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TEMPORAL AIRWAY MICROBIOME CHANGES RELATED TO VENTILATOR ASSOCIATED PNEUMONIA IN CHILDREN

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Summary Statement: 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.

Word Count: 3959

Abstract

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

Using a multicenter prospective study of children 31 days to 18 years requiring mechanical ventilation (MV) support for > 72 hours, daily tracheal aspirates were collected and analyzed by sequencing of the 16S rRNA gene. VAP was assessed using 2008 CDC pediatric criteria. The association between microbial factors and VAP was evaluated using joint longitudinal time-to-event modeling, matched case-control comparisons, and unsupervised clustering.

Of 366 eligible subjects, 66 (15%) developed VAP at a median of 5 (IQR: 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 (HR: 0.39; 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.

Word Count: 248

INTRODUCTION

Mechanically ventilated children are at high risk for ventilator-associated pneumonia (VAP). Children who develop VAP have an increased risk of mortality (1) and morbidities such as prolonged intubation and pediatric 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 defense. Endogenous bacteria (4, 5) are likely critical regulators of both pathogen behavior 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 alpha 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 multi-center cohort study of mechanically ventilated children were to determine whether 1) compositional differences at the time of intubation, and 2) decreasing lower airway bacterial alpha diversity, increasing bacterial burden, and compositional change of the microbiome (increasing pathogen abundance) over time, are associated with development of VAP. Further, 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 were previously reported in abstract form (30).

MATERIAL AND METHODS

Study Design and Subjects

We conducted a prospective cohort study of mechanically ventilated children admitted to the 8 PICUs in the National Institute of Child Health and Human Development's Collaborative Pediatric Critical Care Research Network (CPCCRN) from February 2015 to December 2017. Children ages 31 days – 18 years who were expected to require mechanical ventilation (MV) via endotracheal tube (ETT) >72 hours were eligible. Exclusion criteria included: children in whom an ETT aspirate was not obtained within 24 hours 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 MV during the hospitalization, previous enrollment into this study; and limitations of care.

Eligible patients and their legal guardians were approached for consent within 96 hours of intubation. Delayed consent was granted, allowing for tracheal aspirate (TA) samples collected from standard of care suctioning of the ETT via sterile specimen trap and stored at -

80^{o}C until informed consent could be obtained. Specimens from non-consenting patients were destroyed. The study was approved by the University of Utah central IRB.

Initial specimens were collected within 24 hours of intubation, and subsequent samples were collected daily until the first attempted extubation or for up to 14 days. Specimens were frozen at -80^{o}C until analysis. Clinical data were prospectively collected as detailed in the supplementary material. Subjects were screened daily to identify VAP defined by the pediatric 2008 Center for Disease Control (CDC) criteria (31) in blinded fashion to the caregivers. Physicians were also separately 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 MV for >72 hours 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. Total bacterial load (TBL) was estimated by 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 (RA) of each taxon was calculated (number of sequences for specific taxon/total number of sequences*100). Shannon diversity and evenness indices characterized alpha diversity. Morisita-Horn characterized beta 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) 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 MV. For this analysis, the day of VAP diagnosis in VAP subjects was designated as “Day 0” and a corresponding day of MV was assigned in the non-VAP subjects. Mixed effects models were used to evaluate the changes in microbial factors and beta 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

Of 1,542 subjects screened, 514 were enrolled, 454 were ventilated >72 hours and had TA samples available for analysis, and 366 subjects met criteria to be included in the supervised analytic cohort (Figure 1). Tables 1 and S1 describe the cohort characteristics, and subject specific reports can be found at https://wkayla.shinyapps.io/subject_specific/ (Figure S1). For the supervised analytic cohort, median age was 17 (inter-quartile range [IQR]: 5 - 66) months, and 58% were male. Infection was the presenting diagnosis for 284 (78%) patients, of whom

203 (55%) were lower respiratory tract infection (LRTI) and 45 (12%) were sepsis. Clinically performed viral PCR testing within 48 hours of admission was positive in 127 (35%) subjects. Severity of Illness (median PRISM III score: 5 [IQR: 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. Sixty-six (18%) subjects developed VAP at a median of 5 days (IQR: 3 - 5) post-intubation (Figure S2). VAP subjects were more likely to be placed on extracorporeal membrane oxygen (ECMO) support, have longer duration of MV 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).

Sample collection description

For the whole cohort (n=454), there was a total of 4,031 ventilator days, and 2,987 (74%) TA samples were collected. Of those, 2,202 (74%) samples had sufficient bacterial DNA present to obtain robust sequence data (Supplementary methods). Similar values were observed for the supervised analytic cohort (Table S2, 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* taxonJF146818 (Table S3). The first sample with sequencing data within 48 hours of intubation revealed a dominant organism (taxon with relative abundance >50%) in 187 (51%) subjects, of which 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 8 (19%) of the cases. Of the VAP subjects with a dominant organism, 45% had the dominant organism present in the initial TA sample. For 24% of VAP subjects, the most abundant taxon represented <50% relative abundance.

Beta diversity measures quantify the divergence in communities within individual subjects over time. Evaluation of trends in beta diversity indicated that VAP subjects had a bimodal pattern of higher divergence in their bacterial communities early (days 3-4) and late (days 10—11) compared to non-VAP subjects (Figure 2 A 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 2 C 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) modeled over time during MV 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 (Table S4). Multiple measures of antibiotic exposure were associated with diversity and evenness but not with TBL (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 MV, measures of antibiotic exposure, age, and PRISM III score, only TBL was associated with development of VAP (Table S4, Figure 3B). Surprisingly and counterintuitively, higher TBL on each sequential day of MV was associated with a lower hazard (HR: 0.39; CI: 0.23, 0.64) for developing VAP (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 (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 that included VAP subjects with at least 3 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).

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 MV (match quality is reported in 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 MV day) are provided in Figure S5. Applying the beta 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, $p = 0.06$; Figure S6). We observed lower diversity and evenness in the VAP cases at day -2, but not at days -3, -1, or 0 (Table S7 and 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 (Table S7, 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 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 (Figure S8), indicated several tight, small clusters of patients with similar microbial composition and clinical characteristics. The contribution of 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 TA samples dominated by *Haemophilus,* the majority of whom had an infectious diagnosis and low PRISM III scores upon intubation. Of this subset, 4 subjects were diagnosed with VAP with variation in the dominant organism at or near the time of VAP (2 with *Streptococcus mitis/pneumoniae*, 1 with *Bacilli,* and 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 amongst the other clusters.

DISCUSSION

In this prospective multi-center cohort study of mechanically ventilated children at high risk for 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, 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 for developing VAP. Additionally, within relatively homogeneous clusters of subjects, there were no discernable changes in the bacterial community or antibiotic usage patterns that were associated with VAP, suggesting other unmeasured factors may contribute to development of VAP.

The hypothesis that VAP, like other infections, is characterized 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 MV 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 colonization and subsequent infection, but this explanation would require more detailed 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 characterize 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. Approximately 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 centered 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 TA 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 empiric 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 etiologies of VAP.

The taxonomic composition identified in in tracheal aspirates is similar to those identified in our previous study of tracheal aspirates and ETT biofilms and consist largely of oropharyngeal bacteria (51). Unsurprisingly, these findings likely 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 pediatric 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 that true bacterial burden. 2) Amplification of 16S genes may result in bacterial detection bias, and some bacteria were only identified to the genus level. Further, 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 by the pediatric 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 heterogenous 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 recognize. 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 MV 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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FIGURE LEGENDS

Figure 1: CONSORT diagram

Figure 2: Comparison of beta diversity between VAP and non-VAP subjects in the supervised analytic cohort. Plots A and C present the raw Morisita Horn (MH) values for each individual (grey lines) and the average trends for the VAP groups using smoothing splines (colored curves). Plots B and D include the estimates and the 95% confidence intervals from the mixed model. The solid lines are included in the CI for the other group, indicating there is no significant difference between groups. Plots A and B correspond to the MH between consecutively collected samples within an individual. Plots C and D 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.

Figure 3. Parameter estimates from the three joint models, each of which includes time to VAP and a microbial factor modeled 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 4 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 TBL. In the subset of subjects with sequencing data, younger age is associated with shorter time to VAP after adjusting 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 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-axis. CrI: credible interval.

Figure 4: Average trajectories of change in microbial factors reveal subtle statistical differences between subjects who developed 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 supplement for details). Comparisons are displayed for Shannon Diversity (A) and Total Bacterial Load (B), Shannon Evenness not shown, for up to 3 days preceding Day 0. Comparison of diversity between VAP cases and controls at each of three days prior to Day 0 indicated a lower diversity in the VAP cases at day -2 prior to VAP diagnosis compared to controls (* indicate p-value < 0.05), however, significant differences were not present on days -3, -1 or 0.

Figure 5: Clustering analysis does not reveal high-risk VAP phenotypes. The patient clusters represented in 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 color 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 hours 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 hours prior to extubation in non-VAP subjects. Abbreviations LOS: length of hospital stay, TBL: total bacterial load, Length MV: Length of mechanical ventilation, Mortality: in-hospital mortality, VAP: ventilator associated pneumonia, RA: relative abundance.

Table 1. Baseline Characteristics and Outcomes.

Patient Characteristics	All Subjects (n=454)	Supervised Analytic Cohort (n=366)			Subjects with Physician diagnosed or suspected VAP not meeting CDC criteria (n=88)
		No VAP (n=300)	VAP (n=66)	P-value	
Gender (Male)	257 (57%)	166 (55%)	45 (68%)	0.076	46 (52%)
Age at Intubation (months)	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[1]				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[2]	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[3]	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[3]	10 (7, 15)	9 (7, 12)	14 (10, 26)	<0.0001	12 (9, 19)
Hospital LOS[3]	17 (11, 29)	16 (11, 24)	20 (14, 37)	0.001	21 (14, 34)
Hospital mortality[1]	27 (6%)	11 (4%)	7 (11%)	0.027	79(10%)

ECMO during ICU Stay[1]	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[1]	66 (14%)	36 (12%)	14 (21%)	0.006	16 (18%)
Total number of days on oxygen[3]	10 (7, 14)	9 (7, 12)	14 (9, 26)	<0.001	11 (8, 18)

[1]Fisher's exact test was used to compare groups

[2]Specific comorbidities are listed in Table E1

[3]Values presented as median (Interquartile range), Wilcoxon rank sum test was used to compare groups

[4]P-value for comparison between no VAP and VAP subjects in the supervised analytic cohort

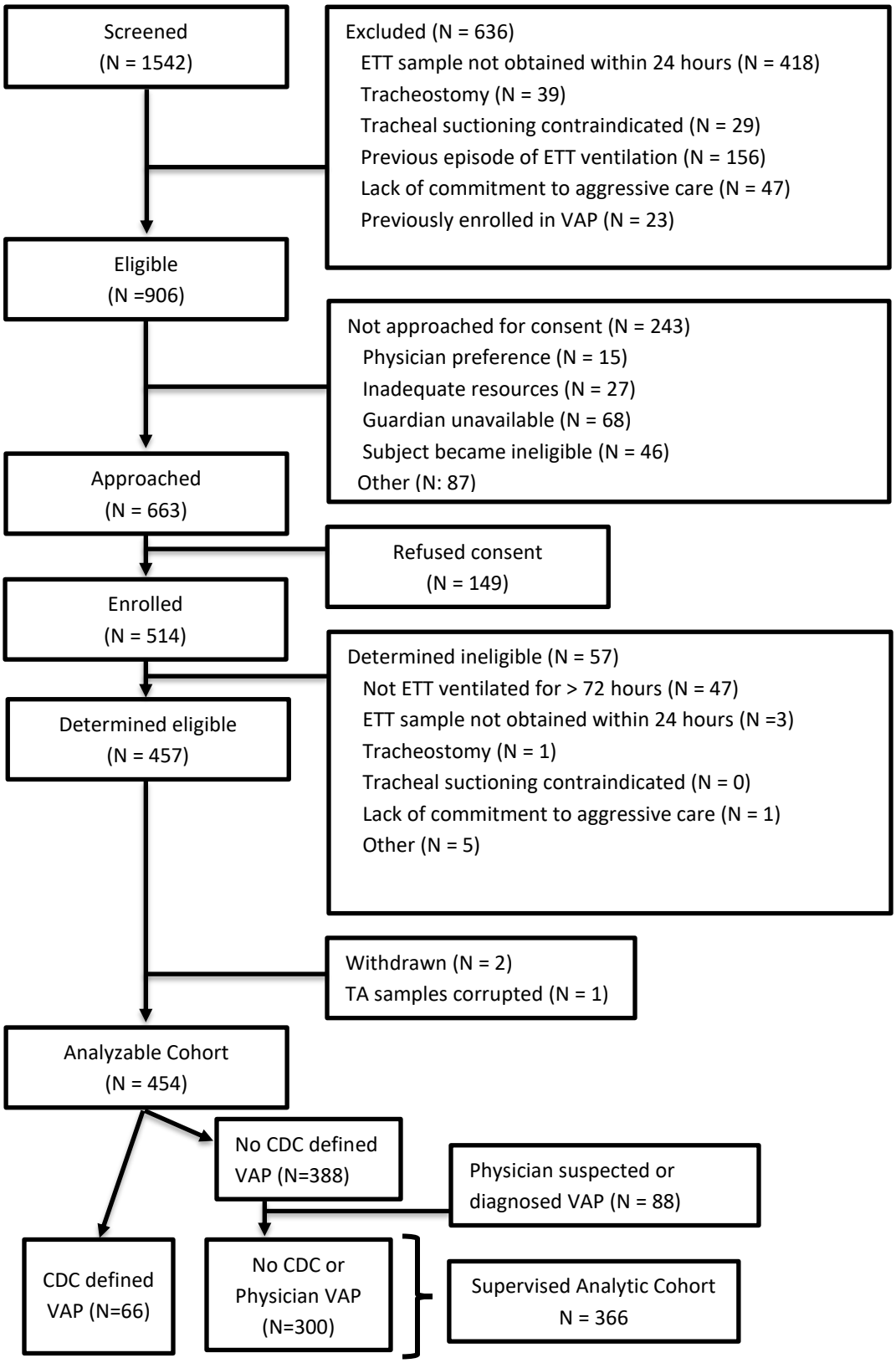
Abbreviations: PRISM: Pediatric Risk of Mortality; PICU: pediatric intensive care unit; LOS, length of stay; ECMO: extracorporeal membrane oxygenation.

