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Airways Disease

Characterization of ocular and nasopharyngeal microbiome in allergic rhinoconjunctivitis

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Abstract

Background: Allergic rhinoconjunctivitis (ARC) is a prevalent allergic condition in the pediatric population. Microbial dysbiosis has increasingly been recognized to influence on host immunity and allergic diseases. However, the microbial profile of ARC has not been characterized. This cross-sectional study aims to evaluate the changes in nasal and ocular surface microbiome of children with ARC.

Methods: Ocular and nasopharyngeal swabs were collected from controls and pediatric ARC cases for 16S rRNA amplicon sequencing. The bacterial community profile was analyzed. The correlation of the microbial diversity with the ARC-related clinical scores was studied.

Results: A total of 23 patients with ARC and 17 healthy controls were recruited; 30 were ocular samples (15 controls vs 15 ARC), while 40 were nasal samples (17 controls vs 23 ARC). The alpha diversity of nasopharyngeal microbiome was significantly higher in ARC patients than healthy controls ($P < 0.01$), but not for ocular microbiome. The clinical scores in all subjects were negatively correlated with the Shannon diversity for ocular ($P = 0.014$) and positively correlated with nasopharyngeal ($P = 0.010$) microbiome. While the ocular microbiome remained significantly distinct from nasopharyngeal microbiome in terms of both alpha and beta diversity in both healthy subjects and ARC patients, significant differences of relative abundance of certain phyla (Bacteroidetes, Cyanobacteria, and Deinococcus-Thermus) and genera (Dolosigranulum and Moraxella) between nasal and ocular surfaces were only detected in healthy controls, but not in the ARC subjects, suggesting the microbial composition at both body sites becoming more similar at disease state.

Conclusion: This study reported (a) a higher alpha diversity in ocular than nasopharyngeal microbiome in both ARC patients and controls, and (b) nasopharyngeal microbiome became more diverse in ARC patients than in controls. Our results suggested an interaction of the microbiome between ocular and nasal compartments in patients with ARC.

KEYWORDS

16S rRNA sequencing, allergic rhinoconjunctivitis, microbiome, nasopharyngeal, ocular

1 | INTRODUCTION

Allergic rhinoconjunctivitis (ARC) is a common allergic disease with a globally average incidence rate of 10%-20% with a greater prevalence among the pediatric populations.¹ The proportion of children with ARC is on the rise in the past few decades and up to 40% of the children are affected nowadays.²

Allergic rhinoconjunctivitis is mainly caused by hypersensitivity to specific aeroallergens, leading to local IgE synthesis and histamine release.³ Consequently, the affected children develop inflammatory symptoms in upper airway mucosa and conjunctiva, resulting in rhinorrhea, nasal congestion, chronic or repeated sneezing, watery red eyes, and itchiness arisen from the nasal and ocular regions.^{4,5} While current medications aid temporary symptomatic relief, ARC is not curable and poses severe impact on the quality of life of the patients. Thus, there is still a need to understand the ARC pathophysiology for developing better management strategies.

Recently, it was demonstrated that the gut microbiome plays a critical role in the pathogenesis of various allergic diseases such as asthma and eczema.⁶ Despite the low biomass issue, studies also attempted to study the microbial composition of other body sites for more direct linkage to the extraintestinal diseases.^{7,8} As for ARC, the relationship between nasopharyngeal and ocular microbiome and the disease remained unexplored. In the present study, the nasopharyngeal and ocular microbiome from both healthy subjects and ARC patients were studied using 16S amplicon sequencing.

2 | METHODS

2.1 | Study design and participants

Children aged 6-18 years were recruited from the Paediatric and Ophthalmology Clinics at Prince of Wales Hospital and The Chinese University of Hong Kong Eye Centre between May and July in 2017. The exclusion criteria of the study were as follows: (a) active ocular inflammation or infection; (b) history of ocular injury or (c) intercurrent epistaxis; (d) use of topical or systemic steroids, antibiotics, or immunomodulatory medications; (e) poorly controlled asthma; and (f) inability to cooperate with the study protocol for any reason, at the time of samplings. Ethical approval was obtained from the Joint Clinical Research Ethics Committee of the Faculty of Medicine, The Chinese University of Hong Kong and New Territories East Cluster, Hospital Authority. Written informed consents were obtained from all subjects or their parents or guardians. ARC or its absence was determined by an ophthalmologist. The Score of Allergic Rhinitis (SFAR) was used to screen for subjects with ARC. The allergic disease quality of life score, a Chinese version of the Paediatric Allergic Disease Quality of Life Questionnaire (PADQLQ), was also obtained. Demographic variables such as family history of atopy, close contact with pets, passive smoking, and incense burning practice were recorded.

Key Message

Allergic rhinoconjunctivitis (ARC) is a common allergic disease in the pediatric population. Although recent studies demonstrated the influence of microbiome on allergic diseases, there were limited studies on ARC given its multiple body sites involvement and low biomass issues. This cross-sectional study characterized the microbiome in both nasopharyngeal and ocular compartments in patients with ARC, using 16S rRNA sequencing. Our findings suggested possible channeling of microbiome between the eye and nose in ARC cases.

2.2 | Sample collection

Ocular microbiome was sampled from the inferior palpebral conjunctiva using a cotton swab. Nasal samples were taken at the nasopharyngeal mucosa using COPAN FLOQSwabs (COPAN Diagnostics Inc). The swab heads were incised and soaked with ST solution (0.15 M NaCl and 0.1% Tween-20). Samples were placed temporarily at 4°C on-site and then transited to -80°C deep freezer for long-term storage.

2.3 | DNA extraction and 16S rRNA gene sequencing

Microbial genome was extracted from swabs using PureLink Microbiome DNA Purification Kit (ThermoFisher Scientific) according to the manufacturer instructions. DNA concentration was measured using Qubit dsDNA HS Assay Kit. If both side of the conjunctiva or nasopharyngeal mucosa were successfully collected, only samples of higher DNA yield were proceeded. The V3-V4 hypervariable region of 16S rRNA gene was amplified using the fusion primers 314F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3').⁹ Libraries were sequenced in two separate runs on Illumina MiSeq sequencing platform to generate 2 × 300 bp paired-end reads.

2.4 | Metagenomic data analysis

Sequencing data were imported to QIIME2 version 2018.11. DADA2 on R 3.5.0 was used for reads filtering and trimming, sequence variants (SVs) inference, and taxonomic assignment using default settings.^{10,11} Reads were filtered using a maximum expected error of two. The forward reads were trimmed at 283 bp, while reverse reads were cut at 220-bp position to maintain a minimal of 20-bp nucleotides for read pair overlapping. Approximately 385k forward and 520k reverse reads were used to estimate the error model via unsupervised machine learning. The taxonomy table was then constructed using the SILVA 132 database.¹² Phyloseq (v 1.24.0) on R 3.5.0 was used to analyze taxonomy table, sample metadata, and phylogenetic tree for

Category	Group 1 vs Group 2	Total SVs	Sampling depth	No. of samples excluded
Control	Ocular (n = 15) vs Nasal (n = 17)	1860	31 390	EL18
ARC	Ocular (n = 15) vs Nasal (n = 23)	2218	31 790	N-1, NR34, ER43
Ocular	ctrl (n = 15) vs case (n = 15)	2492	21 054	EL18
Nasal	ctrl (n = 17) vs case (n = 23)	1650	36 098	N-1, NR34

Abbreviations: case, ARC patients; ctrl, healthy controls; SVs, sequence variants.

