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Original Article

Characteristics and associations of microbial community in the eye, anterior nares, and oropharynx of healthy adults: eye-nose microbiota transmission

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Abstract: Purpose: We compared the microbial communities of the eye, anterior nares (ANs) and oropharynx (OP) of healthy adults to provide a basic understanding of the microbial associations among the three sites. Methods: The name of the registry of our prospective study was "Study on the diversity of microbial flora in the eye, nose and oropharynx of healthy people". The trial number is ChiCTR2300067724 (https://www.chictr.org.cn/index.aspx). Swabs were collected from the eye, ANs and OP of 48 healthy adult participants for 16S rRNA gene amplicon sequencing. The bacterial community profiles and their functional associations were compared among the three sites. Results: At a phylum level, the basic bacterial compositions in the eye and ANs were generally similar, and the predominant phyla were *Actinobacteria, Proteobacteria* and *Firmicutes*. In contrast, the OP microbiota was characterized by an increased abundance of *Bacteroidetes*. At a genus level, *Corynebacterium, Cutibacterium* and *Staphylococcus* were the most abundant in the eye and ANs. *Prevotella-7, Alloprevatella, Haemophilus* and *Streptococcus* were more abundant in the OP. Correlation analysis of the eye and ANs microbiota suggested that *Cutibacterium* and *Micrococcus* may migrate from the eye to nose ($P < 0.05$). Conclusions: The bacterial flora composition and function predictions of eye and ANs were similar, but differed from those of the OP in healthy adults. The OP bacterial flora distribution was markedly different, showing characteristics similar to that of the digestive tract flora. Thus, the eye and ANs microorganisms may be related in healthy individuals. *Cutibacterium* and *Micrococcus* may migrate from the eye to the nose.

Keywords: Ocular surface microbiota, nose microbiota, 16S rRNA gene sequencing, bacterial transmission

Introduction

Anatomically, the eyes and nasopharynx are connected. The eye, anterior nares (ANs) and oropharynx (OP) are open to the air and form an "open ecosystem". The three niches are protected by the mucosal immune system and are vulnerable to multiple external environmental factors, such as delivery and feeding methods, growth environment, nutritional status, host immunity, smoking, trauma and surgery, inflammation, and use of antibiotics and other drugs [1, 2].

The ocular surface is continuously exposed to the external environment and harbors various commensal microbiota that play fundamental roles in the modulation of host physiology, in the induction and development of the immune system, and in the host's defense against pathogen invasion [3]. The nasolacrimal system provides a bridge between the ocular and respiratory systems via the nasopharynx, providing a conduit for microbiota exchange between these sites [4]. Tears are secreted from the lacrimal gland and are distributed on the anterior surface of the eyeball. They are then gathered in the conjunctival sac and lacrimal lake temporarily, via the lacrimal puncta and canaliculi, into the lacrimal sac and through the nasolacrimal duct into nose [5]. Cavuoto et al. assumed that there may be a correlation between microorganisms in the eye and nose [6]. Mucociliary clearance is a major self-clearing process in the nasal cavity. It functions by capturing particles and microorganisms in mucus and delivering the mucous film to the OP, where it is eliminated via coughing or swallowing [7].

In general, microorganisms are likely transported through the nasolacrimal duct and then enter the nasopharynx, OP, and respiratory and gastrointestinal systems [8]. Current studies suggest that the nasopharynx is even a major "opportunistic pathogen reservoir" [9, 10] and harbors opportunistic pathogens, which can then spread to the upper respiratory tract, and promote the development of diseases.

Recent studies have been conducted on microbial flora in different niches of health and disease. It has been indicated that the microbiota in other niches of humans, such as the cephalic and gut microbiome, are associated with the pathophysiology of some ophthalmic diseases [11-13]. The oral microbiota of patients with glaucoma showed a much higher count than that of the control groups, which suggests that increased bacterial loads in the oral cavity might be related to microglial activation in the optic nerve and retina, eventually leading to neurodegeneration [14].

The microbial composition of a niche is determined by the characteristics of the given niche, such as the eye, ANs and OP. However, there have been few reports on the comparison and association of nearby anatomic niche microbes in healthy hosts. In addition, some studies have compared microbial flora in different sampling sites of the nasopharynx and upper respiratory tract in healthy individuals. They found that the ANs and OP are highly representative sites for sampling, as other ecological niches are not easily accessible [15-19].

In this study, we compared the composition and associated functions of the microbial communities in the eye, ANs and OP of healthy adults, using 16S rRNA gene sequencing, to provide a baseline for understanding the microbial associations among these three sites that can be used as reference in future work.

Materials and methods

Patients and sample collection

This is a prospective study. Forty-eight healthy adults were recruited from the Shanxi Eye Hospital between July and August 2021. Informed consent was obtained from all participants. All methods adhered to the principles of the Declaration of Helsinki (SXYYLL20210614). The inclusion criteria were the absence of infectious eye disease and passable irrigation of the lacrimal passage. The exclusion criteria were as follows: eye and nasopharynx infectious diseases; history of surgery and trauma; local or systemic use of antibiotics, immunosuppressants, eye drops and nasal sprays in the past 6 months; nasopharyngeal organic disease or lacrimal duct diseases (such as lacrimal atresia, dacryocystitis); contact lens wearing within the past 6 months; dry eye; systemic diseases (diabetes, hypertension, etc.); and pregnancy or lactation. All participants underwent a complete ophthalmic examination under a slit-lamp biomicroscope and complete nasal and oropharyngeal examinations by the same doctor.

Microbial samples from the conjunctival sac of the lower eyelid, ipsilateral ANs and OP were collected by an experienced clinician with specific swabs (EYE: 4N6FLOQSwabs, COPAN Diagnostics, Murrieta, CA; ANs/OP: Sterile Swabs, KangJian, China). Sterile swabs were collected early in the morning, prior to tooth brushing and eating breakfast. OP swabs were obtained by swabbing the left and right tonsillar surfaces and posterior wall of the tonsil. All samples were stored frozen at -80°C after sampling.

16S rRNA gene sequencing

For the analysis of the microbiota, genomic DNA was extracted using the DNeasy PowerSoil® Pro Kit (QIAGEN, Hilden, Germany). The V3 and V4 hypervariable regions of bacterial 16S rRNA genes were amplified from extracted DNA samples. All polymerase chain reactions (PCRs) were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 2 μM of forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles each consisting of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, and a final extension step at 72°C for 5 min. The PCR products were mixed at equidense ratios. The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, Dusseldorf, Germany). Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the standard protocols, and index codes were added. After

assessing the library quality using the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system, the library was sequenced on an Illumina NovaSeq platform following the manufacturer's recommendations, and 250-bp paired-end reads were generated.

Statistical and bioinformatics analyses

Tag generation and filtering: Sequencing data were assigned to samples based on their unique barcodes and were truncated by cutting off barcode and primer sequences. Each read pair was merged into a continuous tag using FLASH (V.1.2.7) [20] and overlap relationship information. Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags [21] according to the QIIME (V.1.9.1, http://qiime.org/scripts/split libraries fastq.html) [22] quality control process. The tags were compared with the Silva database (https://www.arb-silva.de/) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) [23] to detect chimera sequences, which were subsequently removed [24]. The tags were further aligned to the human genome GRCh38 using bwa-mem (https://github.com/lh3/bwa). Tags with more than 200 bp aligned to the human genome were considered contaminated and removed.

Data normalization, operational taxonomic unit (OTU) clustering, and annotation: Tags were clustered into OTUs according to the recommended research pipeline. Rarefication curves were generated to evaluate whether sequencing data were sufficient. For comparison, tags from each site were downsampled to 24 k for downstream analysis. Sites with tags less than 24 k were removed. Additionally, subjects with incomplete data were removed.

