

Respiratory microbiome profiles differ by recent hospitalization and nursing home residence in patients on mechanical ventilation

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Abstract

Background: Healthcare-associated pneumonia (HCAP) is a heterogeneous disease. We redefined nursing-home- and hospital-associated infections (NHA1) group by revising existing HCAP risk factors. The NHA1 group comprised nursing home residents with a poor functional status, or recent (past 90 days) hospitalization or recent (past 180 days) antibiotic therapy. Our aim was to determine whether respiratory microbiota profiles are related to newly defined NHA1 group in critically ill patients on mechanical ventilation.

Methods: The 180 endotracheal aspirates (ETAs) from 60 mechanically ventilated ICU patients (NHA1 group, n = 24; non-NHA1 group, n = 36) were prospectively collected on days 1, 3 and 7 in a university hospital. The bacterial community profiles of the ETAs were explored by 16S rRNA gene sequencing. A phylogenetic-tree-based microbiome association test (TMAT), generalized linear mixed models (GLMMs), the Wilcoxon test and the reference frame method were used to analyze the association between microbiome abundance and disease phenotype.

Results: The relative abundance of the genus *Corynebacterium* was significantly higher in the pneumonia than in the non-pneumonia group. The microbiome analysis revealed significantly lower α -diversity in the NHA1 group than in the non-NHA1 group. In the analysis of β -diversity, the structure of the microbiome also differed significantly between the two groups (weighted UniFrac distance, Adonis, $P < 0.001$). The abundance of *Corynebacterium* was significantly higher, and the relative abundances of *Granulicatella*, *Staphylococcus*, *Streptococcus* and *Veillonella* were significantly lower, in the NHA1 group than in the non-NHA1 group.

Conclusions: The microbiota signature of the ETAs distinguished between patients with and without risk factors for NHA1. The lung microbiome may serve as a therapeutic target for NHA1 group.

Background

Among the recommendations of the 2016 and 2019 American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) guidelines was the removal of the category of healthcare-associated pneumonia (HCAP)[1, 2]. This is in contrast to the 2005 guidelines, which advocated the use of broad-spectrum antibiotic therapy in HCAP and nosocomial pneumonia[3]. The change was based on several studies showing that, while patient characteristics such as comorbidities and age are determinants of the risk of multidrug-resistant (MDR) infection, this is not the case for all of the HCAP criteria [4–6]. Jones et al. showed that acceptance of the HCAP criteria lowered the threshold for broad-spectrum antibiotics for infection with nosocomial pathogens [7]. However, some HCAP criteria, such as admission from a nursing home or recent (preceding 90 days) hospitalization, are independent predictors of MDR infection [5, 8, 9]. For example, the risk of MDR infections in patients with HCAP requiring mechanical ventilation is similarly high to that of nosocomial infections [10, 11].

Based on previous studies, three risk factors may be better than the previous HCAP criteria for predicting the risk of infection with nosocomial pathogens: recent hospitalization, nursing home residence and antibiotic

use in the past 90 days [5, 8, 12]. The application of new, culture-independent methods of characterizing microbial communities, such as 16S rRNA, may lead to better characterization of patient risk groups and more appropriate therapeutic strategies[13]. We hypothesized that the composition of the bacterial microbiome in the respiratory tract during mechanical ventilation would be related to the newly defined risk factors for nursing-home and hospital-associated infections (NHAf), and to the 28-day all-cause and final hospital mortality rates. Therefore, we analyzed the microbial profiles of the endotracheal tube aspirates (ETAs) of intubated patients and determined the relationship between the ETA microbiome and risk factors for NCAf.

Methods

Patients and study design

Patients admitted to the intensive care unit (ICU) of Chuncheon Sacred Hospital (South Korea) between July 2017 and October 2018 were prospectively recruited. All patients placed on mechanical ventilation at the time of ICU admission were included in the study. The exclusion criteria were age < 18 years, initiation of mechanical ventilation > 48 h after ICU admission and a duration of mechanical ventilation < 7 days.

In 2005, the ATS/IDSA guidelines introduced HCAP as a new category of pneumonia requiring therapy similar to that prescribed for nosocomial pneumonia, namely broad-spectrum antibiotics[3]. However, several studies showed that HCAP is a heterogeneous disease, and that broad-spectrum antibiotic therapy may not be necessary for all patients with HCAP[6, 8, 14]. Therefore, attempts were made to refine the definition of HCAP based on risk factors for MDR infections. On the basis of the available data[5, 8, 10, 12, 14, 15], we redefined NHAf patients as those meeting at least one of the following criteria: (1) nursing home resident with poor functional status; (2) recent (past 90 days) hospitalization; or (3) recent (past 180 days) antibiotic therapy.

The 60 patients (41 with and 19 without pneumonia) enrolled in this study were divided into NHAf and non-NHAf groups and prospectively followed. This study was approved by the Institutional Review Board of Chuncheon Sacred Heart Hospital (IRB approval number: 2017-47).