TABLE 1 Overview of sample groupings and data preprocessing

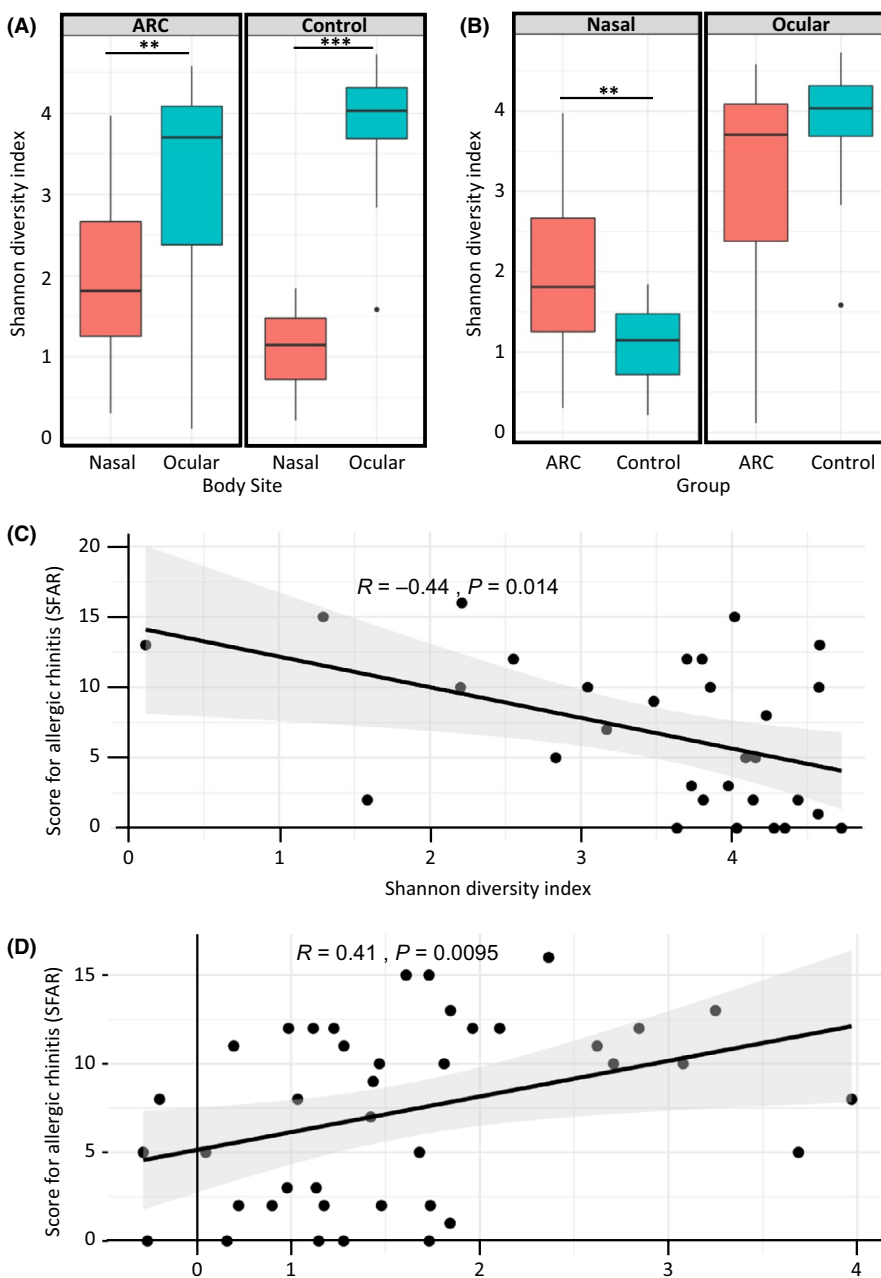
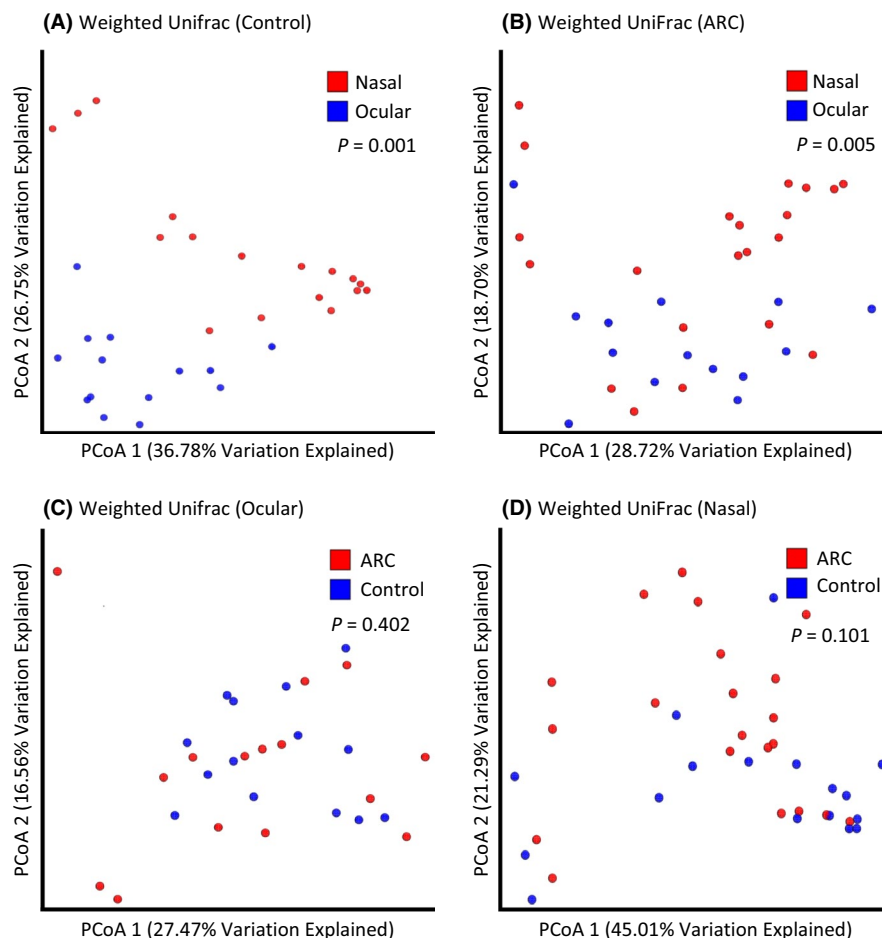


FIGURE 1 Alpha diversity of microbiome samples and correlation with clinical scores. The difference of diversity among (A) nasal and ocular microbiome and (B) Allergic rhinoconjunctivitis (ARC) patients and healthy controls were analyzed using Shannon diversity index. Two-sided Wilcoxon rank-sum test; ** $P < 0.01$, *** $P < 0.001$. Scatter plots showing the Pearson correlations of the Score for Allergic Rhinitis with (C) ocular microbiome and (D) nasopharyngeal microbiome [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Beta diversity analysis. PCoA plots of beta diversity based on weighted UniFrac distance according to body sites (A and B) and disease status (C and D). PERMANOVA test with 999 permutations [Colour figure can be viewed at wileyonlinelibrary.com]



case-control and ocular-nasal comparisons.¹³ The median sequencing depth was used for normalization. SVs that were absent in each comparison were removed. Details are summarized in Table 1.

2.5 | Statistical analysis

Statistical analysis was performed using QIIME2 and R. Alpha diversity was evaluated using Shannon's diversity index, Simpson's diversity index, observed OTUs, Faith's phylogenetic diversity, and Pielou's evenness.¹⁴ Permutational multivariate analysis of variance (PERMANOVA), Jaccard distance, Bray-Curtis dissimilarity, unweighted UniFrac distance, and weighted UniFrac distance were assessed for beta diversity analysis.¹⁵ Analysis of composition of microbiomes (ANCOM) was used to study the differential abundance of microbiome.¹⁶ Wilcoxon rank-sum test was used for the comparison between sample groups. P -value < 0.05 represents statistical significance.

3 | RESULTS

3.1 | Sample groupings, sequencing statistics, and data preprocessing

A total of 23 patients with ARC and 17 healthy controls were recruited. Seventy samples were subjected to sequencing, 30 are ocular samples

(15 controls vs 15 ARC), and 40 are nasal samples (23 controls vs 17 ARC). The sample metadata are listed in Table S1. Overall, 2 658 582 reads were obtained from two sequencing runs. Following DADA2 quality control, 2 299 989 reads remained with an average coverage of 32 857 reads per sample (range = 4957-89 954). There were 3389 SVs identified from the clean reads in total (Table S2).

3.2 | Alpha diversity of ocular and nasopharyngeal microbiome samples

The Shannon diversity of ocular microbiome was significantly higher than that of nasopharyngeal microbiome for controls ($P < 0.0001$) and ARC patients ($P = 0.0019$; Figure 1A). Furthermore, the Shannon diversity of nasopharyngeal microbiome was significantly higher in ARC patients compared to controls ($P = 0.003$). There was no significant difference in the ocular microbiome alpha diversity between ARC patients and controls (Figure 1B). Consistent results were obtained using other alpha diversity metrics (Table S4 and Figure S1).

3.3 | Beta diversity of ocular and nasopharyngeal microbiome samples

The microbiome of the nasal samples was significantly distinct from that of ocular samples in both healthy controls ($P = 0.001$; Figure 2A)

and ARC patients ($P = 0.005$; Figure 2B). Insignificant differences in terms of beta diversity were observed between ARC patients and healthy controls for both ocular and nasopharyngeal microbiome (Figure 2C,D). Similar results were obtained when using other distance metrics (Table 2, Figures S2 and S3).