After downsampling, OTUs were annotated to taxonomy using the Silva database, which is based on the Mothur algorithm [25]. The abundance of microbial genera was calculated and used in subsequent analyses.

The accession code of the microbiome raw sequence data in the NCBI Sequence Read Archive is PRJNA879932 (SAMN30839563-SAMN30839706).

Microbial richness and diversity: The Shannon index, Simpson index, Chao's estimator and Abundance-based coverage estimators (ACEs) were calculated using the vegan package in R software (https://www.r-project.org/). Euclidean distances were used for principal component analysis (PCA: factoextra::fviz_pca). Bray-Curtis dissimilarity was used for principal coordinate analysis (PCoA: ade4::dudi.pco). The linear discriminant analysis (LDA) effect-size method (LEfSe: http://huttenhower.sph.harvard.edu/galaxy) was used to compare the differences in bacterial community structure among the three sites. R version was 4.0.5. Statistical significance was set at $P < 0.05$.

Transfer from eye to nose

The Pearson correlation coefficient (PCC) between the relative abundance of a genus in the eye and that in the ANs was calculated and used as an indicator for eye-AN transfer. The empirical distribution of the PCC was estimated using a simple ideal transfer model (see Supplementary Material for details). Genera with a relative abundance > 1% at either site and with PCC that met the empirical distribution were considered as candidates for exchange between eye and ANs. For each candidate genus, a permutation test was performed to assess statistical significance.

The permutation test was conducted as follows. First, we assumed that the abundance of a given genus in the eye and ANs was independent. Therefore, shuffling the abundance between samples did not affect the PCC. We shuffled the relative abundance of the given genus in the ANs, re-normalized the relative abundance to ensure that they summed to 1, and calculated the PCC between the relative abundance in the eye and in the shuffled ANs data. This PCC was considered a data point in the null hypothesis. The shuffle-normalize-PCC procedure was repeated 1000 times to generate the empirical distribution under the null hypothesis, and the *p*-value was defined as the probability of calculated PCC ≥ observed PCC in the empirical distribution.

Functional prediction of microbial communities at the three sites

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt;

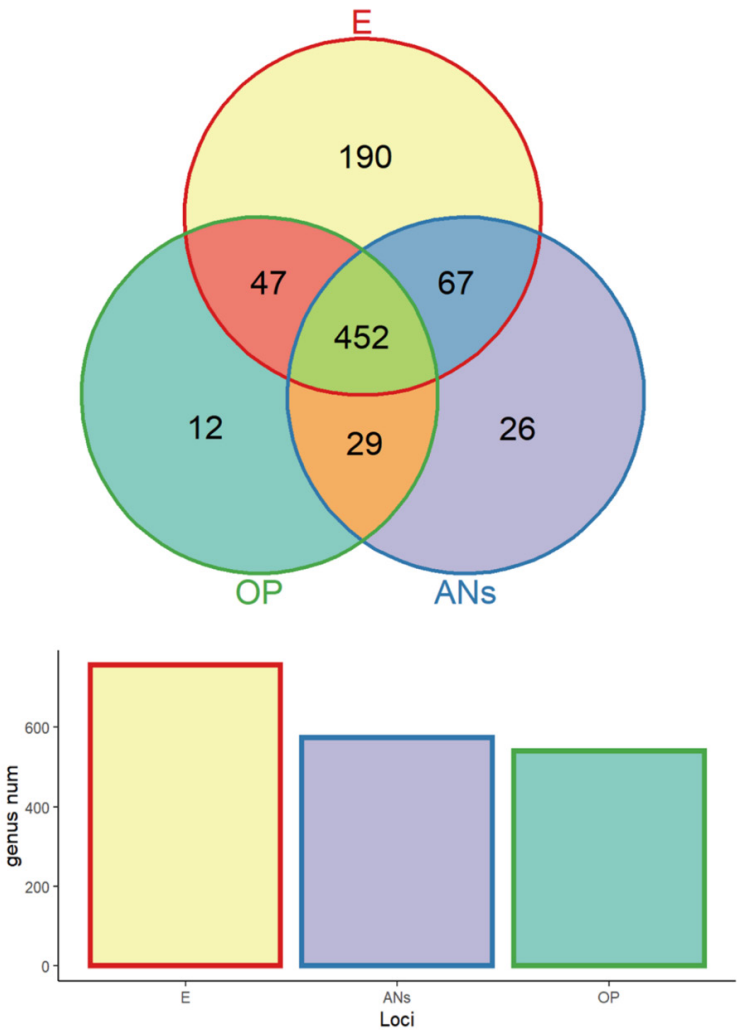


Figure 1. Venn diagram: at genus level, the number of microbial in Eye (E), Anterior nares (ANs) and Oropharynx (OP).

http://picrust.github.io/picrust/) was used to predict the functional profiles of the microbial communities in the eye, ANs and OP based on 16S rRNA gene sequences. In addition, pathways in Kyoto Encyclopedia of Genes and Genomes functional categories were identified.

Results

Subject population

Forty-eight healthy adults (22 females, 26 males) were enrolled in the study. The median age of the participants was 22.3 (range: 17-37) years.

On average, 82.8 k read-pairs were generated for each site, and 59.0 k tags remained after filtering. Rarefication curves (Supplementary Figure 1) were generated to evaluate whether the sequencing data were sufficient. For comparison, tags from each site were downsampled to 24 k. Eight subjects had sites that failed to meet this criterion and were excluded from the downstream analysis. After annotation, 756, 574 and 540 genera were observed at the eye, ANs and OP, respectively (**Figure 1**).

Predominant bacteria across the three sites

In terms of the ocular surface microbial communities of healthy subjects, the phyla Actinobacteria, Proteobacteria, Firmicutes and Bacteroidota had the highest relative abundance (**Figure 2**). At a family level, the top-10 abundant bacteria were Corynebacteriaceae (19.14%), Propionibacteriaceae (13.29%), Staphylococcaceae (12.01%), Moraxellaceae (4.14%), Comamonadaceae (3.29%), Streptococcaceae (2.79%), Micrococcaceae (2.72%), Enterobacteriaceae (2.42%), Rhodobacteraceae (2.06%) and Caulobacteraceae (1.90%). Similarly, the four predominant phyla in the ANs in healthy adults were Actinobacteria, Firmicutes, Proteobacteria and Bacteroidota (**Figure 2**). At a family level, the top-10 most abundant bacteria were Corynebacteriaceae (35.37%), Staphylococcaceae (16.69%), Propionibacteriaceae (7.95%), Enterobacteriaceae (5.69%), Corynebacteriales (4.01%), FamilyXI (3.98%), Caulobacteraceae (3.67%), Alcaligenaceae (2.86%), Comamonadaceae (1.84%) and Pasteurellaceae (1.64%).

