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# The Microbiome And Pneumonia Disease Severity In Asthmatic Children

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# The Microbiome and Pneumonia Disease Severity in Asthmatic Children

A Thesis
Presented to
The Department of Epidemiology of Microbial Diseases
Yale School of Public Health

In Partial Fulfillment
of the Requirements for the Degree
Master of Public Health

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## Abstract:

Background: Pnuemonia is a leading cause of morbidity and mortality worldwide and children diagnosed with asthma have been shown to be at greatly increased risk of recurrent Community-Acquired Pneumonia (CAP). CAP in asthmatic children can incur nearly double the healthcare costs and lead to poorer outcomes during the course of the pneumonia infection.

Objective: This study seeks to determine if sputum (SP) samples may be used in the pediatric population to better understand the microbiome environment during severe pneumonia in place or in conjunction with the more commonly used nasopharyngeal (NP) samples. Additionally, this study seeks to identify features of the microbiome associated with pneumonia severity in asthmatic children.

Methods: Sputum and nasopharyngeal/oropharyngeal (NP/OP) samples were collected from asthmatic children diagnosed with asthma upon admission to a hospital. Bacterial cultures for known CAP pathogens using sputum samples, and PCR detection for viral pneumonia pathogens on the NP/OP samples were performed. To study the microbiome, 16s rRNA analysis of sputum and nasopharyngeal samples was performed and analysis conducted using a variety of single and community-based analyses. Outcomes of interest were LOS > 4 days and admission to the ICU.

Results: High relative abundance of CAP pathogens, including *Moraxella* and *Haemophilus,* were associated with poorer CAP outcomes in both age groups for both ICU admission and longer LOS. Similarly, a positive sputum culture result for *Staphylococcus aureus* was found to be significantly associated with more severe pneumonia. *Bacteroidetes* was associated with shorter LOS and *Rothia* association with longer LOS in several of the analyses. Both conclusions are consistent with previous characterizations of the bacteria in the onset of pneumonia and asthma. *Moraxella* was consistently associated with longer LOS and increased risk of ICU admission, consistent with its characterization as a minor CAP pathogen, but was protective against longer LOS in the younger age group.

Conclusions: First, our study demonstrates that sputum samples may be used in a pediatric population. Our findings demonstrate that many of the microbiome features previously identified as being predictive of, or associated with, CAP, also serve to predict severe pneumonia outcomes in this pediatric population, including longer Length of Stay (LOS) and Intensive Care Unit (ICU) admission. However, certain inconsistencies in the trends in our data highlight the need to perform microbiome analyses using many different approaches to fully understand the complex relationships between the diverse commensal and pathogenic bacteria that comprise the microbiome.

# Acknowledgments

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Next to my friends and family, and my husband Jesse, who have provided me with constant support. I would especially like to thank my twin Jenny and Jesse, who were always there to listen to me when I was stressed and provided encouragement when I needed it. And of course my parents who always believed in me since the beginning and who have pushed and supported me throughout my educational career. You guys always have good advice even if I'm not always ready to hear it.

# Table of Contents

# List of Tables

# List of Figures

## Introduction:

Pneumonia is among the three leading causes of admission for pediatric patients and remains a major cause of morbidity and mortality worldwide in pediatric populations (Parikh, 2014; Vila-Corcoles, 2012). Pneumonia is most commonly found in children under four years of age and adults sixty-five or older (Myles, 2009). From within the community, this so-named Community-Acquired Pneumonia (CAP) has been primarily linked to four bacterium when of bacterial etiology: *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus* and *Moraxella catarrhalis* (Cevey-Macherel, 2009). Recently, the widespread use of the 13-valent conjugate pneumococcal vaccine targeting *S. pnuemoniae* has reduced the prevalence of pneumonia in the population. Yet there remains a significant burden of pneumonia, as well as possible concerns regarding continued efficacy of the vaccine due to increasing burden of non-vaccine *S. pneumoniae* serotypes (Maldonado, 2014). Within the U.S. population, asthmatics represent a community who are at increased risk for developing pneumonia (Obert, 2012; Talbot, 2005; Maldonado, 2014; Patria, 2013). Asthma exacerbations are associated with increased health care costs and poorer outcomes among patients, and these exacerbations can often be caused by infections with CAP pathogens (Ivanova, 2012).

This relationship between asthma and pneumonia has been supported by a broad range of biological data demonstrating an increased susceptibility towards infection, however the mechanism of this increased susceptibility is not yet clear. In the pediatric population, asthmatic patients have been shown to have a deficient cellular and humoral immune response to *Mycoplasma pneumoniae* infection compared with non-asthmatic controls (Atkinson, 2009). Some studies have looked at a genetically-determined immune response present in asthmatics, mediating the susceptibility to pneumonia while others have looked at structural changes in the respiratory tract (McKeever, 2013). However, asthma diagnoses have also been associated with the presence of pneumonia pathogens such as Human Rhinovirus (HRV), *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* (Lu, 2014; Specjalski, 2011), as well as increased diversity within the bronchial microbiota, mainly attributed to the presence of pathogenic bacteria (Hilty, 2010; Huang, 2013). Pathogenic proteobacteria, particularly *Haemophilus spp*., were far more common in asthmatic patients, both adults and children, while *Bacteroidetes*, especially *Prevotella spp*., were less frequent in the asthmatic population and possibly played a protective role against invasion (Hilty, 2010). Disturbed microbiota have often been implicated as a risk factor for susceptibility toward invading pathogens and the development of pneumonia. In this study we seek to investigate the relationship between the microbiome and asthma by looking at the severity of disease within pneumonia patients. Most studies pneumonia have focused on the relationship between the microbiome and the risk of developing pneumonia (Boutin, 2013). To the best of our knowledge, no study has yet looked at the relationship between the microbiome and the severity of pneumonia within the asthmatic population.

Previous culture based analyses have also revealed specific etiologies associated with pneumonia and the onset of pneumonia. Within pediatric patients, *S. pneumoniae* (46) and rhinovirus (29) were found to be the most common microbes detected in CAP (Lahti, 2009). A prospective cohort study found that neonatal airway colonization by *S. pneumoniae, H. influenzae* or *M. catarrhalis* led to increased risk of pneumonia and bronchiolitis in the first three years of life, independent of asthma diagnosis (Vissing, 2013). When nasopharyngeal samples were used to diagnose the etiology of CAP in pediatric patients diagnosed with acute asthma, 35% of the pediatric patients were diagnosed with viral pneumonia (Isaacs, 1989). However, in the same study radiographic tests and clinical criteria were unable to distinguish between viral and bacterial pneumonia, highlighting the need for other tests in the clinical diagnosis of the pneumonia pathogen.

Additionally, there are questions surrounding the validity of various diagnostic tests used to detect and diagnose pneumonia. Nasopharyngeal samples are often used in diagnosis, but may be susceptible to underdiagnosis of *S. aureus* and overdetection of asymptomatic pathogenic bacteria that are capable of colonizing the nasopharynx (Isaacs, 1989; Nolte, 2008; Kumar, 2008). Induced sputum samples have previously been used to test for the presence or absence of CAP pathogens in pneumonia patients (Specjalski, 2011). Sputum samples have been proposed as an alternative strategy to enhance detection of some pathogens. Sputum samples were successfully used in a recent study of pediatric populations diagnosed with CAP and were used to microbiologically identify the causative agent of the pneumonia in 90% of cases (Lahti, 2009). Moreover, one-quarter of all of the identified bacterial pathogens were only detected in the sputum, not in the nasopharyngeal samples (Lahti, 2009). In this study we seek to assess the relative value of the sputum and the nasopharyngeal samples in predicting pneumonia disease severity and their value as surrogates for evaluating the lung microbiome.

## Methods:

These data and samples were all from the recently conducted Centers for Disease Control (CDC) study on the Etiology of Pneumonia in the Community (EPIC) (Jain, 2015). The EPIC study was a prospective, multi-site, population-based active surveillance study to determine the incidence and etiology of CAP in children (Jain, 2015). Subjects under 18 years of age were identified and eligible for entry into the study when seeking medical care for respiratory illness to Le Bonheur Children's Hospital (LBCH) or the LBCH catchment area in Memphis Tennessee. Patients were examined for clinical evidence of pneumonia, and the final study sample was limited to only those patients with an abnormal chest X-ray confirming the presence of pneumonia. Upon enrollment in the study, patients completed a full medical history questionnaire, and provided samples of whole blood, induced sputum sample (SP), blood culture, nasopharyngeal swab (NP), oropharyngeal swab (OP) and serum samples at admission (acute). The following tests were performed on the specimens: culture of blood and NP/OP secretions for bacteria, real-time polymerase chain reaction (PCR) for influenza viruses A and B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza virus (PIV) 1, 2 and 3, coronaviruses 229E, OC43, NL63 and HKU1, adenovirus, rhinoviruses, *S. pneumoniae, Legionella pneumophila, M. pneumoniae,* and *Chlamydia pneumoniae* (NP/OP secretions), PCR for *S. pneumoniae* and Group A *Streptococcus* (whole blood) , Enzyme Immunoassay (EIA) (Remel) for *M. pneumoniae*, Microimmunofluorescence (MIF) for *C. pneumoniae* (serum), Enzyme-Linked Immunosorbent Assay (ELISA) for influenza viruses A and B, RSV, hMPV, and PIV 1, 2, 3, PCR for *S. aureus* using whole blood, and the culture of induced sputum for bacteria in children. The final sample size was 208 pediatric patients when the sample was limited only to children who had both their NP/OP and SP sample successfully amplify for the 16S ribosomal analysis, as well as having X-ray confirmed pneumonia, and a confirmed history of asthma.

