Compositional alteration of the nasal microbiome and Staphylococcus aureus–characterized dysbiosis in the nasal mucosa of patients with allergic rhinitis

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Running title: Dysbiosis of nasal microbiome in allergic rhinitis

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Acknowledgement

This work was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI15C0694 to WY Kim). This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2016R1D1A1B01014116 and 2019M3C9A6091945 to HJK). This research was also supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare of the Republic of Korea (HI20C0546 to HJK).

Author contributions

Conceptualization: HJK, WK. Data curation: J-HK. Formal analysis: SH. Methodology: HJK, J-HK. Project administration: HJK, WK. Visualization: HJK. Writing–original draft: HJK. Writing– review & editing: WK
Conflict of interests

The authors declare that they have no conflict of interest.

Financial disclosure

No financial support or disclosures to declare.
Highlights

- A significantly distinctive colonization pattern of *Firmicutes*, *Actinobacteria*, and *Proteobacteria* phyla in the nasal mucus of patients with allergic rhinitis (AR) compared to healthy participants.

- Nasal commensals classified into both unclassified *Enterobacteriaceae* and *Lactococcus* genera were more abundant in the nasal mucus of AR patients. The distribution of *Staphylococcus* genus also exhibited the noticeable alterations between healthy participants and patients with AR.

- The dysbiosis of *S. aureus* was characterized in the nasal mucus of AR patients and the abundance of *S. aureus* in AR nasal mucus depended on AR patients’ age, height, and the sensitization of house dust mites.
Abstract

Objectives. Host–microbial commensalism can shape the innate immune responses in
the nasal mucosa, and the microbial characteristics of the nasal mucus directly impact
the mechanisms of initial allergic responses in the nasal epithelium. We sought to
determine alterations of the microbial composition in the nasal mucus of patients with
allergic rhinitis (AR) and to elucidate the interplay between dysbiosis of the nasal
microbiome and allergic inflammation.

Methods. A total of 364,923 high-quality bacterial 16S ribosomal RNA–encoding gene
sequence reads from 104 samples from the middle turbinate mucosa of healthy
participants and patients with AR was obtained and analyzed using the Quantitative
Insights into Microbial Ecology pipeline.

Results. We analyzed the microbiota in samples of nasal mucus from patients with AR
(n = 42) and clinically healthy participants (n = 30). Proteobacteria (Ralstonia genus)
and Actinobacteria (Propionibacterium genus) phyla were predominant in the nasal
mucus of healthy subjects, whereas the Firmicutes (Staphylococcus genus) phylum was
significantly abundant in the nasal mucus of patients with AR. Especially, Ralstonia
genus were significantly dominant in the clinically healthy subjects. Additional
pyrosequencing data from 32 subjects (healthy participants: N=15, AR patients: N=17)
revealed a greater abundance of Staphylococcus epidermidis, Corynebacterium
accolens, and Nocardia coeliaca, accounting for 41.55% of mapped sequences in the
nasal mucus of healthy participants. The dysbiosis of nasal microbiome was more
pronounced and Staphylococcus aureus exhibited the greatest abundance (37.69%) in
the presence of microbial distribution in the nasal mucus of patients with AR depending on the positive response to house dust mites and patient age and height.

Conclusions. This study revealed alterations in the nasal microbiome that occur in the nasal mucus of patients with AR at the levels of microbial genera and species. S. aureus–dominant dysbiosis was distinctive in the nasal mucus of patients with AR, suggesting a role of host–microbial commensalism in allergic inflammation.

Keywords: Allergic rhinitis; nasal mucosa; Dysbiosis; Nasal microbiome; Staphylococcus species.
Introduction

Allergic rhinitis (AR), an immunoglobulin (Ig) E- and T helper (Th)2-mediated inflammatory nasal disease, is caused by sensitized immune responses to inhaled allergens such as pollens, environmental fungi, dust mites, bacteria, and animal dander. This allergen-specific immune response is thought to arise from an imbalance in Th1–Th2 immune regulation that results in increased levels of Th2 cytokines [1,2]. Nasal epithelial cells exposed to external allergens induce Th2 inflammatory responses that then spread to the upper airway mucosa [3,4]. The allergen-mediated inflammatory immune response begins with increased secretion of epithelial cell–derived cytokines, and the process of allergen sensitization involves the participation of antigen-presenting cells, T lymphocytes, and B lymphocytes and depends on environmental allergen exposure. Sensitization results in generation of allergen-specific IgE, which circulates in the peripheral blood and attaches itself to the surface of all mast cells and basophils, including those of the nasal mucosa [1,2]. Subsequent nasal exposure to allergen activates these cells; through the release of classic mediators of the allergic reaction, acute nasal symptoms are triggered. Therefore, the nasal epithelium might be responsible for the vast majority of allergic inflammation in response to inhaled allergens, and research about regulation of epithelial cell–derived cytokines is needed to develop a more effective approach to treat AR [4,5].