We also identified four main bacterial phyla in the OP of healthy subjects: Bacteroidota, Firmicutes, Proteobacteria and Fusobacteria (**Figure 2**). At a family level, the top-10 most abundant bacteria were Prevotellaceae (34.84%), Pasteurellaceae (16.09%), Streptococcaceae (10.27%), Fusobacteriaceae (5.43%), Veillonellaceae (4.19%), Porphyromonadaceae (3.09%), Campylobacteraceae

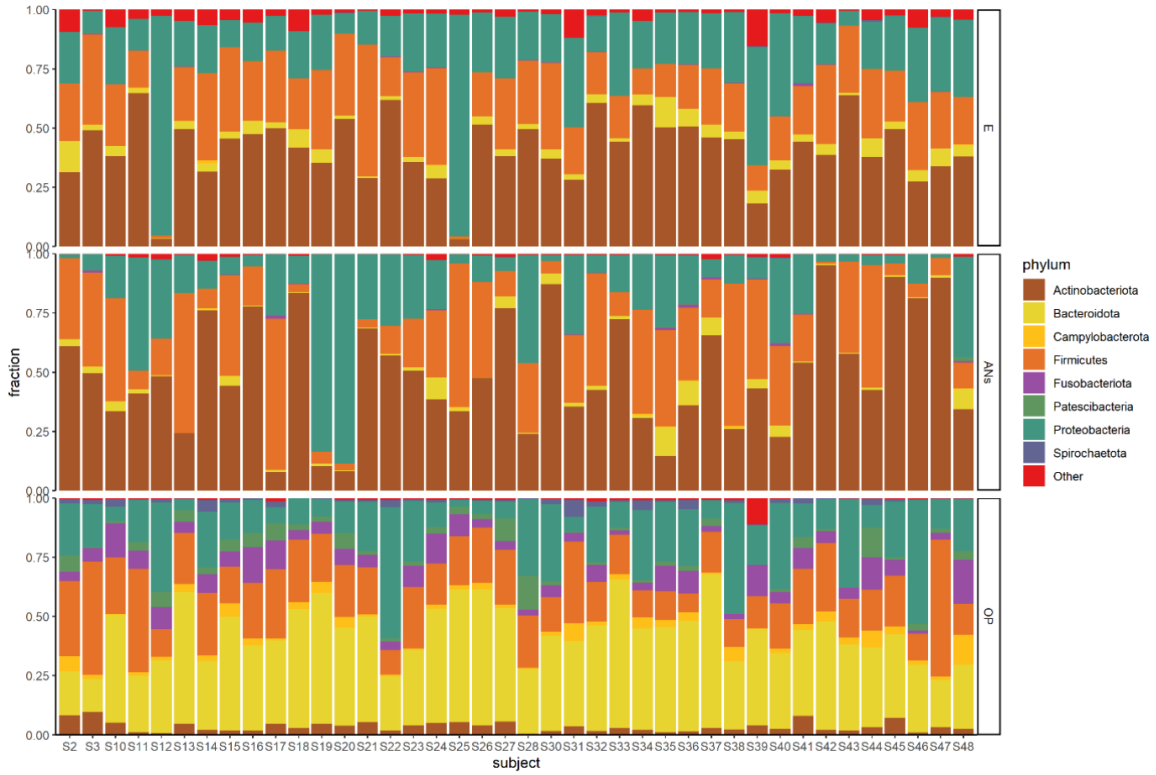


Figure 2. The overall microbiota structure at the phylum of Eye (E), Anterior nares (ANs) and Oropharynx (OP) of healthy adults.

(2.92%), Neisseriaceae (2.25%), Leptotrichiaceae (1.49%) and Spirochaetaceae (1.45%).

Common and different bacteria across the three sites

In **Table 1**, we show the mean bacterial abundance of genera with an abundance of more than 1% in the three sites. The bacteria common to the eye and ANs were *Corynebacterium, Cutibacterium, Staphylococcus, Acinetobacter, Streptococcus, Comamonas, Micrococcus, Brevundimona, Pseudomona* and *Citrobacter*. At a genus level, *Corynebacterium* had the highest abundance in the ANs, and this abundance in the ANs was higher than that in the eyes (35.37% vs. 19.14%).

In addition, the bacteria common to both the ANs and OP were *Haemophilus* and *Streptococcus* spp.

LEfSe analysis of the eye and ANs revealed that the *Corynebacterium* genus was abundant in the ANs, while the *Proteobacteria* phylum was enriched in the eye. Compared with that in the ANs, Bacteroidota was enriched in the OP (**Figure 3**).

Analysis of microbial richness and diversity in eye, ANs and OP

Rarefaction analysis was used to compare the richness and diversity of bacterial species. For the Chao and ACE indices, the number of species in the eye was higher than that in the ANs and OP. At the genus level, considering community evenness and richness, the Shannon and Simpson index indicated that the microbial diversity of the ocular surface was higher than that of the ANs and OP. The microbial diversity of the ANs was lower than that of the OP. There were significant differences in the Shannon index among the three groups ($P < 0.0001$). The differences in the Simpson index between the eye and ANs and between the ANs and OP were significant; however, the difference of that between the eye and OP was not significant (**Figure 4A**).

PCA and PCoA were used to analyze the bacterial regularity of elements and structural differ-

Table 1. The mean abundance of eye, AN and OP major microbiota in healthy subjects

genus	mean abundance	genus	mean abundance	genus	mean abundance
	Eye		ANs		OP
Corynebacterium	19.14%	*Corynebacterium*	35.37%	*Prevotella_7*	13.63%
Cutibacterium	13.16%	*Staphylococcus*	16.69%	*Alloprevotella*	12.03%
Staphylococcus	12.01%	*Cutibacterium*	7.79%	*Haemophilus*	11.76%
Acinetobacter	3.04%	*Enterobacteriaceae_unclassified*	4.53%	*Streptococcus*	10.27%
Streptococcus	2.70%	*Corynebacteriales_unclassified*	4.01%	*Prevotellaceae_unclassified*	8.81%
Comamonas	2.58%	*Brevundimonas*	3.66%	*Fusobacterium*	5.43%
Corynebacteriales_unclassified	2.00%	*Peptoniphilus*	1.64%	*Veillonella*	3.90%
Micrococcus	1.90%	*Comamonas*	1.62%	*Porphyromonas*	3.09%
Brevundimonas	1.84%	*Castellaniella*	1.62%	*Actinobacillus*	3.07%
Pseudomonas	1.62%	*Anaerococcus*	1.46%	*Campylobacter*	2.92%
Citrobacter	1.54%	*Haemophilus*	1.35%	*Neisseria*	2.12%
Alcaligenaceae_unclassified	1.32%	*Pseudomonas*	1.30%	*Streptobacillus*	1.49%
Psychrobacter	1.10%	*Acinetobacter*	1.29%	*Treponema*	1.44%
Paracoccus	1.08%	*Alcaligenaceae_unclassified*	1.23%	*Absconditabacteriales_(SR1)_ge*	1.44%
Lachnospiraceae_unclassified	1.04%	*Alloiococcus*	1.23%	*Actinomyces*	1.34%
				Saccharimonadales_ge	1.25%
				Planococcaceae_unclassified	1.22%

Note: anterior nares (ANs) and oropharynx (OP).

ences between the groups. At the genus level, the OP bacterial community formed a cluster of points well-separated from that of the eye and ANs. There was a significant overlap in bacterial composition between the eye and ANs samples (**Figure 4B**, **4C**). The results of PCA and PCoA were consistent with those of the LEfSe analysis (LDA cutoff = 4).

Transfer of bacteria from eye to nose

The similarity between the microbiota in the eye and ANs suggested that there may be microbiome communication between the two sites. Although the relative abundance of a given species could be affected by other microbes in the community, a simple ideal transfer model suggested that the PCC could have a non-random distribution when the difference in abundance between the considered species and the background species was less than one magnitude (Supplementary Material). Therefore, we focused on identifying genera that could communicate between the two sites. The PCC and its empirical distribution under the ideal model were used to screen candidates. Candidate communicating genera were further verified using a permutation test. These results strongly suggested that *Cutibacterium* and *Micrococcus* might communicate between the two sites (**Table 2**).

Prediction and analysis of functions of microbiota in the eye, ANs and OP

Microbiota assume essential physiological functions in the host. Therefore, PICRUSt was used to predict the potential function of bacteria in the eye, ANs and OP. According to these results, pathways in Kyoto Encyclopedia of Genes and Genomes functional categories were used. Microbiota functions involving metabolism, cellular processes, genetic information processing, organismal systems, environmental information processing and human diseases were similar between the microbial communities in the eyes and ANs, but were different from those in the OP (**Figure 5**).