Data collection and clinical outcome measures

Data on demographic characteristics (age, sex, preexisting comorbidities), the indications for intubation, the presence of acute respiratory distress syndrome (ARDS), the arterial oxygen tension/fraction of inspired oxygen (SaO₂/FiO₂) ratio, and the Glasgow Coma Scale (GCS), Acute Physiology and Chronic Health Evaluation (APACHE II) and Sequential Organ Failure Assessment (SOFA) scores were recorded. The Charlson Comorbidity Index (CCI) score was calculated as described previously[16]. The clinical outcomes of interest in this study were the 28-day all-cause and final hospital mortality rates. Respiratory samples were acquired by endotracheal aspiration, with serial samples collected 1, 3 and 7 days after the initiation of mechanical ventilation.

DNA extraction, PCR and sequencing

Genomic DNA was extracted using a commercial microbial DNA isolation kit (MP Biomedicals, USA) and then amplified using primers targeting the V3–V4 region of the prokaryotic 16S rRNA gene. The amplicons were purified using XSEP MagBead (CELEMICS), and then subjected to PCR using the Nextera Index Kit (Illumina, USA) following the manufacturer's instructions. The resultant product was further purified using XSEP MagBead (CELEMICS). The prepared bacterial amplicon library was then quantified, mixed with multiple libraries and sequenced using the MiSeq v3 platform (Illumina).

The raw sequencing data files were preprocessed before downstream data analysis. To remove sequences with low-quality scores, the raw sequence reads were pre-filtered using PRINSEQ[17]. Adapter sequences were removed using CUTADAPT[18]. Paired-end reads were merged using PEAR[19] and then filtered with PRINSEQ. Chimeric sequences and singletons were screened and reduced using USEARCH[20]. Finally, 180 samples were analyzed and an average of 133,219 reads per sample were obtained (minimum, 29,247; maximum, 587,337). The downstream data analysis was carried out using QIIME[21] with the EzBioCloud 16S rRNA gene sequence database[22]. Operational taxonomic units (OTUs) were defined as clusters of sequences with $\geq 97\%$ identity.

Data processing and statistical analysis

Various Microbial community comparisons were performed using Quantitative Insights in Microbial Ecology software (QIIME, v. 1.8) and the R package Vegan.

The microbial sequences from the ETAs represented 554 genera and 2,009 species. Each subject was sequenced twice at two different time points (day 1 and day 3 or 7). Absolute OTU of each subject for both time points was combined, and species were filtered by two criteria; 1) eliminating strains that appear only in a few patients, 2) removing strains that account for less than 1000 OTU. Finally, 11 predominant genera (*Acinetobacter*, *Streptococcus*, *Corynebacterium*, *Staphylococcus*, *Prevotella*, *Neisseria*, *Veillonella*, *Mycoplasma*, *Granulicatella*, *Actinomyces*, *Campylobacter*) and 8 predominant species (*Acinetobacter baumannii*, *Streptococcus mitis*, *Corynebacterium ulcerans*, *Staphylococcus caprae*, *Veillonell adispar*, *Granulicatella adiacens*, *Streptococcus parasanguinis*, *Streptococcus lactarius*) were included in the statistical analysis.

Ace, Chao1, the Shannon index and the Simpson index were used to express α -diversity; β -diversity (inter-sample diversity) was defined as the extent of the similarity between microbial communities based on the degree of structural overlap. Nonmetric multidimensional scaling plots based on weighted UniFrac distances were used to visualize the differences between groups in microbial community structure. Principle coordinate analysis was conducted using a permutational multivariate ANOVA (PERMANOVA), performed via the Adonis function of the R package vegan (1,000 permutations).

We conducted statistical analysis to identify the association of pneumonia and NHA1 risk factor on the abundance with each OTU. Each subject was measured at two different time points and generalized linear mixed effects models (GLMM) was utilized to handle the repeatedly observed measurement. Pneumonia and NHA1 risk factor were considered as response variables. Relative abundances of OTUs at each time point were Log2 transformed, and its effect was evaluated by including it as a covariate. We also

considered the paired and longitudinal distance-based approach (Pldist) to detect the longitudinal changes in the microbiome over time with various outcome types[23]. It can also calculate differences in non-phylogenetic dissimilarities such as Gower's distance, Bray-Curtis dissimilarities, Jaccard distance and Kulczynski distance between time points. Last we found that pneumonia and NHA1 risk factor are time-invariant and thus considered three methods for cross-sectional data; phylogenetic tree-based microbiome association test (TMAT), Wilcoxon test and reference frame method[24, 25]. For the cross-sectional methods, means of log-transformed read count per million (CPM) at two different point were considered as the response variables. Among all approaches, only GLMM and TMAT can adjust the effect of the other covariates and age, sex, APACHE score and CCI were included as covariates for both. GLMM was conducted with SAS 9.4 (Cary, NC), and the other analyses were done with R software. All test results were considered significant using a p value of ≤ 0.05 .