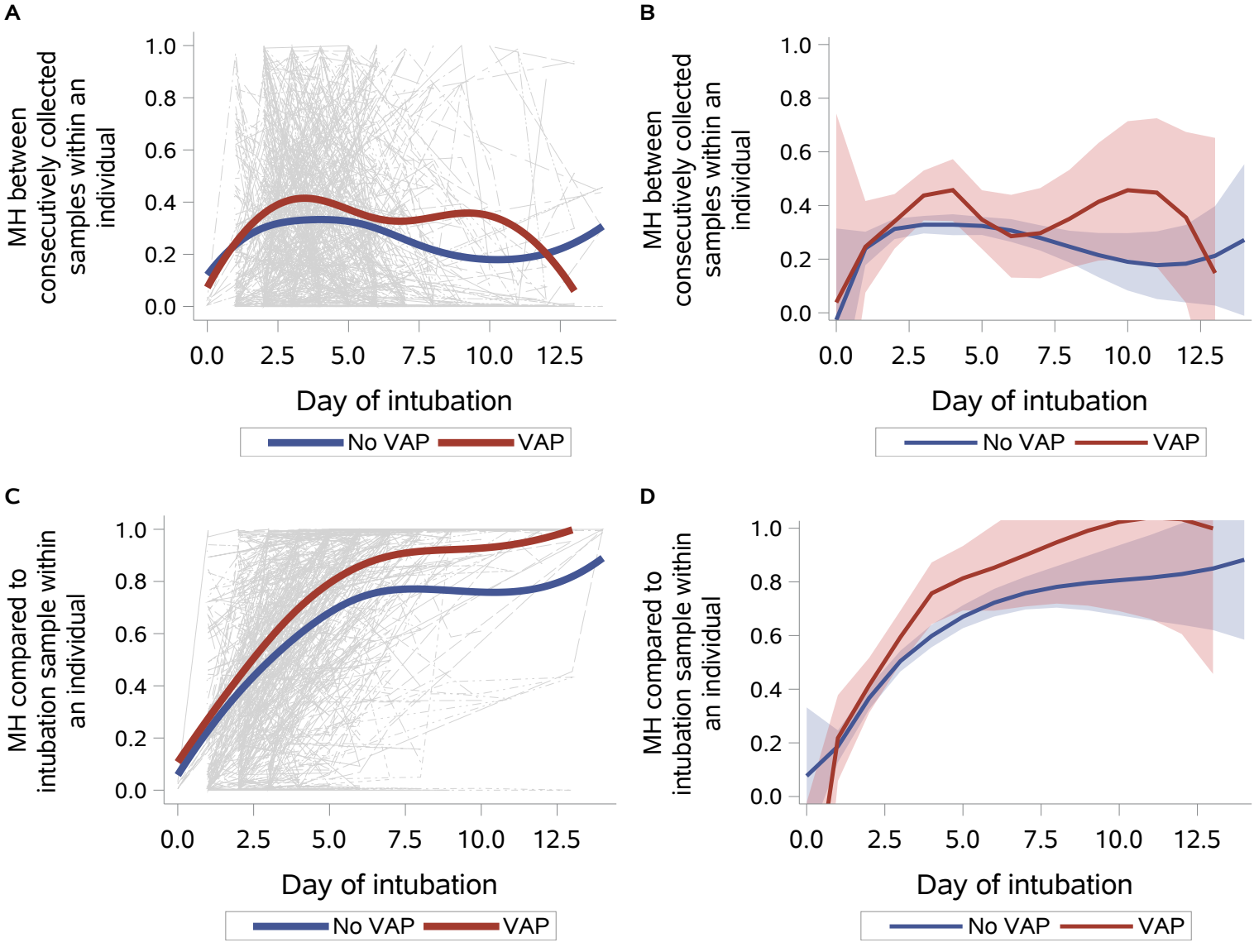
Table 2: Dominant Taxon[1] in VAP patients on day ± 1 of diagnosis

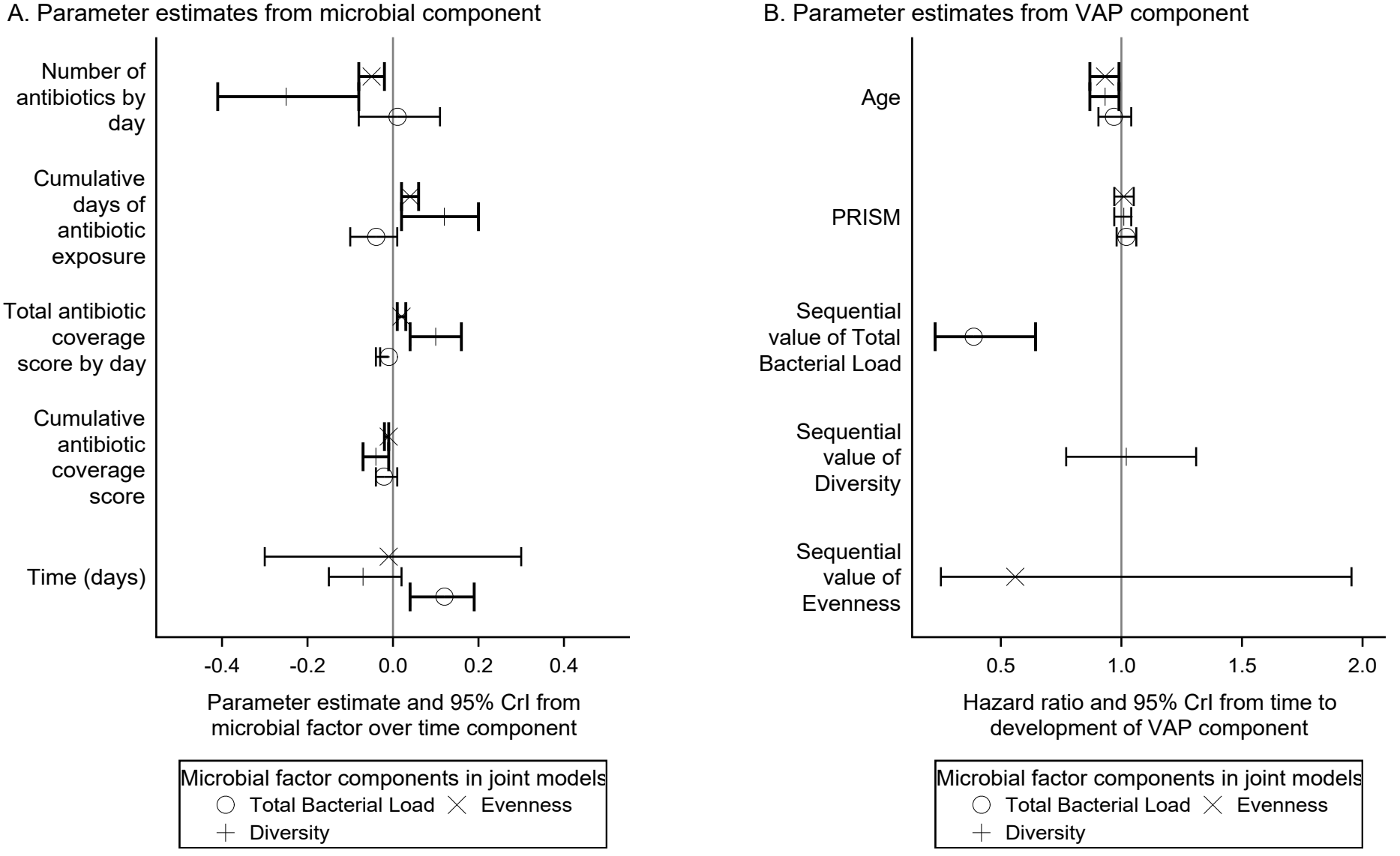
Taxon	Number of VAP cases[2] (n=42)
Pseudomonas aeruginosa	6
Prevotella melaninogenica	4
Streptococcus mitis/pneumoniae	4
Enterobacteriaceae spp.	3
Mycoplasma spp.	3
Haemophilus spp.	3
Streptococcaceae spp.	2
Moraxella spp.	2
Ureaplasma spp.	2
Staphylococcus epidermidis	2
Stenotrophomonas spp.	2
Escherichia/Shigella spp.	1
Staphylococcus aureus	1
Bacilli spp.	1
Acinetobacter spp.	1
Neisseria mucosa	1
*Prevotella oral taxon299 strF0039**	1
*Prevotella oral taxon306 strF0472***	1
Prevotella taxon JF146818	1
Prevotella spp.	1

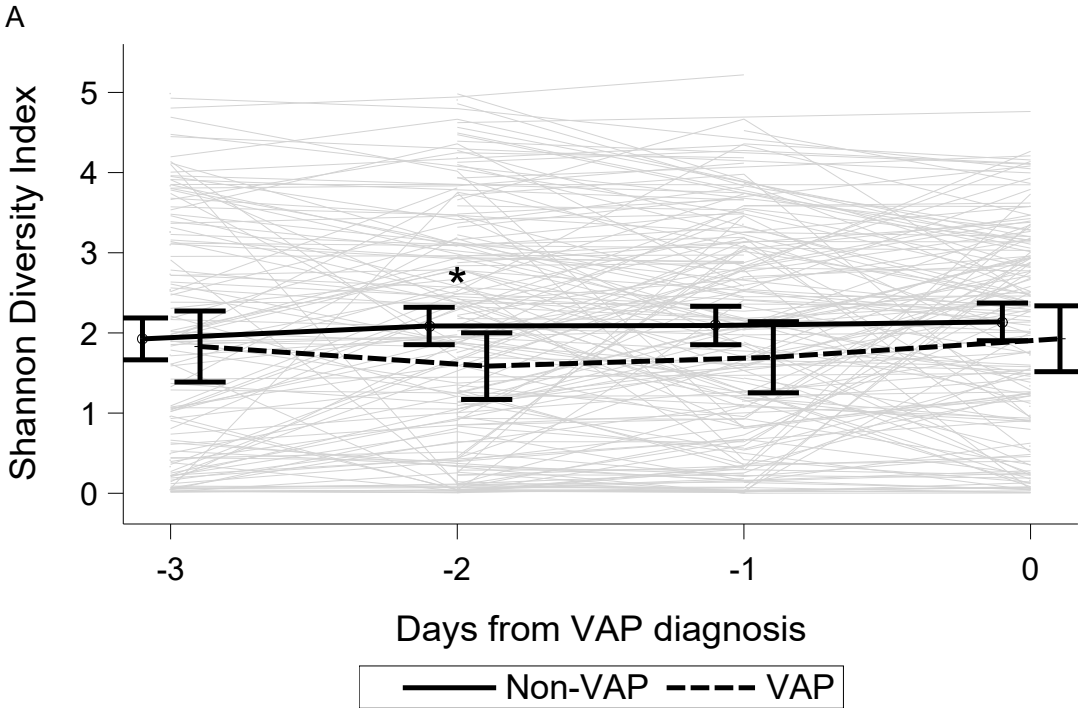
[1]Dominant taxa are defined as those with >50% relative abundance. [2]Fifty-five VAP cases had samples/sequence data available with ± 1 day of diagnosis, 13 had top ranked taxa < 50%.