Discussion

The microbiota plays an important role in the maintenance of homeostasis and health in humans [26]. Normal microbiota can protect host niches either by adhering to surfaces or by producing antimicrobial materials to stimulate human defense systems [27]. Most studies to date have suggested that disorders of human microbial flora are related to several disease states, such as infectious eye diseases and nasopharyngeal diseases [1, 28-30]. However, the exact association of the microbiota of the eye, ANs and OP in healthy individuals remains

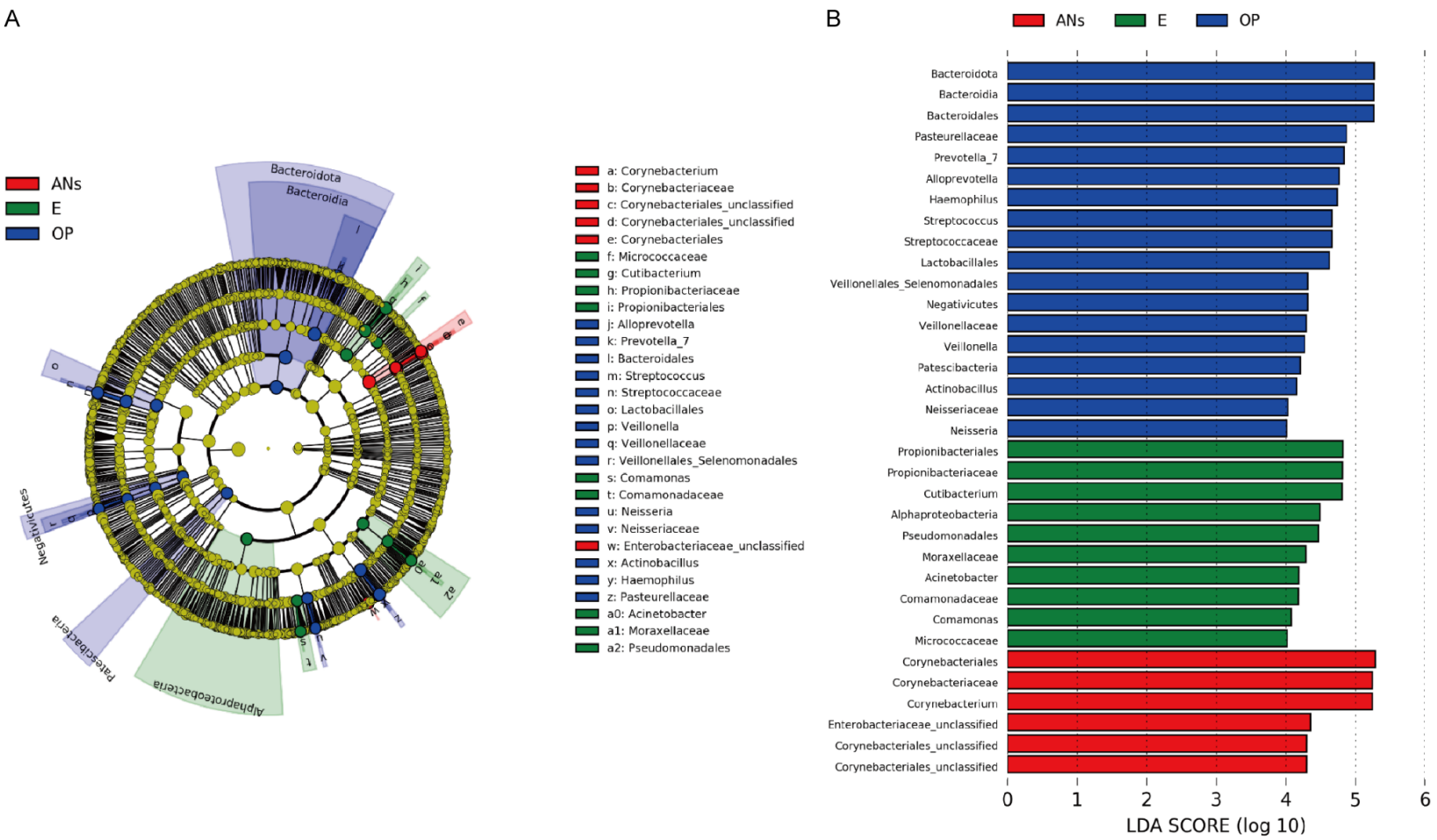
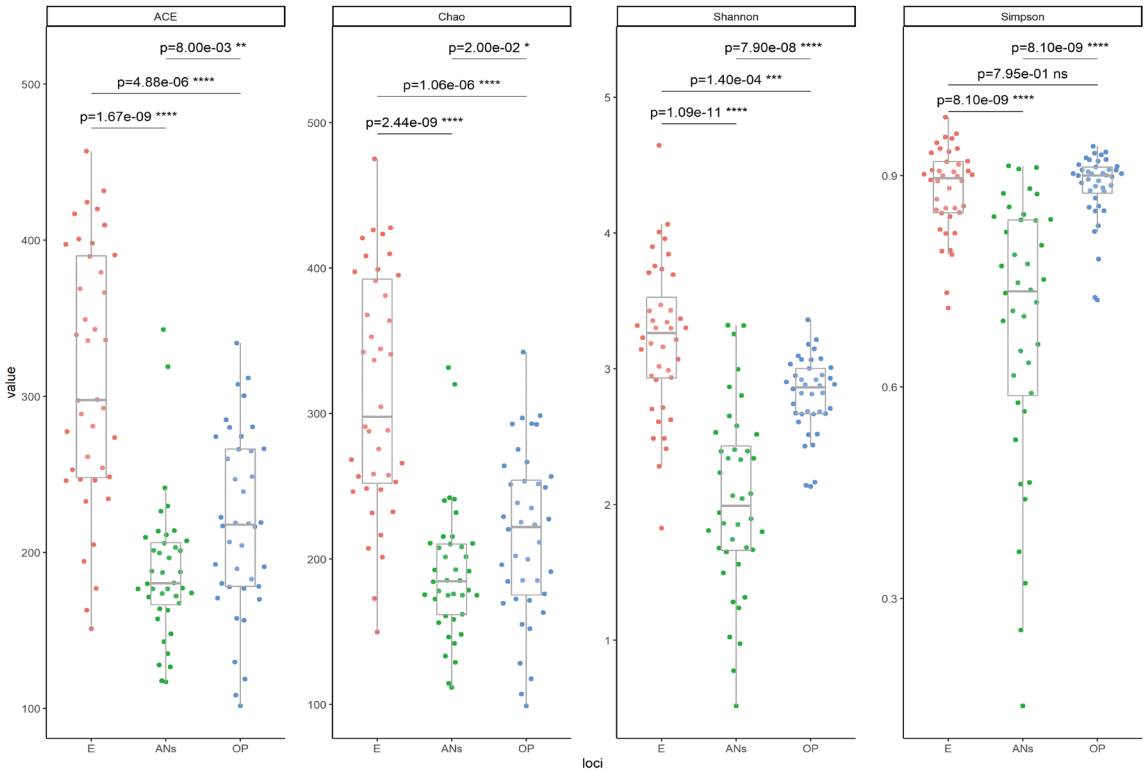


Figure 3. Linear discriminant analysis effect size (LEfSe) analysis among the three sites. A. LEfSe tree diagram; B. Difference contribution analysis chart.