### *16s RNA Analysis:*

The microbiome samples were analyzed through the hypervariable regions V4 of the 16S ribosomal RNA using bar-coded 16sRNA primers as previously described (Caporaso, 2012**)**. Initial cleaning, binning, and processing of sequence reads were done using Btrim (Kong, 2011). Sequences were scanned for linkers and primers while allowing for two errors, and primer sequences were removed from each sequence read. Reads missing the 5'-end primer were removed from the data set. Bar codes were identified, allowing for one error, and trimmed after sequence reads were binned into separate FASTA files.

Sequences were aligned using Infernal 1.1 available from the Ribosomal Database Project (RDP) (Cole, 2009). Strict alignment criteria were set to minimize the number of chimeras: all sequences had to align to at least 200 bp of the 16S rRNA gene, and any sequence aligning outside of the 27 or the 338 position of the 16s rRNA gene was discarded. Taxonomic identification of samples was achieved using the RDP Bayesian classifier tool at 90% confidence from the RDP pipeline on the RDP database project. Those bacteria that failed to classify to an order were labeled as "unclassified". Following classification, sequence reads were then clustered (RDP pipeline) into Operational Taxonomic Units (OTU's), defined in an iterative process beginning first with genus level identification, and then moving up to the next taxonomic level when the lower level grouping was not available. Individual samples were assessed for measures of microbiome diversity, calculating the evenness and Shannon Index (RDP pipeline).

*Statistical Analysis:*

The population was stratified into older than and younger than five years of age for all analyses due to differences between the populations and potential complications with differences in diagnostic practices for asthma in these age groups (asthma is not considered to be an appropriate diagnosis prior to five years of age). Disease severity was measured through hospital Length of Stay (LOS) as a dichotomous categorical variable, categorizing those with LOS >4 days as having the outcome of interest. This cutoff was chosen because the top quartile of LOS (q4 >4.29 days) closely correlated with those having LOS greater than four days (26.6% of study population had LOS >4 days). Intensive Care Unit (ICU) admission was also used in a subgroup analysis as a dichotomous variable in patients older than five years of age; the analysis was only performed in the older age group as the frequency of ICU visitation was not high enough in the lower age group to provide adequate power to the analysis. Unadjusted associations for outcome variables LOS and ICU admission, as well as potential confounders, presence of cultured pathogens, and diversity measures were calculated using a chi–square test or t-test as appropriate.

For all subsequent analyses, a cutoff of 0.6% mean relative abundance within either the NP/OP or the SP samples was used for each taxa. This left 26 taxa for subsequent analyses and all remaining taxa or unclassified sequences were combined into the category “meta_other”. We used several measures of microbial community structure. 1) Principal component analysis (PCA) was performed in *SAS* 9.4 (SAS Institute, Cary NC) on both the SP and NP/OP 16S RNA samples to determine associated groups of bacteria, and the resulting factor scores being used in the logistic regression. 2) Linear Discriminant Analysis (LDA) was performed using *LefSE* to determine significantly differentiated taxa between those with and without severe disease, as well as between the NP/OP and SP samples within each category (Segata, 2011). Each participant was then assigned as being either in the lower three or top quartile with respect to the relative abundance of each of the significantly enriched taxa. These dichotomous categorical variables for significantly enriched taxa were then input into a logistic regression model. 3) Lastly, a community typing analysis using *SimClust* (Walesiak, 2014) in *R* (R Core Team, 2014) was used to cluster patients into groups with similar microbiome profiles. *RandomForest* (Liaw, 2012) in *R* was then used to determine the significantly discriminant features of the community types.

All logistic regressions were stratified by age and performed separately by outcome variable in *SAS* 9.4, resulting in three models, unless stated otherwise: (i) LOS outcome in the < 5 age group, (ii) LOS outcome in the ≥ 5 age group and lastly (iii) ICU outcome in the ≥ 5 age group. The first logistic regression models were created using the diversity measures for evenness and the Shannon Index. Community types were used as a categorical variable in logistic regression models. Additionally, heat maps of the community types were created in *R* by determining the proportion of patients of each community type in the fourth quartile for relative abundance of each taxa. Potential confounders included sex, race, education, type of facility the patient was admitted from, presence of co-morbidities, prior antibiotic use, oral steroid use, inhaled steroid use, and vaccination history for seasonal influenza, *pneumococcal*-7 or 13 conjugate and *H. influenza* B. Pathogens included *S. pneumoniae, S. aureus, M. catarrhalis, H. influenzae, M. pneumoniae,* Rhinovirus, RSV, adenovirus, coronavirus, *H. parainfluenzae,* and hMPV.

## Results and Discussion:

### 1. Study Population

All of the analyses were performed upon an age-stratified population, where children were grouped into the younger category less than five years of age, and the older age group of children from five to less than eighteen years of age. This cutoff was selected due to the difficulties and controversy in diagnosing asthma in those under five years of age (Mayo Clinic Staff, 2015). Official guidelines dictate that asthma cannot be properly diagnosed in children under the age of five as many of these children will eventually grow out of the asthma as they become older. This has several implications for this study, namely that the population of children diagnosed with asthma under the age of five may well be a different population than those who maintain their asthma diagnosis past the age of five. Thus, we elected to stratify the analysis by age for all of the analyses in this study.

Demographic, vaccination and clinical characteristics of the stratified study population are described in Table 1 by LOS and Table 2 by ICU admission. Within both age groups, there were slightly more males than females, with 44.4% and 42.9% of the population being female in the younger and older age groups respectively. Most of the population was admitted into the hospital directly from home, 82.5% and 63.6% coming from home in the younger and older age group respectively. Although 7.1% and 10.4% came from outpatient clinics in the younger and older age groups respectively; and 9.5% of the younger and 26.0% of the older age group came from another hospital. The majority of the study's population was black, with 82.5% and 88.3% of the population identifying as black in the younger and older age groups respectively, with the rest of the population roughly evenly split between white and other races. Race does appear to be a significant unadjusted association with LOS in the younger age group (p-value = 0.014). There was no significant unadjusted association found between LOS and ICU admission with education, presence of co-morbidities, prior antibiotic history, oral steroid use, inhaled steroid use, and *H. Influenzae* vaccination. Nearly all of the study's population had previously received the *Pneumococcal*-7 or 13 conjugate vaccine, though coverage was not as high among the older age group (81.8%), with the *pneumococcal* vaccine appearing to have a significant unadjusted association with LOS in the older age group.

Tables 3 and 4 present the unadjusted associations between the different culture results for the CAP bacterial pathogens, while Tables 5 and 6 presents the same for the PCR tests for the viral pathogens and bacteria. There did not appear to be any association between a positive culture for *S. pneumoniae, M. catarrhalis, H. influenzae,* and *M. pneumoniae* and either of the disease outcomes in the analysis. However, a positive *S. aureus* culture did appear to have a significant association with increased LOS in the younger age group ($p<0.001$). Similarly, there was no significant association found between a positive PCR viral test for rhinovirus, RSV, adenovirus, coronavirus, PIV, or hMPV, and either of the pneumonia severity markers, LOS or ICU admission.

## 2. Differences between the NP/OP and SP samples within the population.

### *Relationship between diversity measures and disease severity within NP/OP and SP samples:*

There were significant differences found between the NP/OP and SP samples with respect to the diversity indices. The SP samples were consistently less diverse with respect to both evenness and the Shannon Index in the total study population (Evenness: $p=0.031$; Shannon: $p < 0.001$) as well the younger age group (Evenness: $p < 0.001$; Shannon: $p < 0.001$) (Table 7). However, within the older age group, the SP samples were actually found to be significantly more diverse than the NP/OP samples with respect to the samples' evenness ($p=0.018$), though there was no significant difference found in the Shannon Index. Thus the SP sample does appear to be capturing a different microbiota than the NP/OP samples. However, despite the differences between the two sample types, neither of the measures of microbiota diversity tested were found to be significantly associated with either ICU admission or LOS in the adjusted analyses within either age group.

### *Top taxa from the 16s rRNA data:*

Table 8 shows the top 26 taxa that had the highest mean relative abundance from either the SP or NP/OP samples. *Streptococcus* was the most prevalent taxa in both samples comprising roughly 19 of the reads in both the NP/OP and SP samples. Genera previously implicated to play a role or have increased abundance in the presence of CAP, including *Prevotella, Veillonella, Rothia,* *Neisseria, Fusobacterium, Haemophilus, Moraxella* and *Actinomyces*, were all found to rank highly in relative abundance here as well (Chen, 2013; Garzoni, 2013; Sakwinska, 2014).