Human mucosal surfaces are in direct contact with the external environment and are susceptible to invasion and colonization by various allergens and pathogens [6]. The respiratory mucosa is exposed constantly to inhaled pathogens and allergens that directly impact mucosal immune mechanisms [7]. Studies focusing on interaction
between the mucosal microbiome and the host increasingly consider the contribution of mucosal immune responses and specific microbiome-mediated protection from external pathogens to integrate environmental signals [7]. The most commonly studied symbiont microbiota are those of the intestinal mucosa, which are essential for formation of intestinal immune responses in health and disease and dysbiosis of the intestinal mucosal microbiota and disruption of its close interactions with the host can destroy this steady-state immune balance in concert with development of chronic inflammatory diseases [8,9]. The lungs and airways harbor diverse composition of microbes and to date, many studies have confirmed that the microbiome of respiratory tract can not only influence susceptibility or causes of respiratory diseases but be affected by disease activities of respiratory diseases as well as in responses to treatment [10-12]. The presence of microbiota in the respiratory mucosa and the relation between alterations in the constituents and the respiratory immunity suggest that the airway microbiota plays a role in chronic airway diseases, such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis [13,14].

Inhaled allergens first encounter the host immune system in the nasal mucosa, and microbial characteristics of the nasal mucus directly impact the mechanisms of initial allergic responses in the nasal epithelium [15]. Research into the role of the microbiome in the sinonasal diseases is rapidly expanding and insights into the microbiota and dysbiosis of the allergic nasal mucosa provide fundamental information regarding susceptibility to allergens and their relationship to allergic inflammation [16,17]. Further studies must be conducted to verify potential functions or modifications of the airway microbiota in AR, but current evidence points to effects on disease progression and
exacerbation. Therefore, alterations to the airway microbiota can influence the consequences of AR and investigating the microbial balance might provide new insights into the pathogenesis of these diseases and at fundamental therapeutic strategies. Based on the current data, we identified *Ralstonia, Staphylococcus*, and *Propionibacterium* spp. as the most abundant constituents in healthy nasal mucus and contributors to the significant dysbiosis of microbial composition in the nasal mucus of patients with AR. Our study presents evidence that colonization of *Staphylococcus* spp. was altered most significantly in the nasal mucus of patients with AR, and *Staphylococcus aureus* contributes to dysbiosis in allergic nasal mucus in a manner dependent on patient clinical factors.
Materials and Methods

Participant Recruitment

A total of 104 participants (45 healthy participants, 59 AR patients) were enrolled in this study, and the difference of nasal microbial genus was first analyzed using the nasal mucus from 72 participants (30 healthy participants and 42 patients with AR), stratified by sex as 44 men (mean body mass index, 21.8 kg/m²) and 28 women (mean body mass index, 22.3 kg/m²) with a mean age of 32.5 years. Eligible individuals for inclusion in this study included those referred to the Department of Otolaryngology Chung-Ang university college of medicine from November 2013 to April 2014. Intranasal endoscopy, computed tomography of the paranasal sinus, and AR tests were performed before intranasal sampling; none of the study participants showed signs of upper airway infection. Subjects who had taken any kind of antibiotics two months prior; were pregnant or smoker; had diseases and medication histories related to asthma; and/or had any other chronic diseases, such as atherosclerosis, hypertension, arrhythmia, congestive heart failure, diabetes mellitus, osteoporosis, hepatitis, cancer, and autoimmune or neurological diseases, were excluded. Study participation was voluntary, and written informed consent was obtained from all participants. The Institutional Review Board (IRB) approved the protocol of this study (IRB approval no. C2013158 [1118]). The nasal microbial composition at the species level was further analyzed using nasal mucus from 32 participants, stratified as 15 healthy participants (mean age, 34.3 years) and 17 patients with AR (mean age, 37.1 years), who were referred to the Department of Otorhinolaryngology Seoul national university college of medicine primarily for septal surgery between March 2018 and January 2019 (IRB approval no.
To confirm AR, they underwent a skin prick test or a multi-allergen simultaneous test (MAST) using the optigen allergen specific IgE assay system korean inhalent panel (Minaris Medical America, Inc, CA, USA) for the detection of allergens and specific IgEs. Among them, forty-five subjects were classified into healthy subjects who were negative for all antigens and had no symptoms such as runny nose, nasal obstruction, itching sensation and sneezing. Fifty-nine who were confirmed positive for a specific antigen and showed symptoms of rhinitis were classified as AR subjects.