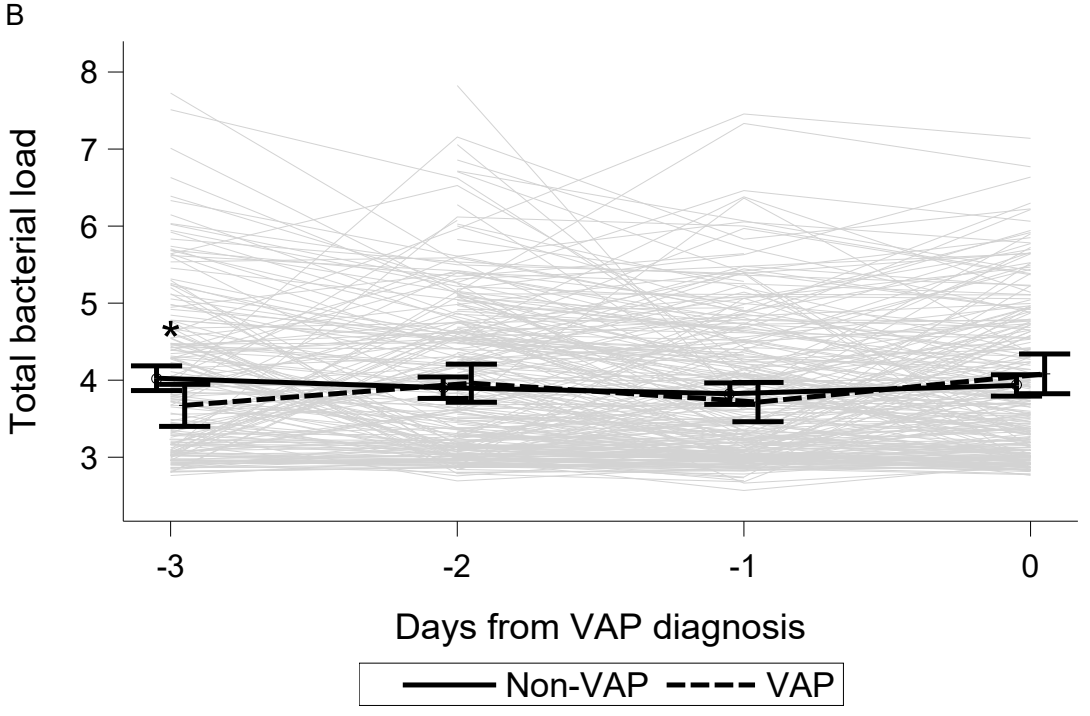
Figure 1. Consort Diagram

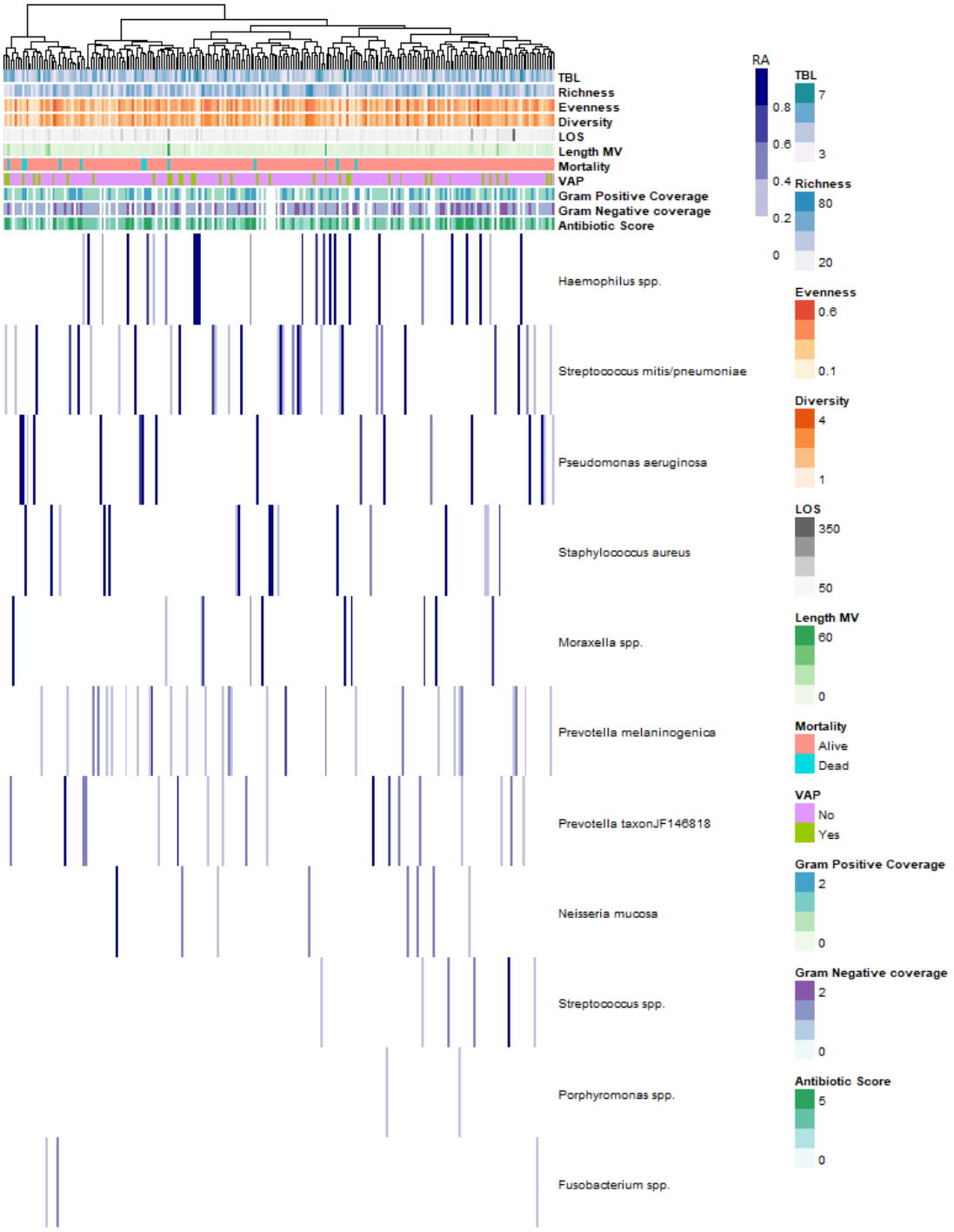



A
MH between consecutively collected samples within an individual
1.0
0.8
0.6
0.4
0.2
0.0
0.0
2.5
5.0
7.5
10.0
12.5
Day of intubation
No VAP
VAP
B
MH between consecutively collected samples within an individual
1.0
0.8
0.6
0.4
0.2
0.0
0.0
2.5
5.0
7.5
10.0
12.5
Day of intubation
No VAP
VAP
C
MH compared to intubation sample within an individual
1.0
0.8
0.6
0.4
0.2
0.0
0.0
2.5
5.0
7.5
10.0
12.5
Day of intubation
No VAP
VAP
D
MH compared to intubation sample within an individual
1.0
0.8
0.6
0.4
0.2
0.0
0.0
2.5
5.0
7.5
10.0
12.5
Day of intubation
No VAP
VAP


A. Parameter estimates from microbial component
Number of antibiotics by day
Cumulative days of antibiotic exposure
Total antibiotic coverage score by day
Cumulative antibiotic coverage score
Time (days)
-0.4
-0.2
0.0
0.2
0.4
Parameter estimate and 95% CrI from microbial factor over time component
Microbial factor components in joint models
Total Bacterial Load
Evenness
Diversity
B. Parameter estimates from VAP component
Age
PRISM
Sequential value of Total Bacterial Load
Sequential value of Diversity
Sequential value of Evenness
0.5
1.0
1.5
2.0
Hazard ratio and 95% CrI from time to development of VAP component
Microbial factor components in joint models
Total Bacterial Load
Evenness
Diversity


A
Shannon Diversity Index
5
4
3
2
1
0
*
-3
-2
-1
0
Days from VAP diagnosis
Non-VAP
VAP


B
Total bacterial load
8
7
6
5
4
3
*
-3
-2
-1
0
Days from VAP diagnosis
Non-VAP
VAP


TBL
Richness
Evenness
Diversity
LOS
Length MV
Mortality
VAP
Gram Positive Coverage
Gram Negative coverage
Antibiotic Score
Haemophilus spp.
Streptococcus mitis/pneumoniae
Pseudomonas aeruginosa
Staphylococcus aureus
Moraxella spp.
Prevotella melaninogenica
Prevotella taxonJF146818
Neisseria mucosa
Streptococcus spp.
Porphyromonas spp.
Fusobacterium spp.
RA
0.8
0.6
0.4
0.2
0
TBL
7
3
Richness
80
20
Evenness
0.6
0.1
Diversity
4
1
LOS
350
50
Length MV
60
0
Mortality
Alive
Dead
VAP
No
Yes
Gram Positive Coverage
2
0
Gram Negative coverage
2
0
Antibiotic Score
5
0

TEMPORAL AIRWAY MICROBIOME CHANGES RELATED TO VENTILATOR ASSOCIATED PNEUMONIA IN CHILDREN

ONLINE DATA SUPPLEMENT

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Figure S5: Matched case-control analyses: Comparison of microbiota communities at time of intubation and VAP diagnosis in matched analysis

Figure S6: Comparison of beta diversity (MH) between VAP and non-VAP subjects relative to Day 0

Figure S7. Matched case-control analyses: Comparison of microbial factor changes from intubation between subjects who do and do not develop VAP

Figure S8. Clustering analyses: Hierarchical clustering of subjects at intubation

Figure S9: Background comparison of average relative abundance of taxa based on the 20 most abundant taxa in controls.

Figure S10: Background comparison of average relative abundance of taxa based on the 20 most abundant taxa in subject samples.

Supplemental Tables

Table S1. Specific Chronic Diagnoses

Table S2: Description of sample collection

Table S3: Comparison of microbial factors between VAP and non-VAP patients at Intubation (entire cohort)

Table S4: Parameter estimates from the joint model of longitudinal outcome assuming a linear trend over time and time to VAP diagnosis that includes the sequential value of the longitudinal outcome

Table S5: Time to event analysis: Hazard ratios from joint model

Table S6: Matched case-control analyses: Description of group matched cohort

Table S7. Matched case-control analyses: Results

MATERIALS and METHODS

VAP Diagnosis

Subjects were screened daily to identify VAP defined by the pediatric 2008 Center for Disease Control (CDC) criteria (1). VAP diagnosis was assigned on the day all criteria were met within the CDC defined window. Chest radiographs obtained for clinical purposes during the study period and the accompanying radiologist interpretations were reviewed by the site investigators to determine whether the radiographic criteria were met. Presence of fever and white blood cell count were obtained from the electronic medical record. Other criteria were obtained by daily surveys of bedside nurses. Physicians (attending or fellow) were also surveyed daily to determine whether they initiated antibiotics for suspected or physician diagnosed VAP. They were asked: “Is the subject receiving antibiotics today for a suspected or diagnosed hospital acquired lower respiratory tract infection?” If the question was answered in the affirmative, they were then asked whether the antibiotics were being administered for “rule out of infection” (suspected VAP) or for a “dedicated treatment course” (physician diagnosed VAP).

Data Collection

Prospectively collected clinical data were recorded in a web-based research database maintained by the Collaborative Pediatric Critical Care Research Network (CPCRRN) data coordinating center at the University of Utah. Clinical data included demographics, primary diagnosis, chronic illnesses/disabilities, baseline functional status score (FSS) (2), Pediatric Risk of Mortality (PRISM) III score (3), antibiotic administration, physician suspected and diagnosed VAP, and data elements utilized to define VAP based on the Center for Disease Control (CDC) pediatric definition (1). Outcome measures included use of extracorporeal membrane oxygenation (ECMO) support, length of MV and other respiratory support, PICU and hospital lengths of stay, PICU discharge FSS, and hospital survival status.

Antibiotics

Antibiotic data were collected for each patient and included the drug name, administration route, and start and stop date. These data were summarized in two ways, 1) the number of drugs given on each day of intubation and 2) total coverage score that was based on whether the antibiotic included coverage against gram-positive bacteria, gram-negative bacteria and/or anaerobic bacteria. Coverage in each area was graded on a 3-point scale, 0 for no activity, 1 for narrow activity, and 2 for broad activity. For patients treated with multiple antibiotics simultaneously, the score for each antibiotic received on a given day was summed. Cumulative scores were summed across days. The calculation of the score for each drug is included as supplementary data (supplementary data file 1). These summary measures resulted in four covariates that were all included the statistical analysis. Two were daily variables (i.e. number of antibiotics administered, and coverage score on each sequential day of MV) and two were cumulative variables (i.e. the cumulative number of antibiotics administered, and the coverage score from intubation to the day of assessment).

Protocol for Specimen Collection

The first tracheal aspirate specimens were collected with routine suctioning of the endotracheal tube (ETT) as soon as possible after intubation, but within 24 hours. Subsequent collections were obtained with the first morning routine suctioning performed by the clinical caregiver (nurse or respiratory therapist). All sites utilized in-line suctioning equipment for routine ETT suctioning. All clinical personnel at each site were trained in the collection procedure utilizing universal training materials and processes. Primary site leads were trained via webinar, and they in turn, trained the remaining local site personnel. To collect the tracheal aspirate, a 40 mL sterile suction trap was attached to the in-line suctioning tubing. Per routine clinical suctioning protocol, the suction catheter was inserted through the ETT to 0.5 cm below the tip of the ETT and

suction applied as catheter is slowly withdrawn. If contents of suction did not enter the suction trap, then 0.5 mL of sterile saline could be instilled to clear the suction catheter of retained secretions into the suction trap. The trap was then aseptically sealed and delivered to the clinical research coordinator for processing and storage. Briefly, under sterile conditions, the tracheal aspirate specimen was pipetted from the suction trap and transferred to a 2 mL sterile microtube labeled with the subject ID and immediately frozen. Specimens were permitted to be placed in a 20°C Freezer for up to 72 hours prior to moving to -80°C freezer, where specimens remained until shipping to the centralized Pediatric Microbiome Laboratory at the Children's Hospital of Colorado.

Microbiome Methods

DNA extraction and Quantitative PCR: DNA extractions were performed using the Qiagen EZ1 Advanced automated extraction platform (Qiagen Inc., Valencia, CA) with the bacterial card and tissue extraction kit per manufacturer's instructions. Total bacterial load was measured using a quantitative real-time PCR assay that has been previously published (4), and evaluated for use in human airway samples (5). DNA extracts were diluted 1:40, and 4 µl of the diluted DNA (dilution factor 10) was used as template in triplicate. A cloned 16S rRNA gene was used to establish copy number based on a standard curve (10^3 to 10^8 copies). Fidelity of the molecular biology preparation was monitored using triplicate blanks in each plate.

