Geographic Difference Shaped Human Ocular Surface Metagenome of Young Han Chinese From Beijing, Wenzhou, and Guangzhou Cities

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PURPOSE. Microbial ecosystems interact with the human body and affect human health. The microbial community on the ocular surface remains an underexplored territory despite its importance as the first line of defense barrier that protects the eye and ultimately sight. We investigated how age and sex affected human ocular surface microbiome, and in the present study wanted to understand how geographic difference shaped the microbiome in the ocular surface.

METHODS. We collected conjunctival specimens of 172 eyes from 86 healthy volunteers living in three Chinese cities, namely, Guangzhou, Wenzhou, and Beijing. Using the direct metagenomic shotgun sequencing approach, we characterized how geographic difference affected the human ocular microbiome.

RESULTS. We surveyed the taxonomic composition and metabolic function of the microbiota on human ocular surface. We showed that the ocular surface microbiota was composed of bacteria, viruses, and fungi. A geographical difference in both composition and function of the conjunctival microbiome suggests that the environment people lived in shapes their conjunctival microbiome, especially the dominate species.

CONCLUSIONS. Our study provides a reference catalog of the healthy conjunctival metagenome and raises a concern for environmental influences on the ocular surface microbiome.

Keywords: metagenome, ocular surface, geographic difference, microbial communities, healthy conjunctiva

Diverse microbial communities consider the human body their home.[1] Despite the strong local antimicrobial environment, the healthy ocular surface also has a commensal microbiota.[2] Like their counterparts found on other mucous membrane surfaces, ocular microbiota plays a key role in maintaining the physiology of the ocular surface, and dysbiosis of local microbial community can lead to ocular surface diseases.[3–7]

Detection of ocular microbes has been heavily dependent on traditional culture-based methods that can find only a limited number of microbial species, including *Staphy-lococci*, *Propionibacterium*, and *Corynebacterium* at various isolation rates.[3] These methods are influenced by sample collection and culture conditions.[3] Recently, high-throughput sequencing of 16S rRNA genes suggests a much higher diversity of commensal microbiota than previously reported.[8–11] Interestingly, many previously defined ocular pathogens, such as *Pseudomonas*, *Propionibacterium*, and *Staphylococcus*, were found on the ocular surfaces of healthy individuals, contact lens wearers, and patients with infectious eye disease.[3] These findings raise concerns about the false negativity and positivity of ocular surface pathogens

TABLE. Demographic Characteristics of Study Participants and Summary of Direct Metagenomic Shotgun Sequencing Reads

Content	Total	Guangzhou City	Wenzhou City	Beijing City
No. of participants	86	48	18	20
Age, years	26.9 ± 0.3	27.9 ± 0.4	24.4 ± 0.4	26.9 ± 0.6
Male/female	44/42	23/25	11/7	10/10
No. in sample	268	192	36	40
No. in sample passed filter	248	172	36	40
Average no. of total reads	51,609,179	51,147,732	55,342,300	50,233,593
Average no. of nonhuman reads	1,470,425	1,476,867	1,575,322	1,348,316
Average % of nonhuman reads	2.9	2.9	2.8	2.7

detected by standard culture-based diagnostic methods.[12] In addition, studies based on 16S rRNA genes can only reveal the core taxonomic characteristics of the microbial community, with limited ability to accurately quantify the composition of the ocular surface microbiome due to the prevalence of horizontal gene transfer and the varying copy number of the ribosomal operon in bacteria.[13] Importantly, it is unclear whether bacteria are the only kingdom to inhabit the ocular surface of healthy individuals. Therefore, as suggested by the Human Microbiome Project,[14] it is necessary to survey the human ocular surface microbiome using a metagenomic shotgun sequencing approach.

Recent studies have proposed that the ocular commensal population regulates local immunity against infections.[4,6] However, it is unclear whether the environmental changes modulate the symbiosis of the ocular surface microbiota. Because human eyes, particularly the conjunctiva, are exposed to the constantly changing environment, it is important to determine whether a discernable difference in microbiota can be found on ocular surfaces of people living in different cities. Therefore, using the direct metagenomic shotgun sequencing approach, we surveyed the composition and function of the conjunctival microbiome on a total of 172 eyes from 86 healthy participants living in three major Chinese cities, Guangzhou, Wenzhou, and Beijing.