3.4 | Correlations between ARC clinical scores and microbial diversity in all subjects

The clinical score of ARC depicted by score of allergic rhinitis (SFAR) correlated negatively with ocular microbiome alpha diversity ($P = 0.014$; Figure 1C and Figure S6A). In contrast, the score was significantly positively correlated with nasopharyngeal microbiome alpha diversity ($P = 0.0095$; Figure 1D and Figure S6B). Similarly, there was a significantly positive correlation of the PADQLQ score with nasopharyngeal microbiome Shannon and Simpson diversities, but not with the diversity indexes of the ocular microbiome (data not shown).

3.5 | Characterization of ocular and nasopharyngeal microbiome in healthy controls and ARC patients

The most abundant phyla were Proteobacteria (nasal 54.73%; ocular 41.07%), Firmicutes (nasal 23.82%; ocular 30.55%), and Actinobacteria (nasal 19.87%; ocular 15.81%), accounting for over 85% of SVs across most ocular and nasal samples regardless of the disease status (Figure 3A and Table S3). Other phyla

accounting for more than 1% of SVs included Bacteroidetes (nasal 1.01%; ocular 8.12%), Fusobacteria (nasal 0.23%; ocular 1.08%), and Deinococcus-Thermus (nasal 0.07%; ocular 1.18%). Of note, the ratio of Bacteroidetes ($W = 16$), Cyanobacteria ($W = 13$), and Deinococcus-Thermus ($W = 12$) relative to the remaining phyla was significantly higher in ocular than nasal in healthy control subjects but not in ARC patients (Figure 3C and Figure S4A). At the genus level, the top five most prevalent candidates were Moraxella (nasal 44.58%; ocular 3.35%), Corynebacterium (nasal 17.64%; ocular 4.57%), Streptococcus (nasal 4.59%; ocular 13.86%), Staphylococcus (nasal 6.64%; ocular 10.54%), and Dolosigranulum (nasal 10.82%; ocular 0.30%; Figure 3B and Table S3). Moraxella was the most abundant genus for nasopharyngeal microbiome (control 52.65%; ARC 32.20%), whereas Streptococcus was the most abundant genus for ocular microbiome (control 13.22%; ARC 14.49%). Healthy controls had significantly more Moraxella (52.65% vs 3.31%; $W = 423$) and Dolosigranulum (10.94% vs 0.46%; $W = 424$) in the nasal compartment compared to the ocular (Figure 3D). As with ARC patients, only one genus showed significantly different abundance between ocular and nasal samples, and this genus belonged to the order Micavibrionales with the relative abundance <1% for all samples (Figure S4B). In the search of taxa with differential abundance between controls and ARC patients, neither candidate was identified from any taxonomic level for both ocular and nasal samples (Figure S4C,D). The relative abundance of all phyla and genera in each individual sample is shown in Figure S5.

4 | DISCUSSION

Allergic rhinoconjunctivitis is one of the common inflammatory disorders with several symptoms involving both eye and nose. Although previous studies attempted to examine microbiome dysbiosis along the respiratory tract and the ocular surface in various diseases, these studies only focus on either nasal or ocular microbiome.^{7,17} For the first time, the microbial community in context of both body sites (ocular and nasal) and disease status (ARC or not) was delineated and compared simultaneously.

In line with the previous findings, bacteria identified at the nasopharyngeal mucosa were dominated by Moraxella, Corynebacterium, Staphylococcus, Dolosigranulum, Streptococcus, and Haemophilus in children.¹⁸ Notably, the level of Moraxella and Dolosigranulum was significantly higher in the nasopharyngeal samples than in the ocular microbiome in control subjects, suggesting an advantage of these genera colonizing the nasal area over the eyes. The detection of Streptococcus, Staphylococcus, Corynebacterium, Propionibacterium, and Haemophilus at the conjunctiva in this cohort was also coherent with other 16S amplicon sequencing-based studies.^{19,20} As these genera were shared by most ocular samples, our data supported the existence of core microbiome in human conjunctiva in the pediatric population.²¹ Besides, more Bacteroidetes, Deinococcus-Thermus, and Cyanobacteria were present in ocular surfaces than the nasopharyngeal ones among the controls.

TABLE 2 Analysis of beta diversity

Group	Metric	pseudo-F	P value
OC (n = 14) vs NC (n = 17)	Jaccard distance	2.11	0.001
	Bray-Curtis dissimilarity	4.86	0.001
	Unweighted UniFrac distance	5.77	0.001
	Weighted UniFrac distance	10.5	0.001
OA (n = 14) vs NA (n = 21)	Jaccard distance	1.17	0.048
	Bray-Curtis dissimilarity	2.73	0.002
	Unweighted UniFrac distance	1.55	0.072
	Weighted UniFrac distance	3.31	0.005
OC (n = 14) vs OA (n = 15)	Jaccard distance	0.956	0.786
	Bray-Curtis dissimilarity	1.21	0.126
	Unweighted UniFrac distance	1.10	0.307
	Weighted UniFrac distance	1.00	0.402
NC (n = 17) vs NA (n = 21)	Jaccard distance	1.13	0.127
	Bray-Curtis dissimilarity	1.06	0.385
	Unweighted UniFrac distance	1.75	0.056
	Weighted UniFrac distance	2.11	0.101

Abbreviations: NA, nasal-ARC patients; NC, nasal-healthy controls; OA, ocular-ARC patients; OC, ocular-healthy controls.

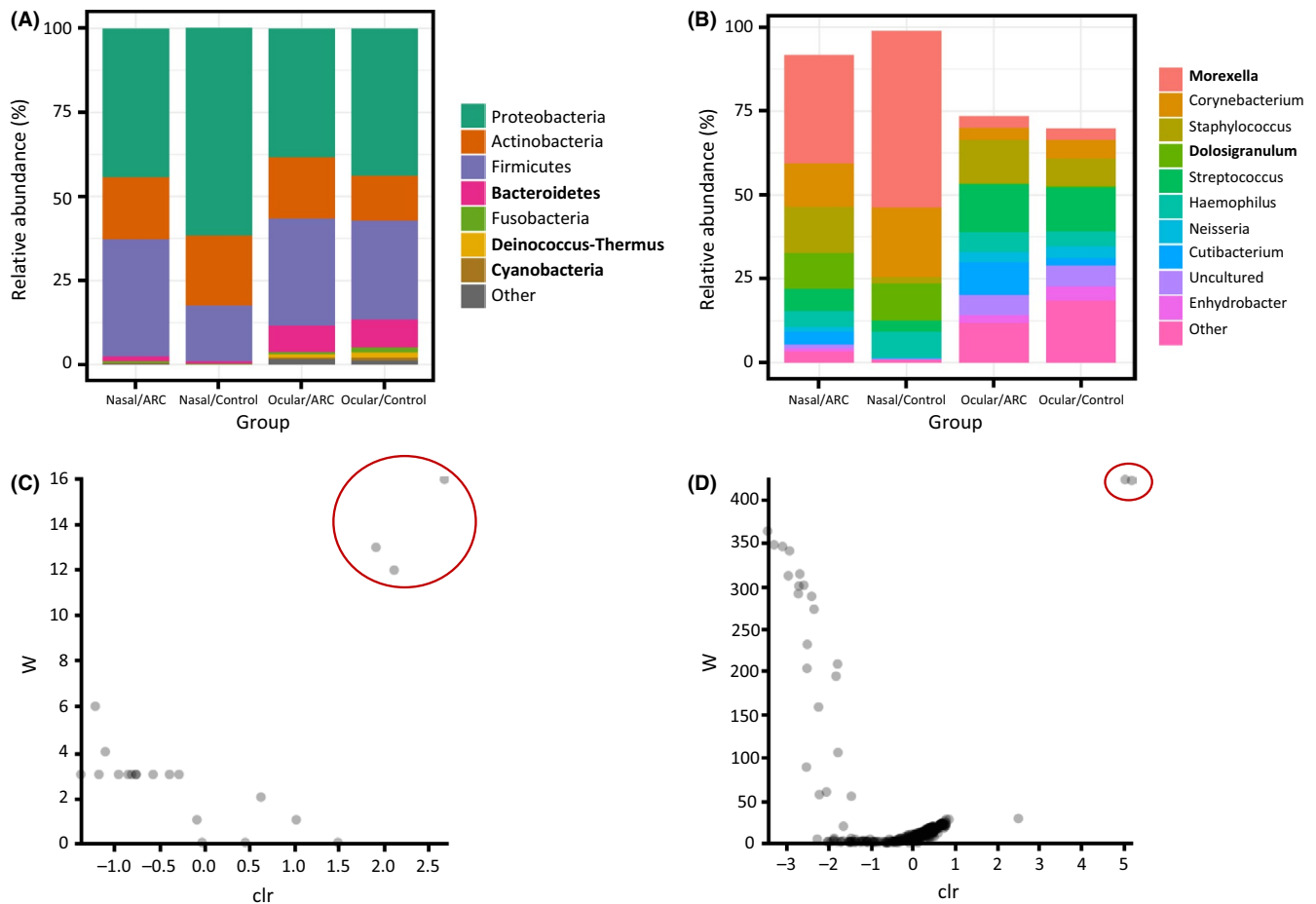


FIGURE 3 Taxonomical analysis of the ocular and nasopharyngeal microbiome. Histogram showing the mean relative abundance at (A) the phylum and (B) the genus level. Genera with the 90th percentile relative abundance > 1% were indicated. “Uncultured” refers to the sequence variants (SVs) that have no match in the Silva 132 database. They are classified in the Neisseriaceae family. The volcano plot indicated the differential abundant (C) phylum and (D) genus using ANCOM test. Clr, centered log ratio transformation on the relative abundance of each SV. The SVs with differential abundance between ocular and nasal microbiota for healthy controls were bold in the panels in (A) and (B) [Colour figure can be viewed at wileyonlinelibrary.com]