In an LDA analysis comparing the taxa between the NP/OP and the SP samples, genera *Atopobium*, and family *Actinomycetaceae* were significantly enriched in the NP/OP samples of both the older and the younger age groups, while phylum *Bacteroidetes* was significantly enriched in the SP samples of both the older and the younger age group (Figure 1). Within the younger age group, 7 taxa, along with the grouped rare taxa, were significantly enriched in the NP/OP samples, and 3 taxa were significantly enriched within the SP samples. The older age group had 4 taxa with significantly higher relative abundance in the NP/OP samples, and eight significantly enriched in the SP samples.

### 3. Associations between individual taxa in SP samples and pneumonia disease severity.

Within the younger age group, *Corynebacterium* and *Actinomyces* were significantly enriched in those children who had LOS shorter than four days (Figure 2a). In the adjusted analysis, patients in the top quartile for relative abundance of *Corynebacterium* and *Actinomyces* were found to be associated with shorter LOS after pneumonia diagnosis (OR= 0.30: 0.10-0.93, p=0.0372; OR= 0.30: 0.10-0.93, p=0.0372 respectively). Positive *S. aureus* culture was also found to be a significant covariate in the younger age group and with a positive culture being associated with LOS longer than four days (OR=4.96: 1.83-13.42, p=0.002). In the older age group, *Streptococcus*, *Atopobium*, *Moraxella*, family *Actinomycetaceae*, *Leptotrichia* and family *Veillonellaceae* were all significantly enriched in the samples of those children who had shorter LOS (Figure 2b). However, in the adjusted analysis, none of the taxa were significantly associated with longer LOS. Within the older age group, the high relative abundance of *Moraxella* was found to be significantly associated a risk of ICU admission (OR=4.97: 1.10-

22.50, p=0.037). In the final adjusted model for ICU admission in the older age group, recent flu vaccination was found to be a significant covariate, with recent flu vaccination being positively correlated with ICU admission (OR=4.83: 1.01-23.13, p=0.049). This was unexpected, but may be due to those receiving the vaccination being more likely to have severe underlying conditions; they may have been sicker to begin with.

### 4. Relationships between correlated taxa and pneumonia severity.

Within the microbiota, members of the commensal flora may co-aggregate or be more likely to co-colonize based on similar nutritional requirements or the member's use of various waste products and secondary metabolites (Rickard et al., 2003; Willing et al., 2011). Thus, PCA was used to identify groups of taxa (factors) that correlated with each other within each of the stratified samples. The SP samples in the younger age group produced four independent factors (Table 9), while PCA for the SP samples in the older age group produced five independent factors (Table 10).

*Stratified analysis in patients younger than five:*

**(i) *Atopobium, Veillonella*, *Actinomycetaceae*, and *Actinomyces.*** As a group, the relative abundance of these Factor 1 taxa, genera *Atopobium*, *Veillonella*, *Actinomyces* and the family *Actinomycetaceae*, did not differ significantly by LOS outcome.

**(ii) *Leptotrichia*, *Capnocytophaga*, *Neisseria*, *Lactobacillales*. *Betaproteobacteria*, *Moraxella*, and rare taxa.** The PCA Factor 2 taxa include genera *Leptotrichia*, *Capnocytophaga*, *Neisseria*, *Moraxella*, suborder *Lactobacillales*, class *Betaproteobacteria*, and the rare taxa. However, the relative abundance of these taxa as a group was not found to significantly differ with LOS outcome.

**(iii) *Mycoplasma*, *Veillonellaceae*, *Leptotrichiaceae* and *Bacteroidetes*.** Genera *Mycoplasma*, families *Veillonellaceae, Leptotrichiaceae* and phylum *Bacteroidetes* are all positively correlated with PCA factor 3. As a group, the taxa in factor 3 are significantly associated with shorter LOS and appear to be protective (OR= 0.49: 0.26-0.91, p=0.023).

**(iv) *Moraxella*, *Corynebacterium*, *Dolosigranulum*, *Fusobacterium*, *Leptotrichiaceae* and *Bacteroidales*.** The taxa in PCA factor 4, comprised of genera *Moraxella*, *Corynebacterium*, *Dolosigranulum*, *Fusobacterium*, family *Leptotrichiaceae* and suborder *Bacteroidales*, appears to be protective and is significantly associated with shorter LOS (OR=0.536: 0.30-0.96, p=0.035). Genus *Fusobacterium*, family *Leptotrichiaceae* and suborder *Bacteroidales* are negatively correlated with the factor 4, and a decrease in their relative abundance appears to be protective against longer hospital stays. In contrast, the genera *Moraxella*, *Corynebacterium* and *Dolosigranulum* are positively correlated with factor 4 in the younger children and an increase in their relative abundance appears to be protective against longer LOS.

*Stratified analysis in patients older than five:*

**(i) *Veillonella*, *Atopobium*, *Actinomyces*, *Prevotella*, *Neisseria*, and *Pasteurellaceae*.** The genera *Veillonella*, *Atopobium*, *Actinomyces*, *Prevotella*, *Neisseria*, and family *Pasteurellaceae* comprise PCA factor 1 in the older children (Table 10). Taken as a group, the relative abundance of Factor 1 taxa does not significantly differ by LOS or ICU admission.

**(ii) *Dolosigranulum*, *Corynebacterium*, and *Rothia*.** PCA factor 2 is comprised of *Dolsigranulum*, *Corynebacterium* and *Rothia,* all of which are positively associated with the factor. These taxa appear to be, as a group, associated with increased odds of longer LOS in the older age group (OR=3.18: 1.15-8.85, p=0.026). In the final model, a 7-conjugate pneumonia vaccine was also found to be a significant protective covariate, with reduced odds of longer LOS (OR=0.09: 0.02-0.36, p=<0.001).

**(iii) *Porphyromonadaceae*, *Lactobacillales*, and *Bacteroidales.*** Taken as a group, the taxa in Factor 3, including the genera *Dolosigranulum*, *Corynebacterium*, and *Rothia* do not differ significantly by LOS or ICU admission in the older age group.

**(iv) *Leptotrichia*, *Fusobacterium*, and *Leptotrichiaceae.*** The genera *Leptrotrichia, Fusobacterium*, and family *Leptotrichiaceae* comprised PCA factor 4, and are positively associated with the factor. As a group, the taxa in PCA factor 4 were found to be protective and have a significantly decreased relative abundance in older children with longer LOS (OR=0.491: 0.242-0.999, p=0.049).

**(v) *Streptococcus*, *Gemella*, and *Prevotella*.** The taxa PCA Factor 5 were also not found to significantly differ as a group with either ICU admission or LOS in the older age group.

### 5. Relationship between SP microbiota community types, predictive features of the community types and pneumonia disease severity.

A separate approach to addressing the considerable interpersonal variation in the composition of the lung microbiota is to use enterotypes or community types. Community typing acts by clustering samples into bins based upon their taxonomic similarity, and reflect a network of co-occurring bacterial populations (Ding, 2014). Community types have been found to be associated with a wide variety of environmental and behavioral characteristics, such as diet and sexual activity, as well as diseases such as psoriasis and *Trichomonas vaginalis* (Ding, 2014). Even more tellingly community types at one body site have been shown to be predictive of community types at adjacent body sites (Ding, 2014). We generated community types for each stratified population using the partitioning around the medoid approach (PAM). This resulted in three distinct community types being generated for the SP samples in the younger age group, and two distinct community types in the older age group (Figure 3). In a logistic analysis, there was no significant association between any of the community types and disease outcomes in the younger age group. However, in the older age group, community type 2 was found to be highly predictive of a longer LOS; though it was not possible to run the logistic model due to the presence of small cell values (Table 11).

Using Random Forest plots, *Moraxella, Dolosigranulum, Prevotella, Haemophilus and Corynebacterium* are all significantly discriminant features, and taken together account for 79% of the total predictive value of the taxa to distinguish between community types 1 and 2 (data not shown). *Moraxella, Prevotella, Dolosigranulum, Corynebacterium* and *Haemophilus* are all more highly enriched in Community Type 2 than 1 (Figure 4) and are thus significantly associated with a longer LOS. These findings appear to be both in support and contradiction with some of the earlier findings; namely *Corynebacterium*'s presence runs counter to the LDA results where the taxa was associated with shorter LOS in the older age group. Similarly, *Prevotella*'s presence in Community Type 2 is in conflict with the earlier finding from the factor analysis in which *Prevotella* positively associated with a protective Factor against longer LOS in the older age group (Table 9). However, *Dolosigranulum*'s inclusion in Community Type 2 is completely consisted with the PCA results in the older age group finding a positive association with increased LOS.

**Discussion:**

Consistent with expectations, there were significant differences in the microbiota make-up between the SP samples to the NP/OP samples. Interestingly, the NP/OP samples appeared to be significantly more diverse within the younger age group, though the opposite effect, though less pronounced, was true for the older age group. It is not clear why this might be the case. Despite these inter-sample differences, microbial diversity did not appear to have any significant impact on the severity of pneumonia in either age group. Increased diversity of the microbiota is often thought of as protective, as demonstrated by a recent study showing both decreased richness and diversity was associated with pneumonia diagnosis in CAP pediatric patients (Sakwinska, 2014). However, in pneumonia pathogenesis the results have been inconsistent and often inconclusive (Toma, 2014; Hilty, 2010; Huang, 2013). A recent study examining high-risk vs. low-risk patients for clinical pulmonary infection score in hospitalized adults found no significant correlation between risk and the diversity of lung microbiota (Toma, 2014).