METHODS

Participant Recruitment

As described in our previous study,[15] the bacterial Shannon diversity index score of old people was higher than that of young, which implied the instability of bacterial community in a certain degree in the old group. To alleviate this influence, healthy nonsmoker volunteers having a medical history free of current systemic and ocular diseases and no recent contact lens wear or antibiotic treatment (in the past 6 months) were recruited from Guangzhou, Wenzhou, and Beijing city, China, between February 2015 and December 2015. The basic demographic information was listed in the Table. This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Zhongshan Ophthalmic Center (ZOC), Sun Yat-sen University (protocol #2015MEKY011). All participants provided written informed consent before participation.

Sampling of Conjunctiva

The conjunctival impression cytology samples from inferior bulbar conjunctiva were obtained using a protocol described as follows. (1) Topically anesthetize the eye with one to two drops of Alcaine Eye Drop (Alcon, Fort Worth, TX) and keep the eye closed for several minutes. (2) Use disposable tweezers to place the MF Membrane filter (Millipore, Burlington, MA; REF: HAWP01300, 0.45 μm) on the inferior bulbar conjunctiva with the edge of the membrane clear of the lower tear meniscus and gently press for 10 to 15 seconds. (3) Remove the membrane and store it immediately in a sterile Eppendorf tube with 300 μL Tissue and Cell Lysis Solution containing 1 μL of proteinase K, provided in the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) under –80°C. (4) Apply one to two drops of neomycin sulfate eye drops (Alcon) to each examined eye. The conjunctival specimens were collected again within a 3-week interval from the same individual.

Conjunctiva Sample Culture

A standardized culture procedure developed in the Clinical Laboratory of Zhongshan Ophthalmic Center was performed using the following procedure. Conjunctival sac swabs were aseptically collected by one clinical laboratory technician from all healthy young volunteers. After topical anesthetization (0.5% Alcaine, Alcon), the inferior conjunctival sac was swabbed with a sterile premoistened eSwab (Copan Diagnostics, Brescia, Italy) for aerobic bacterial culture or a premoistened flocked swab (FLOQSwab, Copan Diagnostics) for anaerobic bacterial culture. The technician used a continuous stroke from the nasal to temporal side and then a second stroke from temporal to nasal side avoiding contact with the eyelid or lashes. The same procedure was performed on the other eye. One conjunctival specimen was randomly used for subsequent aerobic bacterial culture, and the other was for anaerobic bacterial culture, both of which were processed in the Clinical Laboratory of Zhongshan Ophthalmic Center.

Aerobic Bacterial Culture

A nylon-flocked eSwab (eSwab, Copan Diagnostics) was used to collect conjunctival sac specimen for aerobic bacterial culture. After specimen collection, the swab was inserted into the screw-top tube containing liquid amies medium and incubated in 5% CO_2 at 35°C to 36°C for 24 hours. The bacteria, if any, would be subcultured onto a Columbia agar plate supplemented with 5% sheep blood (BioMerieux, Durham, NC) using sterile inoculation loop and incubated at 35°C to 36°C for another 48 hours.

Anaerobic Bacterial Culture

A flocked swab (FLOQSwab, Copan Diagnostics) was used to collect conjunctival sac specimen from a participant. A Columbia agar plate supplemented with 5% sheep blood

(BioMerieux, Shanghai, China) was inoculated with the swab immediately after the specimen collection and incubated at 35°C to 36°C for 7 days in an anaerobic bag with indicator.

Identification of bacteria species was carried out using an automated microbial identification system VITEK MS (BioMerieux).

DNA Extraction

DNA extraction was carried out following the protocol of MasterPure Complete DNA and RNA Purification Kit (Epicentre) provided by the manufacture. Briefly, the conjunctival impression cytology samples were thawed and vigorously vortexed for 10 minutes, followed by an incubation at 65°C for 15 minutes. The RNA was removed by 5 µg RNase A and metagenomic DNA was extracted by MPC Protein Precipitation Reagent and isopropanol-ethanol precipitation procedure. Five blank samples were subjected to the same procedure without any tissues.