16S rRNA sequencing: Bacterial profiles were determined by broad-range amplification and sequence analysis of 16S rRNA genes (5, 6). Amplicons were generated using primers that target approximately 300 base pairs of the V1/V2 (27F/338R) variable region of the 16S rRNA gene. This fragment was selected for numerous reasons. First, the highest level of sequence variation is in the V1-3 region of the 16S rRNA gene. Sequence variation is the most critical issue for taxonomic resolution, which is the main limitation of sequence length constraints from

next generation sequencing platforms. Second, novel diversity was anticipated, and targeting the 5' or 3' ends of the gene would allow primer design to attempt capture of near full-length sequences. Finally, simulation studies performed to target high-throughput sequencing primers during the transition from Sanger sequencing to 454 identified the region ~100 – 350 of the 16S rRNA gene as the best performing fragment compared to full length sequences (7, 8). More recently, ability to assemble the paired-end reads generated by MiSeq was used as a proxy for sequence quality in our workflow when we transitioned from 454 to Illumina. The original 454 validation experiment described in Hamady et al. (9) used samples that had existing V1-5 Sanger data, and the community composition observed by 454 sequencing was very similar. This agreement, presumably driven by the 27F primers sequence common across all sequencing platforms, allowed for comparison between 454 studies and earlier studies based on Sanger sequences. Our internal data confirms that transition from 454 to Illumina platforms did not significantly impact community composition from a range of sample types. PCR was performed using dual indexed primers in triplicate along with a negative PCR control for each sample. Reagent and processing controls were included and evaluated identically to the project samples. The triplicate PCR for each sample was combined, and product was evaluated by agarose gel electrophoresis along with the negative control. Any reaction that exhibited amplification in the negative control was repeated. PCR and DNA extraction controls were performed in parallel using the same PCR design as samples. There was no amplification apparent when assessed by agarose gel electrophoresis. *PCR* products were normalized using agarose gel densitometry by combining approximately equimolar amounts into sequencing pools (PCR and extraction controls were added at maximum volume since there was no band available to assess concentration). The pooled amplicons were gel purified and concentrated using a DNA Clean and Concentrator Kit (Zymo, Irvine, CA). Pooled and concentrated amplicons were quantified using Qubit Fluorometer 2.0 (Invitrogen, Carlsbad, CA). The pool

was diluted to 4nM and denatured with 0.2 N NaOH at room temperature. The denatured DNA was diluted to 20pM and spiked with 10% of the Illumina PhiX control DNA prior to loading the sequencer. Illumina paired-end sequencing was performed on the MiSeq platform using a 500-cycle version 2 reagent kit.

Analysis of Illumina Paired-end Reads. Illumina MiSeq paired-end reads were aligned to human reference genome Hg19 with bowtie2 and matching sequences discarded (10). As previously described, Illumina MiSeq paired-end sequences were sorted by sample via barcodes in the paired reads with a python script (11). Sorted paired end sequence data were deposited in the NCBI Short Read Archive under accession number PRJNA533819 (data generated from methods development [PRJNA436139] and comparison of gastric and tracheal aspirates [PRJNA508231] were also utilized by this study). The sorted paired reads were assembled using phrap (12, 13). Pairs that did not assemble were discarded. Assembled sequence ends were trimmed over a moving window of 5 nucleotides until average quality met or exceeded 20. Trimmed sequences with more than 1 ambiguity or shorter than 200 nt were discarded. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32) (14) using the Schloss (15) Silva reference sequences were removed from subsequent analyses. Assembled sequences were aligned and classified with SINA (1.2.11) (16) using the 418,497 bacterial sequences in Silva 115NR99 (17) as reference configured to yield the Silva taxonomy. Sequences with identical taxonomic assignments were clustered into Operational taxonomic units (OTUs). This process yielded 182,567,651 sequences from 2,202 samples (average size: 82,948 sequences/sample; min: 6,266; max: 494,800). The median Goods coverage score was ≥ 99.4% at the rarefaction point of 6,266 with 100 resamplings. The software package Explicet (v2.9.4, www.explicet.org) (18) was used to calculate rarefied Good's coverage, alpha diversity measures (Shannon diversity and evenness index) and beta diversity (Morisita-Horn index). These indices were chosen a priori based on the relatively equal weighting between evenness

and richness (19). Given the depth of sequencing with current approaches, we chose to use indices that assign less weight to rare taxa.

Species Calls. Rather than applying an arbitrary distance cutoff (e.g., 97% or 99% identity) across all genera, we pre-compute such differentiation criteria for each genus of interest based on the genomic reference sequences provided in Silva and using only the subsequences of these genomic sequences bounded by the primer pair employed in the study. We then apply these individual genus-specific species classification criteria to all sequences falling within the genera of interest. The pre-computation process flags which species can be differentiated within a genus for a given primer pair and which cannot by evaluation of aligned positional differences; the latter sequences remain classified only to the genus level. This is a very conservative process requiring that the MiSeq derived sequences are essentially exact matches to the genomic subsequences in order to achieve a given species level attribution. In some cases the best that can be reported is that, e.g., the species is likely to be in a group of similar species within the genus. Binomial names, when available, or accession numbers are used to identify species groups.

Background assessment. Background was assessed using the 20 most common taxa observed in control data (Figure S9) compared by extraction controls, PCR controls and samples. Likewise, we have provided the top 20 taxa observed in samples (Figure S10) to demonstrate the limited overlap observed between controls and samples.

To limit the impact of background we excluded samples with no evidence of bacterial DNA. Initially, we attempted PCR for all samples irrespective of TBL, but samples with less than approximately 1.5x the TBL background were not successful in amplification as assessed using agarose gel electrophoresis (n=319). The two independent results showing little/no signal to detect were interpreted as quantity not sufficient (QNS) for microbiota determination. Once this

threshold was established, we did not attempt PCR for 505 TA samples with inadequate load to obtain clear amplification.

Statistical Analyses

Children with VAP and without VAP were compared using t-test or Wilcoxon rank sum test as appropriate for continuous variables and chi-square or Fisher's Exact test for categorical variables. To account for differences in sequencing depth, the relative abundance (RA) of each taxon was calculated (number of sequences for specific taxa/ total number of sequences*100).

Change in microbial factors prior to development of VAP: Time to event modeling

The association between changes in microbial factor measures (TBL, Shannon Diversity Index [diversity], and Shannon Evenness [evenness] over time and development of VAP was estimated using a joint longitudinal time to event model (JointModel package in R, R Foundation, Vienna). The longitudinal model included covariates for number of antibiotics administered on each successive day of MV, the cumulative number of antibiotics administered up to the day of assessment in the model, total coverage score on each successive day of MV, and cumulative coverage score up to the day of assessment in the model. Random intercepts and slopes were included for subjects. The survival model included age at intubation and PRISM III score as covariates. Use of a joint model accounts for change and variability in the longitudinal outcome and measurement error. Several approaches to incorporate the longitudinal outcome (TBL, diversity, evenness,) in the time to event model were evaluated and included using the corresponding sequential value, lagged days' values (from 1 or 2 days before the current sequential day), or slope. Models that included splines were also evaluated to assess results that did not include a linear trend constraint.

Matched Case-Control Analysis

In addition to the joint model which used data on all subjects up to extubation or VAP diagnosis, a sub-analysis of subjects with similar characteristics was performed. To make comparisons, we assigned a reference day for each non-VAP subject to correspond with the day of diagnosis for matched VAP subjects. To ensure comparisons at a similar stage of illness, the controls had to remain mechanically ventilated for at least 2 additional days past their assigned reference day. For example, a case who developed VAP on day 6 could only be matched to a control patient who was mechanically ventilated for a minimum of 8 days. Specifically, the assignment was made after sorting the controls, in descending order, by number of days intubated. The distribution of the identified reference day in the non-VAP group was targeted to match the distribution of the day of VAP diagnosis in the VAP group for our comparisons to be as useful as possible. For presentation purposes, results for the analyses evaluating microbial risk factors for VAP are presented in relation to the day of diagnosis (in VAP subjects) or the reference day (in non-VAP subjects), which was termed “Day 0”. We group matched non-VAP subjects to VAP subjects to also have similar distribution of the following characteristics: age at intubation, PRISM III score (3), and infectious admitting diagnosis. Twenty-eight non-VAP subjects were removed because they were ventilated for less than 4 days. An additional 80 non-VAP subjects were removed to make the distribution of the infectious admitting diagnosis similar between cases and controls. Priority for inclusion in the matched analysis was given to controls with longer ventilation times and higher PRISM III scores because these were less frequent occurrences in the cohort. The proportion of cases that were diagnosed for each day was multiplied by the total number of controls to obtain the number of controls that needed to be assigned to match the distribution of case diagnosis days. The resulting group matched cohort consisted of 280 controls and 66 cases.

Mixed effects models were used to evaluate the changes in microbial factors over time. These models were adjusted for antibiotic exposure and included a random subject intercept.

Least squared means for the microbial factors were compared between non-VAP and VAP groups up the three days prior to Day 0. Because sequence data were missing on Day 0 for some VAP patients (n =18 [27%]), a sensitivity analysis was performed that included subjects with at least 3 samples, with the requirement that VAP subjects had one of the samples available on the day of or the day prior to VAP diagnosis. Morisita-Horn (MH, Beta-diversity) for pairwise samples within each subject were calculated and compared across the VAP groups using a mixed model with B-splines to estimate trends over time. Time was included as a continuous variable and modeled using cubic B-splines with internal knots placed at the quintiles and boundary knots placed at the extremes. The model included a random individual intercept and slope, which were assumed to have a multivariate normal distribution and heterogeneous within individual variances that differed by VAP diagnosis. Cholesky decomposition was used to constrain the covariance matrix of the random effects to be positive-definite. Methods for modeling ecological measures over time are described in further detail in Wagner et al (20)

Clustering analysis: Identifying VAP phenotypes

To understand the relationship between clinical and microbial factors at intubation, an unsupervised random forest clustering algorithm was performed. Factors included in the random forest were baseline characteristics (age at intubation, PRISM III score, primary diagnosis, infectious diagnosis, and site of enrollment) and microbial factors (total bacterial load, presence of a pathogen (supplementary data file 2), and dominant taxa in sample). The resulting proximity matrix was transformed into a distance (21) and Ward agglomeration clustering using complete linkage and was applied to generate a hierarchical clustering of subjects.