Results

Characteristics of the study participants

The study population consisted of 60 patients who had been placed on mechanical ventilation, including 41 patients diagnosed with pneumonia and 24 who had risk factors for NHA1. Table 1 shows the patient characteristics. The proportions of ARDS, a high C-reactive protein (CRP) level and an abnormal paO_2/FiO_2 ratio were higher in patients with than without pneumonia, as expected. Patients in the NHA1 and non-NHA1 groups did not differ in terms of sex, severity index (APACHE and SOFA) scores, or the GCS score. However, patients in the NHA1 group were older and had more comorbidities than the non-NHA1 group. There were no significant differences between the two groups in either the 28-day all-cause or final hospital mortality rate.

Table 1
Baseline characteristics of the study participants.

	Pneumonia	Non-pneumonia	<i>P</i> value	NHAI	Non NHAI	<i>P</i> value
	n = 41	n = 19		n = 24	n = 36	
Age ¶	73 (61–79)	76 (59–81)	0.744	78 (72–85)	71 (57–77)	0.004
Male †	30 (73.2%)	12 (63.2%)	0.431	15 (62.5%)	27 (75.0%)	0.391
ARDS	8 (19.5%)	0 (0%)	0.047	4 (16.7%)	4 (11.1%)	0.702
Pneumonia				23 (95.8%)	19 (52.8%)	< 0.001
Charlson Comorbidity Index ¶	3 (1–4)		0.085	3 (2.3-5.0)	1.5 (0-2.8)	< 0.001
Cause of intubation			< 0.001			< 0.001
Cardiac arrest	1 (2.4%)	3 (15.8%)		0 (0.0%)	4 (11.1%)	
Neurological distress	5 (12.2%)	13 (68.4%)		1 (4.2%)	17 (47.2%)	
Post-operative status	0 (0%)	1 (5.3%)		0 (0.0%)	1 (2.8%)	
Respiratory	35 (85.4%)	2 (10.5%)		23 (95.8%)	14 (38.9%)	
PaO ₂ /FiO ₂ ratio	212 (133.5–299)	431 (321–458)	< 0.001	222 (131.3–301.0)	323 (184.3–442.5)	0.022
Severity						
APACHE II score ¶	20 (16–24)	22 (17–25)	0.202	20.5 (16.3–24.0)	21 (17–25)	0.634
SOFA score ¶	7 (6–9)	6 (4–9)	0.117	7 (6–9)	7 (5–9)	0.569
GCS ¶	8 (6–11)	6 (5–9)	0.074	8.5(6.0-10.8)	7.5 (6–9)	0.303
Extubation success in 3 weeks	23 (56.1%)	11 (57.9%)	0.999	14 (58.3%)	20 (55.6%)	0.999
28-day all-cause mortality	12 (29.3%)	6 (31.6%)	0.999	7 (29.2%)	11 (30.6%)	0.999
Final hospital mortality	19 (46.3%)	6 (31.7%)	0.4	11 (45.8%)	14 (38.9%)	0.606
MV duration ¶	13 (8–18)	10 (7–16)	0.335	13 (8-17.5)	10 (7.0-16.8)	0.384

¶ median (interquartile range) †frequency (%) § median (range)

	Pneumonia	Non-pneumonia	<i>P</i> value	NHAI	Non NHAI	<i>P</i> value
	n = 41	n = 19		n = 24	n = 36	
CRP (mg/dL)	133 (45-213.5)	21 (5-132)	0.008	124.5 (62.8-192.0)	94 (8.0-194.0)	0.127
¶ median (interquartile range) †frequency (%) § median (range)						

Differences in the microbial composition of the ETAs between patient groups (pneumonia vs non-pneumonia, NHAI vs. non-NHAI) are shown in the taxonomic summary (Fig. 1). The average abundance of the genera *Corynebacterium*, *Staphylococcus* and *Pseudomonas* was higher in the pneumonia group than in the non-pneumonia group. The relative abundances of *Streptococcus* and *Prevotella* were higher in the non-NHAI group, whereas the relative abundance of *Corynebacterium* was higher in the NHAI group.

Respiratory microbiota during mechanical ventilation in patients with and without pneumonia

Neither α -diversity nor β -diversity differed significantly between patients with and without pneumonia. The association between pneumonia and microbial taxa was analyzed (Table 2), adjusting for the effects of age, sex, and APACHE and CCI scores in the TMAT, optimal microbiome-based association test (OMiAT), and GLMM, but not in the Wilcoxon test. A correlation between *Corynebacterium* and pneumonia was demonstrated by the TMAT, while the GLMMs identified a significant difference in the abundance of *C. ulcerans* between patients with and without pneumonia ($P = 0.035$).

Table 2
Bacterial genera associated with pneumonia.