Metagenomic Sequencing

A total of 100 ng DNA from conjunctival impression cytology samples was sonicated into fragments of 300 to 400 bp using Bioruptor (Diagenode, Seraing, Belgium) and subjected to sequencing library preparation following the standard protocol provided by the manufacture using KAPA LTP Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA). DNA libraries were sequenced to a depth of $\sim$ 50 million reads per sample using HiSeq PE Cluster Kit v4 and HiSeq SBS V4 250 cycle kit (Illumina, San Diego, CA) on the Illumina HiSeq2500 sequencer and subjected to initial processing using CASAVA (v1.8.2) (Illumina). All five blank samples were subjected to the same sequencing library preparation process.

Data Analysis

Preprocessing of Sequencing Reads. All reads were first evaluated by FastQC for quality control. To maintain the consistency of alignment accuracy among all microbial reads, we first used Cutadapt (v1.9.1) to filter adapter, as well as to trim low-quality reads, and then filtered all reads lower than 75 bp using PrinSeq (v0.20.4), which provided a best Q30 in our sample set. Paired-end reads from each sample were combined into one single file and treated as single-end reads. Low quality reads, replicated reads, and potential adapter sequences were removed using Fastx toolkit (v0.0.12). The reads containing more than 10% of ambiguous bases were depleted using PrinSeq (v0.20.4). Human reads were then removed from the subsequent analysis using HiSAT2 (v2.0.1)[16] and DeconSeq[17] to obtain clean nonhuman sequences.

Sequence Analysis. The nonhuman sequences were mapped against our custom microbial genome collection using Burrows-Wheeler Aligner (BWA0.7.5a)[18] with three mismatches. Our genome collection included bacterial genomes (containing 1432 species and 2974 strains, downloaded from ftp://ftp.ncbi.nlm.nih.gov/genomes/HUMAN_MICROBIOM/Bacteria/, on July 7, 2014), viral genomes (containing 4984 strains, downloaded from ftp://ftp.ncbi.nlm.nih.gov/genomes/Viruses/, in October 2015), and the fungal genomes (containing 68 species and 75 strains, downloaded from http://fungidb.org/fungidb/, on March 12, 2015). Sequences were assigned to taxonomic units

according to their species level classification. The relative abundance of each species was calculated by the ratio of the total mapped reads of each species, normalized by their genome size and the total mapped microbial reads within each sample. Community diversity (Shannon index) was calculated according to the method described in the Mothur[19] program after using a subsampling cutoff of 500 bacterial sequences per sample. The HMP Unified Metabolic Analysis Network (HUMAnN2)[20] was used to analyze the abundance of microbial KEGG pathways. Principal component analysis was performed on the relative abundance of bacterial species and microbial KEGG pathways using Ade4 package in R statistical software (v3.1.1) after using a subsampling cutoff of 500 bacterial sequences per sample. LDA effect size[21] was used to identify species and functional pathways characterizing the differences among sample groups. In this study, because of many antibacterial components in tears,[22] some samples contained less than 500 bacterial reads. Thus, to make the analysis more specific and more accurate, we filtered these samples for all following analyses. This cutoff point was based on the ROC curve (cutoff, 501.5; sensitivity, 1; specificity, 0.990; area under the curve, 0.990).

Statistical Analysis

All statistical analysis was performed in the SPSS software (v17.0; SPSS Inc, Chicago, IL). Data were represented as mean $\pm$ SE unless otherwise indicated. For all boxplots, center lines represented the median and the box edges represented the first and the third quartiles. The parametric (Student's) t or nonparametric (Mann-Whitney U and Wilcoxon rank-sum) tests were used.

RESULTS

### Specimen Collection and Metagenomic Data Processing

A total of 86 nonsmoker healthy volunteers (42 female) were enrolled in the current study from three major Chinese cities (Guangzhou, Wenzhou, and Beijing), with an average age of 26.9 years (Table), and 268 specimens were collected from both eyes of the healthy volunteers. An average of 51.6 million single-end reads per sample was obtained and the majority of all reads were of human origin in all conjunctival samples. We further applied a quality control procedure on all reads and obtained an average of about 1.5 million nonhuman quality-filtered microbial sequence reads (Table). As described in our previous published study,[15] there were no differences of the bacterial composition in both eyes, so that the samples coming from both eyes of an individual were processed as two independent samples for the following studies.