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Figure S1. Subject specific reports. Individual summary reports are generated for each subject in the analysis cohort and are accessible at https://wkayla.shinyapps.io/subject_specific/. A report for a single subject is included here as an example. The clinical data for the subject are printed at the top of the report, followed by graphs displaying the bacterial composition, culture data, total bacterial load, antibiotics administered, and ecological measures over time during intubation for all available samples.

```
[1] "Subject: 1031376, Primary Admitting Diagnosis: Trauma, Age at Intubation
: 4.0246, VAP Case?: No, VAP Day of Diagnosis: 4, PRISM Score: 7 Matched?: NA
"
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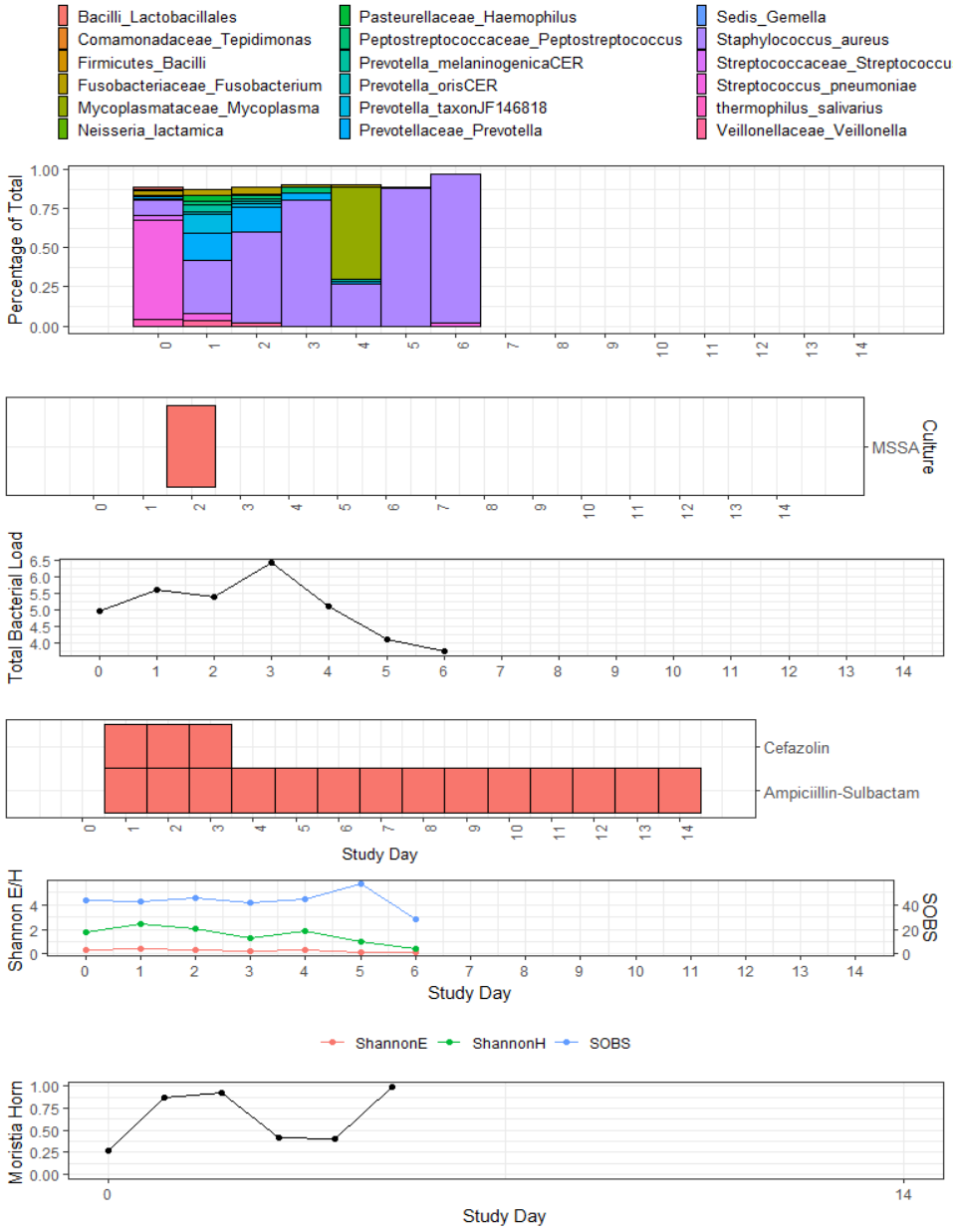


Figure S2. Distribution of VAP diagnosis day. The median day of VAP diagnosis for the 66 subjects diagnosed with VAP was 4.5 days (Range 2 - 14 days).

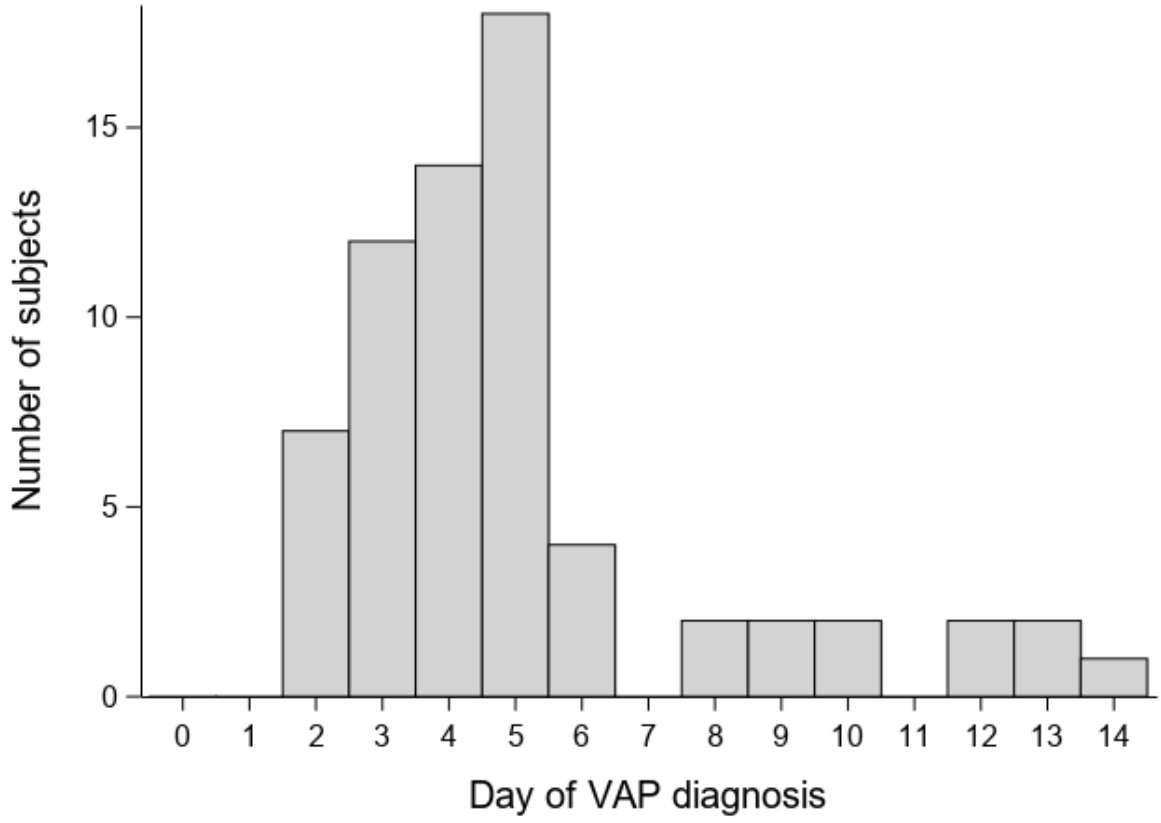


Figure S3. Sample Collection Distribution. Upper panel: Histogram of the proportion of daily samples collected during the first intubation period for each of the 366 subjects in the supervised analytic cohort. 2,330 samples (85%) were collected out of a possible 2,739 ventilator days. The figure shows the distribution of the proportion of daily samples collected during the first intubation period, the y-axis corresponds to the number of subjects and the x-axis represents the proportion of possible samples collected. There were 225 (61%) subjects with at least 75% of their total daily samples collected. Lower panel: The distribution of the proportion of samples with sequencing data, corresponding to 1,693 samples (73% of collected samples). The y-axis corresponds to the number of subjects and the x-axis represents the proportion of possible samples that had sequencing data.

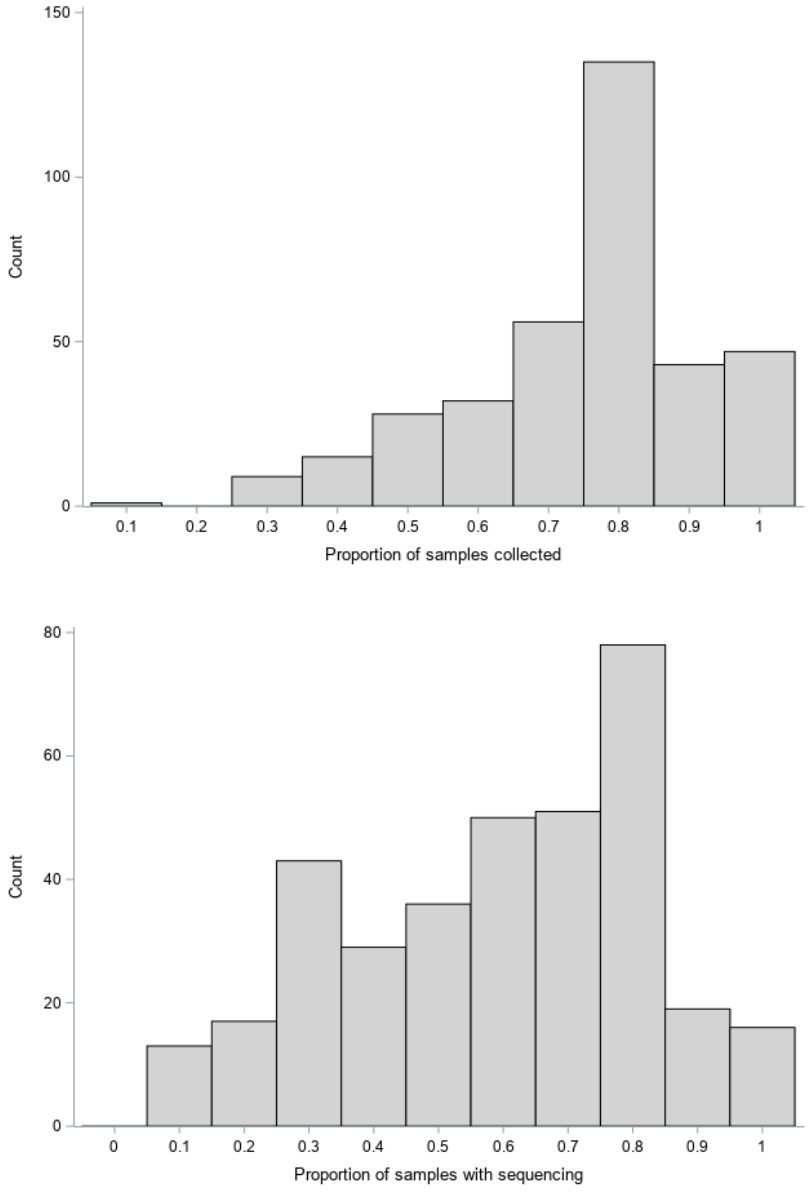


Figure S4: Distribution of total bacterial load for tracheal aspirate samples. The distribution of total bacterial load across samples are displayed in comparison to the values from 348 total negative controls using PCR water as template run in triplicate on each plate (N = 116) of qPCR assay (blue). Samples with sequencing available are shown in red, and samples that failed to amplify sequence are shown in green. Lower panel shows the distribution of TBL does not vary depending on the age of the subject.

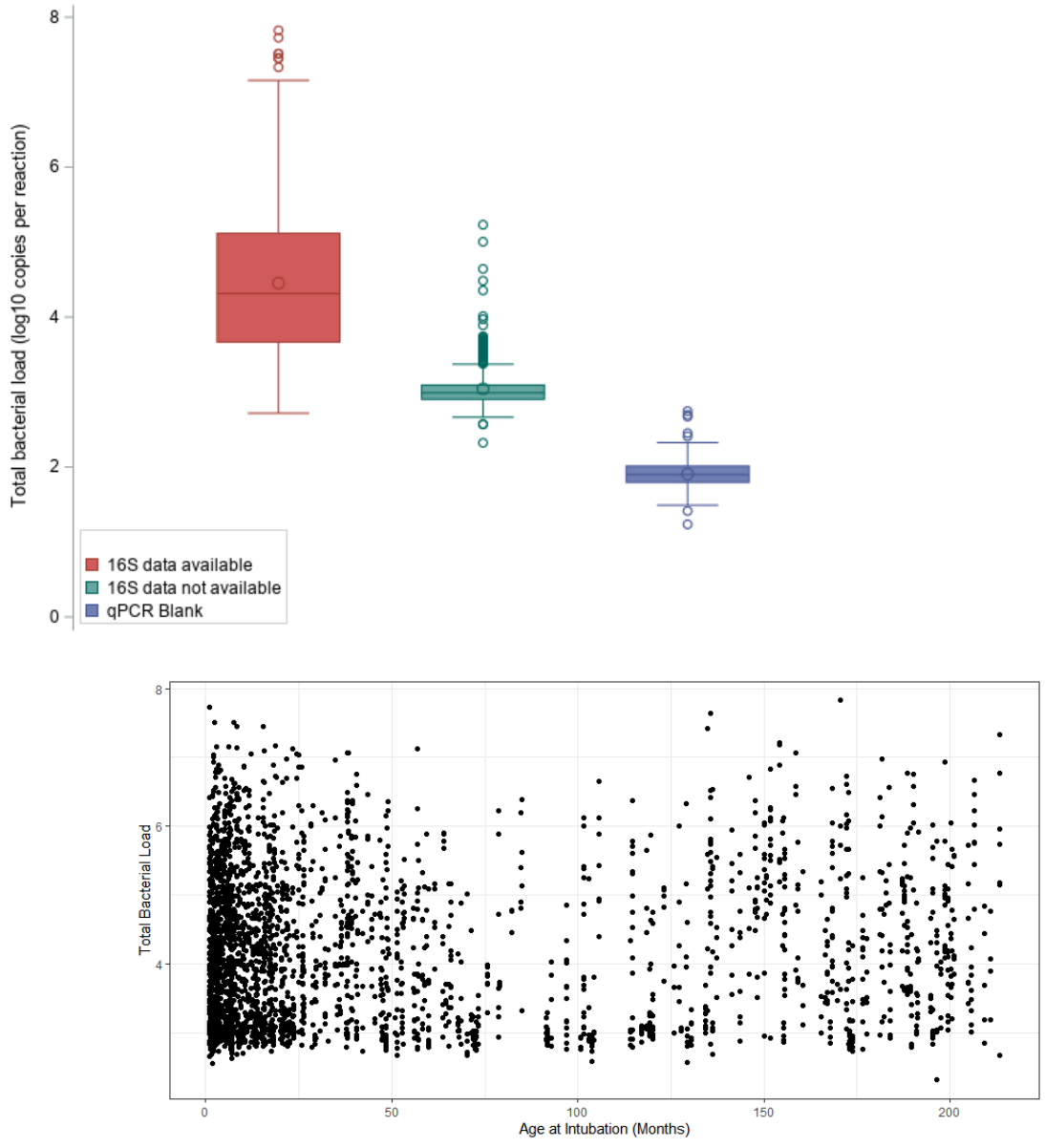


Figure S5: Matched case-control analyses: Comparison of microbiota communities at time of intubation and Day 0. Heatmap comparing microbiota communities at time of intubation (left) and Day 0 (VAP group: day of VAP diagnosis, non-VAP group: corresponding MV day; right) in matched analysis. Samples from non-VAP cases are displayed in columns to the left of the vertical lines (annotated with the blue bar below) and VAP cases are displayed to the right (annotated with the red bar below). Prominent taxa (at least 80% relative abundance (RA) in one sample) for each sample are displayed in rows. The darker the color of the bar, the higher the RA. Microbial taxa appear similar between VAP and non-VAP samples at intubation. Dominant taxa detected at VAP diagnosis are also prominent in non-VAP samples. There does not appear to be a single taxon that contributes to VAP. Often, these taxa are similarly represented in the non-VAP samples as well, suggesting that they may not be pathogenic in some subjects.

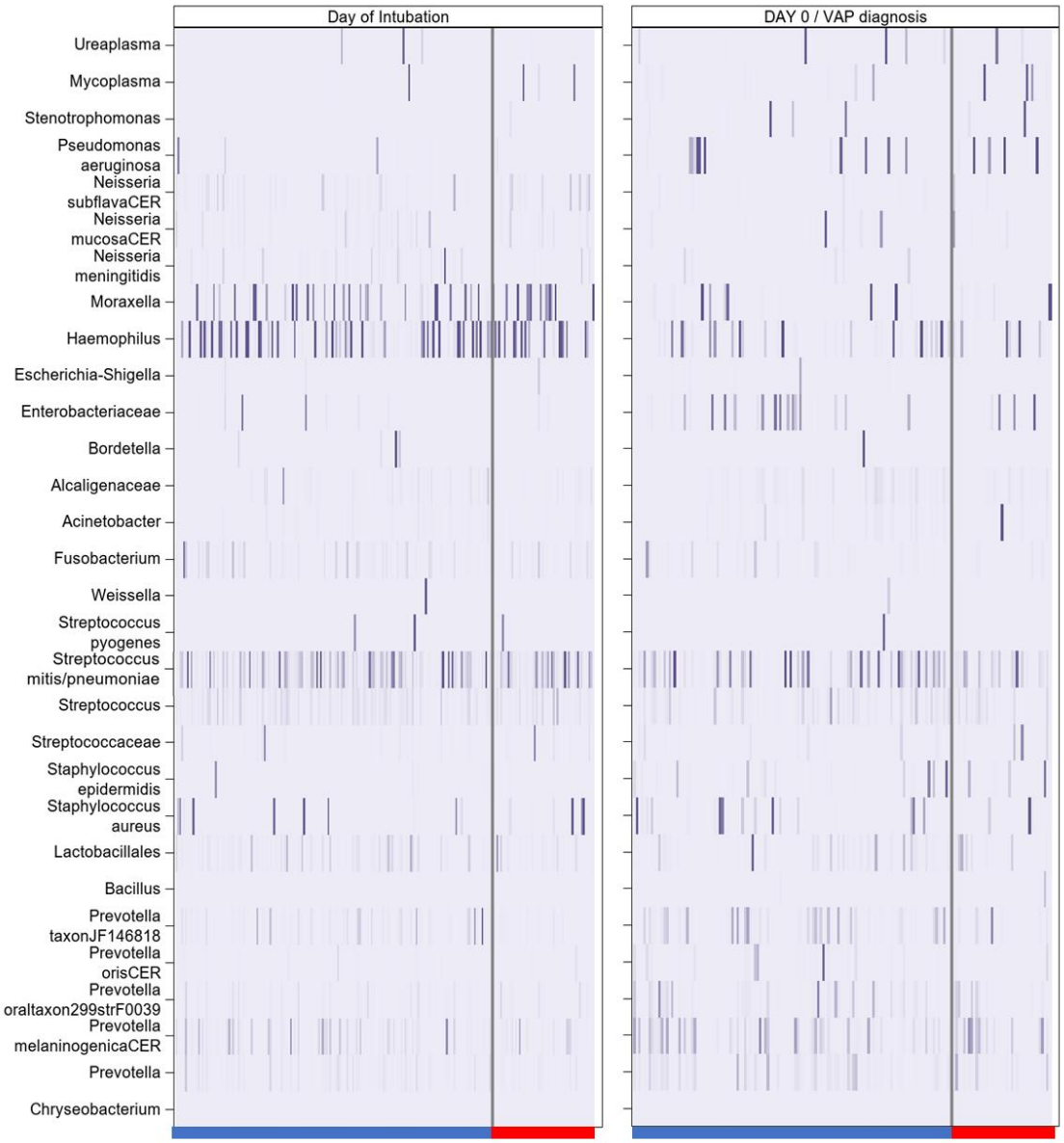
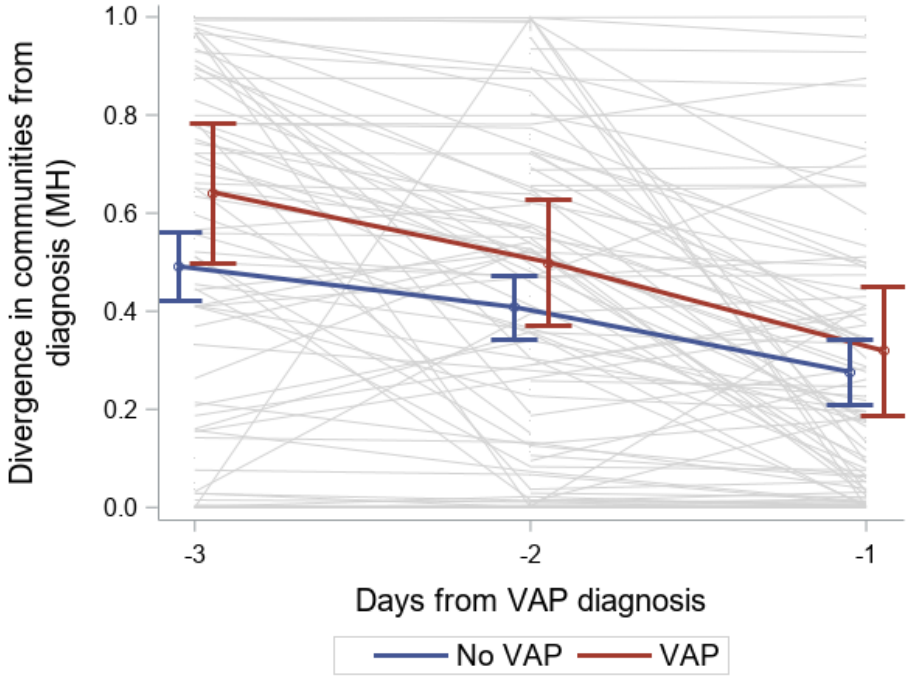
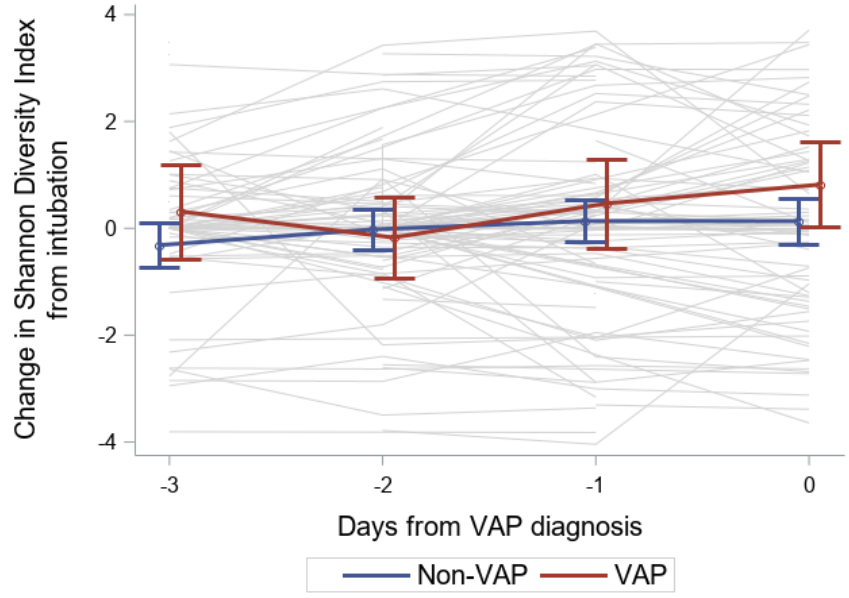


Figure S6: Comparison of beta diversity (MH) between VAP and non-VAP subjects relative to Day 0. Each individual (grey lines) and the average trends for the VAP groups (colored lines) denote the MH between each time point sample (Days -1 to -3) and the Day 0 sample. Day 0 denotes day of diagnosis in VAP cases (n = 66) and the reference day of mechanical ventilation in controls (n=227). Bars represent 95% confidence intervals. The table below presents the statistical comparison between groups at each time point. The degree of divergence relative to Day 0 samples was greatest 3 days prior to Day 0 and was more divergent in the VAP subjects, but was not statistically higher in comparison to non-VAP subjects.



Day relative to Day 0	Estimate (difference between VAP and No VAP)	Standard Error	Pr > |t|	Lower	Upper
-3	-0.1513	0.08043	0.0619	-0.3102	0.007624
-2	-0.09139	0.07320	0.2138	-0.2360	0.05324
-1	-0.04272	0.07401	0.5647	-0.1890	0.1035