<p>NHAI: nursing-home- and hospital-associated infections</p> <p>ARDS: acute respiratory distress syndrome</p> <p>APACHE II: Acute Physiology and Chronic Health Evaluation II</p>								
<p>SOFA: Sequential Organ Failure Assessment</p>								
<p>GCS: Glasgow Coma Scale</p>								
<p>MV: mechanical ventilation</p> <p>CRP: C-reactive protein</p>								
Genus	FDR- OMiAT	Beta TMAT	FDR- TMAT	Estimate- RF	FDR- RF	FDR- Wilcoxon	β- GLMM	FDR- GLMM
<i>Acinetobacter</i>	0.7832	0.02	0.7321	NA	NA	0.6809	0.014	0.7855

FDR: false discovery rate

OMiAT: optimal microbiome-based association test

TMAT: phylogenetic-tree-based microbiome association test

RF: reference frame

GLMM: generalized linear mixed model

NHAI: nursing-home- and hospital-associated infections								
ARDS: acute respiratory distress syndrome								
APACHE II: Acute Physiology and Chronic Health Evaluation II								
<i>Corynebacterium</i>	0.2791	0.1	0.0226	-2.03	0.9985	0.005	0.102	0.4899
<i>Granulicatella</i>	0.7832	-0.04	0.3564	-1.89	0.9985	0.3817	-0.104	0.4899
<i>Staphylococcus</i>	0.7832	-0.01	0.8202	0.09	0.9985	0.9556	0.026	0.725
<i>Streptococcus</i>	0.2791	-0.03	0.7321	0	0.9985	0.3817	-0.04	0.6638
<i>Veillonella</i>	0.7832	-0.03	0.708	0.35	0.9985	0.6809	-0.065	0.5494
FDR: false discovery rate								
OMiAT: optimal microbiome-based association test								
TMAT: phylogenetic-tree-based microbiome association test								
RF: reference frame								
GLMM: generalized linear mixed model								

Figure 2a shows the increased relative abundance of the genus *Corynebacterium* in the pneumonia than in the non-pneumonia group. The difference of this genus abundance was mainly caused by a species named *C. ulcerans*. No other *Corynebacterium* species detected in the samples showed meaningful difference between the pneumonia and non-pneumonia groups. Figure 2b shows the larger increase in *C. ulcerans* in the pneumonia than in the non-pneumonia group, both on day 1 and on day 7. A trend towards a larger difference in the OTU abundance of *Corynebacterium* in the pneumonia group compared to the non-pneumonia group on day 7 was also identified.

Respiratory microbiota during mechanical ventilation in patients with and without risk factors for NHAI

An analysis of α -diversity revealed significant differences between the NHAI and non-NHAI groups (expressed as median [interquartile range]; Fig. 3a). Ace (67.5 [48.2, 108.9] vs. 139.9 [106.2, 215.7]; $P < 0.001$), Chao (64.4 [45.3, 112.0] vs. 137.5 [106.2, 230.6]; $P < 0.001$), Shannon index (2.3 [0.7, 2.9] vs. 3.8 [2.3, 4.7]; $P < 0.001$) and Simpson index (0.7 [0.2, 0.8] vs. 0.9 [0.6, 0.9]; $P < 0.001$) values were lower in the NHAI group than in the non-NHAI group. The microbiome structure of the NHAI group could also be clearly distinguished from that of the non-NHAI group based on the β -diversity (PERMANOVA, adjusted $P < 0.001$, Fig. 3b).

The microbial taxa associated with NHA1 were identified using the methods shown in Table 3. Adjustments were made for age, sex, and the APACHE and CCI scores in the TMAT, OMiAT, and GLMMs, but not in the Wilcoxon test. The TMAT and Wilcoxon analyses revealed that *Granulicatella*, *Streptococcus* and *Veillonella* were negatively correlated with NHA1, while *Corynebacterium* was positively correlated (Table 3).

Table 3
Bacterial genera associated with NHA1.

Genus	FDR-OMiAT	β -TMAT	FDR-TMAT	Estimate-RF	FDR-RF	FDR-Wilcoxon	Beta-GLMM	FDR-GLMM
<i>Acinetobacter</i>	0.5861	-0.08	0.0807		NA	0.52	-0.039	0.6165
<i>Corynebacterium</i>	0.1788	0.2	0.0004	3.21	0.3302	< 0.05	0.17	0.2166
<i>Granulicatella</i>	0.1911	-0.18	0.0002	-1.15	0.8524	< 0.05	-0.318	0.0385
<i>Staphylococcus</i>	0.2129	-0.14	0.023	-2.22	0.8524	0.344	-0.142	0.2166
<i>Streptococcus</i>	0.2129	-0.09	0.0132	3.1	0.8524	0.0045	-0.067	0.4439
<i>Veillonella</i>	0.5861	-0.11	0.0101	-0.39	0.8611	0.0045	-0.136	0.2166
FDR: false discovery rate								
OMiAT: optimal microbiome-based association test								
TMAT: phylogenetic-tree-based microbiome association test								
RF: reference frame								
GLMM: generalized linear mixed model								

Similarly, the GLMMs revealed that the genera *Granulicatella* and *Prevotella* were more abundant in the non-NHA1 group, while at the species level *C. ulcerans* was more abundant in the NHA1 group and *Granulicatella adiacens* was more abundant in the non-NHA1 group (Supplementary Table 1).

Supplementary Fig. 1 shows the longitudinal trends in microbial taxa according to the risk factors for NHA1. In ETAs from days 1 and 7, the OTU abundances of *Veillonella*, *Granulicatella* and *Streptococcus* were significantly higher in the non-NHA1 than in the NHA1 group, whereas *Corynebacterium* was more abundant in the NHA1 group. However, none of the longitudinal changes in microbial taxa differed significantly by disease phenotype.