Although the percentages of these phyla were not high and were not considered as part of the substantial core, these phyla accounted for >1% of SVs, suggesting that it is less likely the detection of these phyla was due to environmental contamination. The difference of the age range of subjects recruited might explain the discrepancy of proportion of these phyla compared to the other studies.²⁰

Among children with ARC, the nasopharyngeal microbiome displayed a higher alpha diversity compared to that of healthy subjects. Higher diversity of the nasal samples was associated with greater SFAR and PADQOL scores. Severity of ARC was suggested to be evaluated based on the nasal and non-nasal symptom severity and quality of life.²² Intriguingly, SFAR and PADQOL included questions on these components and the two scores had a positive correlation (data not shown). If given more information on the frequency of ARC-related medical consultation and the effectiveness of medication for the patients, the relationship between disease severity and microbiome could be confirmed.

The finding of an increase of nasopharyngeal microbiome diversities was out of expectation at first sight. It is more common to observe

a reduction in diversity during various diseases.⁶ Nevertheless, as demonstrated in studies related to asthma and food allergy, dysbiosis in terms of decrement of microbial diversities was not always true.^{23–25} Asthmatic patients had greater bacterial diversity in their induced sputum and bronchial samples when compared to non-asthmatics controls.^{23,24} In some adult patients with ARC, an increase of bacterial and fungal diversity in middle meatus and nasal vestibule was reported.^{26,27} A possible mechanism of microbiome modulating inflammation is through microbial metabolites. For example, short-chain fatty acids released by gut microbes induce Treg cells and dendritic cell precursors, which in turn suppress inflammation and alleviate allergy.²⁸ Both asthma and ARC involve inflammation of different parts of the respiratory tract, and over one-third of patients with ARC experienced asthma. Further study is needed to differentiate whether such an increase of microbial diversity precedes these airway allergies or the microbial alterations are just consequences of the diseases.

The nasal and ocular microbiome remained unique to one another in terms of microbial diversities, independent of ARC status.

Yet, significant differences between nasopharyngeal and ocular microbiome in certain phyla and genera could only be found in the control samples. Our result suggested that for the patients with ARC, the bacterial community at the nasopharyngeal mucosa became more similar to conjunctiva, when compared to the non-ARC subjects. Together with an increase of alpha diversities for the nasopharyngeal microbial samples from patients with ARC, it is suspected that the dynamics of nasopharyngeal microbiome might be driven by the ocular microbiome. The nasolacrimal duct bridges the lacrimal sac of the eye and the nasal cavity anatomically and plays a role in innate immunity.²⁹ Hence, tearing as well as frequent rubbing of eyes and noses in patients with ARC might facilitate conjunctival bacteria reaching the nasal cavity. It would be exciting to examine the viability of different microbes detected in the tear fluid and the possible interactions of commensals between one body site and the other.

In the past, there were some controversies on the impact of conjunctival microbiome on ocular health due to its low biomass.^{21,30,31} With more sequencing platform of high sensitivities available, alterations of ocular microbiome were found in several eye problems.^{17,32} In patients with blepharitis, bacterial communities found in the tear samples in patients were distinct from those in healthy individuals.³² Although the detailed mechanism of how the ocular microbiome cross-linked with the inflammatory pathways is still unknown, in a recent mouse model study, an ocular commensal elicited IL-17 production and protected the eyes from infection, indicating a role of the microbes in localized immune response.³³ The alpha diversity of ocular samples in our cohort decreased with SFAR, suggesting the linkage of ocular microbiome and ARC. A reason why the alpha diversity of ocular microbial community in ARC patients did not show significant difference compared to non-ARC controls might be that a large proportion of ARC patients enrolled had relatively mild ocular symptoms of ARC and the statistical power of the small sample size was reduced. In the future, a similar cross-sectional study could be conducted, with an increase of sample size, more cases with moderate-to-severe ARC, to confirm the role of ocular microbiome and to see whether particular genera of microbes are associated with the disease.

Possible factors shaping microbiome included gender, age, use of medications, and disease comorbidity.^{7,34-36} Little studies were conducted to explore these factors on conjunctival microbiome particularly in the pediatric populations. After adjusting for these factors, the result did not change dramatically (Figure S7). Nevertheless, one should always take these variables into considerations when establishing a similar cohort with a bigger sample size given the former studies.

The reliability and reproducibility of metagenomic data depend a lot on the design of the bioinformatics pipeline. The current pipeline aimed at high accuracy in taxonomic assignment and minimizes false discoveries. Compared to conventional operational taxonomic unit (OTU) binning methods where all reads of >97% similarity were classified as the same unit, DADA2 approach infers exact SVs with a finer resolution up to single nucleotide level. Moreover, compositionality

of microbiome data was often neglected in previous studies.³⁷ During differential abundance testing, ANCOM takes this issue into account without sacrificing statistical power and sensitivity while maintaining low false discovery rates in contrast to other tools.³⁷ Therefore, these two methods were chosen for the present study.

Overall, our finding that the nasopharyngeal microbiome becomes similar to ocular microbiome in ARC patients while remaining significantly distinct between the two for healthy people suggests the potential interaction between nasal and ocular microbiome of these patients. Further studies are warranted to further investigate any specific bacterial species that are beneficial to host immunity and can be targeted for developing therapeutic strategies to treat ARC.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

K.L.H. supervised the work and conceived the project. J.W.Y. designed the study and prepared the clinical samples for sequencing; J.P.H. was responsible for the computational tools and codes; J.W.Y. and J.P.H. analyzed and interpreted the data as well as prepared the manuscript; S.K.W.T., T.F.L. and V.J. contributed to result interpretation and manuscript revision. J.W.Y., N.S.C., J.C.Y. and K.W.K. helped in clinical assessment and sample acquisition.

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REFERENCES

1. Passali D, et al. The International Study of the Allergic Rhinitis Survey: outcomes from 4 geographical regions. *Asia Pac Allergy*. 2018;8:1-15.
2. De Groot H, Brand P, Fokkens WF, Berger MY. Allergic rhinoconjunctivitis in children. *Br Med J*. 2007;335:985-988.
3. Broide DH. The pathophysiology of allergic rhinoconjunctivitis. *Allergy Asthma Proc*. 2007;28:398-403.
4. Gelardi M, Leo ME, Quaranta VN, et al. Clinical characteristics associated with conjunctival inflammation in allergic rhinoconjunctivitis. *J Allergy Clin Immunol Pract*. 2015;3:387-391.e1.
5. Brehler R, Stöcker B, Grundmann S. Allergologie - aktuelles zur prävention und diagnostik von soforttyp-allergien und zur therapie der allergischen rhinokonjunktivitis. *J Dtsch Dermatol Ges*. 2015;13:747-764.
6. Lynch SV. Gut microbiota and allergic disease: new insights. *Ann Am Thorac Soc*. 2016;13:S51-S54.
7. Yang HJ, LoSavio PS, Engen PA, et al. Association of nasal microbiome and asthma control in patients with chronic rhinosinusitis. *Clin Exp Allergy*. 2018;48(12):1744-1747.
8. Watters GA, Turnbull PR, Swift S, Petty A, Craig JP. Ocular surface microbiome in meibomian gland dysfunction. *Clin Exp Ophthalmol*. 2017;45:105-111.

9. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 2013;41:1-11.
10. Callahan BJ, McMurdie PJ, Rosen MJ, et al. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods.* 2016;13:581-583.
11. R Core Team. *R: A Language and Environment for Statistical Computing.* Vienna, Austria: R Foundation for Statistical Computing.
12. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 2013;41:D590-D596.
13. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* 2013;8:e61217.
14. Hill T, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. *FEMS Microbiol Ecol.* 2003;43:1-11.
15. Anderson MJ. Permutational multivariate analysis of variance (PERMANOVA). Wiley StatsRef: Statistics Reference Online 1-15. 2017. <https://doi.org/10.1002/9781118445112.stat07841>
16. Mandal S, Van Treuren W, White RA, et al. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis.* 2015;26:27663.
17. Zhou Y, Holland MJ, Makalo P, et al. The conjunctival microbiome in health and trachomatous disease: a case control study. *Genome Med.* 2014;6:1-10.
18. Man WH, De Steenhuijsen Piters W, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol.* 2017;15:259-270.
19. Dong Q, Brulc JM, Iovieno A, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci.* 2011;52:5408-5413.
20. Cavuoto KM, Banerjee S, Miller D, Galor A. Composition and comparison of the ocular surface microbiome in infants and older children. *Transl Vis Sci Technol.* 2018;7(6):16.
21. Kugadas A, Gadjeva M. Impact of microbiome on ocular health. *Ocul Surf.* 2016;14:342-349.
22. Spector SL, Nicklas RA, Chapman JA, et al. Symptom severity assessment of allergic rhinitis: Part 1. *Ann Allergy Asthma Immunol.* 2003;91:105-114.
23. Huang YJ, Nelson CE, Brodie EL, et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol.* 2011;127:372-381.e3.
24. Marri PR, Stern DA, Wright AL, Billheimer D, Martinez FD. Asthma-associated differences in microbial composition of induced sputum. *J Allergy Clin Immunol.* 2013;131:346-352.e3.
25. Fazlollahi M, Chun Y, Grishin A, et al. Early-life gut microbiome and egg allergy. *Allergy Eur J Allergy Clin Immunol.* 2018;73:1515-1524.
26. Choi CH, et al. Seasonal allergic rhinitis affects sinonasal microbiota. *Am. J. Rhinol. Allergy.* 2014;28:281-286.
27. Jung WH, Croll D, Cho JH, Kim YR, Lee YW. Analysis of the nasal vestibule mycobiome in patients with allergic rhinitis. *Mycoses.* 2015;58:167-172.
28. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell.* 2016;165:1332-1345.
29. Knop E, Knop N. Lacrimal drainage-associated lymphoid tissue (LDALT): a part of the human mucosal immune system. *Invest Ophthalmol Vis Sci.* 2001;42:566-574.
30. Willcox M. Characterization of the normal microbiota of the ocular surface. *Exp Eye Res.* 2013;117:99-105.
31. Zegans ME, Van Gelder RN. Considerations in understanding the ocular surface microbiome. *Am J Ophthalmol.* 2014;158:420-422.
32. Lee SH, Oh DH, Jung JY, Kim JC, Jeon CO. Comparative ocular microbial communities in humans with and without blepharitis. *Invest Ophthalmol Vis Sci.* 2012;53:5585-5593.
33. St. Leger AJ, Desai JV, Drummond RA, et al. Ocular commensal protects against corneal infection by driving an interleukin-17 response from mucosal $\gamma\delta$ T cells. *Immunity.* 2017;47:148-158.e5.
34. Cavuoto KM, Mendez R, Miller D, Galor A, Banerjee S. Effect of clinical parameters on the ocular surface microbiome in children and adults. *Clin Ophthalmol.* 2018;12:1189-1197.
35. Hagymási K, Bacsárdi A, Egresi A, et al. The role of gut microbiota in chronic liver diseases, and treatment possibilities. *Orv Hetil.* 2018;159:1465-1474.
36. Ozkan J, Nielsen S, Diez-Vives C, et al. Temporal stability and composition of the ocular surface microbiome. *Sci Rep.* 2017;7:1-11.
37. Weiss S, Xu ZZ, Peddada S, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome.* 2017;5:1-18.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Fig. S1 Alpha diversity of microbiota based on Simpson's diversity index.

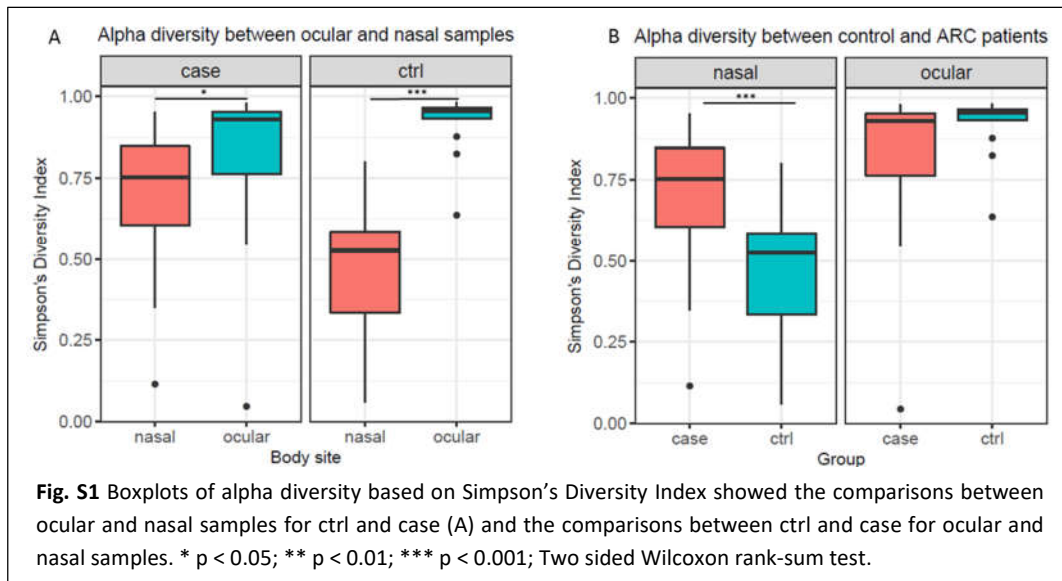


Fig. S2 PCoA plots showing the analysis of beta diversity based on Unweighted UniFrac distances

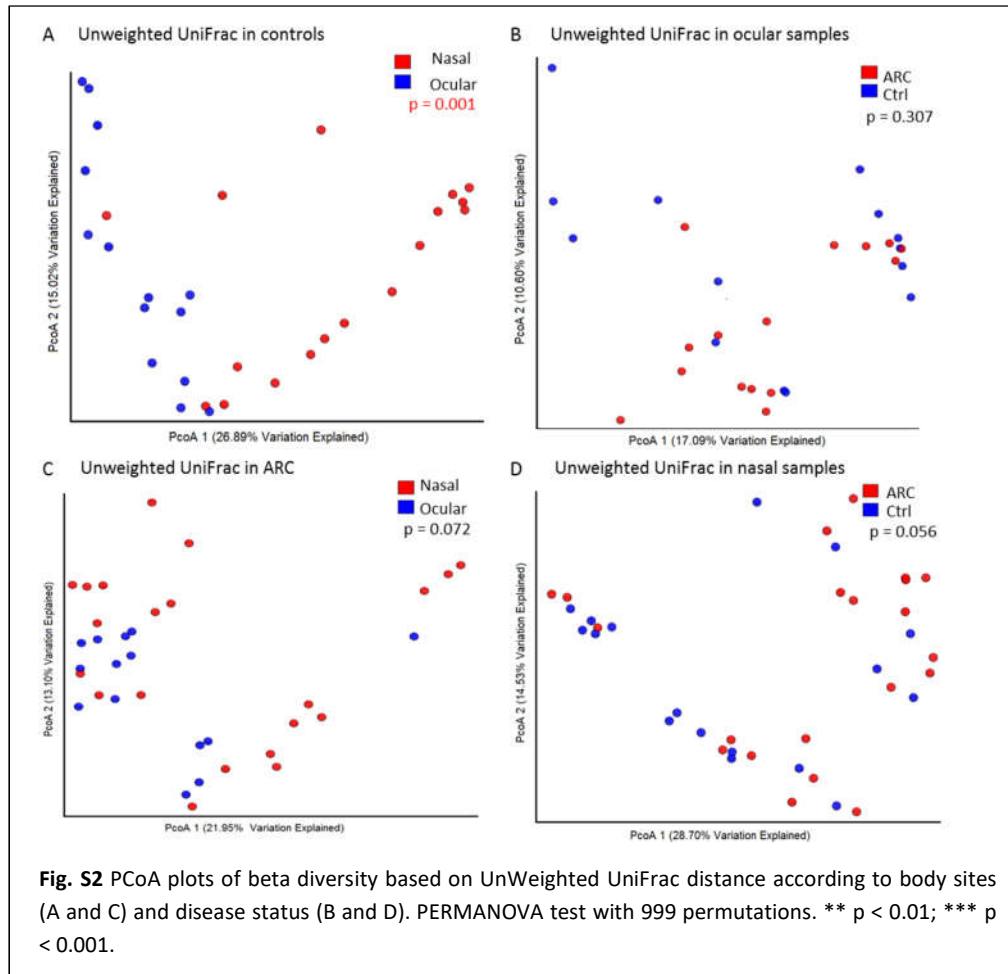


Fig. S3 PCoA plots showing the analysis of beta diversity based on Bray-Curtis dissimilarity.