Figure S7: Matched case-control analyses: Comparison of microbial factor changes from intubation between subjects who do and do not develop VAP. Mean trajectories of change from intubation in microbial factors do not differ dramatically between subjects who did and did not develop VAP in the matched cohort. Bars represent 95% confidence intervals. Day 0 denotes day of diagnosis in VAP cases (n = 66) and the reference day of mechanical ventilation in controls (n=227). Panels represent comparisons in Shannon Diversity and Total Bacterial Load (Shannon Evenness not shown) for changes from day of intubation for Days -3 to 0.



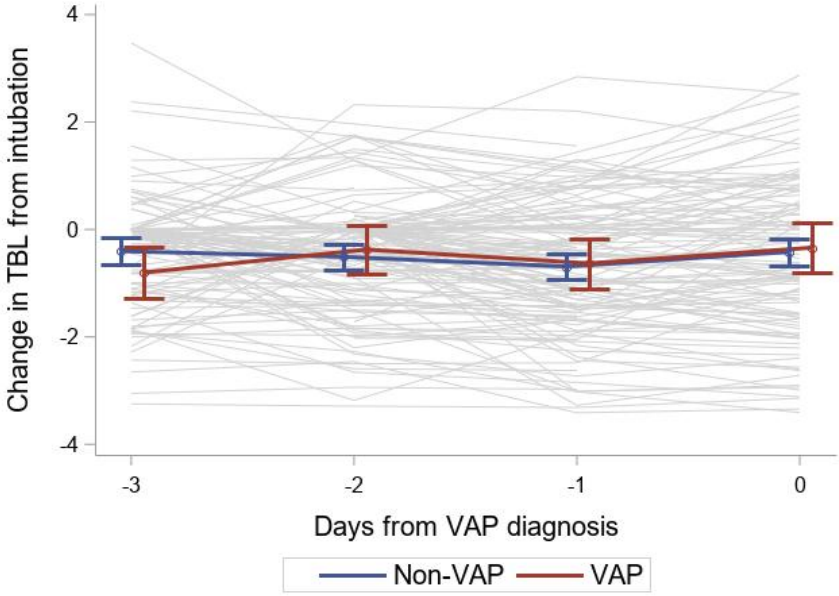


Figure S8: Clustering analyses: Hierarchical clustering of subjects at intubation. We used an unsupervised machine learning algorithm (random forest) to evaluate whether subjects with similar characteristics at the time of intubation cluster together. Subjects with similar characteristics are closest together at the lowest branch of the tree. The features included in the random forest algorithm to define cluster membership are listed on the right side of the dendrogram. Notably, in this heterogeneous cohort, there are no apparent microbial differences across site of enrollment. Abbreviations: Site: site of enrollment; PRISM: Pediatric Risk and Mortality III; TBL: total bacterial load; Infectious Admit: infectious diagnosis at PICU admission; Pathogen: Pathogenic organism present as defined in File 2; Diversity, Shannon diveristy; RA, Relative abundance.

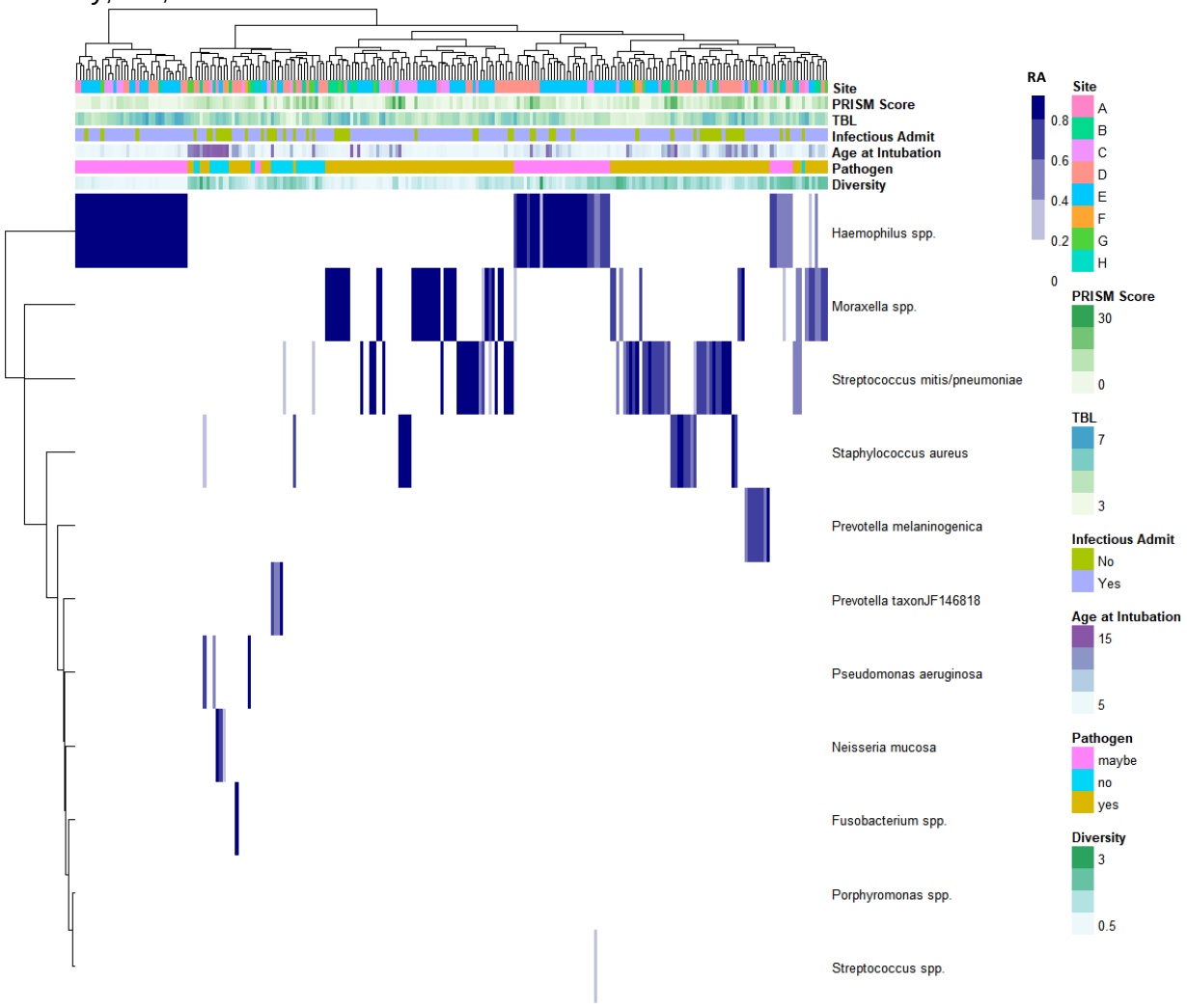


Figure S9: Background comparison of average relative abundance of taxa based on the 20 most abundant taxa observed in controls. Bar plots show PCR and extraction controls for each taxon identified along with the distribution of RA from clinical samples. The majority of taxa observed are consistent with prior reports for background. There was a single predominant taxon associated with the extraction controls (*Alcaligenaceae*). The other taxa identified in the extraction controls appear to originate from the PCR mix, and in two cases (*S. pneumoniae*|*mitis, Haemophilus*) human source (most likely the concurrently tested samples or potentially research staff). There is limited overlap between controls and clinical samples when comparing RA of these taxa. Origin of *Stenotrophomonas* is challenging to interpret. This taxon represents known contaminant sequences and pathogens associated with chronic lung infections. The range observed in samples is higher than any control suggesting both sources contributed to detection of this taxon. *Pseudomonas stutzeri* is represented by accession number (CP007509).

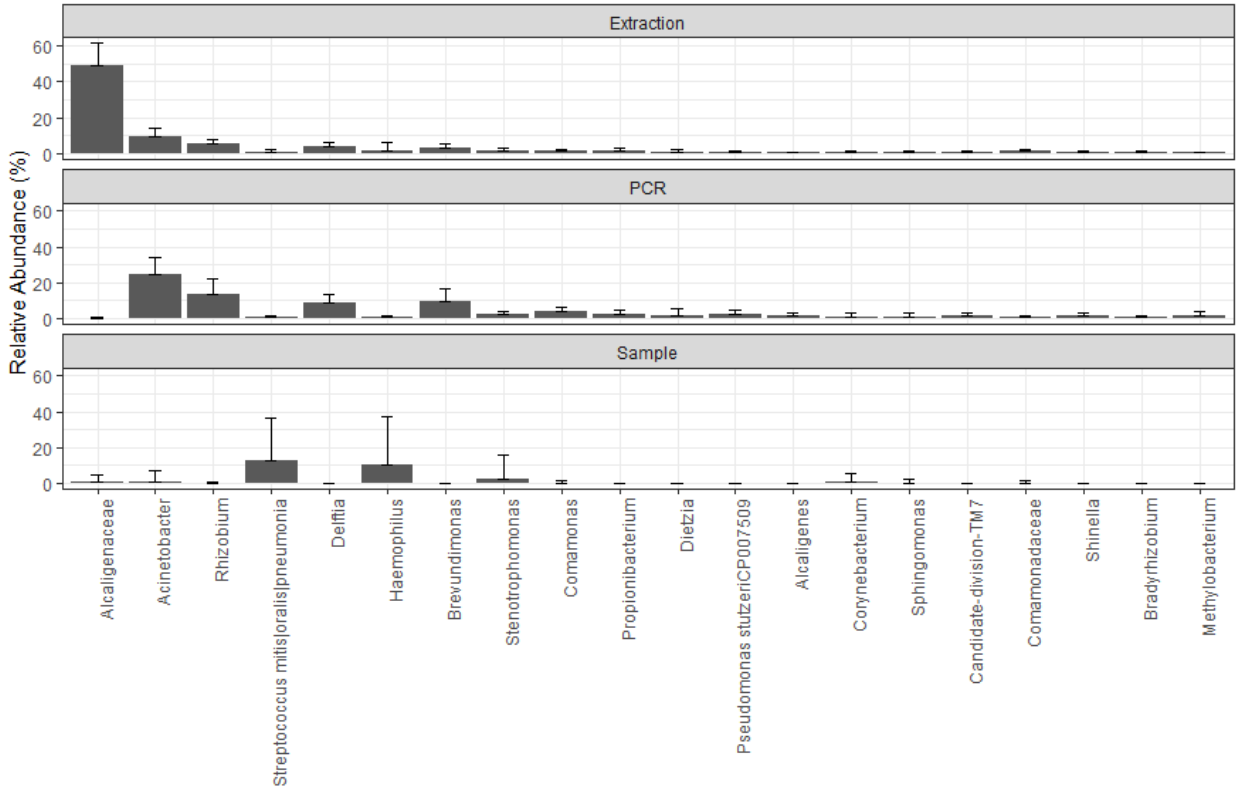


Figure S10: Background comparison of average relative abundance of taxa based on the 20 most abundant taxa in subject samples. Bar plots show PCR and extraction controls for each taxon identified along with the distribution of RA from clinical samples. There is limited overlap between controls and clinical samples when comparing RA of these taxa. The predominant taxa observed in the clinical samples are also present at low levels in controls. None of the controls demonstrated amplification by agarose gel electrophoresis, and overall sequence counts obtained from these samples were much lower than those from TA samples that demonstrated amplification. Exact source tracing is challenging and was most likely due to very low levels of transfer during PCR. However, we can't rule out low background during the extraction step either. Sequences that did not match to any available species sequences were reported at the Genus level (e.g. *Streptococcus*).

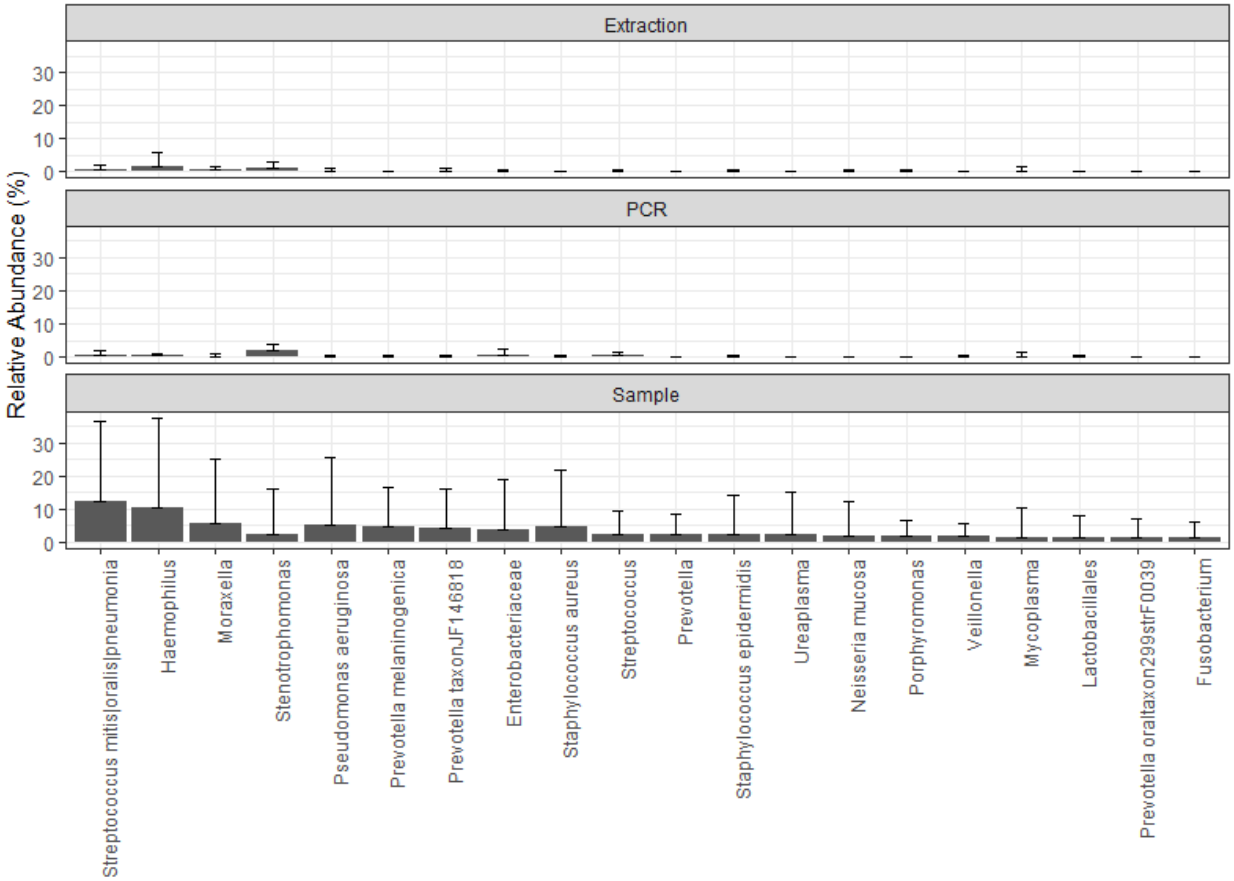


Table S1: Specific Comorbid Diagnoses

Comorbidities	All Subjects (n=454)	Supervised Analytic Cohort (n=366)		Subjects with Physician diagnosed or suspected VAP not meeting CDC criteria (n=88)
		No VAP (n=300)	VAP (n=66)	
Airway/lung disease	60 (27%)	40 (13%)	10 (29%)	10 (11%)
Cardiovascular disease	37 (17%)	19 (6%)	7 (20%)	11 (12%)
Neurological disease	32 (15%)	21 (7%)	3 (9%)	8 (9%)
Congenital anomaly or chromosomal defect	23 (10%)	13 (4%)	9 (26%)	1 (1%)
Cancer	17 (8%)	12 (4%)	3 (9%)	2 (2%)
Immunological disorders	9 (4%)	6 (2%)	0 (0%)	3 (3%)
Liver disease	8 (4%)	7 (2%)	0 (0%)	1 (1%)
Metabolic disease	7 (3%)	3 (1%)	1 (3%)	3 (3%)
Renal disease	3 (1%)	2 (1%)	1 (3%)	0 (0%)
Hematological disease	3 (1%)	2 (1%)	0 (0%)	1 (1%)
Gastrointestinal disease	2 (1%)	2 (1%)	0 (0%)	0 (0%)
Psychiatric disorder	2 (1%)	2 (1%)	0 (0%)	0 (0%)
Transplant	1 (0.5%)	1 (0.3%)	0 (0%)	0 (0%)
Other	15 (7%)	13 (4%)	1 (3%)	1 (1%)

Table S2: Description of sample collection, number of samples sequenced by day of collection

A. Full Cohort (n=454)			
Days after intubation	**Number of subjects intubated**	**Number of subjects with a sample collected**	**Number of subjects with a sequenced sample**
1	454	454 (100%)	406 (89%)
2	454	399 (88%)	273 (68%)
3	425	369 (87%)	260 (70%)
4	347	302 (87%)	216 (72%)
5	280	235 (84%)	169 (72%)
6	214	197 (92%)	146 (74%)
7	151	126 (83%)	95 (75%)
8	113	94 (83%)	66 (70%)
9	87	75 (86%)	51 (68%)
10	72	62 (86%)	43 (69%)
11	61	49 (80%)	36 (73%)