Longitudinal change in the respiratory microbiome according to clinical outcome

The associations between non-phylogenetic dissimilarities (based on pldist) and the clinical outcomes (the 28-day all-cause and final hospital mortality rates) were analyzed using the microbiome regression-based kernel association test (MiRKAT). The results showed that the variability in the respiratory microbiome was not associated with the clinical outcome (Table 4). The GLMM analysis yielded similar results. There was

no association of microbial OTU abundance with the 28-day all-cause or final hospital mortality rate, in either the pneumonia or non-pneumonia group (Supplementary Table 2).

Table 4

Association between microbiome variability and clinical outcome. P values are from the MiRKAT. The quantitative and qualitative analyses (Bray-Curtis, Jaccard, Kulczynski and Gower analyses) were adjusted for sex, age, and APACHE II and CCI scores.

	28-day all-cause mortality		Final hospital mortality	
	Species	Genus	Species	Genus
Total (n = 60)				
Bray-Curtis	0.15	0.63	0.45	0.24
Jaccard	0.33	0.57	0.48	0.22
Kulczynski	0.34	0.58	0.42	0.32
Gower	0.29	0.45	0.43	0.23
Pneumonia (n = 41)				
Bray-Curtis	0.3	0.76	0.54	0.36
Jaccard	0.51	0.63	0.43	0.26
Kulczynski	0.5	0.69	0.28	0.3
Gower	0.29	0.63	0.38	0.44
Non-pneumonia (n = 19)				
Bray-Curtis	0.65	0.14	0.65	0.14
Jaccard	0.66	0.5	0.66	0.5
Kulczynski	0.75	0.62	0.75	0.62
Gower	0.71	0.13	0.71	0.13

Discussion

Patients with HCAP are a heterogeneous group[14]. Because the existing HCAP criteria did not reflect the risk of nosocomial pathogen infection and led to overuse of broad-spectrum antibiotics, they were omitted from the 2016 hospital-acquired pneumonia/ventilator-associated pneumonia (HAP/VAP) guidelines [1, 7]. Attempts to develop alternative criteria for HCAP, and a specific therapy [8, 11, 14], included our concept of NHA1, which may better predict the occurrence of MDR infections and consequences of continued exposure to hospital and other healthcare environments.

Recent studies using conventional microbiologic culture tests have shown different patterns of infection among countries and geographic regions [26–29]. Studies from the USA and the Asia/Pacific region reported that HCAP is characterized by high frequencies of infections with drug-resistant pathogens,[26–28, 30] whereas other studies showed similarities in the involved pathogens between HCAP and community-acquired pneumonia (CAP).[29]

Culture-independent 16S rRNA gene sequencing is useful for detecting fastidious bacteria, the abundance of which tends to be underestimated; this method is more sensitive than conventional culture methods [31]. To our knowledge, this is the first study to use microbiome sequencing to determine the associations of healthcare/hospital-related factors with the composition of the respiratory microbiome of intubated patients.

The most important finding of this study was that the ETA microbiome profile of intubated patients was significantly associated with newly defined risk factors for NHA1. Generally, microbial diversity was higher in patients without than with risk factors for NHA1. The analysis of β -diversity, based on weighted UniFrac distances, showed distinct clusters of samples, identified as NHA1 and non-NHA1 groups. Beyond microbial diversity, our study showed the association of specific bacterial genera with continued exposure to healthcare and hospital environments.

The abundance of *Corynebacterium* increased, whereas the abundances of *Granulicatella*, *Streptococcus*, *Staphylococcus* and *Veillonella* decreased, in intubated patients with risk factors for NHA1. In particular, members of the genus *Corynebacterium* were significantly more abundant in intubated patients with than without pneumonia, and the abundance of *C. ulcerans* in the pneumonia group tended to increase over time. Although *Corynebacterium* spp. are among the commensal flora of the nasopharynx[32], a causative role in pneumonia was recently described[33–36]. The identification of *Corynebacteria* subspecies is now possible using new diagnostic technologies, such as matrix-assisted laser desorption ionization time of flight mass spectrometry and 16S rRNA gene sequencing[13, 33–35, 37]. Based on such studies, Clariot et al. suggested that *Corynebacterium* spp. may be responsible for pneumonia in mechanically ventilated patients, similar to our results[33], while Yasuda et al. reported that *C. ulcerans* produces diphtheria toxin and causes severe pneumonia complicated by diffuse pseudomembrane formation in the central airways[36]. *Corynebacterium* spp. may also be responsible for HAP. An analysis of bronchoalveolar lavage fluid (BALF) obtained from Japanese patients revealed *Corynebacterium* spp. in the samples of 11.8% of the patients with HAP, and in 4.9% of those from patients with HCAP; however, *Corynebacterium* spp. was present in only 1.6% of the samples from patients with CAP[37]. Our results also demonstrate the need to consider *Corynebacterium* spp. as a possible healthcare/hospital-associated pathogen.