A


ACE
Chao
Shannon
Simpson
p=8.00e-03 **
p=4.88e-06 ****
p=1.67e-09 ****
p=2.00e-02 *
p=1.06e-06 ****
p=2.44e-09 ****
p=7.90e-08 ****
p=1.40e-04 ***
p=1.09e-11 ****
p=8.10e-09 ****
p=7.95e-01 ns
p=8.10e-09 ****
value
loci
E
ANs
OP

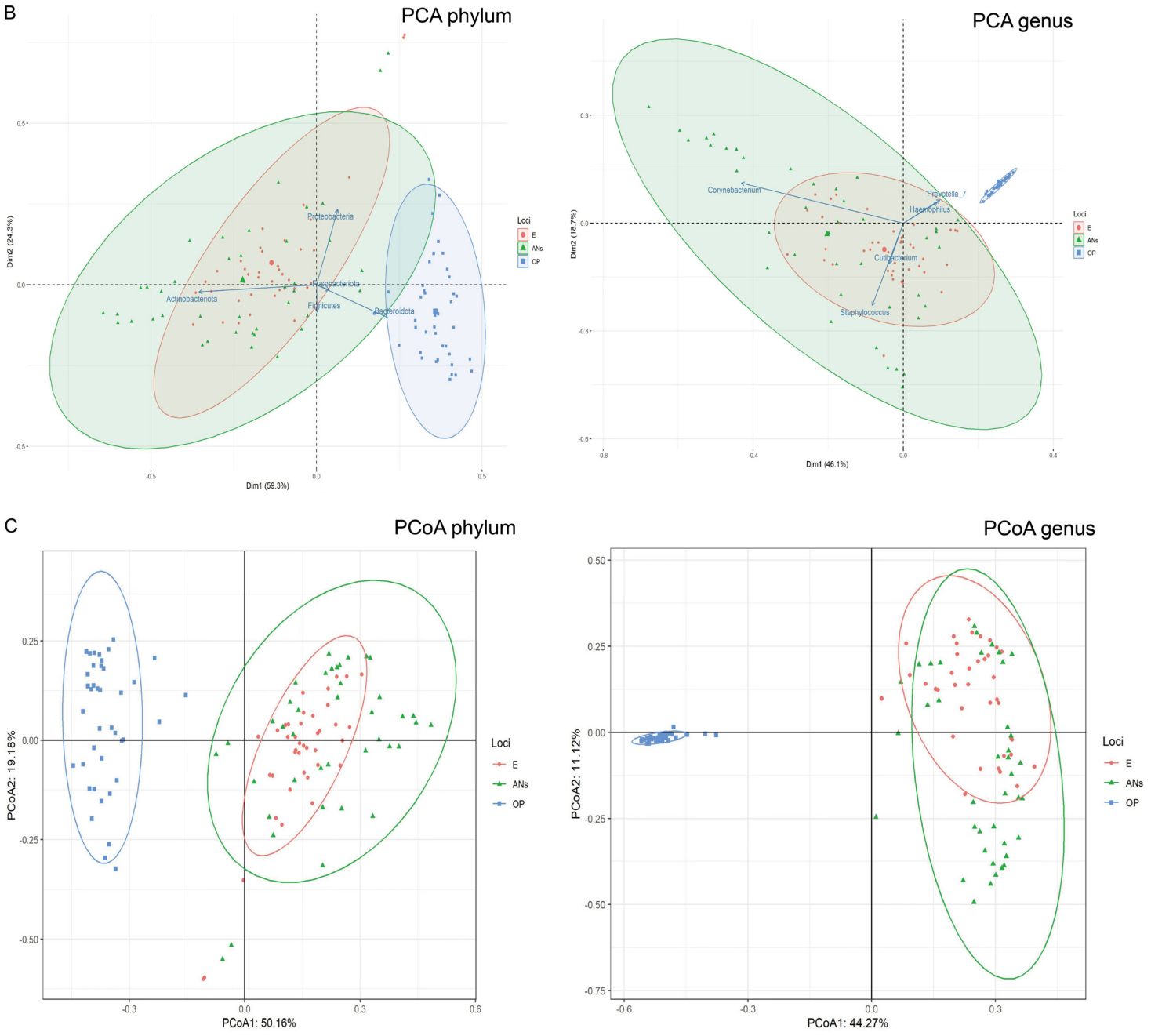

B
PCA phylum
Dim2 (24.3%)
Dim1 (59.3%)
Proteobacteria
Actinobacteriota
Firmicutes
Bacteroidota
Loci
E
ANs
OP
PCA genus
Dim2 (18.7%)
Dim1 (46.1%)
Corynebacterium
Prevotella_7
Haemophilus
Cutibacterium
Staphylococcus
C
PCoA phylum
PCoA2: 19.18%
PCoA1: 50.16%
PCoA genus
PCoA2: 11.12%
PCoA1: 44.27%

Figure 4. Alpha and beta diversity indices of microbiota across different sites. A. Alpha-diversity indices of microbiota across different sites for ACE index, Chao index, Shannon index and Simpson index; B. Principal Component Analysis (PCA) based on Euclidean distances among the three sites; C. Principal Co-ordinates Analysis (PCoA) based on Bray-Curtis dissimilarity among the three sites. *: $0.01 < p \leq 0.05$; **: $0.001 < p \leq 0.01$; ***: $0.0001 < p \leq 0.001$; ****: $0.00001 < p \leq 0.0001$.

Table 2. Correlation of eye and ANs species abundance

genus	mean abundance		correlation coefficient	*p*.value
	Eye	ANs		
Corynebacterium	19.14%	35.37%	-0.148	0.715
Staphylococcus	12.01%	16.69%	-0.094	0.704
Cutibacterium	13.16%	7.79%	0.546	0.001***
Corynebacteriales_unclassified	2.00%	4.01%	0.271	0.078
Brevundimonas	1.84%	3.66%	-0.013	0.354
Enterobacteriaceae_unclassified	0.36%	4.53%	-0.052	0.481
Acinetobacter	3.04%	1.29%	0.014	0.356
Comamonas	2.58%	1.62%	-0.071	0.584
Streptococcus	2.70%	0.55%	0.201	0.114
Pseudomonas	1.62%	1.30%	-0.117	0.787
Alcaligenaceae_unclassified	1.32%	1.23%	-0.094	0.723
Citrobacter	1.54%	0.93%	-0.068	0.543
Castellaniella	0.57%	1.62%	-0.138	0.956
Micrococcus	1.90%	0.28%	0.550	0.004**
Anaerococcus	0.72%	1.46%	0.191	0.099
Peptoniphilus	0.41%	1.64%	-0.193	0.898
Haemophilus	0.66%	1.35%	0.193	0.134
Lachnospiraceae_unclassified	1.04%	0.37%	0.222	0.075
Psychrobacter	1.10%	0.24%	0.257	0.070
Alloiococcus	0.03%	1.23%	0.049	0.148
Paracoccus	1.08%	0.15%	0.074	0.178

Note: **: $0.001 < p \leq 0.01$; ***: $0.0001 < p \leq 0.001$; anterior nares (ANs).

unknown. In our investigation, we demonstrated that some bacteria were prone to localize to specific sites and revealed the relationships of the microbiota of these three sites in healthy adults. Our results indicated that the microbiota composition and functional predictions were similar in the eye and the ANs, but differed from those of the OP in healthy adults. This implies that eye and AN microorganisms may be related, while the OP microbiota was more similar to digestive tract flora. Moreover, our results indicate that, in healthy individuals, *Cutibacterium* and *Micrococcus* may migrate from the eye to the nose.

A previous study [31] identified a similar predominant microbiota composition at phylum level in the eye as found in our study: *Actinobacteria* (46%), *Proteobacteria* (24%) and *Firmicutes* (22%). *Corynebacterium* was the most abundant genus, representing 16.2% of all samples in their study and 19.14% in our study. In a recently study conducted by Heleen et al. [32], the core microbiota of the ocular surface were described. The most-represented, "core" bacterial flora on the ocular surface belonged to the following genera: *Corynebacterium, Acinetobacter, Staphylococcus, Pseudomonas, Propionibacterium* (now classified as *Cutibacterium*) and *Streptococcus*. This is consistent with the results of a similar study. Our study supported the finding of a core ocular surface bacterial community, as posited by other authors [11, 33-36].