**Mucus Collection and DNA Extraction**

Mucus was collected from the middle turbinate individually using sterile 3M Quick swabs (3M Microbiology Products, St. Paul, MN, USA) from 72 participants (30 healthy participants and 42 patients with AR) using a rigid 0° endoscope in an operating room. The cotton swabs were inserted into the nasal cavities of the participants without touching either the nostril or the anterior part of the inferior turbinate, which are lined with stratified squamous epithelium, and were gently rotated around the middle turbinate lined with respiratory epithelium. The swabs with mucus were fixed in a fixative solution and transported immediately to the laboratory for identification and microbial analysis. Each swab sample was centrifuged at 12,000 × g for 15 minutes, and the supernatant was removed. To detect contamination, negative controls were prepared and subjected to the same procedures. The first step in the analysis was extraction of DNA from the bacterial pellet; here, bacterial DNA from middle turbinate mucosal swab samples was extracted with the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). The extracted DNA was dissolved in sterile water containing 40 µg/mL RNase A
and quantified with a Nano Quant Infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland) as the ratio of absorbance values at 260 and 280 nm (A260/A280).

**Microbiome Analysis**

DNA from each swab sample was amplified in the hypervariable V1–V3 region of the 16S ribosomal RNA (rRNA) gene before pyrosequencing. V1–V3 amplification involved a specific primer set: 8F (5′-CTGCTGCTYCCGTA-3′) as the forward primer and 530R (5′-GTATTACCGCGGTGTGCTG-3′) as the reverse primer, with a 10-bp MID sequence. Polymerase chain reaction (PCR) was conducted on a T-professional Thermal Cycler (Biometra, Goettingen, Germany) under the following conditions: initial denaturation at 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 58°C for one minute, extension at 72°C for one minute, and a final extension at 72°C for 10 minutes. The PCR products were purified using the AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea) and analyzed by electrophoresis in a 1.2% agarose gel. Equal amounts of purified PCR products were pooled for 454 pyrosequencing, which was performed using a Roche 454 GS-FLX Titanium system (Roche Molecular Systems, Branchburg, NJ, USA). After a sequencing run was finished, the sequence and quality data were recovered as MID sequences. Quality-control efforts were conducted to eliminate trimmed sequences with ambiguous base calls, low-quality sequence ends, read lengths < 200 bp, and quality scores < Q-25.

**Sequence Analysis**
Bacterial composition was estimated from the data using the Quantitative Insights into Microbial Ecology (QIIME 1.9.1) pipeline [18]. Briefly, QIIME was used to assess phylogenetic and operational taxonomic units (OTUs). First, it was used to de-multiplex the barcoded reads and perform chimera filtering. Filtered sequence reads were grouped into OTUs at a sequence similarity level of 97%, which approximates species-level phylotypes. Next, the taxonomy of the OTUs was assigned, and sequences were aligned with Greengenes and PyNAST. The bacterial taxonomic abundance data were imported into R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) with the phyloseq package to analyze richness, diversity, and ordination plot [19]. Also, the lme4 package in R was used for fitting linear mixed-effects models [20]. The compositional differences of nasal microbiome among healthy volunteers and AR patients were evaluated by non-parametric Wilcoxon test and the value has been adjusted by Holm-Bonferroni method for multiple comparisons. A $P$-value of $< 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA, USA) to identify relative frequencies of differences in microbial genera. Data were considered significantly different at $P < .05$. 
**Results**

**Differences in microbial phyla and genera in the nasal mucus of healthy participants and patients with AR**

A total of 364,923 sequence reads of 16S rRNA genes derived from the nasal mucus swab samples of the middle turbinate was obtained from the genomic DNA samples of 42 patients with AR and 30 healthy participants. The raw data were submitted to the National Center for Biotechnology Information Sequence Read Archive (NCBI-SRA; http://www.ncbi.nlm.nih.gov/sra) under accession number SRA236631. In terms of alpha-diversity, using the qualified sequences reads, although we found the typical healthy participant to demonstrate greater genus-richness than patients with AR (Wilcoxon test, $P < .001$), there were no significant differences in Shannon index in the microbial community of the nasal mucus (Wilcoxon test, $P = .39$; Fig. 1A). This means that the occurrence of AR might be not explained by alpha dysbiosis in the microbiota of the nasal mucus.

The bacterial compositions in the nasal mucus from both the healthy participants and AR patient group showed variable composition patterns. Taxa with an average abundance of at least 1% are shown; those with less than 1% were reported as "other." Although the numbers of genera to which the symbiotic microorganism belonged were more abundant in AR patient group than healthy participants, three phyla (*Firmicutes*, *Proteobacteria*, and *Actinobacteria*) were the most dominant in the nasal mucus of both groups. (Fig. 1B). Specifically, *Proteobacteria* (42.34% ± 34.4%) and *Actinobacteria* (33.73% ± 28.0%) phyla were represented relatively well in the healthy participants,
while *Firmicutes* (41.23 ± 33.6%) phylum was predominant in patients with AR based on linear mixed-effects models (Table 1).