*Streptococcus* was the most prevalent taxa in both samples comprising roughly 19% of the reads in both the NP/OP and SP samples. This is consistent with previous findings from 16s rRNA data of CAP patients in both SP and NP/OP samples (Chen, 2013; Garzoni, 2013, Sakwinska, 2014). Genera *Prevotella, Veillonella, Rothia, Neisseria, Fusobacterium, Haemophilus* and *Actinomyces* have all been previously reported to be highly prevalent in the NP/OP and SP samples of CAP patients and were all found to rank highly here as well in relative abundance (Chen, 2013; Garzoni, 2013). However, a recent Swiss study using NP/OP samples in CAP pediatric patients found 92% of the reads were from *Moraxella, Haemophilus* and *Streptococcus* alone (Sakwinska, 2014). While *Streptococcus, Moraxella,* and *Haemophilus* are ranked first, fourth and ninth respectively in the NP/OP samples, and first, second and sixth in the SP samples, the combined relative abundance of these pathogens are only 40.87% in this study. The presence of genera *Prevotella Veillonella* and *Rothia* appear to be more consistent with the previously mentioned studies of the U.S. young adult CAP population and may reflect a different etiology of pneumonia in different areas. In contrast, *Moraxella* is commonly found in patients with pulmonary infections across studies and is found ranking highly in both the NP/OP and SP samples, as well as *Rothia* (Chen, 2013, Sakwinska, 2014, Garzoni, 2013).

The results from the SP LDA and the community based analyses implicated several known CAP pathogens as being associated with increased pneumonia severity. Previous studies have found that *Moraxella*, *S. pneumonia* and *S. aureus* are major causes of CAP within a younger population (Hashemi, 2010). Here in this study, positive *S. aureus* culture was strongly associated with longer LOS in the fully adjusted analysis using LDA in the younger age group. It is not terribly surprising to find that the presence of a known CAP pathogen would be associated with poorer outcomes, especially given studies indicating the increased risk associated with co-infections of known CAP pathogens. From the community typing, *Moraxella* and *Haemophilus* were more highly enriched in Community Type 2, which was strongly associated with a longer LOS in the older age group. *Moraxella* and *Haemophilus* are both known CAP pathogens, and are known to be associated with CAP in the pediatric population (Hashemi, 2010; Chen, 2013; Vissing, 2013). Previous studies have shown that *Moraxella* is a pnuemonia causing pathogen in children with CAP (Hashemi, 2010). *Moraxella* is absent in the lower respiratory tract of healthy individuals, but recent studies have found it to be present in patients diagnosed with CAP (Chen, 2013). Most of the results in this study are consistent with the idea that *Moraxella* is a CAP pathogen, including the LDA analysis in the older children finding *Moraxella* to be positively associated with ICU admission, and the Community Typing results again in the older children. However, within in the younger children, PCA factor results pointed towards *Moraxella* being a part of a factor associated with shorter LOS. A note of caution should enter here that the community analyses can only infer that a factor as a whole is associated with a protective role, and that it may not be *Moraxella* driving the association seen in this result, but the other taxa in the factor. Given that lung colonization of *Moraxella* has been shown to be associated with an increased risk of pneumonia in neonates and early life children independent of asthma (Vissing, 2013), caution should be employed in the interpretation of this result. Overall, the weight of the evidence from the various community analyses do point to *Moraxella* playing a role in CAP severity, and increased bacterial load is associated with more severe infection and pneumonia severity.

Interestingly, the PCA SP analysis in the younger children indicated that the phylum *Bacteroidetes*, positively correlated with the protective PCA Factor 3, was associated with reduced LOS. This phylum has previously been shown to play a protective role against pneumonia severity (Huang, 2013) and has previously been described as being less frequently found within asthmatic populations, attributed to its function of preventing against pathogenic invasion (Huang, 2013). These findings suggest that *Bacteroidetes* may play a similar role in pneumonia severity, possibly by interfering with the colonization of the lung by pathogenic bacteria. Similarly, previous studies have implicated *Prevotella* in playing a protective role against pathogenic invasion in pediatric patients, the presence of *Prevotella* was implicated as playing a protective role in the PCA analysis in the older age group, where it was positively associated with Factor 4 which was protective against longer LOS (Huang, 2013). However, *Prevotella* was also a significantly predictive feature of Community Type 2 in the older children, and associated, in this analysis, with longer LOS. Again, this result highlights the need for caution in interpreting the results from the community analyses and suggest that there are likely complex interactions at work that will require multiple different analytical approaches to determine their full extent.

Other taxa that emerged from the SP analysis include *Rothia* and *Dolosigranulum*, which here was found to be associated with Factor 2 in the older age group that was significantly associated with longer LOS, and consistently *Dolosigranulum* was again found to be associated with Community Type 2 from the community typing. These results were consistent with previous studies where *Rothia* was identified as a possible infective agent of CAP, although it is a normal part of the flora in the oral cavity and upper respiratory tract (Chen, 2013). The increased presence of *Rothia* in those with longer LOS may be an indication of a more severe pneumonia infection.

Lastly, *Corynebacterium* was found in increased relative abundance in Community Type 2 and thus potentially associated with longer LOS in older kids, however this was in contrast to the LDA results where *Corynebacterium* and *Actinomycs* were associated with a shorter LOS in the younger age group. Here, the community typing results were more consistent with previous findings that the phylum *Actinobacteria,* of which both *Corynebacterium* and *actinomyces* are a part, was positively associated with a CAP diagnosis as compared to a healthy population (Chen, 2013). Taken together, all of these finding highlight the complexity of the relationships between various microbes and raises the need for caution in being to quick to interpret the results from various microbiome analyses. However, despite this, there was still remarkable consistency between many of the findings in this study and previous literature investigating the role of the microbiome and pneumonia etiology with the risk of developing pneumonia.

## Conclusion:

The asthmatic pediatric population represents an at-risk population for recurrent CAP pneumonia (Patria, 2013). A disturbed lung microbiota has been implicated to play a role in the onset and progression of asthma (Lu, 2014; Specjalski, 2011; Hilty, 2010; Huang, 2013) as well as being associated with, and increasing the risk of developing pneumonia (Vissing, 2013; Sakwinska, 2014). Thus, it is likely that the altered status of the lung microbiota in asthmatic patients plays a role in the susceptibility of asthmatics to pneumonia. This study is the first to look at significant predictors within the microbiome of pneumonia disease severity within the pediatric asthmatic population.

Taken as a whole, this study found that many of the previously identified features of the microbiome known to be predictive of developing CAP or associated with the presence of CAP, were the same features that were predictive of poor CAP disease outcomes in the pediatric population, such as a longer LOS and ICU admission. These include increased relative abundances of the known CAP pathogens, including *Moraxella, S. pneumoniae, S. aureus* and *Haemophilus*, being associated with increased odds of poorer disease outcomes, both LOS and ICU admission. Similarly, *Bacteroidetes*' association with shorter LOS and *Rothia*'s association with longer LOS are both consistent with previous characterizations of the bacteria in the onset of pneumonia and asthma (Huang, 2013; Chen, 2013).

*Corynebacterium* was twice found to be associated with poor disease outcomes in the older age group; once in the Community Type analysis with longer LOS, and once as a part of PCA factor 2 with longer LOS; though it appears that *Corynebacterium* was positively associated with the protective PCA factor 4 in the younger age group. It is not clear why this disparity emerged between the two age groups, but previous studies have shown *Corynebacterium* is positively associated with CAP diagnosis (Chen, 2013). *Moraxella* was another taxa frequently present in the analyses.

*Moraxella*, a known minor pneumonia causing pathogen, including in the pediatric CAP population (Hashemi, 2010; Chen, 2013), had a similar finding to that of *Corynebacterium*. *Moraxella* was associated with increased risk of ICU admission in the older age group from the LDA analysis, and with increased odds of longer LOS in the older age group through its association with Community Type 2. However, from the PCA analysis in the younger age group, *Moraxella* was positively associated with Factor 4, which was found to be protective against a longer LOS. These inconsistencies in the data analysis highlight the complex nature of the relationships between the different bacteria in the microbiota. Each type of analysis has its own strengths and weaknesses, and seeks to highlight a different aspect of the relationship within the microbiome. It is possible that some of the differences in the role of each taxa is influenced by the lens through which it is examined. Prior studies have demonstrated microbiome results are sensitive to the methods being used, and that methods vary in their ability to detect clusters (Kuczynski, 2010). Alternative explanations may be that the younger age group represents a different population of asthmatics than the older children, and that taxa found to be associated with poorer pneumonia outcomes in older children may perform a different role within the younger age group.