Healthy Conjunctiva Harbors a Unique Microbiota

Our metagenomic analysis identifies all detectable microbial species without the limitations of marker gene-based methods. Previous studies surveyed the microbiome on healthy and diseased conjunctiva using the 16S rRNA sequencing approach and identified mainly bacterial species.[8–10] Our data showed that, within the healthy conjunctival metagenome of the sampled population, on average 77.5% of microbial reads were of bacterial origin, whereas 19.5%
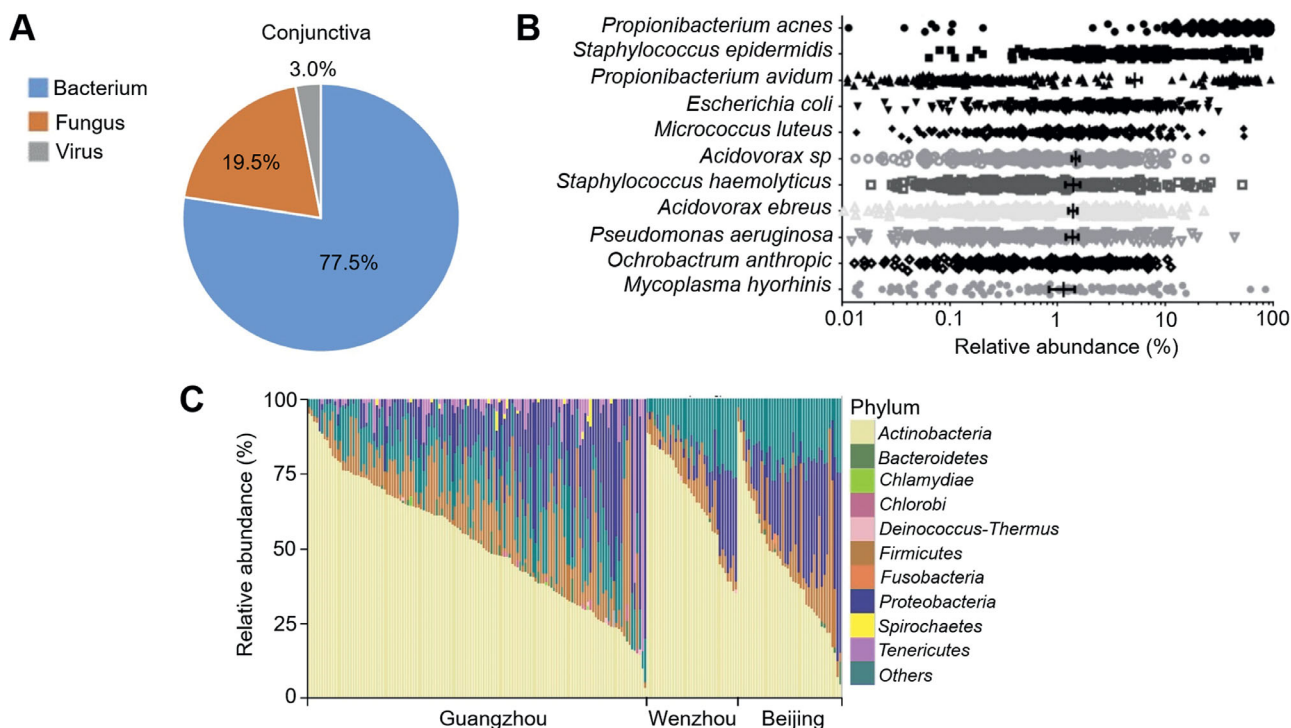
FIGURE 1. The microbiota on healthy human conjunctiva (**A**). Relative composition of major kingdoms on human conjunctiva. (**B**) Relative abundance of the core bacterial species (average >1%) on conjunctiva. (**C**) Relative abundance plots of core bacterial taxa by sampling groups.
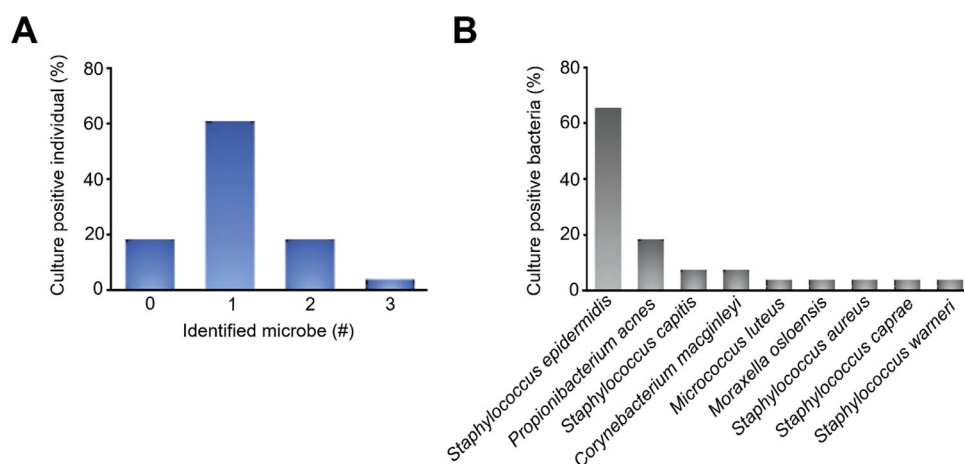