12	57	47 (82%)	37 (79%)
13	51	30 (59%)	23 (77%)
14	32	11 (34%)	9 (82%)
B. Supervised Analytic Cohort (n=366)			
Days after intubation	**Number of subjects intubated**	**Number of subjects with a sample collected**	**Number of subjects with a sequenced sample**
1	366	366 (100%)	330 (90%)
2	366	321 (88%)	218 (68%)
3	339	297 (88%)	206 (69%)
4	270	239 (89%)	169 (71%)
5	212	183 (86%)	133 (73%)
6	156	149 (96%)	108 (72%)
7	103	84 (82%)	60 (71%)
8	75	62 (83%)	40 (65%)
9	59	51 (86%)	32 (63%)
10	49	40 (82%)	28 (70%)
11	43	33 (77%)	24 (73%)
12	40	32 (80%)	25 (78%)
13	36	21 (58%)	16 (76%)
14	22	7 (32%)	5 (71%)

Table S3: Comparison of microbial factors between VAP and non-VAP subjects at intubation. Total Bacterial Load (TBL), Shannon diversity, and Shannon evenness were tested using a two-sample t-test, means and standard deviations are reported. Taxa were tested using a Wilcoxon Rank Sum test, medians, and 75th percentile and 100% percentile (maximum relative abundance) of the highest-ranking taxa are reported. Diversity and evenness at intubation are lower in VAP subjects as compared to non-VAP subjects. No differences in dominant taxa were found between the two groups.

	All Subjects (n=454)	Supervised Analytic Cohort (n=366)			Subjects with Physician diagnosed or suspected VAP not meeting CDC criteria (n=88)
Microbial factor Means (std) or median (75%, 100%)		**No VAP (n=300)**	**VAP (n = 66)**	**P-value**	
Shannon Diversity	1.74 (1.18)	1.83 (1.19)	1.35 (1.16)	0.005	1.7 (1.08)
Shannon Evenness	0.32 (0.2)	0.34 (0.2)	0.25 (0.2)	0.004	0.32 (0.18)
Total Bacterial Load	4.65 (0.99)	4.58 (0.97)	4.47 (0.95)	0.394	5.04 (1.02)
Moraxella	0.0 (0.3, 99.6)	0.0 (0.2, 99.6)	0.0 (2.7, 98.9)	0.754	0.0 (0.2, 98.3)
Haemophilus	0.7 (6.7, 100)	0.7 (5.3, 100)	0.5 (33.7, 100)	0.356	0.9 (15.1, 99.9)
Streptococcus mitis/pneumoniae	3.7 (18.3, 99.9)	3.4 (19.2, 99.9)	3.2 (15.2, 99.7)	0.879	5.6 (17.8, 98.9)
Staphylococcus aureus	0.0 (0.0, 99.7)	0.0 (0.0, 99.6)	0.0 (0.0, 99.7)	0.632	0.0 (0.0, 92.2)
Prevotella melaninogenica	0.2 (2.5, 93.4)	0.2 (3.0, 93.4)	0.1 (0.9, 42.0)	0.17	0.5 (3.1, 74.2)
Ureaplasma	0.0 (0.0, 84.3)	0.0 (0.0, 84.3)	0.0 (0.0, 0.4)	0.238	0.0 (0.0, 0.8)
Mycoplasma	0.0 (0.0, 99.5)	0.0 (0.0, 99.5)	0.0 (0.0, 97.6)	0.759	0.0 (0.0, 18.5)
Pseudomonas aeruginosa	0.0 (0.0, 99.5)	0.0 (0.0, 63.7)	0.0 (0.0, 99.5)	0.928	0.0 (0.0, 0.4)
Neisseria subflava	0.0 (0.6, 97.4)	0.0 (0.6, 97.4)	0.0 (0.1, 30.7)	0.073	0.0 (0.9, 62.0)
Shigella	0.0 (0.0, 86.8)	0.0 (0.0, 9.2)	0.0 (0.0, 20.8)	0.123	0.0 (0.0, 86.8)
Enterobacteriaceae	0.0 (0.0, 0.93)	0.0 (0.0, 93.0)	0.0 (0.0, 4.8)	0.342	0.0 (0.0, 12.5)
Bordetella	0.0 (0.0, 99.8)	0.0 (0.0, 99.8)	0.0 (0.0, 0.0)	0.366	0.0 (0.0, 0.0)
Alcaligenaceae	0.0 (0.3, 60.5)	0.0 (0.4, 60.5)	0.0 (0.3, 5.9)	0.404	0.0 (0.1, 9.2)
Acinetobacter	0.0 (0.1, 4.3)	0.0 (0.2, 4.3)	0.0 (0.2, 1.2)	0.899	0.0 (0.0, 4.3)
Fusobacterium	0.1 (1.5, 82.6)	0.1 (1.6, 82.6)	0.0 (0.5, 20)	0.078	0.2 (1.9, 12.6)
Weissella	0.0 (0.0, 98.4)	0.0 (0.0, 98.4)	0.0 (0.0, 0.2)	0.398	0.0 (0.0, 0.0)
Streptococcus pyogenes	0.0 (0.0, 99.6)	0.0 (0.0, 97.1)	0.0 (0.0, 64.8)	0.637	0.0 (0.0, 99.6)
Streptococcus	0.8 (3.8, 29.7)	1.0 (4.1, 23.7)	0.2 (1.5, 29.7)	0.02	0.9 (4.2, 28.3)
Staphylococcus epidermidis	0.0 (0.0, 61.4)	0.0 (0.0, 57.1)	0.0 (0.0, 0.5)	0.896	0.0 (0.0, 61.4)
Lactobacillales	0.1 (1.4, 92.1)	0.1 (1.6, 92.1)	0.0 (0.6, 64)	0.03	0.2 (0.8, 10.5)
Bacillus	0.0 (0.0, 0.3)	0.0 (0.0, 0.3)	0.0 (0.0, 0.1)	0.343	0.0 (0.0, 0.1)

Prevotella oraltaxon299strF0039	0.0 (0.4, 66.4)	0.0 (0.4, 23.2)	0.0 (0.1, 66.4)	0.052	0.1 (0.7, 13.2)
Prevotella	0.2 (1.4, 38.4)	0.3 (1.4, 33.6)	0.1 (0.4, 4.8)	0.012	0.4 (2.2, 38.4)
Prevotella taxonJF146818	0.1 (0.9, 83.1)	0.1 (1.0, 83.1)	0.0 (0.2, 3.3)	0.019	0.1 (1.2, 12.8)
Prevotella oris	0.0 (0.0, 40.7)	0.0 (0.0, 40.7)	0.0 (0.0, 6.5)	0.184	0.0 (0.0, 37.7)

Table S4: Parameter estimates from the joint model of longitudinal outcome assuming a linear trend over time and time to VAP diagnosis that includes the sequential value of the longitudinal outcome.

	Variable	Total Bacterial Load		Shannon Diversity		Shannon Evenness	
		Est (Interval)	**p-value**	**Est (Interval)**	**p-value**	**Est (Interval)**	**p-value**
	Intercept	4.31 (4.20, 4.43)	**<0.01**	2.25 (2.08, 2.42)	**<0.01**	0.38 (0.31, 0.45)	**<0.01**
Predictors of Longitudinal Outcomes (Microbial Factors)	Time (days)	0.12 (0.04, 0.19)	**<0.01**	-0.07 (-0.15, 0.02)	0.12	0.01 (-0.30, 0.30)	0.99
	Number of antibiotics by day	0.01 (-0.08, 0.11)	0.83	-0.25 (-0.41, -0.08)	**<0.01**	-0.05 (-0.08, -0.02)	**<0.01**
	Cumulative days of antibiotic exposure	-0.04 (-0.10, 0.01)	0.08	0.12 (0.02, 0.20)	**0.01**	0.04 (0.02, 0.06)	**<0.01**
	Cumulative antibiotic coverage score	-0.02 (-0.04, 0.01)	0.10	-0.04 (-0.07, -0.01)	**0.02**	-0.01 (-0.02, -0.01)	**<0.01**
	Total antibiotic coverage score by day	-0.01 (-0.04, -0.03)	0.83	0.10 (0.04, 0.16)	**<0.01**	0.02 (0.01, 0.03)	**<0.01**