However, simply detecting the presence of a microbe within the upper respiratory tract does not necessarily mean that it is the cause of the pneumonia nor that it is inhabiting the lower airways (Murdoch, 2012). Previous studies have shown that SP cultures have a high sensitivity and specificity for pneumococcal pneumonia in adults, however it is still uncertain whether these specimens are of equal value for children (Murdoch, 2012). SP samples, if collected poorly, can be highly contaminated with upper respiratory tract microbes, though tests exist to evaluate the quality of the specimens. Similarly, some pneumonia pathogens identified from nasopharyngeal tract specimens may often been associated with incidental carriage, while other, more invasive, serotypes have a higher positive predictive value for pneumonia etiology (Murdoch, 2012). However, NP/OP samples have a high negative predictive value and are of still of value in a clinical setting. In our study, the microbiota present in the SP samples appeared to be consistently less rich and diverse than those of the NP/OP samples and were successfully colonized by many of the known CAP pathogens.

### *Limitations*

There are several limitations to this study: (i) The data in this study was cross-sectional and taken after the onset of disease, and thus this study can only determine that certain taxa or groups of taxa are associated with poorer pneumonia outcomes in asthmatic children. Longitudinal studies are needed to determine the temporal associations and infer causality. (ii) Due to the difficulties surrounding asthma diagnosis in children under the age of five, it is possible that the younger age group likely represents a more heterogeneous population with unknown factors affecting their risk for pneumonia and may limit the ability to interpret the differences between the two age groups.

Overall, this study demonstrates that SP samples have the potential to be used within the pediatric population as a non-invasive surrogate for the lung microbiome. Though further studies are needed to refine and further understand the complicated relationships between the various taxa, and care should be used in deciding which population of children are being assessed. However, this study has shown that many of the same features that are associated with the onset and presence of CAP may also be used to predict the progression of the disease. Asthma exacerbations are associated with increased health care costs among patients, and these exacerbations can often be caused by infections with CAP pathogens (Ivanova, 2012). These excess costs of treating an asthmatic patient with CAP result in nearly double the costs as compared to those patients without asthma, representing a significant opportunity for targeted interventions to avoid excess medical and productivity costs (Polsky, 2012).

**Table 1.** Age-stratified descriptive statistics for demographic characteristics, clinical characteristics and vaccination history, including unadjusted associations with LOS >4 days.

| | Age <5 years | | | Age ≥ 5 years | | |
|---|---|---|---|---|---|---|
| | < 4 days N=91[+] | ≥ 4 days N=35[+] | p-value | < 4 days N=58[+] | ≥ 4 days N=19[+] | p-value |
| Sex | | | 0.563 | | | 0.321 |
| Female | 39 (42.9) | 17 (48.6) | | 23 (39.7) | 10 (52.6) | |
| Male | 52 (57.1) | 18 (51.4) | | 35 (60.3) | 9 (47.4) | |
| Race | | | 0.014 | | | 0.188 |
| White | 7 (7.7) | 4 (9.4) | | 7 (12.1) | 0 (0.0) | |
| Black | 80 (87.9) | 24 (68.6) | | 49 (84.5) | 19 (100.0) | |
| Other | 4 (4.4) | 7 (20.0) | | 2 (3.5) | 0 (0.0) | |
| Education* | | | 0.653 | | | 0.315 |
| <high school | 8 (9.0) | 5 (14.3) | | 6 (10.7) | 0 (0.0) | |
| High school/Some College | 72 (80.9) | 26 (74.3) | | 35 (62.5) | 14 (73.7) | |
| College and graduate | 9 (10.1) | 4 (11.4) | | 15 (26.8) | 5 (26.3) | |
| Admitted From | | | 0.629 | | | 0.675 |
| Home | 74 (82.2) | 30 (85.7) | | 41 (71.9) | 13 (68.4) | |
| Outpatient Clinic | 6 (6.7) | 3 (8.6) | | 5 (8.8) | 3 (15.8) | |
| Another Hospital | 10 (11.1) | 2 (5.7) | | 11 (19.3) | 3 (15.8) | |
| Co-morbidities | | | 0.087 | | | 0.127 |
| Yes | 18 (19.8) | 12 (34.3) | | 6 (10.3) | 5 (26.3) | |
| No | 73 (80.2) | 23 (65.7) | | 52 (89.7) | 14 (73.7) | |
| Prior Antibiotics* | | | 0.598 | | | 0.476 |
| Yes | 25 (27.5) | 8 (22.9) | | 8 (13.8) | 4 (21.1) | |
| No | 66 (72.5) | 27 (77.1) | | 50 (86.2) | 15 (79.0) | |
| Oral Steroids* | | | 0.407 | | | 0.157 |
| Yes | 13 (14.4) | 7 (20.6) | | 7 (12.1) | 5 (26.3) | |
| No | 77 (85.6) | 27 (79.4) | | 51 (87.9) | 14 (73.7) | |
| Inhaled Steroids* | | | 0.597 | | | 0.900 |
| Yes | 19 (24.1) | 6 (19.4) | | 27 (50.9) | 10 (52.6) | |
| No | 60 (76.0) | 25 (80.7) | | 26 (49.1) | 9 (47.4) | |
| **Vaccines:** | | | | | | |
| Seasonal *Influenza* * | | | 0.254 | | | 0.956 |
| Yes | 21 (24.1) | 12 (34.3) | | 11 (19.3) | 3 (15.8) | |
| No | 66 (75.9) | 23 (65.7) | | 46 (80.7) | 16 (84.2) | |
| *Pneumococcal* -7 or 13 conjugate* | | | 0.720 | | | 0.004 |
| Yes | 89 (98.9) | 35 (100.0) | | 52 (89.7) | 11 (57.9) | |
| No | 1 (1.1) | 0 (0.0) | | 6 (10.3) | 8 (42.4) | |
| *H. influenza* B * | | | 1.000 | | | 0.435 |
| Yes | 87 (96.7) | 34 (97.1) | | 57 (98.3) | 18 (94.7) | |
| No | 3 (3.3) | 1 (2.9) | | 1 (1.7) | 1 (5.3) | |

* Numbers may not sum to total due to missing values

+ Restricted to population to those diagnosed with asthma, radiologically confirmed pneumonia and having both np and sp samples.

**Table 2.** Descriptive statistics for demographic characteristics, clinical characteristics and vaccination history, including unadjusted associations for ICU admission in the older age group.

| | ICU Admission | | |
|---|---|---|---|
| | Age ≥ 5 years | | |
| | Not Admitted<br>n = 68$^+$ | Admitted<br>n = 9$^+$ | p-value |
| Sex | | | 0.413 |
| Female | 28 (41.2) | 5 (55.6) | |
| Male | 40 (58.8) | 4 (44.4) | |
| Race | | | 0.509 |
| White | 7 (10.3) | 0 (0.0) | |
| Black | 59 (86.8) | 9 (100.0) | |
| Other | 2 (2.9) | 0 (0.0) | |
| Education* | | | 0.156 |
| <high school | 5 (7.6) | 1 (11.1) | |
| High school/Some College | 41 (62.1) | 8 (88.9) | |
| College and graduate | 20 (30.3) | 0 (0.0) | |
| Admitted From | | | 0.833 |
| Home | 47 (70.2) | 7 (77.8) | |
| Outpatient Clinic | 7 (10.5) | 1 (11.1) | |
| Another Hospital | 13 (19.4) | 1 (11.1) | |
| Co-morbidities | | | 0.469 |
| Yes | 9 (13.2) | 2 (22.2) | |
| No | 59 (86.8) | 7 (77.8) | |
| Prior Antibiotics* | | | 0.694 |
| Yes | 11 (16.2) | 1 (11.1) | |
| No | 57 (83.8) | 8 (88.9) | |
| Oral Steroids* | | | 0.559 |
| Yes | 10 (14.7) | 2 (22.2) | |
| No | 58 (85.3) | 7 (77.8) | |
| Inhaled Steroids* | | | 0.090 |
| Yes | 30 (47.6) | 7 (77.8) | |
| No | 33 (52.4) | 2 (22.2) | |
| **Vaccines:** | | | |
| Seasonal *Influenza* * | | | 0.032 |
| Yes | 10 (14.9) | 4 (44.4) | |
| No | 57 (85.1) | 5 (55.6) | |
| *Pneumococcal* -7 or 13 conjugate * | | | 0.738 |
| Yes | 56 (82.4) | 7 (77.8) | |
| No | 12 (17.7) | 2 (22.2) | |
| *H. influenza* B * | | | 0.088 |
| Yes | 67 (98.5) | 8 (88.9) | |
| No | 1 (1.5) | 1 (11.1) | |

* Numbers may not sum to total due to missing values

\+ Restricted to population to those diagnosed with asthma, radiologically confirmed pneumonia and having both np and sp samples.