Several studies using molecular techniques have revealed the presence of unexpected microorganisms in specific phenotypes of pneumonia. For example, Steenhuisen et al. found an increase in *Lactobacillus* and *Rothia* in CAP, while Zarkharkina et al. reported a decrease in these microorganisms, in patients with VAP [38, 39]. Similar to our own findings, a study of adult ICU patients identified *Prevotella* as being dominant in controls, whereas *Corynebacteria* species were uniquely present in VAP cohorts [40].

Segal et al. reported that a lung microbiome containing taxa from the oral cavity (i.e., *Streptococcus*, *Veillonella*, *Granulicatella*, and *Prevotella*) induced host cellular mucosal immunity of the Th17/neutrophilic phenotype and blunted TLR4 responses [41]. In the study of Kitsios et al., the dominance of oral taxa within lung communities was strongly associated with culture negativity and weaker host immune responses [42]. From these data, it can be inferred that non-NHAI patients in whom oral taxa dominate the lung microbiome will have a weaker immune response than NHAI patients, which would also explain why patients with HCAP present with more severe disease and have clinical outcomes resembling those of nosocomial pneumonia [26, 28, 43].

Our data showed no difference in microbial community according to clinical outcome; this is in contrast to Dickson et al., who reported that the lung microbiome predicts outcomes in critically ill patients [44]. This discordance may be due to differences in study population and sampling method (ETA vs. BALF).

Our study had several limitations. First, it used a single-center design and the sample size was small. Multicenter studies with larger populations are needed to validate our results. Second, we did not obtain BALF samples, although this was in accordance with ATS guidelines recommending non-invasive testing[1]. Nevertheless, analyzing BAL and ETA samples may enable comparison with the results of other studies, such that more robust conclusions could be drawn. Third, although our findings support the concept of an NHAI risk factor-associated microbiome, its clinical significance should be investigated via functional analyses, for example. Such studies would allow conclusions to be drawn regarding the association between an NHAI-specific microbiome and outcome/immune metabolic responses.

The strengths of our study included the homogeneity of the study population in terms of age (all elderly patients), and the multiple serial samples acquired from each patient. The analysis showed that the composition of the NHAI-related microbiome was similar regardless of antibiotic use or time of day.

Conclusions

In this prospective observational cohort study of mechanically ventilated patients, the loss of diversity and dysbiosis of the respiratory microbiome were more profound in patients with than without risk factors for NHAI, which were in turn positively associated with the presence of *Corynebacterium*, and negatively associated with that of *Granulicatella*, *Streptococcus*, *Staphylococcus* and *Veillonella*. Prospective studies are needed to determine the clinical relevance of microbial community profiling, and to identify novel therapeutic targets in critically ill elderly patients with risk factors for NHAI.

Additional information

The supplementary figures and tables can be found in the Supplemental Materials section of the online article.

List Of Abbreviations

HCAP: Healthcare-associated pneumonia

NHA: nursing home and hospital associated infection

ICU: intensive care unit

TMAT: tree-based microbiome association test

GLMM: generalized linear mixed effects models

ETA: endotracheal tube aspirates

OTU: Operational taxonomic units

CAP: community acquired pneumonia

VAP: ventilator associated pneumonia

MDR: multidrug resistant

APACHE II: Acute Physiology, Age, Chronic Health Evaluation II

SOFA: Sequential Organ Failure Assessment

CCI: Charlson comorbidity index

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Chuncheon Sacred Heart Hospital (IRB approval number: 2017-47). Informed consent was obtained from all patients or their legal representatives. This research was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions:

MB, CB, SW, YK and HY had the idea for and designed the study and had full access to all data in the study and take responsibility for the integrity of the data analysis. JYH and JLL wrote the first full draft of the report. MB, NEK, CB, SW, YK, JLL, HY and JYH contributed to critical revision of the report. NEK, SW and HY contributed to the statistical analysis. All authors contributed to data acquisition, data analysis, or data interpretation, and reviewed and approved the final version.

