseudomonas*, *Streptococcus mitis/pneumoniae* and Enterobacteriaceae. However, we also identified microbes not detected by traditional aerobic cultures. *Prevotella* and other anaerobic species represented the most dominant taxa in several VAP cases, corroborating evidence from other reports [42–44]. Almost half of VAP patients had the VAP dominant organism present in the first tracheal aspirate sample, suggesting the possibility that the pathogen was not acquired during mechanical ventilation. As seen in other studies of critically ill children [45], LRTI was the most common admitting diagnosis in our cohort, and children with LRTI developed VAP more frequently. Most of the LRTI cases were viral in origin, which may predispose to secondary bacterial infection [46–49]. Whether broad empirical antibiotic therapy for viral LRTI contributes to the early dominance of anaerobes requires further investigation. *Mycoplasma* and *Ureaplasma* were also identified as the dominant organism in several subjects. Recent adult studies report *Mycoplasma* as a common causative organism for VAP [29, 50], suggesting it should routinely be considered among possible aetiologies of VAP.

The taxonomic composition identified in tracheal aspirates is similar to those identified in our previous study of tracheal aspirates and ETT biofilms and consists largely of oropharyngeal bacteria [51]. Unsurprisingly, these findings probably represent cross-contamination with oral secretions either during the process of intubation or *via* aspiration of oral secretions after intubation. We had previously found that tracheal aspirate specimens and ETT biofilms from the same patients largely shared similar bacterial communities, yet with a substantial minority of patients demonstrating divergent communities between these sites. Thus, the potential role of ETT microbial biofilms in the development of VAP deserves further investigation, but this potential impact was not examined in context of this study.

The strengths of our study include 1) the largest cohort of mechanically ventilated children in whom daily molecular assessments of bacteria were performed on respiratory samples; 2) complementary statistical analytic approaches encompassing a time-to-event analysis, matched case–control approach, and unbiased clustering; 3) prospective and consistent application of the CDC paediatric definition of VAP; and 4) incorporation of clinical factors, including age, severity of illness and antibiotic exposure into our analytic models.

There are several limitations to our study. 1) TBL measurements are subject to variation by dilution from host secretion production and by the addition of small amounts of saline to facilitate sample collection. Thus, it is more a measure of bacterial density than true bacterial burden. 2) Amplification of 16S genes may result in bacterial detection bias, and some bacteria were only identified to the genus level. Furthermore, we only evaluated bacterial composition, which neglects contributions from other microbes (viruses and fungi) to the development of VAP. 3) Our study included gaps in daily sample collection. Daily respiratory samples were not collected in all patients for reasons including inappropriate suctioning

technique, subjects with inadequate secretions and development of a contraindication to suctioning. Additionally, many samples had insufficient bacterial DNA load to robustly sequence without substantial interference of background signals. 4) VAP was diagnosed according to the paediatric CDC criteria, which, despite vigorous prospective application, may still have produced an error rate that adversely impacted our analyses [32]. We attenuated this by excluding subjects with physician-suspected or -diagnosed VAP who did not meet CDC criteria. Yet, it is possible that with a more precise VAP definition, a more direct relationship between microbiome assessments and VAP could be identified. Additionally, it is possible that tracheal aspirates are an inadequate specimen for assessing this relationship, even with a perfect VAP definition. 5) The cohort represents a heterogeneous PICU population, and individual risk for VAP may differ based on the primary admitting diagnosis. The intention of this study was to identify common patterns in the airway microbiome across all PICU patients that could inform the risk and pathogenesis of VAP. While we attempted to control for infectious diagnoses, including LRTI, in our matching analysis, there may be unique patterns within a specific presenting diagnosis group that we failed to recognise. 6) Cultures were performed clinically and were not performed with the same samples as the research samples, precluding direct comparisons between the 16S and clinical culture results. 7) Although a robust antibacterial scoring system was employed, accounting for antibacterial activity and duration, the high variation in antibiotic use may not have been adequately represented in our analyses.

In conclusion, longitudinal analysis of lower airway samples with 16S rRNA gene sequencing in a large, diverse population of critically ill children revealed that the airway microbiome is heterogeneous at intubation with lower Shannon diversity and relative abundance of *Streptococcus*, *Lactobacillales* and *Prevotella* in children who developed VAP. Although there were statistical differences in TBL and diversity between VAP and non-VAP patients during mechanical ventilation support, which may provide some insight into the pathogenesis of VAP, these changes were not suitable for predicting VAP in individual patients. These findings suggest that other factors contribute to VAP risk, and thus focusing exclusively on pathogen burden may not adequately inform VAP diagnosis. Future studies that include comprehensive microbial detection, measures of microbial activity and virulence and assessment of host response may offer greater insight into VAP pathogenesis, provide more accurate prediction models and identify modifiable risk factors to prevent VAP.

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