FIGURE 2. Major bacterial species identified on the ocular surface are viable. Traditional culture of conjunctival swab was carried out in the clinical laboratory at ZOC. The percentage of individuals with positive culture results of varied numbers of bacteria (**A**) and the percentage of each species that was positively cultured among all species (**B**) showed the viability of ocular surface commensals. Aerobic and anaerobic bacteria containing in the conjunctival swab samples had been cultured respectively.

and 3.0% of reads were of fungal and viral origins, respectively (Fig. 1A). Our current analysis focused on the bacterial species that accounted for the majority of the microbial population on ocular surface. Similar to previous reports based on culture of conjunctival swabs, we also found that the conjunctival microbiome of healthy Chinese participants was predominated by *Propionibacterium acnes* and *Staphylococcus epidermidis* (Fig. 1B). Many constituents of the core microbiome (defined as >1% relative abundance) such as *Micrococcus luteus* and *Staphylococcus haemolyticus*, have been frequently identified as ocular surface opportunistic

pathogens. As seen in other body sites, the composition of conjunctival microbiome was also characterized by significant individuality (Fig. 1C). Taken together, our data suggest that commensal microbiota inhabits the ocular surface.

To assess whether the bacteria identified by high-throughput metagenomic sequencing were viable, 28 healthy conjunctival samples, among all sequenced ones, were also subjected to the standard bacterial culture tests performed independently by the clinical laboratory in our hospital. As shown in Figure 2A, 83% of the cultures showed positive for at least one bacterium and the majority of
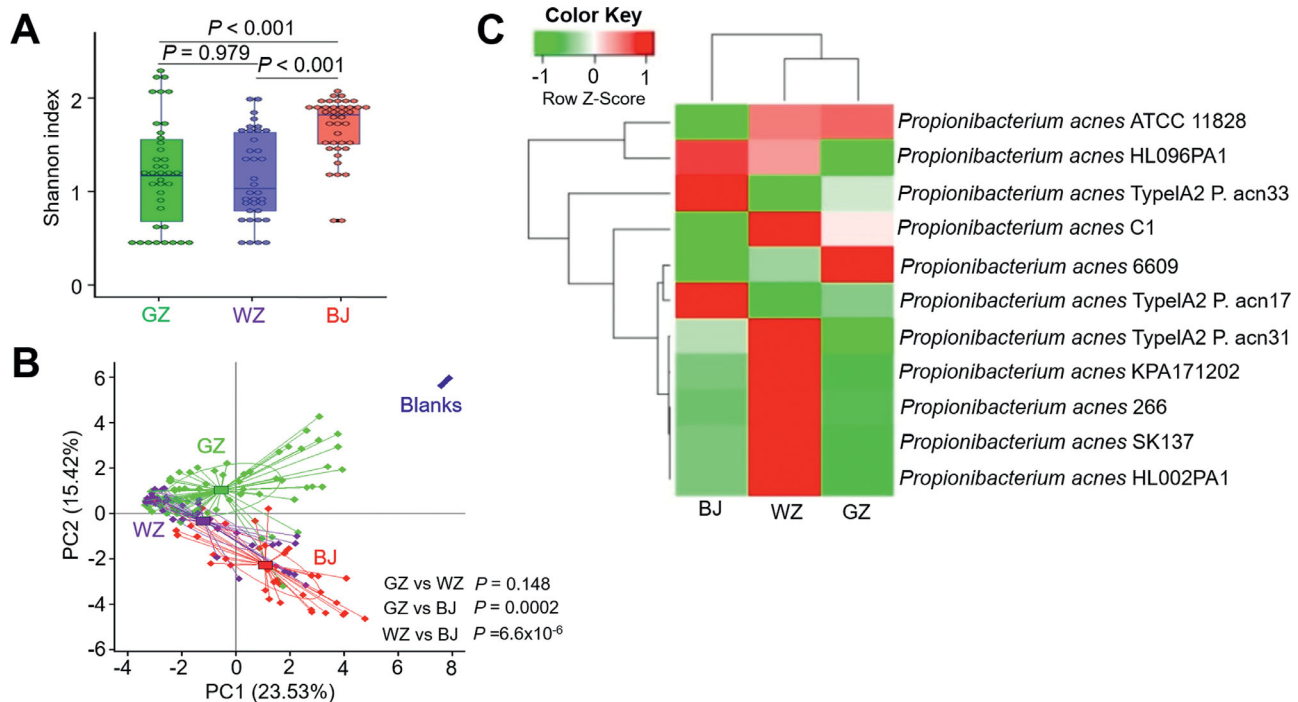
FIGURE 3. The geographical diversity of the conjunctival microbiome. The microbial community diversity of Guangzhou (GZ), Wenzhou (WZ), and Beijing (BJ) samples measured by Shannon index (Mann-Whitney U test) (GZ vs WZ $Z = -0.044$ [$P = 0.979$]; GZ vs BJ $Z = -3.836$ [$P < 0.001$]; WZ vs BJ $Z = -3.979$ [$P < 0.001$]) (**A**) and principal component analysis of bacterial relative abundance (GZ vs WZ $Z = -1.454$ [P = 0.148]; GZ vs BJ $Z = -4.230$ [$P < 0.001$]; WZ vs BJ $Z = -4.589$ [$P < 0.001$]) (**B**). The hierarchical clustering of different *P. acnes* strains was shown (**C**).