**Table 3**. Age-stratified unadjusted associations with LOS >4 days for CAP bacterial sputum cultures.

| | Length of Stay | | | | | |
|---|---|---|---|---|---|---|
| | Age <5 years | | | Age ≥ 5 years | | |
| | < 4 days N=91 | ≥ 4 days N=35 | p-value | < 4 days N= 58 | ≥ 4 days N=19 | p-value |
| *S. pneumoniae* | | | 0.467 | | | 0.571 |
| Yes | 18 (19.8) | 9 (25.7) | | 3 (5.2) | 0 (0.0) | |
| No | 73 (80.2) | 26 (74.3) | | 55 (94.8) | 19 (100.0) | |
| *S. aureus* | | | <0.001 | | | 0.750 |
| Yes | 10 (11.0) | 13 (37.1) | | 12 (20.7) | 3 (15.8) | |
| No | 81 (89.0) | 22 (62.9) | | 46 (79.3) | 16 (84.2) | |
| *M. catarrhalis* | | | 0.655 | | | 1.000 |
| Yes | 20 (22.0) | 9 (25.7) | | 2 (3.5) | 0 (0.0) | |
| No | 71 (78.0) | 26 (74.3) | | 56 (96.6) | 19 (100.0) | |
| *H. Influenzae* | | | 0.183 | | | 0.592 |
| Yes | 12 (13.2) | 8 (22.9) | | 3 (5.2) | 2 (10.5) | |
| No | 79 (86.8) | 27 (77.1) | | 55 (94.8) | 17 (89.5) | |

**Table 4.** Unadjusted associations with ICU admission for CAP bacterial sputum cultures within the older age group.

| | ICU Admission | | |
|---|---|---|---|
| | Age ≥ 5 years | | |
| | Not Admitted n = 68 | Admitted n = 9 | p-value |
| *S. pneumoniae* | | | 0.520 |
| Yes | 3 (4.4) | 0 (0.0) | |
| No | 65 (95.6) | 9 (100.0) | |
| *S. aureus* | | | 0.246 |
| Yes | 14 (20.6) | 1 (11.1) | |
| No | 54 (79.4) | 8 (88.9) | |
| *M. catarrhalis* | | | 0.602 |
| Yes | 2 (2.9) | 0 (0.0) | |
| No | 66 (97.1) | 9 (100.0) | |
| *H. Influenzae* | | | 0.400 |
| Yes | 5 (7.4) | 0 (0.0) | |
| No | 63 (92.7) | 9 (100.0) | |

**Table 5.** Age-stratified, unadjusted associations with LOS for the bacterial cultures of sputum samples and viral PCR results on nasopharyngeal/oropharyngeal samples.

| | Length of Stay | | | | | |
|---|---|---|---|---|---|---|
| | Age <5 years | | | Age ≥ 5 years | | |
| | < 4 days N=91 | ≥ 4 days N=35 | p-value | < 4 days N= 58 | ≥ 4 days N=19 | p-value |
| *Mycoplasma* | | | 1.000 | | | 0.325 |
| Yes | 2 (2.2) | 0 (0.0) | | 5 (8.6) | 0 (0.0) | |
| No | 89 (97.8) | 35 (100.0) | | 53 (91.4) | 19 (100.0) | |
| **Viruses:** | | | | | | |
| Rhinovirus | | | 0.161 | | | 0.432 |
| Combined | 24 (26.4) | 7 (20.0) | | 10 (17.2) | 2 (10.5) | |
| Alone | 18 (19.8) | 3 (8.6) | | 27 (46.6) | 7 (36.8) | |
| None | 49 (53.9) | 25 (71.4) | | 21 (36.2) | 10 (52.6) | |
| RSV | | | 0.911 | | | 0.674 |
| Yes | 38 (41.8) | 15 (42.9) | | 6 (10.3) | 1 (5.3) | |
| No | 53 (58.2) | 20 (57.1) | | 52 (89.7) | 18 (94.7) | |
| Adenovirus | | | 0.331 | | | 1.000 |
| Yes | 23 (25.3) | 6 (17.1) | | 3 (5.2) | 1 (5.3) | |
| No | 68 (74.7) | 29 (82.9) | | 55 (94.8) | 18 (94.7) | |
| Coronavirus | | | 0.987 | | | 0.988 |
| Yes | 10 (11.0) | 3 (8.6) | | 2 (3.5) | 1 (5.3) | |
| No | 81 (89.0) | 32 (91.4) | | 56 (96.6) | 18 (94.7) | |
| Parainfluenzae | | | 0.865 | | | 0.567 |
| Yes | 5 (5.5) | 1 (2.9) | | 4 (6.9) | 0 (0.0) | |
| No | 86 (94.5) | 34 (97.1) | | 54 (93.1) | 19 (100.0) | |
| Metapneumovirus | | | | | | 1.000 |
| Yes | 4 (4.4) | 3 (8.6) | 0.396 | 1 (1.7) | 0 (0.0) | |
| No | 87 (95.6) | 32 (91.4) | | 57 (98.3) | 19 (100.0) | |

Table 6. Unadjusted associations with ICU admission for the bacterial cultures of sputum samples and viral PCR results on nasopharyngeal/oropharyngeal samples.

| | ICU Admission | | |
|---|---|---|---|
| | Age ≥ 5 years | | |
| | Not Admitted n = 68 | Admitted n = 9 | p-value |
| *Mycoplasma* | | | 0.400 |
| Yes | 5 (7.4) | 0 (0.0) | |
| No | 63 (92.7) | 9 (100.0) | |
| **Viruses:** | | | |
| Rhinovirus | | | 0.815 |
| Combined | 10 (14.7) | 2 (22.2) | |
| Alone | 30 (44.1) | 4 (44.4) | |
| None | 28 (41.2) | 3 (33.3) | |
| RSV | | | 0.313 |
| Yes | 7 (10.3) | 0 (0.0) | |
| No | 61 (89.7) | 9 (100.0) | |
| Adenovirus | | | 0.395 |
| Yes | 3 (4.4) | 1 (11.1) | |
| No | 65 (95.6) | 8 (88.9) | |
| Coronavirus | | | 0.234 |
| Yes | 2 (2.9) | 1 (11.1) | |
| No | 66 (97.1) | 8 (88.9) | |
| Parainfluenzae | | | 0.395 |
| Yes | 3 (4.4) | 1 (11.1) | |
| No | 65 (95.6) | 8 (88.9) | |
| Metapneumovirus | | | 0.714 |
| Yes | 1 (1.5) | 0 (0.0) | |
| No | 67 (98.5) | 9 (100.0) | |

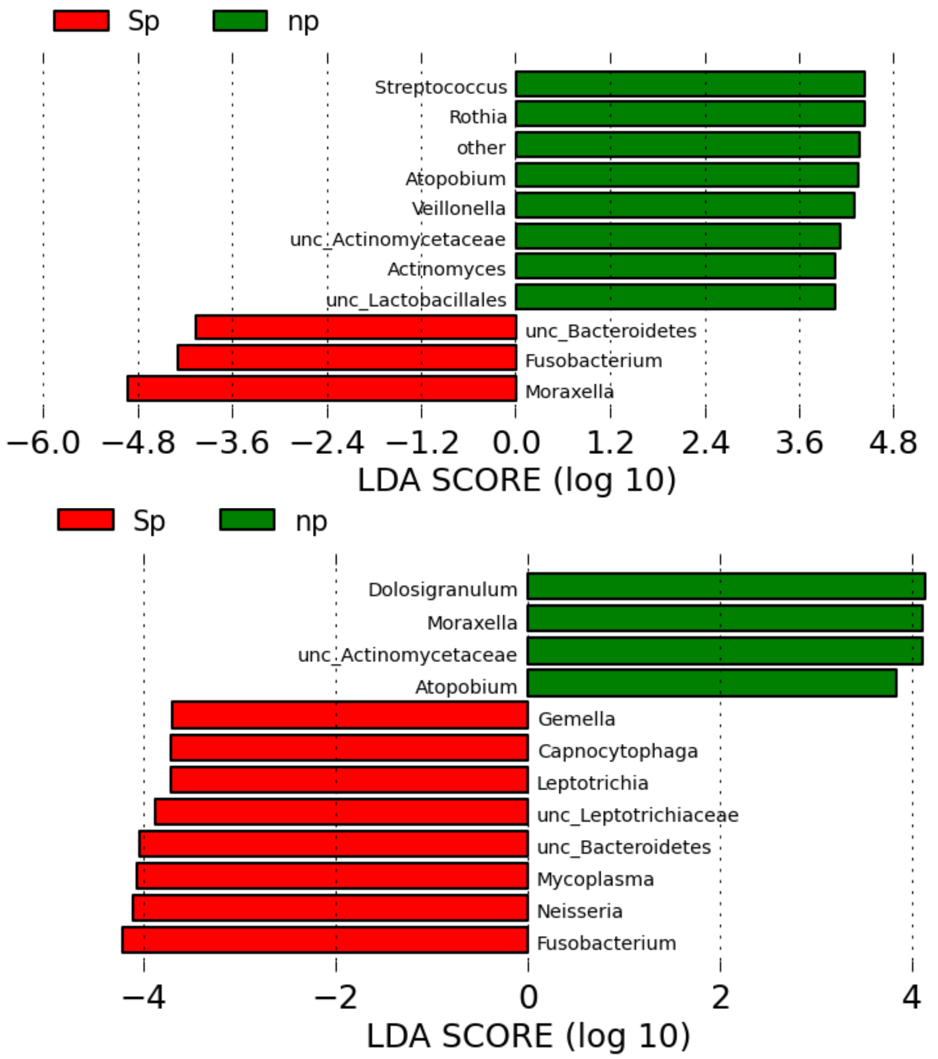
Table 7. Relationship between evenness and Shannon diversity indices and the nasopharyngeal/oropharyngeal (NP/OP) and sputum (SP) samples in the age-stratified population. T-tests were performed on the mean difference between the diversity indices in the SPand NP/OP samples and presented with standard deviation (std) and p-values.