Cavuoto et al. [6] found that the bacterial abundance and OTU richness in the eye were lower than those in pharynx samples. Contrary to Cavuoto's research, we observed that the overall bacterial abundance was the highest in the

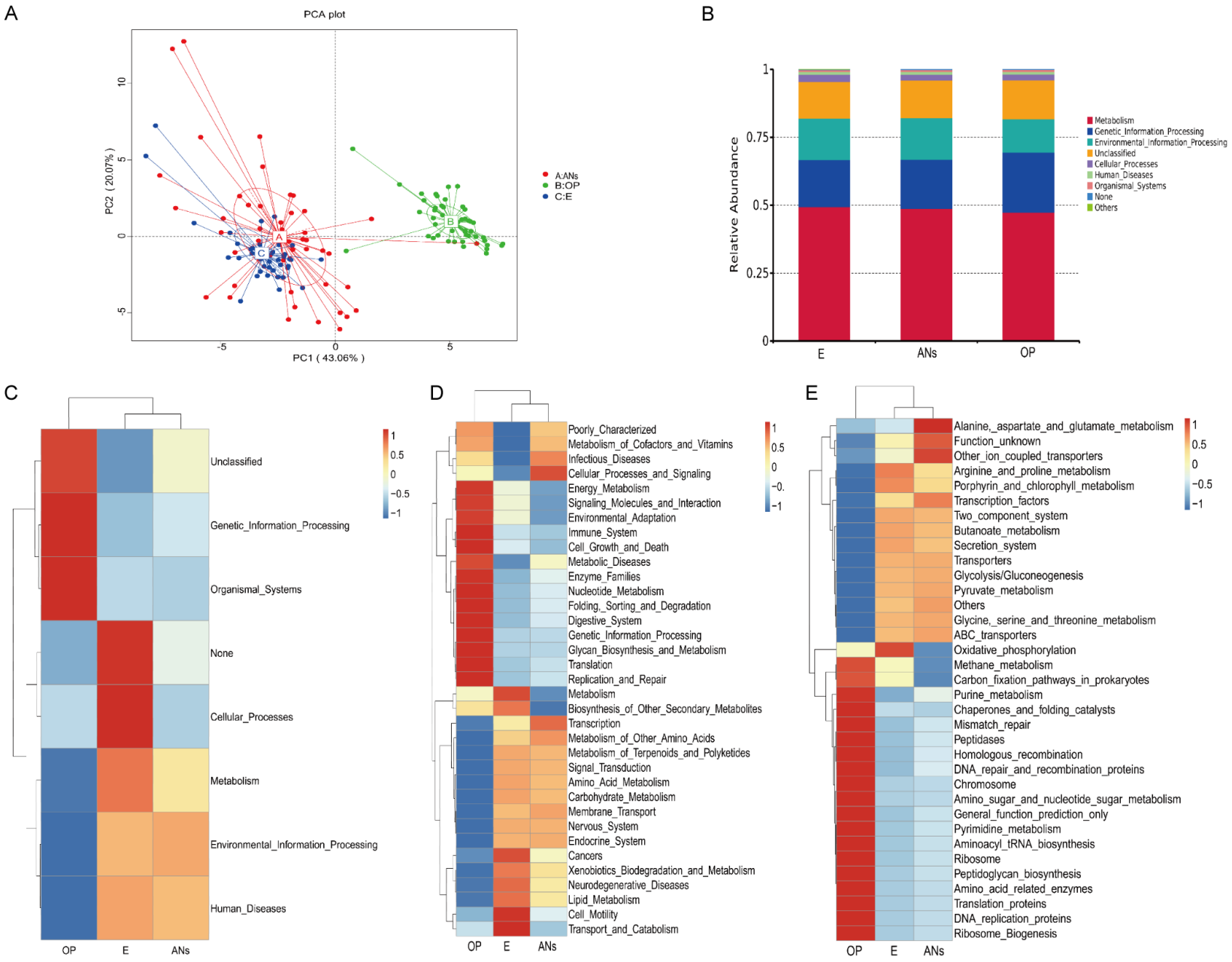

A
PCA plot
PC1 (43.06%)
PC2 (20.07%)
A:ANs
B:OP
C:E
B
Relative Abundance
E
ANs
OP
Metabolism
Genetic_Information_Processing
Environmental_Information_Processing
Unclassified
Cellular_Processes
Human_Diseases
Organismal_Systems
None
Others
C
Unclassified
Genetic_Information_Processing
Organismal_Systems
None
Cellular_Processes
Metabolism
Environmental_Information_Processing
Human_Diseases
D
Poorly_Characterized
Metabolism_of_Cofactors_and_Vitamins
Infectious_Diseases
Cellular_Processes_and_Signaling
Energy_Metabolism
Signaling_Molecules_and_Interaction
Environmental_Adaptation
Immune_System
Cell_Growth_and_Death
Metabolic_Diseases
Enzyme_Families
Nucleotide_Metabolism
Folding,_Sorting_and_Degradation
Digestive_System
Genetic_Information_Processing
Glycan_Biosynthesis_and_Metabolism
Translation
Replication_and_Repair
Metabolism
Biosynthesis_of_Other_Secondary_Metabolites
Transcription
Metabolism_of_Other_Amino_Acids
Metabolism_of_Terpenoids_and_Polyketides
Signal_Transduction
Amino_Acid_Metabolism
Carbohydrate_Metabolism
Membrane_Transport
Nervous_System
Endocrine_System
Cancers
Xenobiotics_Biodegradation_and_Metabolism
Neurodegenerative_Diseases
Lipid_Metabolism
Cell_Motility
Transport_and_Catabolism
E
Alanine,_aspartate_and_glutamate_metabolism
Function_unknown
Other_ion_coupled_transporters
Arginine_and_proline_metabolism
Porphyrin_and_chlorophyll_metabolism
Transcription_factors
Two_component_system
Butanoate_metabolism
Secretion_system
Transporters
Glycolysis/Gluconeogenesis
Pyruvate_metabolism
Others
Glycine,_serine_and_threonine_metabolism
ABC_transporters
Oxidative_phosphorylation
Methane_metabolism
Carbon_fixation_pathways_in_prokaryotes
Purine_metabolism
Chaperones_and_folding_catalysts
Mismatch_repair
Peptidases
Homologous_recombination
DNA_repair_and_recombination_proteins
Chromosome
Amino_sugar_and_nucleotide_sugar_metabolism
General_function_prediction_only
Pyrimidine_metabolism
Aminoacyl_tRNA_biosynthesis
Ribosome
Peptidoglycan_biosynthesis
Amino_acid_related_enzymes
Translation_proteins
DNA_replication_proteins
Ribosome_Biogenesis
OP
E
ANs

Figure 5. Prediction and analysis of functional gene in Eye (E), Anterior nares (ANs), and Oropharynx (OP). A, B. PCA analysis and Bar chart of the functional gene prediction in Eye, ANs, and OP. C-E. Microbiota function basing Kyoto Encyclopedia of Genes and Genomes (KEGG) database in Eye, ANs and OP. C. Pathway 1; D. Pathway 2; E. Pathway 3.

eye. The difference in these results may be related to their focus on only 16S rDNA V4 region sequencing.

Based on the alpha diversity index, we also found that ocular surface microbial diversity was higher than that of the ANs and OP. Our study is similar to that of Yau et al. [37]. Furthermore, we found that microbial diversity in the ANs was lower than that in the OP. The reasons for this finding may be as follows. At the genus level, the dominant bacterial flora in the ANs was *Corynebacterium*, which accounted for 35.37% of the total bacterial community and was found in all samples from healthy noses, while the main bacterial flora in the OP were *Prevotella_7* and *Alloprevotella*, accounting for 13.63% and 12.03%, respectively. Notably, the composition and distribution of OP microbial flora were significantly different from those in ANs and the ocular surface, showing characteristics more similar to those of the gastrointestinal tract bacterial flora. Generally, in the OP, *Bacteroidota* was the most abundant, resembling the gut microbiota distribution. Indeed, food ingestion, reflux [38] and diet alteration [39-41] may affect the OP microbial composition and microenvironment.