To characterize key genera of the microbiota found in nasal mucus collected from healthy participants and patients with AR, the bacterial genera were compared using the non-parametric Wilcoxon test (Fig. 2). The results revealed that 11 nasal microbial genera of AR patients and healthy controls were significantly abundant. Of the identified 11 genera, three taxa were significantly different between the groups and *Ralstonia* genus significantly more common in the nasal microbiome in healthy participants (Fig. 2A). In addition, both unclassified *Enterobacteriaceae* and *Lactococcus* genera more abundant in patients with AR (2B, 2C). Although the abundance of these bacteria was not significantly different, the microbiome of *Staphylococcus* genus was relatively abundant in the nasal mucus of patients with AR and the rate of *Propionibacterium* genus was relatively higher in the nasal mucus of healthy participants (Fig. 2D, 2E).

Although unclassified *Enterobacteriaceae* (*Proteobacteria* phylum) was abundant, the distribution of *Firmicutes* phylum including *Enterococcus* and *Staphylococcus* genera the more characterized in the nasal mucus of AR patients. Based on these findings, we found that the composition of symbiotic microbiome in the nasal mucus was distinctively different in both phylum and genus level from healthy participants and AR patients. We performed the additional microbial analysis focusing on genus level which showed a distinctive distribution in the nasal mucus between healthy participants and AR patients.

Analysis of microbial genera showing differences in the composition of nasal mucus between healthy participants and patients with AR
The comparison of relative abundant microbiome between healthy and AR patient group is shown based on the profiling of strains that dominant or significance. *Staphylococcus* and *Propionibacterium* genera did not show significant difference, but they appeared as dominant strain within each group. In addition, although difference between two groups was confirmed, there might be a possibility that there may be no statistical significance due to the large standard error of healthy participants or AR patient group. As a next step, the linear mixed-effects model (LMEM) analysis was performed to confirm the changing pattern of key genera in the healthy group based on the changing pattern in AR patient group and to prove how the AR patient group affects the healthy group. 11 of the 22 genera such as unclassified_**Enterobacteriaceae** genus, *Staphylococcus* genus, *Lactococcus* genus, *Peptoniphilus* genus, unclassified_**Neisseriaceae** genus, *Curvibacter* genus, *Anaerococcus* genus, *Corynebacterium* genus, *Streptococcus* genus, *Propionibacterium* genus, and *Ralstonia* genus, were identified in the LMEM results (Fig. 3A). Among those bacteria, only *Ralstonia* genus is significantly revealed in the health group (*P < .001*). Further, *Ralstonia* genus was more abundant in the nasal mucus of healthy participants, while unclassified_**Enterobacteriaceae**, and *Lactococcus* genera were more dominant in the nasal mucus of patients with AR.

We examined whether the two groups can be distinguished from each other. To this end, non-metric multidimensional scaling based on Bray–Curtis dissimilarities was used for analysis, and clustering of the nasal microbiome in both healthy participants and patients with AR was observed. Permutational multivariate analysis of variance confirmed sample type as the dominant driver of variation (*P = .001*). In addition, biplot vectors showed the four genera with the strongest contribution to sample dissimilarity.
The NMDS biplot used an extension of vegan library’s bioenv function to find the best set of bacterial taxa with community dissimilarities (bray) based on Pearson correlation and then plotted them as vectors. As expected, *Ralstonia* genus of *Proteobacteria* phylum was found in higher proportions in the nasal mucus of healthy participants in concert with abundance of both *Propionibacterium* genus of *Actinobacteria* phylum and *Enterobacter* genus of *Proteobacteria* phylum, whereas the nasal mucus of patients with AR was predominated by *Staphylococcus* genus of *Firmicutes* phylum with co-presence of a minor bacterium (*Corynebacterium* genus), whereas the nasal mucus of AR patients was predominated by *Corynebacterium* genus of *Actinobacteria* phylum (Fig. 3B).

To confirm the abundance of patterns, Spearman’s correlation analysis was conducted (Fig. 3C, 3D, 3E). The data showed that AR patients with a high distribution of *Ralstonia* genus had a relatively low distribution of *Staphylococcus* genus (Spearman’s rho = −0.1621; *P* = .1737) and AR patients with a high distribution of Propionibacterium genus also had a lower distribution of *Staphylococcus* genus (Spearman’s rho = −0.1471; *P* = .2175). As expected, the distribution of *Ralstonia* and *Propionibacterium* genera showed positive correlation (Spearman’s rho = 0.32; *P* = .0055). These findings demonstrate that the microbial composition of the *Firmicutes* phylum was most abundant in the nasal mucosa of patients with AR, and the distribution of the *Staphylococcus* genus was altered greatly in the nasal mucus of patients with AR. Although statistical significance was not shown in all analyses, the distribution of nasal microbiome classified into *Staphylococcus* genus in the nasal mucosa of AR patients
showed a negative correlation in the distribution of *Ralstonia* and *Propionibacterium* genera microbiomes.