Predictors of VAP	Age	-0.03 (-0.10, 0.04)	0.44	-0.07 (-0.14, -0.01)	**0.02**	-0.07 (-0.14, -0.01)	**0.03**
	PRISM III	0.02 (-0.02, 0.06)	0.35	0.01 (-0.03, 0.04)	0.69	0.01 (-0.03, 0.05)	0.62
	Sequential value for longitudinal outcomes	-0.95 (-1.48, -0.44)	**<0.01**	0.02 (-0.26, 0.27)	0.88	-0.58 (-1.38, 0.67)	0.19

Table S5: Time to event analyses: Hazard ratios. Hazard ratios (95% credible intervals) for association between longitudinal outcomes (TBL, Shannon Diversity and Shannon Evenness) and time to VAP diagnosis from the joint models are presented. Different approaches to incorporate the microbial factors into the time to event model were evaluated to determine if they were informative to VAP diagnosis, including lagged values (evaluating whether microbial factors 1 or 2 days prior to the current sequential value day), slope of the linear trend in sequential values, and splines (a curve function to fit non-linear trends). The hazard ratios are displayed for the different approaches to include the longitudinal variable in the time to VAP model. Inclusion of the sequential or lagged TBL values was associated with time to VAP suggesting that lower TBL is associated with development of VAP. Bolded values indicate the 95% credible interval for the hazard ratio excludes 1.

Model	**TBL**	**Shannon Diversity**	**Shannon Evenness**
sequential value-linear trend	**0.39 (0.23, 0.64)**	1.02 (0.77, 1.31)	0.56 (0.25, 1.95)
Lag 1 day – linear trend	**0.55 (0.34, 0.85)**	0.92 (0.68, 1.20)	0.40 (0.14, 1.16)
Lag 2 day – linear trend	**0.65 (0.42, 0.98)**	0.86 (0.65, 1.10)	0.31 (0.06, 1.20)
Slope – linear trend	**0.52 (0.30, 0.88)**	**1.91 (1.31, 3.03)**	**31.1 (6.7, >100)**
sequential value - spline	0.71 (0.39, 1.14)	1.04 (0.80, 1.34)	0.72 (0.24, 7.00)
Time dependent slopes - spline	1.38 (0.67, 2.51)	**1.90 (1.27, 3.19)**	**44.0 (9.85, >100)**

Table S6: Description of group matched cohort. Subjects that did not develop VAP either by the CDC diagnostic criteria or by physician diagnosis or suspicion of VAP were compared to those who developed VAP by CDC criteria based on PRISM III scores, age at intubation, infection status at PICU admission, and time on mechanical ventilation. Non-VAP subjects who did not exhibit similarities with VAP cases based on these characteristics were not included in the analysis (n =73).

N (%) or median (range)	**No VAP (n = 227)**	**VAP (n = 66)**
Age at intubation	1.3 (0.1 – 17.8)	1.2 (0.1 – 16.6)
Noninfectious admitting diagnosis	21 (9.3%)	6 (9.1%)
PRISM III score	5.0 (0 – 31)	5.5 (0 – 28)
Day of VAP diagnosis or matched sample day for no VAP	4.0 (2 – 14)	4.5 (2 – 14)

PRISM – Pediatric Risk of Mortality; VAP – ventilator-associated pneumonia

Table S7. Matched case-control analyses: Results. Comparisons of microbial factors between those that did and did not develop VAP up to 3 days prior to development of VAP

variable	Day from VAP diagnosis	Control (N = 227)			VAP (n = 66)			p-value[1]
		Mean[2]	95% CI		Mean[2]	95% CI		
Shannon H Diversity Index	-3	1.93	1.66	2.19	1.83	1.39	2.27	0.712
Shannon H Diversity Index	-2	2.09	1.85	2.32	1.59	1.17	2.00	0.040
Shannon H Diversity Index	-1	2.09	1.85	2.33	1.70	1.25	2.14	0.124
Shannon H Diversity Index	0	2.14	1.90	2.37	1.93	1.52	2.34	0.377
Shannon Evenness	-3	0.34	0.29	0.38	0.32	0.25	0.39	0.635
Shannon Evenness	-2	0.36	0.32	0.40	0.28	0.21	0.34	0.040
Shannon Evenness	-1	0.36	0.32	0.40	0.29	0.22	0.36	0.099
Shannon Evenness	0	0.38	0.34	0.41	0.35	0.28	0.41	0.430
Total Bacterial Load	-3	4.03	3.87	4.19	3.67	3.40	3.95	0.027
Total Bacterial Load	-2	3.90	3.76	4.04	3.96	3.71	4.21	0.687
Total Bacterial Load	-1	3.82	3.69	3.96	3.72	3.46	3.97	0.461
Total Bacterial Load	0	3.93	3.79	4.07	4.08	3.82	4.34	0.309
Change from intubation								
Change in Shannon H Diversity	-3	-0.32	-0.73	0.10	0.32	-0.57	1.20	0.196
Change in Shannon H Diversity	-2	-0.02	-0.40	0.35	-0.17	-0.93	0.59	0.725
Change in Shannon H Diversity	-1	0.14	-0.26	0.53	0.47	-0.37	1.31	0.484
Change in Shannon H Diversity	0	0.13	-0.28	0.55	0.82	0.02	1.62	0.131
Change Shannon Evenness	-3	-0.05	-0.12	0.02	0.04	-0.10	0.19	0.257
Change Shannon Evenness	-2	-0.00	-0.06	0.06	-0.02	-0.15	0.10	0.793
Change Shannon Evenness	-1	0.02	-0.04	0.09	0.07	-0.06	0.21	0.500
Change Shannon Evenness	0	0.04	-0.03	0.11	0.16	0.03	0.29	0.114
Change in Total Bacterial Load	-3	-0.40	-0.66	-0.15	-0.80	-1.29	-0.32	0.149
Change in Total Bacterial Load	-2	-0.51	-0.74	-0.27	-0.37	-0.82	0.07	0.597
Change in Total Bacterial Load	-1	-0.69	-0.92	-0.46	-0.63	-1.09	-0.17	0.821
Change in Total Bacterial Load	0	-0.42	-0.67	-0.17	-0.34	-0.80	0.13	0.768

[1]P-values are based on standard linear regression with microbial factors included as the outcome, development of VAP as the primary predictor and time and antibiotics variables as covariates. [2]Values correspond to the least square means from the regression model
Abbreviations: VAP: ventilator-associated pneumonia.