| | Total: Mean Difference (SP-NP/OP: ± std) | | Age Stratification | | | |
|---|---|---|---|---|---|---|
| | | | 0 - < 5 years: Mean Difference (SP-NP/OP: ± std) | | ≤ 5 to <18 years: Mean Difference (SP-NP/OP: ± std) | |
| | n = 203 | p-value | n = 126 | p-value | n = 77 | p-value |
| Eveness | -0.022<br>± 0.141 | 0.031 | -0.056<br>± 0.140 | <0.001 | 0.035<br>± 0.126 | 0.018 |
| Shannon Index | -0.272<br>± 0.993 | <0.001 | -0.530<br>± 1.022 | <0.001 | 0.150<br>± 0.782 | 0.097 |

Table 8. Top 26 mean-ranked taxa within the nasopharyngeal/oropharyngeal (NP/OP) samples and the sputum (SP) samples based upon the 16s rRNA data for the population as a whole. The cutoff for inclusion was 0.6 mean-relative abundance in either the SP or the NP/OP samples.

| **Ranking** | **Sputum Samples** | | | **Nasopharyngeal Samples** | | |
|---|---|---|---|---|---|---|
| | **Taxa** | **Mean (%)** | **STD (%)** | **Taxa** | **Mean (%)** | **STD (%)** |
| **1** | Streptococcus | 24.11 | 18.8 | Streptococcus | 32.68 | 19.2 |
| **2** | Moraxella | 12.22 | 22.9 | Prevotella | 10.91 | 10.6 |
| **3** | Prevotella | 10.23 | 12.6 | Veillonella | 7.74 | 6.08 |
| **4** | Pasteurellaceae[b] | 7.78 | 12.6 | Moraxella | 5.89 | 13.2 |
| **5** | Haemophilus | 6.67 | 14.1 | Rothia | 5.18 | 6.03 |
| **6** | Veillonella | 4.75 | 5.21 | Pasteurellaceae[b] | 2.76 | 5.25 |
| **7** | Neisseria | 3.95 | 6.81 | Neisseria | 2.41 | 4.76 |
| **8** | Fusobacterium | 3.14 | 3.83 | Actinomyces | 2.40 | 2.48 |
| **9** | Porphyromonadace | 2.14 | 3.16 | Haemophilus | 2.30 | 6.42 |
| **10** | Rothia | 1.98 | 3.21 | Lactobacillales[d] | 2.08 | 2.74 |
| **11** | Bacteroidetes[a] | 1.96 | 3.13 | Dolosigranulum | 2.05 | 4.97 |
| **12** | Actinomyces | 1.48 | 2.41 | Corynebacterium | 2.02 | 6.05 |
| **13** | Bacteroidales[d] | 1.44 | 1.97 | Gemella | 1.75 | 3.04 |
| **14** | Leptotrichia | 1.33 | 1.87 | Fusobacterium | 1.55 | 2.23 |
| **15** | Gemella | 1.28 | 1.81 | Porphyromonadaceae[b] | 1.54 | 2.94 |
| **16** | Lactobacillales[d] | 1.00 | 1.94 | Bacteroidales[d] | 1.30 | 1.91 |
| **17** | Capnocytophaga | 0.96 | 2.33 | Leptotrichia | 0.97 | 1.75 |
| **18** | Leptotrichiaceae[b] | 0.91 | 1.57 | Bacteroidetes[a] | 0.93 | 2.04 |
| **19** | Betaproteobacteria[c] | 0.85 | 2.30 | Atopobium | 0.92 | 1.40 |
| **20** | Veillonellaceae[b] | 0.72 | 1.24 | Actinomycetaceae[b] | 0.92 | 1.51 |
| **21** | Mycoplasma | 0.69 | 5.08 | Leptotrichiaceae[b] | 0.70 | 1.52 |
| **22** | Corynebacterium | 0.68 | 1.65 | Capnocytophaga | 0.69 | 2.60 |
| **23** | Dolosigranulum | 0.60 | 1.83 | Veillonellaceae[b] | 0.52 | 0.67 |
| **24** | Actinomycetaceae[b] | 0.32 | 0.74 | Betaproteobacteria[c] | 0.34 | 1.14 |
| **25** | Atopobium | 0.28 | 0.62 | Lactobacillus | 0.10 | 0.69 |
| **26** | Lactobacillus | 0.07 | 0.53 | Mycoplasma | 0.02 | 0.06 |

[a] Phylum
[b] Family
[c] Class
[d] Suborder



**a.**

**b.**

**Figure 1.** Linear discriminant analysis (LDA) showing taxa significantly enriched in the sputum samples (red) and the nasopharyngeal/oropharyngeal samples (green). **a.** LDA results in the younger age group. **b.** LDA results in the older age group.

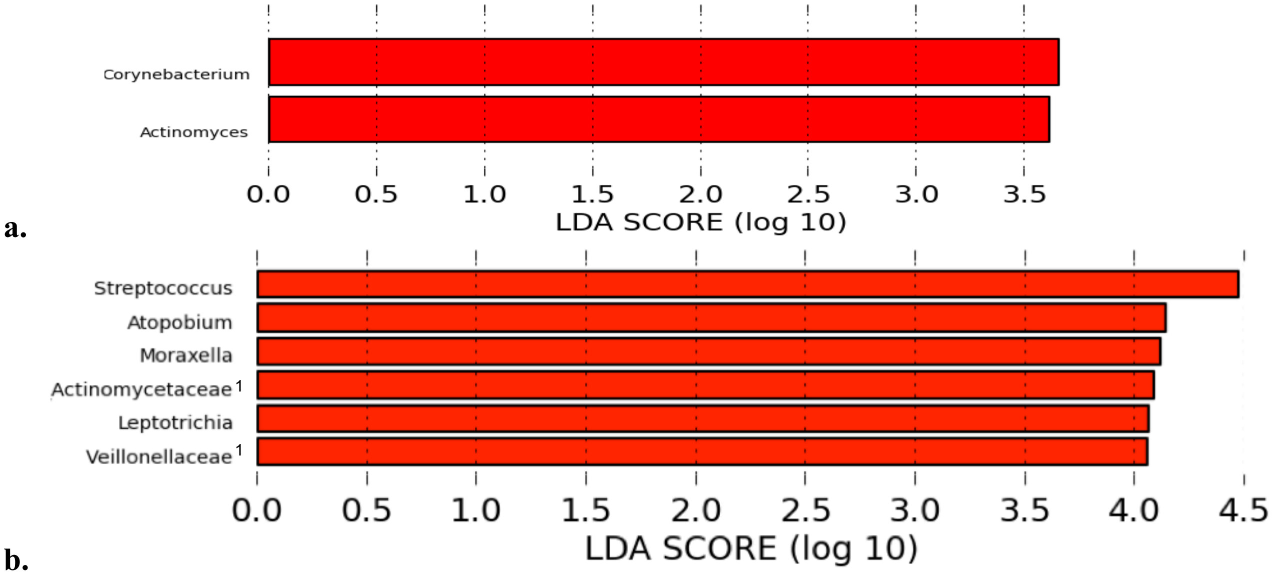


**Figure 2.** Results from the Linear Discriminant Analysis performed on the sputum samples showing significantly enriched taxa in patients with LOS <4 days (red). **a.** LDA sputum results in the younger age group. **b.** LDA sputum results in the older age group.

[1] Family

Table 9. PCA Factors for the sputum samples in the younger age group. [1] Factor significantly associated with shorter LOS in younger age group, $p<0.05$. [2] Category combining rare and unclassified bacteria.

| **Sputum, <5 years** | Factor 1 | Factor 2 | Factor 3 [1] | Factor 4 [1] |
|---|---|---|---|---|
| Atopobium | 88 | | | |
| Veillonella | 83 | | | |
| Actinomycetaceae[b] | 83 | | | |
| Actinomyces | 81 | | | |
| Leptotrichia | | 64 | | |
| Capnocytophaga | | 63 | | |
| Neisseria | | 54 | | |
| Other rare taxa[2] | | 53 | | |
| Lactobacillales[d] | | 49 | | |
| Betaproteobacteria[c] | | 48 | | |
| Moraxella | | -41 | | 41 |
| Veillonellaceae[b] | | | 83 | |
| Bacteroidetes[a] | | | 80 | |
| Mycoplasma | | | 55 | |
| Leptotrichiaceae[b] | | | 50 | -43 |
| Fusobacterium | | | | -50 |
| Bacteroidales[d] | | | | -40 |
| Corynebacterium | | | | 74 |
| Dolosigranulum | | | | 75 |

[a] Phylum

[b] Family

[c] Class

[d] Suborder

Table 10. PCA Factors for the sputum samples in the older age group. [1, 2] Factor significantly associated with shorter and longer LOS in older age group, respectively ($p<0.05$). [3] No factors were significantly associated with ICU admission in older age group.