The dysbiosis of nasal microbial species in AR depending on the clinical characteristics of patients

As a next step, we analyzed alterations in the microbial composition in the nasal mucus of patients with AR at the bacterial species level. To further assess alterations in microbial composition at the levels of bacterial phyla and species, the microbial compositions of the middle turbinate mucus in healthy participants (*n* = 15) and patients with AR (*n* = 17) were evaluated. We re-confirmed results with the same trend as in Fig. 1B, and the *Proteobacteria* (30.2%), *Firmicutes* (14.4%), *Actinobacteria* (14.2%), and *Bacteroidetes* (7.1%) phyla were distributed dominantly in the nasal mucus of healthy participants. Further, pyrosequencing data revealed that the proportions of *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* phyla were reduced in the nasal mucus of patients with AR, and the proportion of *Firmicutes* phylum was elevated (Fig. 4A). Based on a sequence identity of at least 97%, *Nocardia coeliaca* (20.01%, *Actinobacteria* phylum), *Staphylococcus epidermidis* (14.45%, *Firmicutes* phylum), *Corynebacterium accolens* (7.15%, *Actinobacteria* phylum), and *Citrobacter koseri* (6.77%, *Proteobacteria* phylum) were the most abundant microbial species in the nasal mucus of healthy participants. In contrast, the distribution of microbial species was altered greatly in the nasal mucus of patients with AR. The results revealed that *S. aureus* demonstrated the greatest abundance of mapped sequences and the largest expression in microbial distribution in the nasal mucus of patients with AR (37.69% in
AR vs. 0.21% in healthy participants) \((p = .0002, \text{Fig. 4B})\). *C. accolens* and *N. coeliaca* were distributed at relatively high proportions \((p = .012)\), but the composition of *S. epidermidis* was significantly reduced in the nasal mucus of patients with AR (3.24%) \((p = .0036)\). The proportions of *S. epidermidis* and *S. aureus* differed significantly by patient with AR to a maximum of 96.92% and the lowest abundance of *Staphylococcus* genus in the nasal mucus of patients with AR was 0.94% (Fig. 4C).

We questioned whether alteration of *Staphylococcus* species in the nasal mucus of patients with AR might be correlated with clinical characteristics of patients with AR. In eight of 17 patients with AR, *S. aureus* was most abundant in the microbiome of the nasal mucus, and more than 40% of nasal symbiont microorganisms were identified as *S. aureus* (Fig. 4C). Although no significant correlation was found between *S. aureus* proportion and other clinical factors, both the age and height of patients with AR showed a statistically significant correlation with abundance of *S. aureus* in the nasal mucus. Interestingly, 11 patients with AR were confirmed to have strong positive (> 3+) results for *Dermatophagoides pteronyssinus* and *Dermatophagoides farina* based on allergy tests such as allergic skin test or multiple allergen simultaneous test (Table 2). The positive and negative rate of reaction to house dust mites (HDM) showed a statistically significant correlation with more abundance of *S. aureus* in these patients (Fig. 4D, Table 3). Considering these findings, we provide a note of caution that *S. aureus*-derived dysbiosis was distinctive in the nasal mucus of patients with AR, and this dysbiosis might be correlated with patient age, height, and antigen-positivity for HDM.
Discussion

Here, we showed a significantly distinctive colonization pattern of *Firmicutes*, *Actinobacteria*, and *Proteobacteria* phyla in the nasal mucus of patients with AR compared to healthy participants. In addition, significantly different distributions of *Ralstonia*, *Enterobacteriaceae*, and *Lactococcus* genera were observed in the nasal mucus of healthy participants and patients with AR. Our findings clarify that symbiotic dysbiosis was more pronounced in the nasal mucus of patients with AR. *Staphylococcus* genus exhibited the most noticeable alterations in composition between healthy participants and patients with AR, and the composition of *S. aureus* was most abundant in allergic nasal mucus depending on patient age, height, and antigen-positivity for HDM.

The nasal epithelium is the first target organ for environmental allergens, and recent work has highlighted its critical role as a barrier that restricts host exposure to allergens in AR [3,4]. The microbiome colonizes at the nasal mucus, where contact with inhaled pathogens or allergens occurs and might be thought to perform functional crosstalk with the nasal epithelium, especially concerning regulation of immune mechanisms in the upper airway [21]. Therefore, understanding compositional changes in the microbiome and identifying dominant microbial species in the nasal mucus might be essential for understanding a definite pathophysiologic mechanism of upper airway diseases including AR. Recently, as the importance of microorganisms is emphasized, the analysis of the microbial profiles or alterations between health status and diseases became easy to access by development of culture-independent technologies such as
next-generation sequencing (NGS), and research on the distribution of symbiotic microorganisms has become possible up to the species level [22].

In particular, the importance of the nasal microbiome, especially with respect to Th2 cytokine–regulated immune responses, has been recognized increasingly [23]. Therefore, we extended this paradigm to the nasal microbiome in the field of AR and noted marked alterations in microbial composition at the species level in the nasal mucus of patients with AR. To offer more information about the nasal symbiont of the nasal mucus in this study, mucus samples were obtained via swabs of the middle turbinate; nasal mucus is secreted by epithelial cells and was analyzed to determine the composition of the nasal microbiome community that resides in the nasal mucosa. We think that the mucosa around the middle turbinate is correlated highly with the practical microbial environment, with little influence of the external environment, and it is appropriate clinically to analyze the change in distribution of symbiotic microorganisms in nasal diseases such as AR.