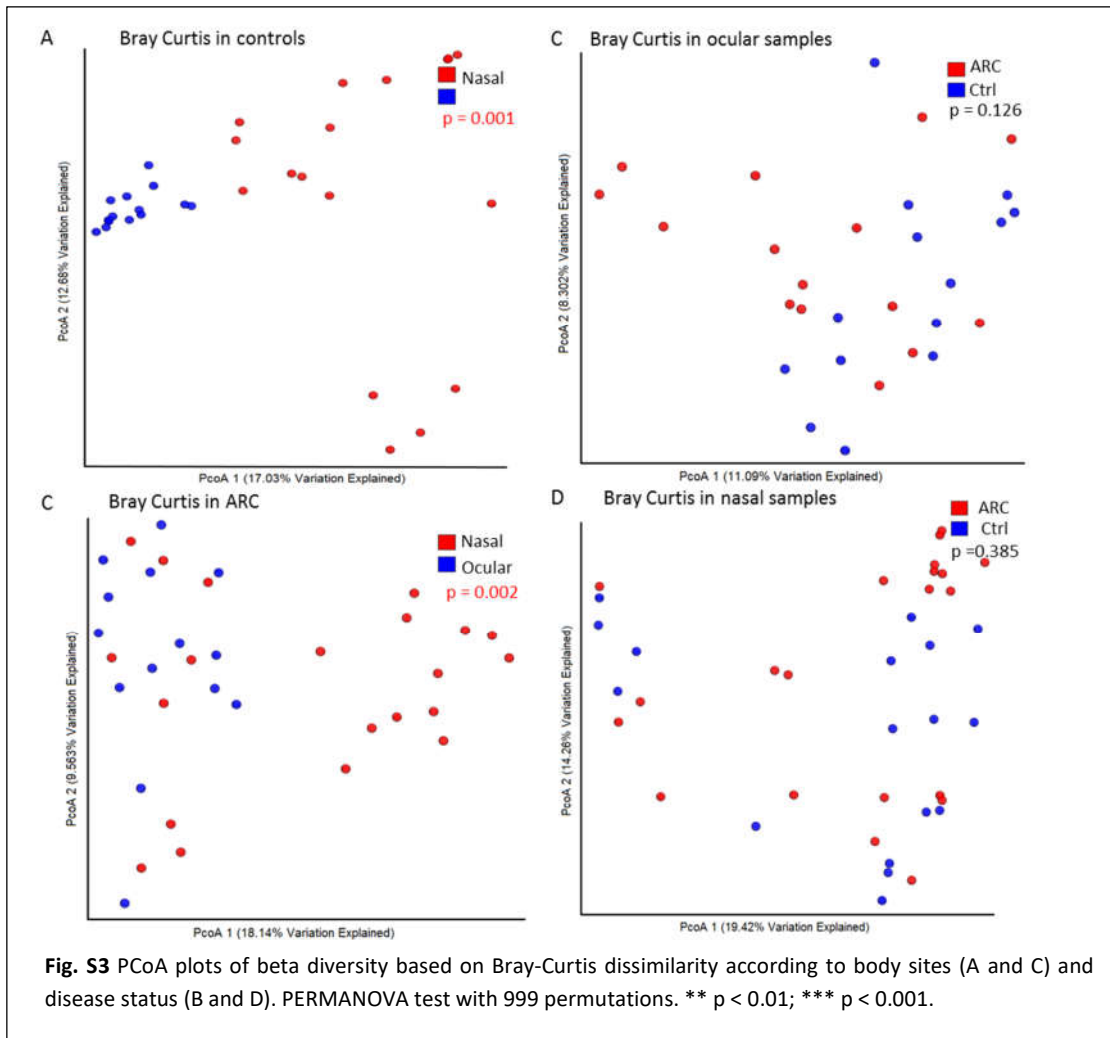


Fig. S4 Differential abundant test at the phylum (A) and the genus (B) level for ARC patients.

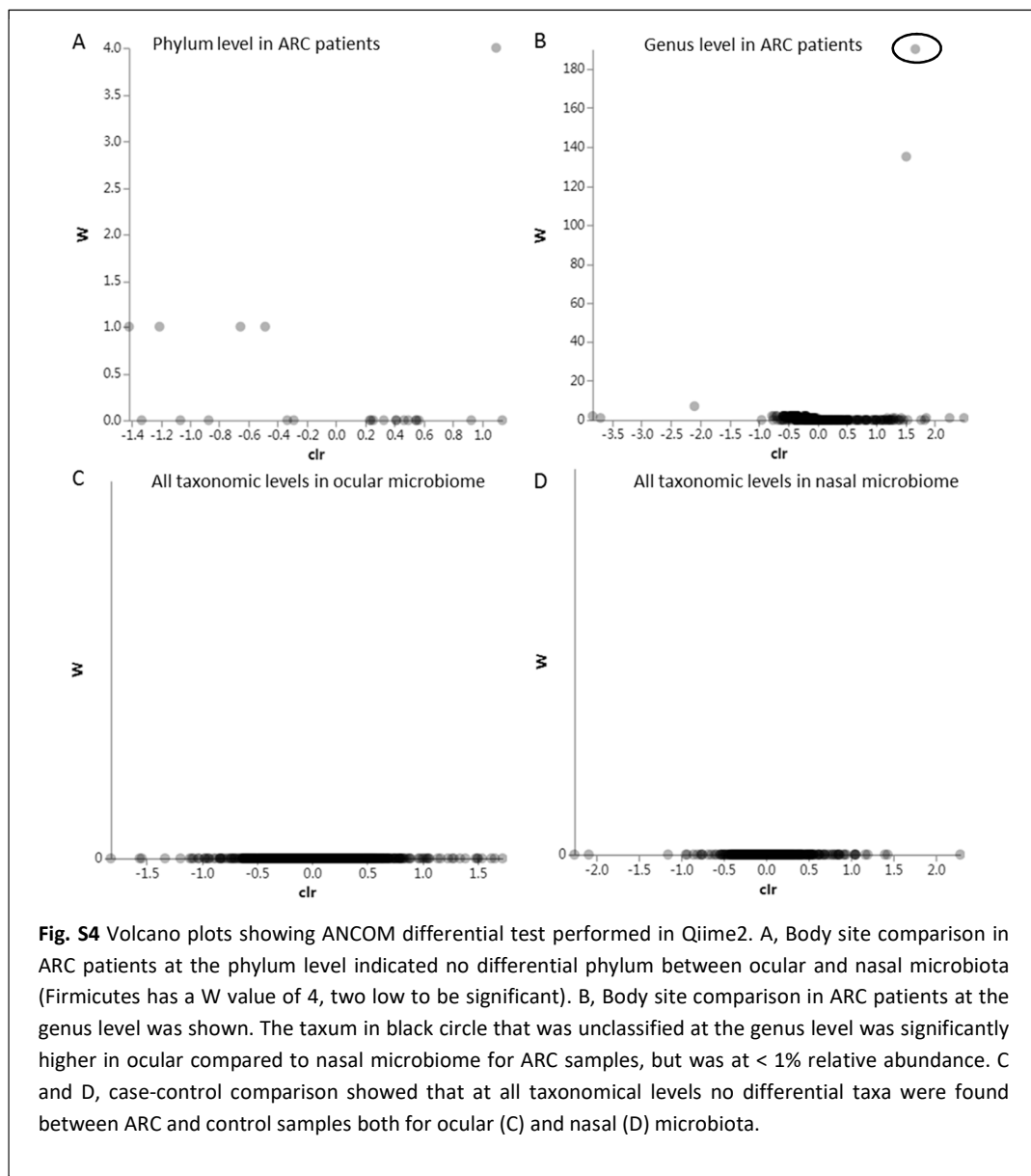


Fig. S5 The relative abundance of ocular and nasal microbiota by individual samples at the phylum (A) and genus (B) level.