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Figures

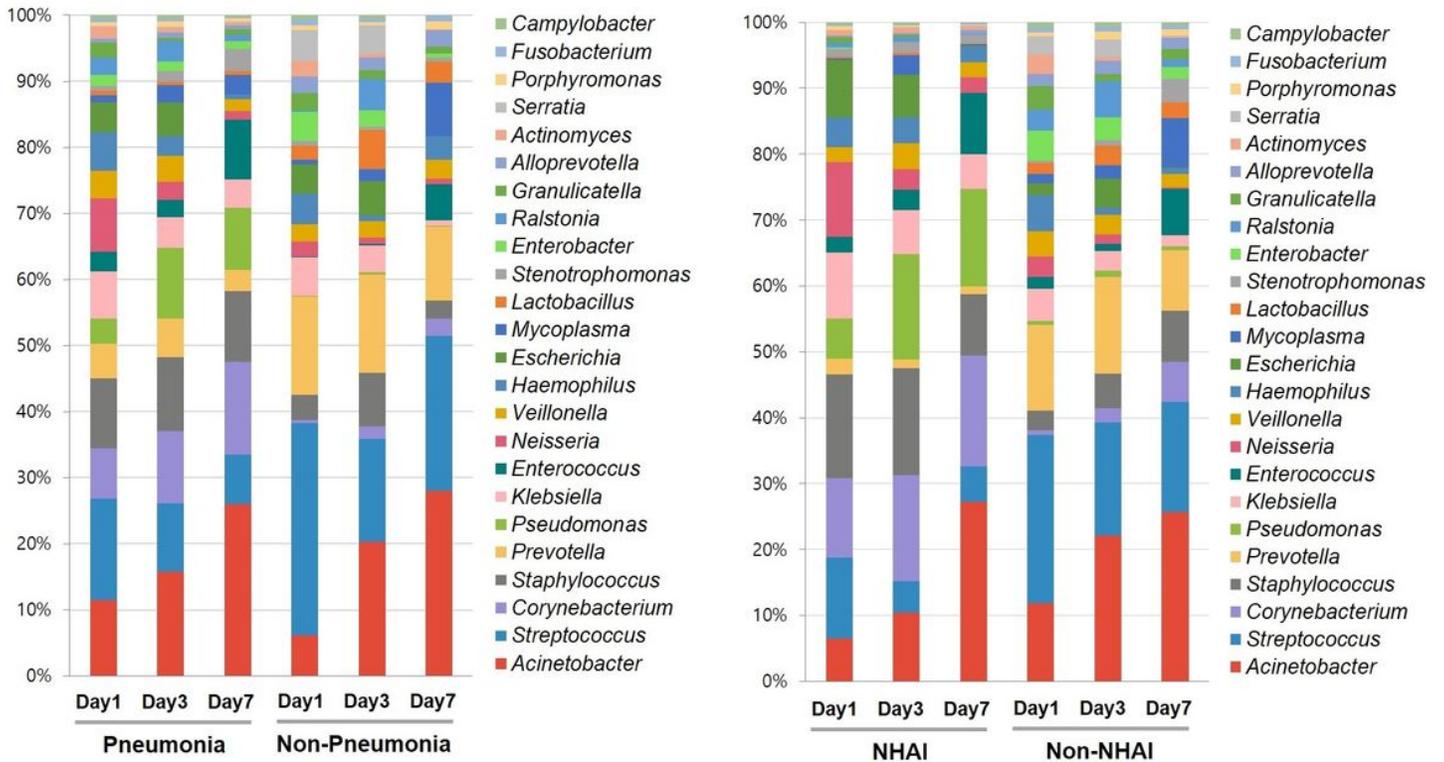


Figure 1

Relative abundance of bacterial communities in the endotracheal aspirates of the study participants over time. Taxonomic summaries of the 180 samples are shown for pneumonia and non-pneumonia patients (a) and for NHAJ and non-NHAJ patients (b).

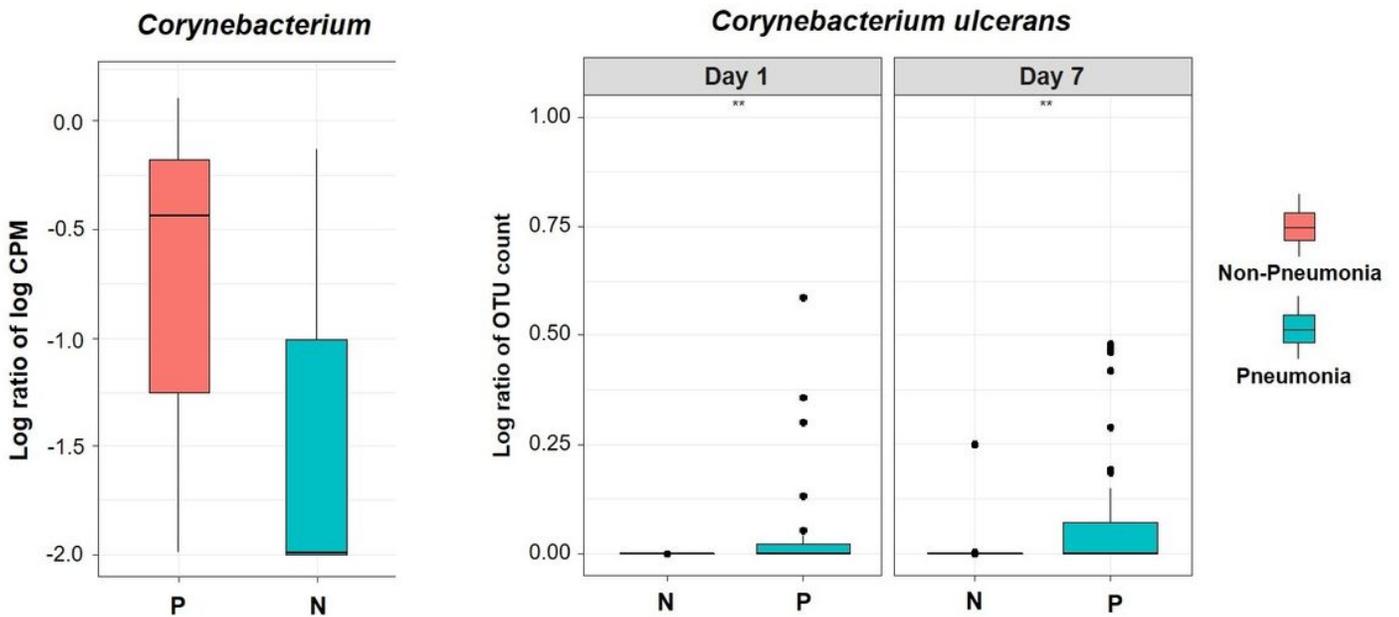


Figure 2

Increased abundance of *Corynebacterium ulcerans* in patients with pneumonia. (a) More frequent occurrence of *Corynebacterium* OTUs in the pneumonia and non-pneumonia groups, respectively. (b) Longitudinal patterns of relative abundance of *C. ulcerans* OTUs in the pneumonia and non-pneumonia groups.