Our data showed that the proportions of microbiome classified into Proteobacteria, Actinobacteria, and Firmicutes phyla were abundant relatively in the nasal mucus of healthy participants. This was observed in healthy participants with a significant abundance of Ralsionia, Staphylococcus, and Propionibacterium genera, and Norcardia coeliaca and S. epidermidis were the most dominant microbial species. Our previous study showed Staphylococcus (Firmicutes phylum) and Corynebacterium (Actinobacteria phylum) genera as the most abundant commensal organisms and S. epidermidis as the most common nasal symbiont of the identified bacterial species in human nasal mucus [24,25]. The current findings revealed that microbial compositions
were altered significantly in the nasal mucus of patients with AR. Interestingly, the proportions of the symbiotic microbiome of *Proteobacterium* and *Actinobacteria* phyla were reduced in the nasal mucus of patients with AR, while the proportion of *Firmicutes* phylum was elevated. This difference also was observed in the microbial genus in the nasal mucus of patients with AR. The proportion of *Ralstonia* genus or *Propionibacterium* genus was decreased and *Staphylococcus* genus was more dominant in the nasal mucus of patients with AR. In addition, the proportions of *Ralstonia* and *Propionibacterium* genera were correlated inversely with that of *Staphylococcus* genus in each patient with AR.

In the end, the nasal mucus of AR patients induces the biologic environment where certain commensal microorganisms can better exist, and more research is needed to analyze the relationship between the dysbiosis of nasal microbiome as a species level and the pathophysiology of AR. In the nasal microbiome of patients with AR, *S. aureus* colonization was dominant most significantly in concert with a high abundance of *Staphylococcus* spp. Our previous study showed that the distribution of *S. aureus* was significantly increased in the nasal mucus of patients with AR to constitute more than 20% of the identified nasal microbiome, and *S. aureus* colonization was abundant most highly relative to the composition of the normal mucus [26]. Thus, we sought to understand the contributions of *Staphylococcus* species to the pathogenic mechanism of AR as nasal symbionts and the synergism of *Staphylococcus* species colonization with Th2-related allergic inflammation in the nasal mucosa. Both *S. epidermidis* and *S. aureus* are well-known human pathogens that cause serious opportunistic infections of the respiratory tract [27,28]. However, *Staphylococcus* species are detected commonly...
in the normal microflora of the skin, intestine, and upper airway, and many studies have implicated a role of Staphylococcus genus as symbiotic commensal organisms involved in allergic diseases [29-32]. Patients with allergic eczema are more likely than healthy participants to have skin colonized with S. aureus, and disease severity is associated with degree of S. aureus colonization [30]. The dominant distribution of S. aureus in the upper respiratory tract of patients with asthma and aspirin sensitivity has been reported up to 87.5%, and greater nasal S. aureus colonization is significantly related to asthma [31]. In addition, patients with AR are colonized more frequently with nasal S. aureus or sensitized to S. aureus enterotoxins than healthy participants [33]. These findings support involvement of S. aureus in allergic immune responses and correlation of S. aureus colonies with positive or negative regulation of Th2 inflammation. Abnormalities in the human microbiota are associated with the etiology of AR and the microbiota of the site of AR, the inferior turbinate sought to be characterized in subjects with allergic rhinitis and healthy controls and to examine the relationship of mucosal microbiota with disease occurrence, sensitized allergen number, and allergen-specific and total IgE levels. Microbial dysbiosis correlated significantly with total IgE levels representing combined allergic responses but not with disease occurrence, the number of sensitized allergens, or house dust mite allergen-specific IgE levels [34].

Our data also indicate that the nasal mucosa of AR might provide a more suitable environment for nasal symbiont S. aureus to survive, resulting in its greatest abundance in the microbiome in the nasal mucus of patients with AR. Intriguingly, the larger proportion of S. aureus in the nasal mucus of patients with AR depended on positive reaction with HDM. We did not determine how nasal Staphylococcus microbiome
colonization is more abundant in the nasal mucus of patients with AR and whether this plays a critical role in regulating the Th2 immune response in patients with AR. Instead, we only surmised that the human nasal mucosa could become a more favorable environment for survival of S. aureus depending on AR patients’ clinical factors such as age or height. In addition, the colonization of S. aureus would be increased when AR patients are sensitized to HDM and we presume that the metabolites secreted by HDM after exposure to nasal mucus and proteolytic enzymes of HDM might induce the alteration of proteomics composition of the nasal mucus. This study evaluated the alterations in nasal microbiome that occur in the nasal mucus of those with AR and documented significant proportional changes in such nasal mucus at the levels of microbial genera and species. Our study highlights the S. aureus-dominant dysbiosis of the nasal mucus of patients with AR and enhances our understanding of microbial alterations related to clinical characteristics of patients with AR, including those related to specific allergens. Future research directions need to include manipulation of the dominant nasal microbiome for therapeutic advances in managing AR and might provide a basis of research on the biologics of AR treatments.
References


FIGURE LEGENDS

**Fig. 1.** Alpha-diversity and relative abundance of bacterial phyla and genera. A: Alpha-diversity richness \( (P < .001) \) and Shannon indexes \( (P = .39) \) among observed species. The values were tested by non-parametric Wilcoxon test. B: The Y-axis of bar graph means the relative abundance of genera of each subject. X-axis represents each subject in healthy participants (N=30) and AR patients (N=42). Only taxa with an average abundance of at least 1% are shown.