| **Sputum, ≥ 5 years** | Factor 1 | Factor 2 [1] | Factor 3 | Factor 4 [2] | Factor 5 |
|---|---|---|---|---|---|
| Veillonella | 79 | | | | |
| Atopobium | 71 | | | | |
| Actinomyces | 55 | | | | |
| Prevotella | 51 | | | | -58 |
| Pasteurellaceae[b] | -50 | | | | |
| Neisseria | -56 | | | | |
| Dolosigranulum | | 86 | | | |
| Corynebacterium | | 75 | | | |
| Rothia | | 72 | | | |
| Porphyromonadaceae[b] | | | 84 | | |
| Lactobacillales[d] | | | 81 | | |
| Bacteroidales[d] | | | 55 | | |
| Leptotrichia | | | | 74 | |
| Fusobacterium | | | | 71 | |
| Leptotrichiaceae[b] | | | | 64 | |
| Streptococcus | | | | | 87 |
| Gemella | | | | | 62 |

[b] Family

[d] Suborder

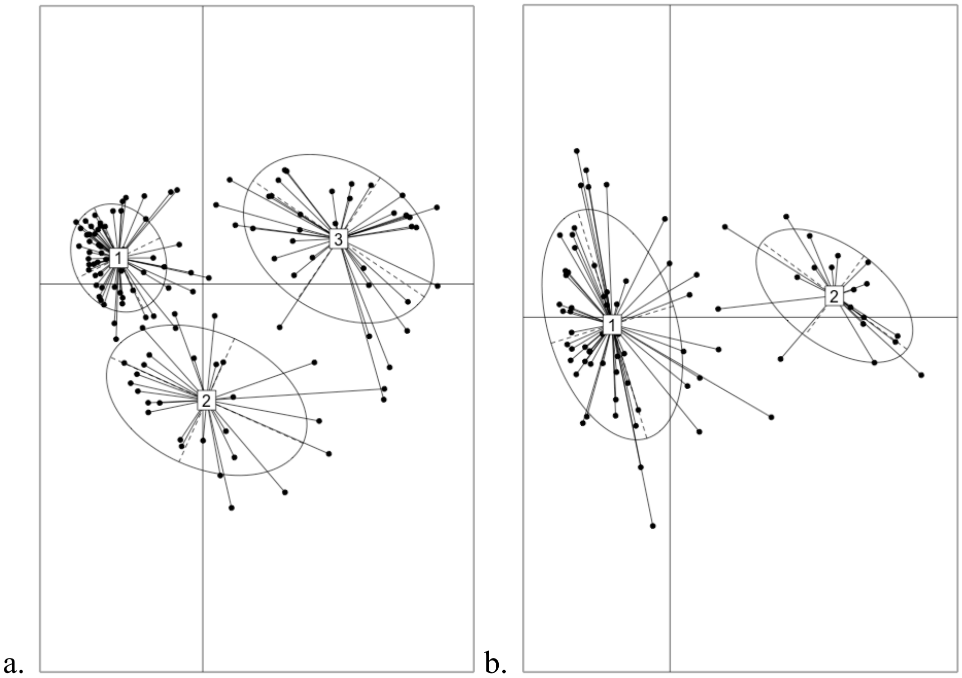
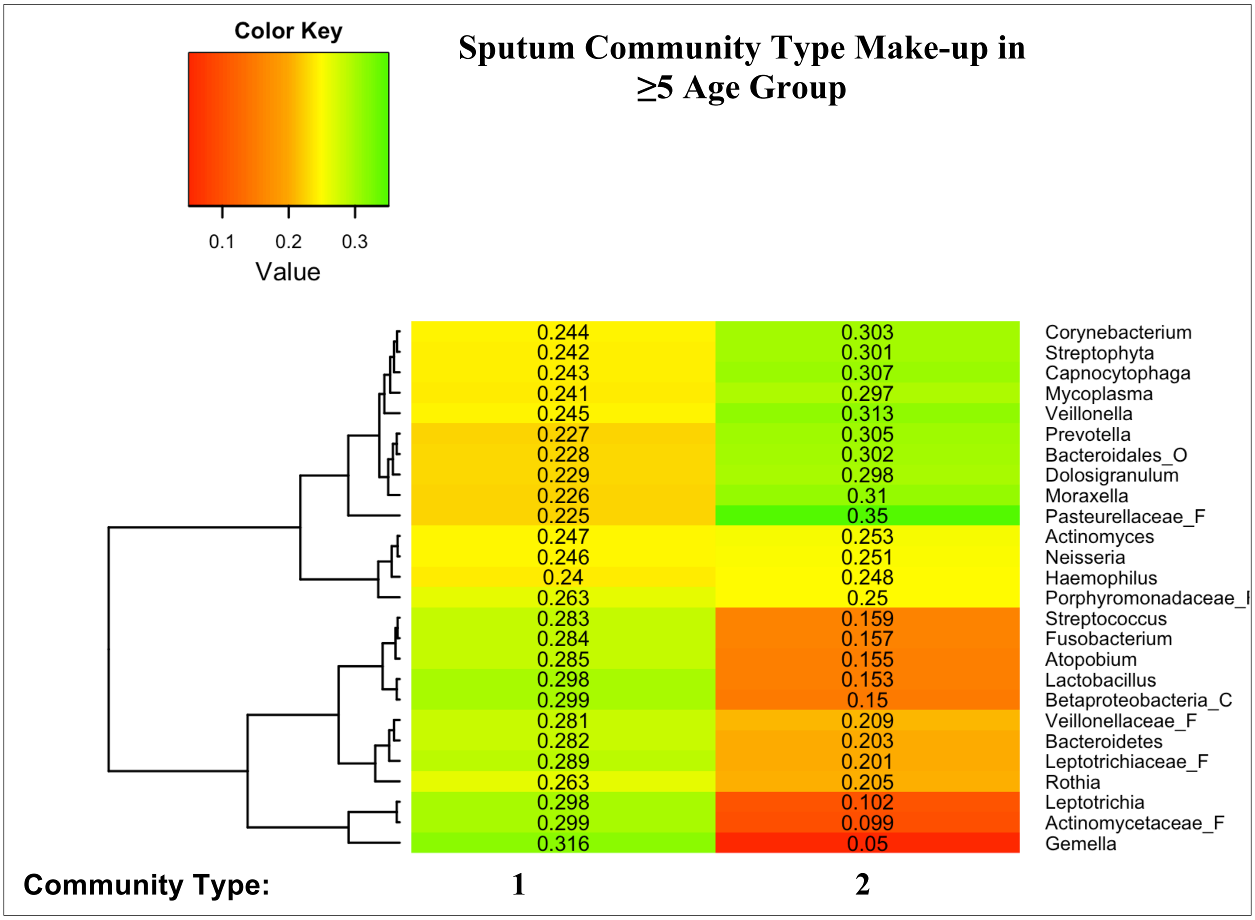


**Figure 3.** Principal Coordinate Analysis generating the community types from the clustering of sputum samples. **a.** Three distinct community types from clustering within the younger age group. **b.** Two distinct community types from clustering within the older age group.



| Taxa | 1 | 2 |
|---|---|---|
| Corynebacterium | 0.244 | 0.303 |
| Streptophyta | 0.242 | 0.301 |
| Capnocytophaga | 0.243 | 0.307 |
| Mycoplasma | 0.241 | 0.297 |
| Veillonella | 0.245 | 0.313 |
| Prevotella | 0.227 | 0.305 |
| Bacteroidales_O | 0.228 | 0.302 |
| Dolosigranulum | 0.229 | 0.298 |
| Moraxella | 0.226 | 0.31 |
| Pasteurellaceae_F | 0.225 | 0.35 |
| Actinomyces | 0.247 | 0.253 |
| Neisseria | 0.246 | 0.251 |
| Haemophilus | 0.24 | 0.248 |
| Porphyromonadaceae_ | 0.263 | 0.25 |
| Streptococcus | 0.283 | 0.159 |
| Fusobacterium | 0.284 | 0.157 |
| Atopobium | 0.285 | 0.155 |
| Lactobacillus | 0.298 | 0.153 |
| Betaproteobacteria_C | 0.299 | 0.15 |
| Veillonellaceae_F | 0.281 | 0.209 |
| Bacteroidetes | 0.282 | 0.203 |
| Leptotrichiaceae_F | 0.289 | 0.201 |
| Rothia | 0.263 | 0.205 |
| Leptotrichia | 0.298 | 0.102 |
| Actinomycetaceae_F | 0.299 | 0.099 |
| Gemella | 0.316 | 0.05 |

**Figure 4.** Heat map of the makeup of the two sputum community types within the older pediatric study population. The heat map represents the proportion of subjects within each community type within the top quartile of each taxa's relative abundances.
(_P) = Phylum
(_F) = Family
(_C) = Class
(_O) = Suborder

**Table 11.** Contingency table for LOS outcome by sputum sample Community Type in the older age group.

| Sputum ≥ 5 years: Community Type | LOS | | Total |
|---|---|---|---|
| | ≥ 4 days | < 4 days | |
| 1 | 5 | 52 | 57 |
| 2 | 19 | 1 | 20 |

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