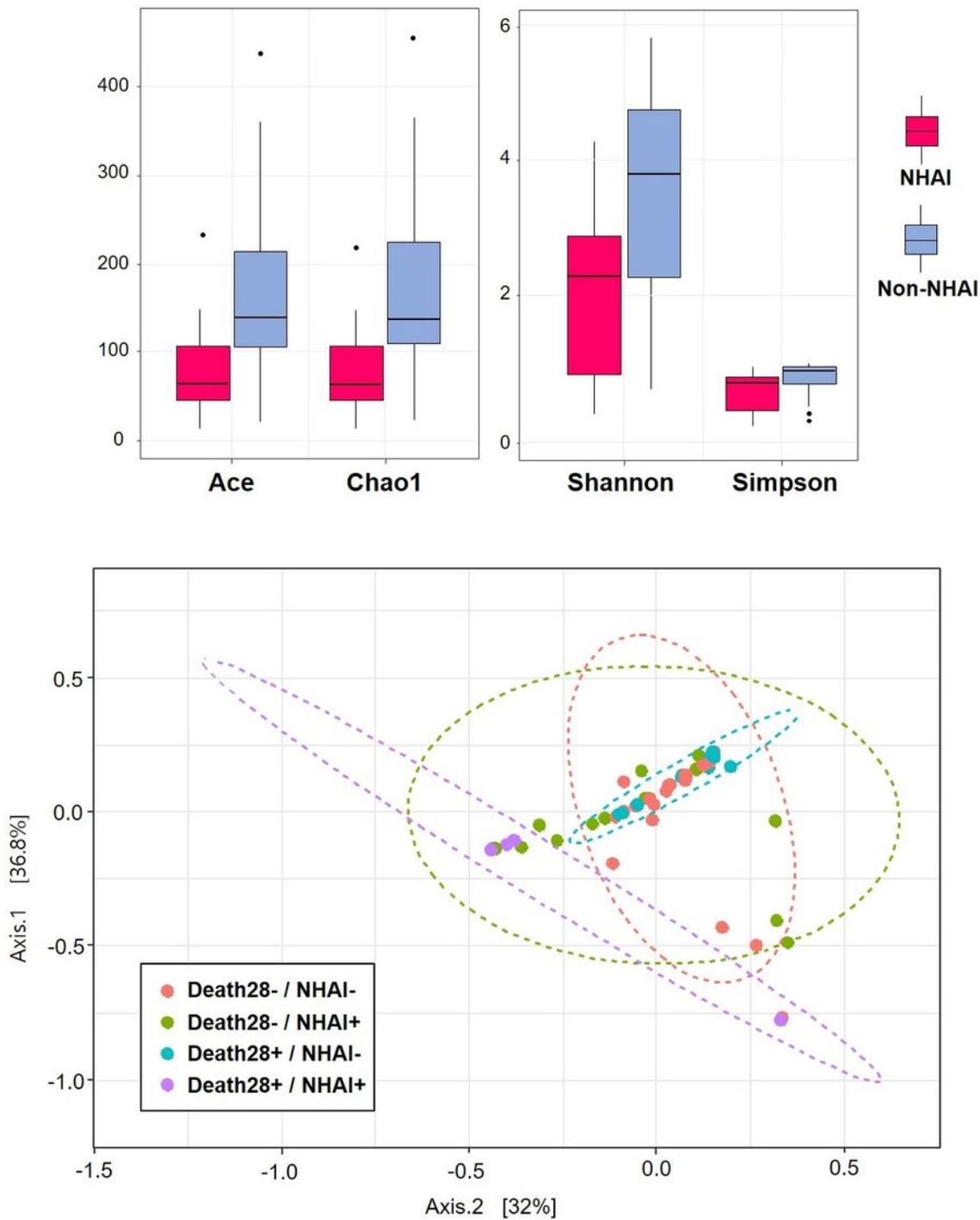


Figure 3

(a) Comparison of the different metrics of α -diversity between the NHA1 group (group 1) and non-NHA1 group (group 0). (b) Microbial community structure in the endotracheal aspirates according to risk factors

for NHAJ based on weighted UniFrac distances. A PERMANOVA, performed using the Adonis function in the R package vegan) was conducted to compare the microbial community structure between the NHAJ and non-NHAJ groups. The analysis was adjusted for age, sex, APACHE score and Charlson Comorbidity Index score.

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