**Fig. 2.** Difference in bacterial abundance in the middle turbinate between healthy individuals and patients with AR using the non-parametric Wilcoxon test.

**Fig. 3.** Overview of the bacteria taxa showing a difference between healthy individuals and patients with allergic rhinitis (AR). A: Linear mixed-effects model for comparing healthy participants and patients with AR. The data shown correspond to patients with AR compared to healthy individuals. Upregulation (not shown), downregulation (blue), and no significance (gray). B: Non-metric multidimensional scaling of bacterial community samples using Bray–Curtis distances. Four genera (*Ralstonia, Propionibacterium, Enterobacter, Enterobacteriaceae* and *Staphylococcus* genera) with the strongest contribution to sample dissimilarity were revealed by biplot analysis. *Corynebacterium* genus was headed over the patients with AR. C, D, E: The correlation between *Ralstonia, Staphylococcus*, and *Propionibacterium* genera. was determined by Spearman's correlation analysis.
Fig. 4. Composition of microbial species in the nasal mucus of healthy participants and patients with allergic rhinitis (AR). (A) Microbial phyla from middle turbinate mucus of healthy participants \((n = 15)\) and patients with AR \((n = 17)\) were identified via 16S rRNA gene sequencing. (B) Microbial species from nasal mucus of healthy participants and patients with AR. Distribution of the 126 identified bacterial species is presented in the graph, and the bar graph presents the relative species abundance of nasal commensal organisms of 17 patients with AR. (C) The distribution of microbial species in the nasal mucus of AR. The bar graph presents the relative species abundance of nasal commensal organisms of 17 patients with AR and distribution of \(S. \text{epidermidis}\) and \(S. \text{aureus}\) of each patient is described (red circle: HDM (+) AR patients). (D) The rate of \(S. \text{aureus}\) in the nasal mucus from middle turbinate of each AR patient \((n=17)\) was compared depending on positive response to house dust mites \((\text{Dp/DF})\) \((\text{Dp, Dermatophagoides pteronyssinus; Df, Dermatophagoides farina}).\)
Table 1. Effect size of phyla in the linear mixed-effects model*

<table>
<thead>
<tr>
<th>Phyla</th>
<th>Estimate</th>
<th>Standard error</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Firmicutes</em></td>
<td>0.17</td>
<td>0.08</td>
<td>2.19</td>
<td>.03</td>
</tr>
<tr>
<td><em>Proteobacteria</em></td>
<td>−0.12</td>
<td>0.08</td>
<td>−1.42</td>
<td>.16</td>
</tr>
<tr>
<td><em>Actinobacteria</em></td>
<td>−0.05</td>
<td>0.06</td>
<td>−0.84</td>
<td>.40</td>
</tr>
</tbody>
</table>

*When the estimate of the value was greater than 0, the phylum was considered to occur at a greater frequency in the samples of patients with allergic rhinitis.
Table 2. Demographics of AR patients included in this study