the culture positive individuals had only one bacterium in their cultures. Among all species that cultured positively, *S epidermidis* seemed to be positive in 64% of the cases, followed by *P. acnes* (Fig. 2B). These data suggest that the bacterial species identified by high-throughput metagenomic sequencing are colonizing organisms.

## Geographic Difference in Human Ocular Surface Metagenome

The ocular surface is constantly exposed to the environment.[2] Therefore, we next examined whether the conjunctival microbiome differed among samples taken from three major Chinese cities (Guangzhou, Wenzhou, and Beijing) that are geographically separated by thousands of kilometers and represent distinct environments. Interestingly, the conjunctival microbiome of Beijing volunteers showed remarkably distinct characteristics as compared to Guangzhou and Wenzhou microbiomes. Beijing conjunctival samples displayed elevated alpha diversity (Fig. 3A) and a unique profile of the microbiome (Fig. 3B), although no difference was found between the diversity of Guangzhou and Wenzhou conjunctival microbiota (Fig. 3). Intriguingly, strain level analysis of the major conjunctival bacterium *P. acnes*. demonstrated the difference in microbiome composition among three cities could be traced by even one bacterial species (Fig. 3C).

The conjunctival microbiomes from Guangzhou and Beijing volunteers featured different prevalent taxa (Fig. 4A), among which *P. acnes*. (Fig. 4B, left) and *Pseudomonas aeruginosa* (Fig. 4B, right) were the most enriched species for Guangzhou and Beijing microbiome, respectively. In addition, different microbial pathways characterized the conjunctival microbiome from Guangzhou and Beijing volunteers (Fig. 5), whereas no genetic difference was identified among Guangzhou, Wenzhou, and Beijing volunteers enrolled in our study (data not shown). These data suggest that the environment people lived in shapes their conjunctival microbiome.