In our study, even at the genus level, the microbiota of the ocular surface and of the ANs were difficult to be distinguished effectively, which suggests a biological relationship between these two microbial communities. We speculated that ocular surface microbiota may be transmitted to the nose through the mechanism of eye-blink and tears via the nasolacrimal system. A study of conjunctival and distal nasal microbiota in an animal experiment on healthy dogs has showed that, although the mucosa of ocular surface and nose secrete variable antimicrobial compounds, conjunctiva-associated lymphoid tissue and lacrimal drainage-associated lymphoid tissue contribute to modify the microbial communities. Thus, the correlations of microbiota between the nearby anatomic sites may also be influenced by similarities in mucosal immunity [42]. Corynebacterium was most abundant in the eyes and ANs of healthy adults. Data from clinical studies indicated that patients with chronic rhinosinusitis have lower *Corynebacterium* levels than healthy subjects [43]. An increasing number of studies have shown that *Corynebacterium* in the nasal cavity can inhibit *Staphylococcus* colonization and virulence via microbial competition, produce antimicrobial substances by commensals, and modulate innate and adaptive immune responses to help maintain microbiota balance [44-47]. Out of our expectations, although the summed abundance of *Corynebacterium* and *Staphylococcus* in the ANs exceeded 50%, *Corynebacterium* and *Staphylococcus* were unlikely to be transmitted between the eye and nose (the correlation coefficient was 0.148 vs. 0.094, *P* value was 0.715 vs. 0.704). In our study, the similarity of the microbial community and the correlation of the abundance of *Cutibacterium* and *Micrococcus* in eye and ANs suggests that there might be microbe communication between these two sites (see Supplementary Material for details).

Interestingly, *Cutibacterium* and *Micrococcus* are common genera on the surface of human skin. *Cutibacterium* consists of four species and three subspecies. *C. acnes* is a lipophilic gram-positive commensal bacterium [48, 49]. Furthermore, it is involved in the regulation of skin homeostasis and has long been considered as an opportunistic pathogen in acne vulgaris. A previous study demonstrated that *Cutibacterium* might be vital as a resident commensal bacterium for the prevention of blepharitis [50]. However, the specific effect of transmission of these bacteria from the eye to the nose requires further study. Besides, *Micrococcus* is isolated from the external environment, such as indoor air, and the different species of *Micrococcus* are difficult to distinguish morphologically [51]. Currently, the significance and clinical implications of the migration of microbial flora and its mechanism are unknown and require further study.

In this study, PICRUSt analysis predicted that the microbial flora in the eye and nose have similar functional genes, and that these are

related to metabolism, cellular processes, genetic information processing, organismal systems and human diseases. Further research is needed to explore the exact functions using meta-genomic analysis.

The limitation of our study is that the ideal transfer model could only provide limited information on the complicated hundred-genera constructed interrelation network. However, it provides the insight that the PCC can be implemented even on relative abundance data, although its inferential power is limited when compared to the utilization of absolute abundance data. Therefore, detailed characterization of eye-nose-associated microbial communities is required. Labeling some bacteria within samples prior to gene sequencing [52] should be used in the future to verify whether migration occurs.

In conclusion, our research may contribute to illuminating the characteristics and associations of the microbiota from different niches (eye, ANs and OP) in healthy adults. Our results suggest that there may be a microbiota connection between the eye and nose through the nasolacrimal duct passage. Our findings imply a new clinical strategy for future therapeutic and preventive modalities through the use of microbiota or their equivalents to modulate diseases of the eye and ANs. Although there is much to learn before implementing microbiota clinically, in future, eye drops may incorporate bacteria [53], which will implant bacteria in specific niches of the body to prevent and treat diseases.

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Disclosure of conflict of interest

None.

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Supplementary Material

Transfer of bacteria from eye to nose

In our study, we inferred that ANs microbiota community may be divided into two parts: ① Microbial immigration: transfer from eye to ANs via nasolacrimal duct or direct contact transmission (Hand-Eye-Nose). ② Micro-aspiration, inhalation of air and direct dispersion along mucosal surface.

Correlation of species abundance between the two sites

Hypothesis: We expected that there is a correlation between the abundance of the same bacterium in two parts. According the interaction between species, we use numerical simulation to estimate the distribution of correlation coefficients.

Ignoring the nasal specific microorganisms, we expect to observe a lot of correlation coefficients, even if the transmission ratio of different microorganisms varies greatly. However, after nasal specific microorganisms are considered, the correlation coefficient was decreased significantly.

In the model, we assume that the abundance of the same microorganism obeys lognormal distribution.

There is no specific microorganism in the ANs

We assume that there are only two microorganisms A and B in the eye, which transmitted to the nose in the proportion of KA and KB respectively. There are no specific microorganisms in the nose. The abundance of microorganisms A and B obeys lognormal distribution.

For different ratio on bacterial abundance of A/B and ka/kb, we simulated the bacterial distribution of the correlation coefficient of B between eye and nose (Supplementary Figure 2).

We can theoretically observe a very good correlation (> 0.85) without considering the specific microorganisms in the nose.

The multiple difference between kb/ka has little effect on the results.

There are some specific microorganisms in the ANs

We supposed that there are some specific microorganisms in the ANs, and its abundance also obeyed lognormal distribution. We assume ka = kb for the purpose of simplify the model.

In addition, we observed that the correlation coefficient of relative abundance of B between eye and nose (Supplementary Figure 3). It can be seen that from left to right, the higher the average abundance of C, the smaller the correlation coefficient observed; From top to bottom, the higher the average abundance of B, the larger the correlation coefficient observed.

The value of correlation coefficient

We analyzed the correlation of the original data. Among the 15 genera which relative abundance greater than 1% in the eye, Cutibacterium and Micrococcus showed significant correlation. Several others showed a certain correlation. In contrast, the correlation between Corynebacterium, which mainly distinguishes in the eye and ANs, is not obvious on PCA diagram.

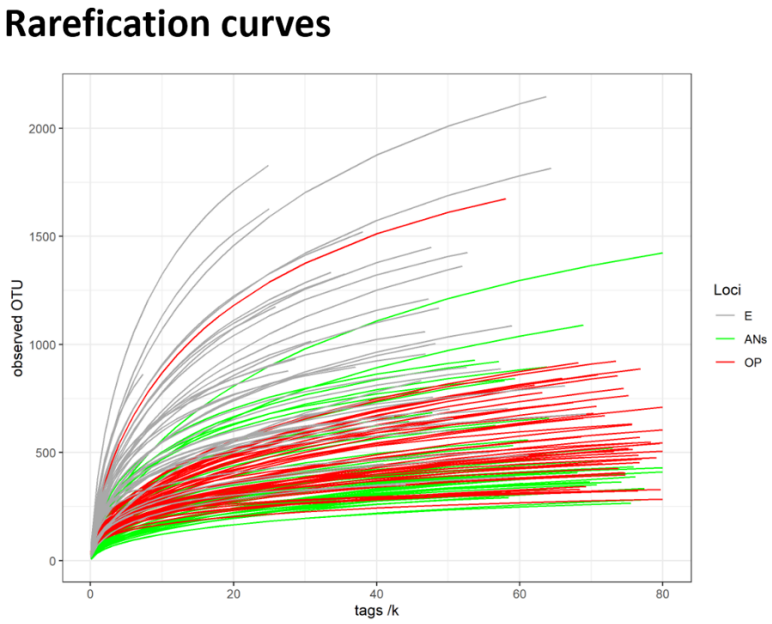
Brief summary

The sum of the abundance of Corynebacterium and Staphylococcus in the nose is more than 50%. We believe that Corynebacterium and Staphylococcus show slight negative correlation or no correlation. It