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>Age</th>
<th>Past disease</th>
<th>Allergy test</th>
<th>Total IgE level</th>
<th>Total IgE level (immunoCAP)</th>
<th>Positive antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>31</td>
<td>none</td>
<td>MAST</td>
<td>87</td>
<td></td>
<td>Dp 4, Df 3</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>25</td>
<td>none</td>
<td>MAST</td>
<td>46</td>
<td></td>
<td>Dp 3, Df 4, Hd 2, shrimp 2</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>21</td>
<td>none</td>
<td>MAST</td>
<td>90</td>
<td></td>
<td>Dp 4</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>20</td>
<td>AD</td>
<td>SPT</td>
<td>204</td>
<td></td>
<td>Mold 1.12, multiple tree pollen</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>62</td>
<td>Cataract</td>
<td>MAST</td>
<td>158</td>
<td></td>
<td>Df 4</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>20</td>
<td>none</td>
<td>MAST</td>
<td>146</td>
<td></td>
<td>Df 4, Mugwort 4, Cat 4, Hd 3, Goldenrod 3, Birch-Alder 3</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>19</td>
<td>none</td>
<td>MAST</td>
<td>142</td>
<td></td>
<td>Dp 4, Df 4</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>25</td>
<td>none</td>
<td>SPT</td>
<td>11.1</td>
<td></td>
<td>Multiple tree pollen</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>24</td>
<td>Asthma</td>
<td>MAST</td>
<td>115</td>
<td></td>
<td>Soy bean 2, Dp 4</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>49</td>
<td>none</td>
<td>MAST</td>
<td>5</td>
<td></td>
<td>Dp 3, Df 2</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>28</td>
<td>none</td>
<td>MAST</td>
<td>60</td>
<td></td>
<td>Dp 3.58, Df 3.64, Cat 2.29, multiple tree pollen</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>23</td>
<td>AS</td>
<td>SPT</td>
<td>649</td>
<td></td>
<td>Df 2</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>38</td>
<td>none</td>
<td>MAST</td>
<td>126</td>
<td></td>
<td>Sallow 4, Hazelnut 3, Bermuda grass 4, Orchard grass 4, Timothy grass 3, Rye 3, Pigweed mix 4, Russian thistle 4 Pigweed mix 3, Russian thistle, mugwort, bermuda grass, orchard grass 2</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>40</td>
<td>none</td>
<td>MAST</td>
<td>81</td>
<td></td>
<td>Dp 2, Df 3</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>59</td>
<td>Bronchiectasis</td>
<td>MAST</td>
<td>166</td>
<td></td>
<td>Shrimp 4, Swallow willow 3, Bermuda grass 4, Orchard grass 4, Pigweed mix 4, Dandelion 4, Cockroach mix 4, Df 4, Dp 3</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>30</td>
<td>None</td>
<td>MAST</td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>62</td>
<td>Hypertension</td>
<td>MAST</td>
<td>166</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
MAST: multiple-allergen simultaneous test, SPT: Skin prick test, AD: atopic dermatitis,
AS: ankylosing spontilitis, Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farina*
Table 3. Comparison of the distribution of *Staphylococcus aureus* with clinical factors of patients with allergic rhinitis (*n* = 17)

<table>
<thead>
<tr>
<th></th>
<th>High level of S. aureus (<em>n</em> = 8)</th>
<th>Low level of S. aureus (<em>n</em> = 9)</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>(6:2)</td>
<td>(9:0)</td>
<td>.490</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.8 (± 8.7)</td>
<td>36.9 (± 15.5)</td>
<td>.041*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.2 (± 16.9)</td>
<td>76.6 (± 14.4)</td>
<td>.657</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166.8 (± 5.1)</td>
<td>175.0 (± 6.4)</td>
<td>.021*</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>27.7 (± 5.8)</td>
<td>25.0 (± 4.4)</td>
<td>.349</td>
</tr>
<tr>
<td>Smoking history</td>
<td>1 (12.5%)</td>
<td>4 (44.4%)</td>
<td>1</td>
</tr>
<tr>
<td>Drinking history</td>
<td>2 (25.0%)</td>
<td>3 (33.3%)</td>
<td>1</td>
</tr>
<tr>
<td>Total IgE level</td>
<td>108.6 (± 44.7)</td>
<td>74.8 (± 51.4)</td>
<td>.270</td>
</tr>
<tr>
<td>Dp sensitization</td>
<td>5 (60%)</td>
<td>2 (22.2%)</td>
<td>.047*</td>
</tr>
<tr>
<td>Df sensitization</td>
<td>6 (80%)</td>
<td>2 (22.2%)</td>
<td>.036*</td>
</tr>
</tbody>
</table>

**Abbreviations:** BMI, body mass index; Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; IgE, immunoglobulin E; S. aureus, *Staphylococcus aureus*.
Figure 1

(A) Box plots showing the comparison of observed and Shannon diversity measures between Healthy and AR Patients. The box plots display the distribution of diversity measures with quartiles and outliers.

(B) Heatmap illustrating the abundance of different bacterial species in Healthy and AR Patient groups. The heatmap uses a color scale to represent the abundance levels, with colors ranging from light grey to dark red. The species categories include Actinobacteria,firmicutes, and proteobacteria, among others.
Figure 3

A. LMEM (Healthy vs AR Patients)

B. allergic - NMDS - bray

C. $r = -0.1621$, $p = 0.1737$

D. $r = 0.3237$, $p = 0.0055$

E. $r = -0.1471$, $p = 0.2175$
Figure 4

A

Healthy subjects vs. Allergic rhinitis patients

- **Proteobacteria**
  - Healthy subjects: 30.2%
  - Allergic rhinitis patients: 23.4%

- **Firmicutes**
  - Healthy subjects: 14.2%
  - Allergic rhinitis patients: 19.7%

- **Actinobacteria**
  - Healthy subjects: 14.4%
  - Allergic rhinitis patients: 11.2%

- **Bacteroidetes**
  - Healthy subjects: 7.1%
  - Allergic rhinitis patients: 6.5%

- **Acidobacteria**
  - Healthy subjects: 6.6%
  - Allergic rhinitis patients: 13.6%
Figure 4

[Bar chart showing the percentage distribution of Staphylococcus epidermidis and Staphylococcus aureus across different patient numbers.]