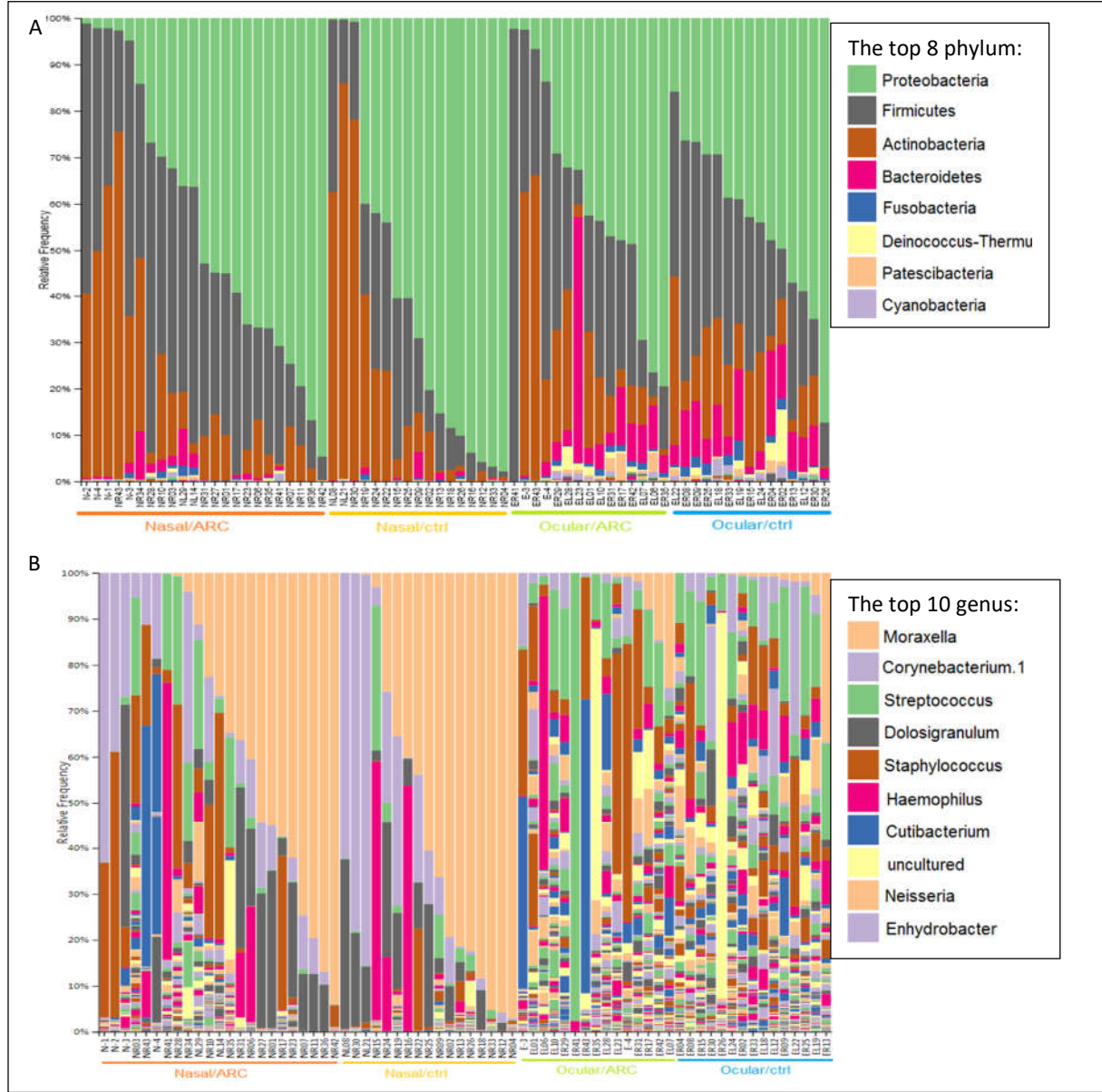


Fig. S6 The correlation between ARC severity and Simpson's diversity index in both nasal and ocular samples

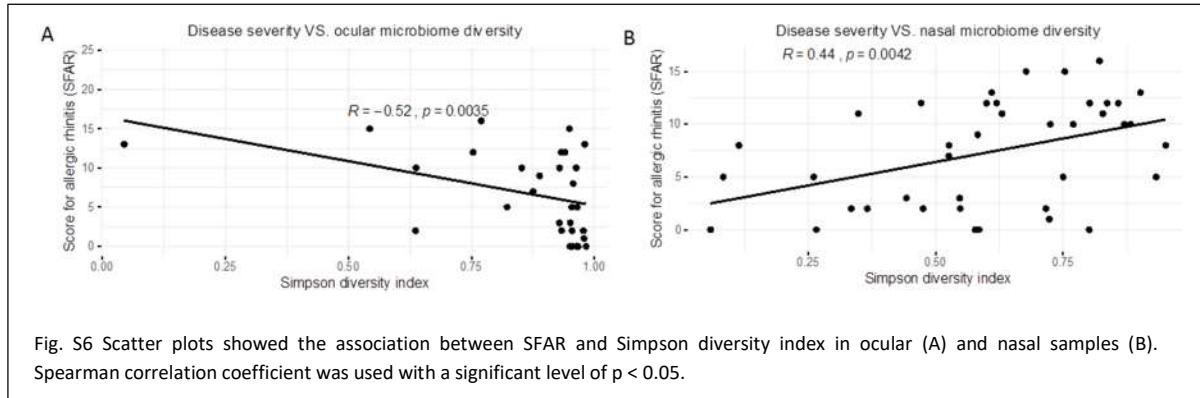
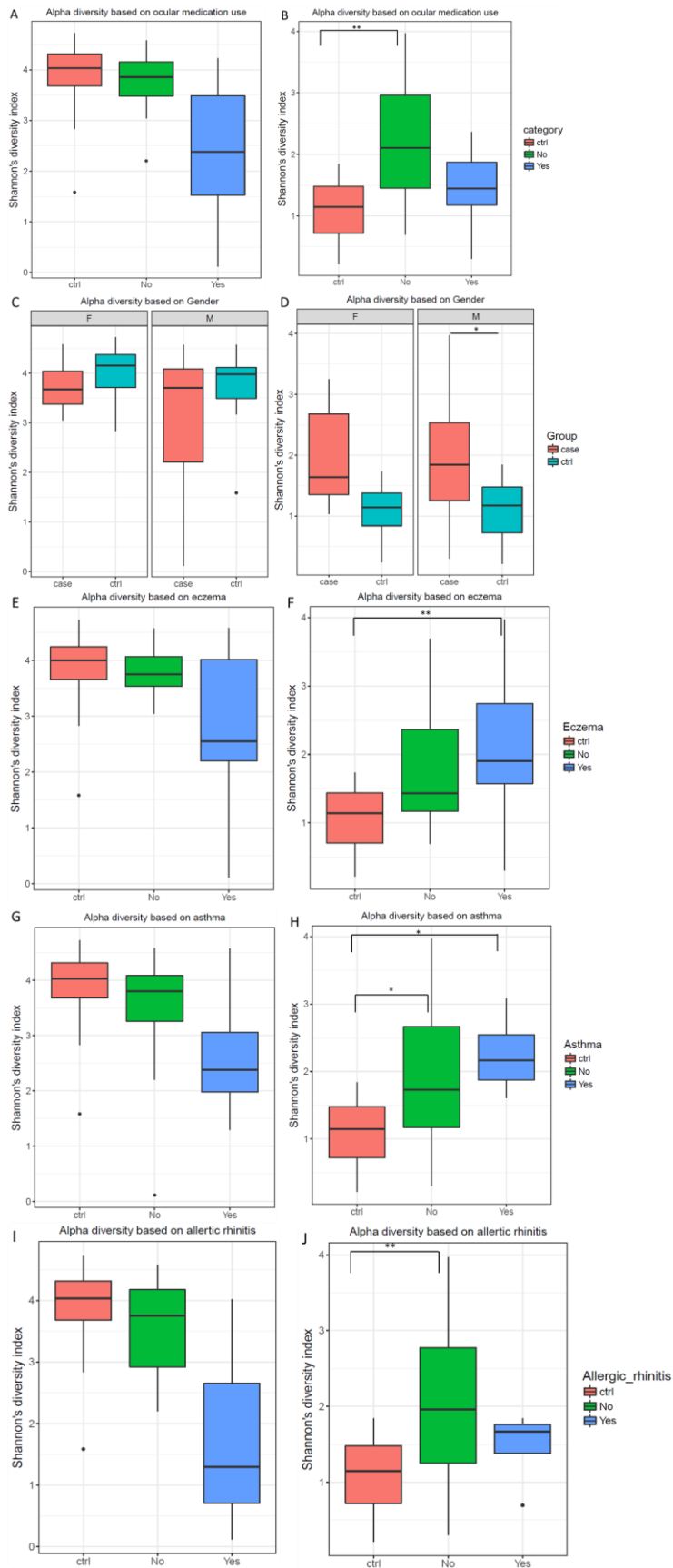