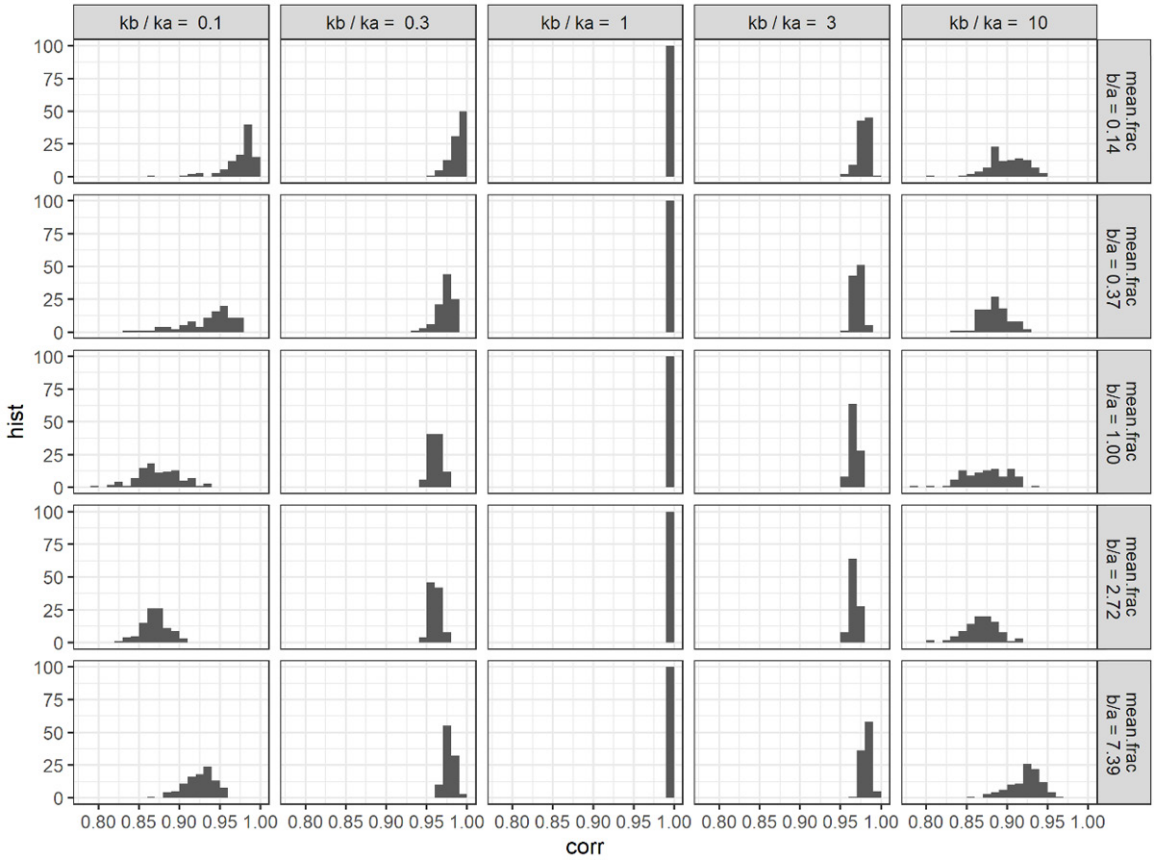
is suggested that there is no transmission between eyes and nose, or the transmission accounts for a small proportion of the nasal composition.

Corresponding to the model, in the three groups of cases close to the diagonal, the main peak of the distribution of correlation coefficient is between 0.5 and 0.75. The closer to the upper right corner, the greater the C/B = (C/A)/(B/A). Similarly, the peak of the correlation coefficient moves to the left accordingly, the smaller the correlation coefficient is expected.

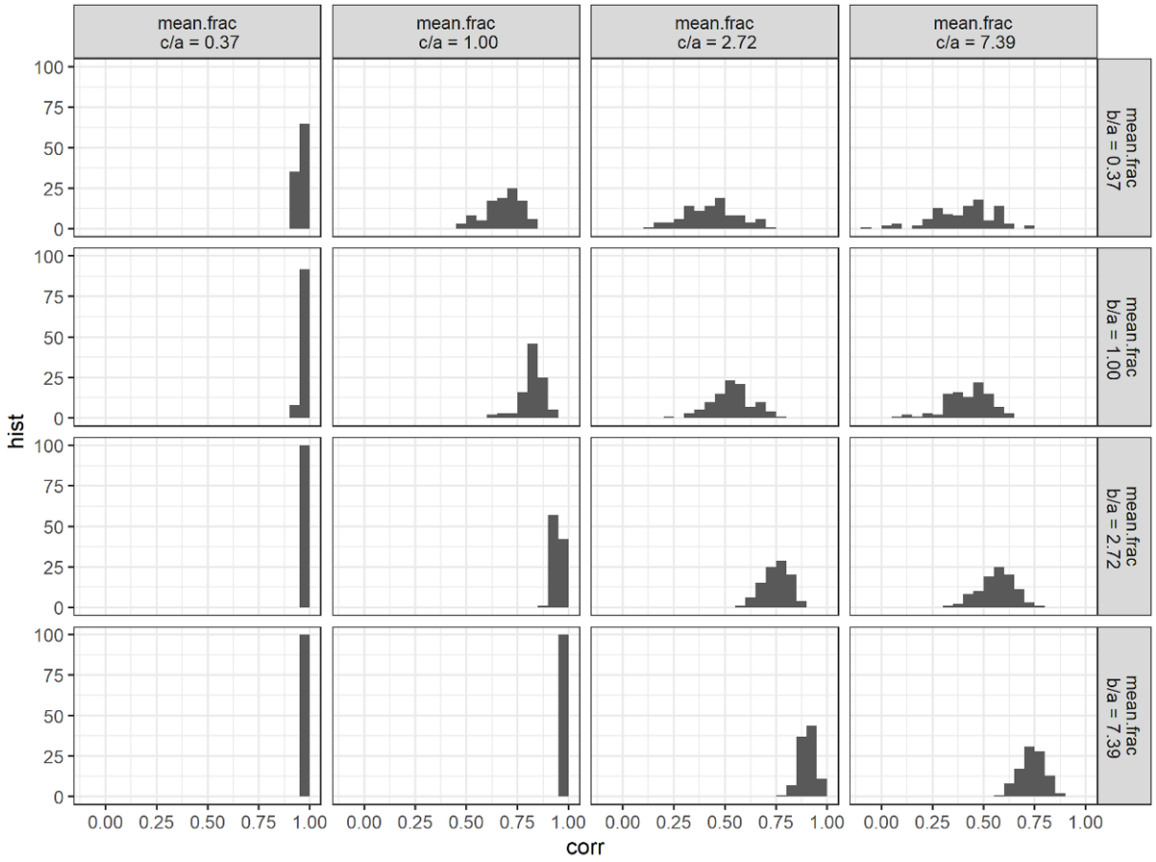
In conclusion, we observed a correlation of > 0.5 in Cutibacterium and Micrococcus. This is very consistent with the correlation coefficient expected in the model. It strongly suggests that they may be transmitted between sites.



Supplementary Figure 1. Rarefication curves of eye, ANs and OP.



Supplementary Figure 2. The theoretial distribution of correlation coefficient of relative abundance of B between eye and nose under Model 1.



Supplementary Figure 3. The theoretial distribution of correlation coefficient of relative abundance of B between eye and nose under Model 2.