Figure S7



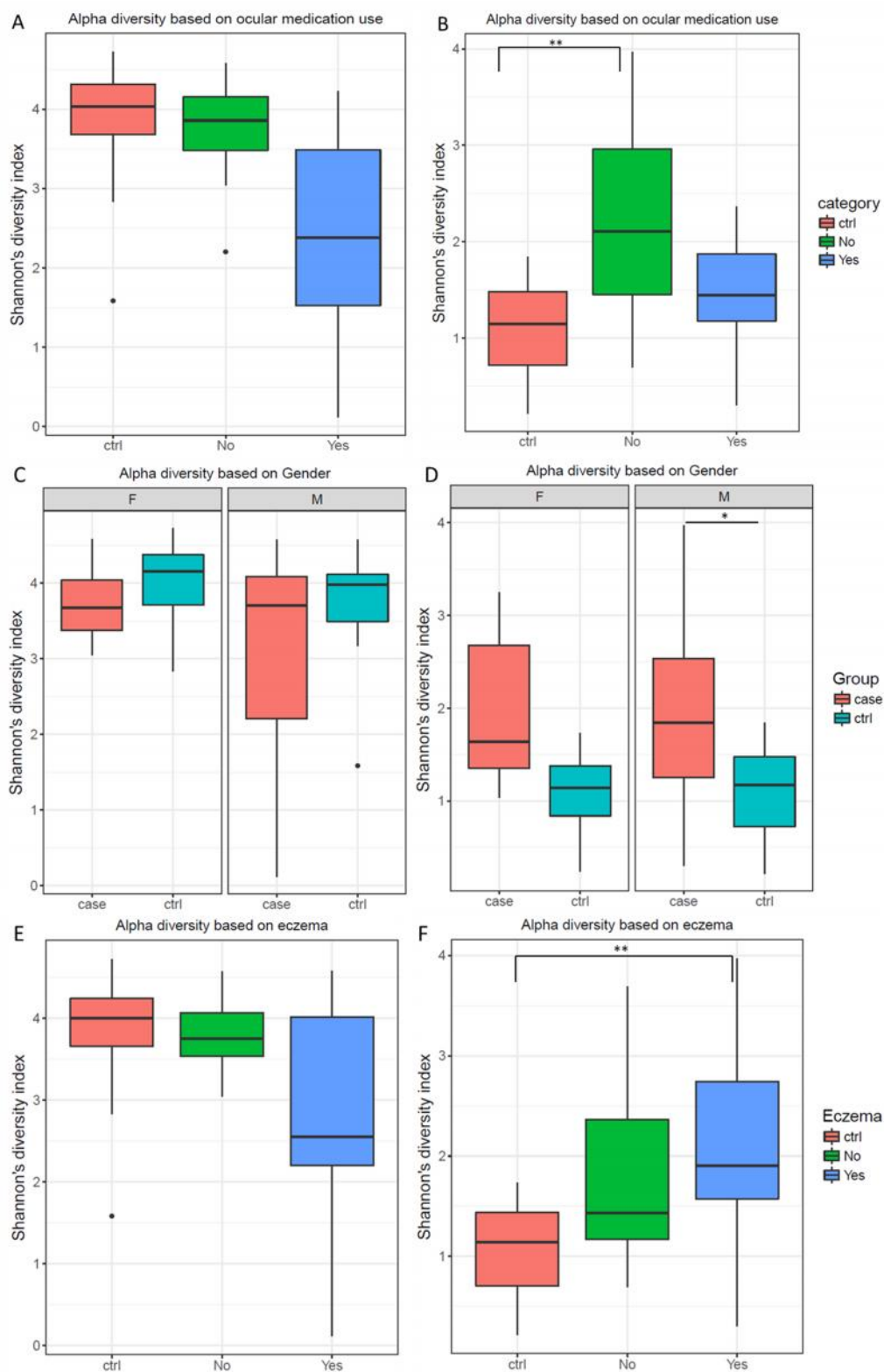


Figure S7 Continue on next page. See next page for the captions.

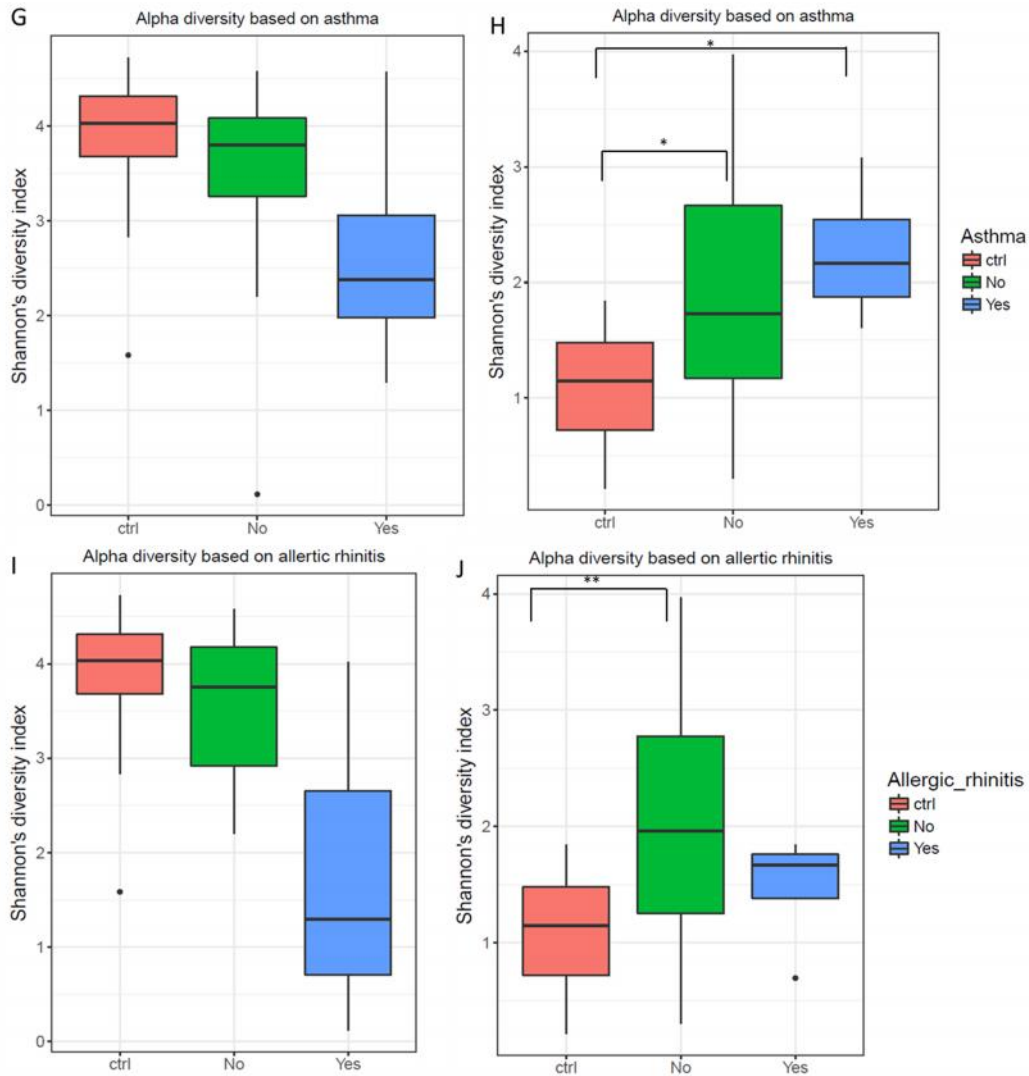


Figure S7. Boxplots showed the alpha diversity of ocular (A, C, E, G, I) and nasal (B, D, F, H, J) microbiome between ARC patients and healthy controls. The Shannon diversity of ocular microbiome between ARC and control still had no significant difference after adjusting for ocular medication use (A), gender (C), and disease comorbidity (E for eczema, G for asthma, I for allergic rhinitis). By contrast, alpha diversity of nasal microbiome was significantly higher in ARC than in controls even after adjusting for the ocular medication use (B), gender (D), and disease comorbidity (F for eczema, H for asthma, J for allergic rhinitis). Kruskal-Wallis Test was used to test the differences across 3 groups. And pairwise Wilcoxon Rank Sum test was used to calculate pairwise comparisons between two groups with Benjamini & Hochberg as multiple testing correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.