Environmental Changes Cause Divergence in the Conjunctival Microbiome

We next addressed the question of whether a short trip to another city within the Guangdong province or a long trip to another province of China, such as Hainan province, Anhui province, Henan province, Hunan province, Hubei province, Zhejiang province, Guangxi province, Yunnan province, Jiangsu province, and Shandong province, would change the ocular surface microbiome. Conjunctival specimens were collected twice, within a 3-week interval, from 48 young volunteers from Guangzhou. The 13 young volunteers had a short trip to nearby cities around Guangzhou within Guangdong province. The data indicated that the composition and function of the microbiome did not significantly change (Figs. 6A, 6B, 6C), and this group of volunteers was considered as "non-travel." However, collected samples of the rest 35 volunteers, who traveled to different Chinese cities in other provinces for at least 2 weeks between sample collections, were clustered into the "travel" group. Significant alternations were found in the taxonomic composition and metabolic functions of the microbiome for the traveled group of volunteers (Figs. 6D, 6E, 6F). Taken together, our results suggest environmental changes may lead to the alteration of the ocular surface microbiome.
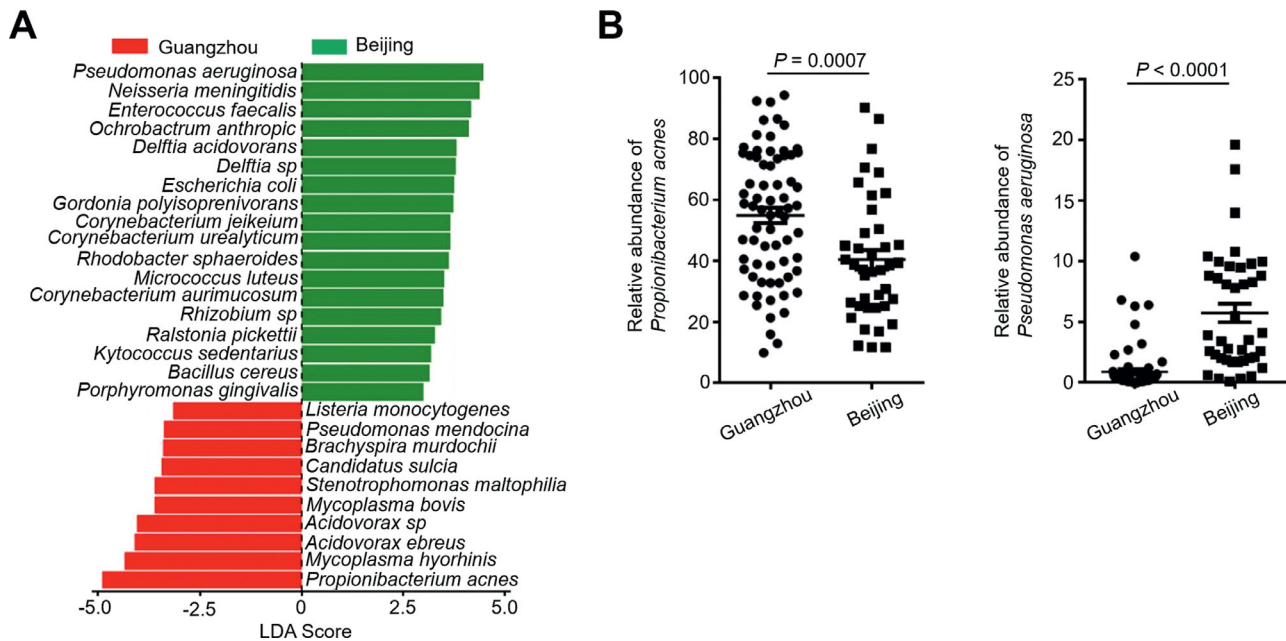
FIGURE 4. The characteristics of Guangzhou and Beijing conjunctival microbiome (**A**). The LDA effect size program was used to find the bacterial species which significantly distinguished Beijing (*green*) and Guangzhou (*red*) microbiomes (LDA score > 3). (**B**) Relative abundance of *Propionibacteruim acnes* (t = 3.450; P = 0.0007; *left*) and *P aeruginosa* (t = 5.209; P < 0.0001; *right*) in the samples from Guangzhou and Beijing cities.
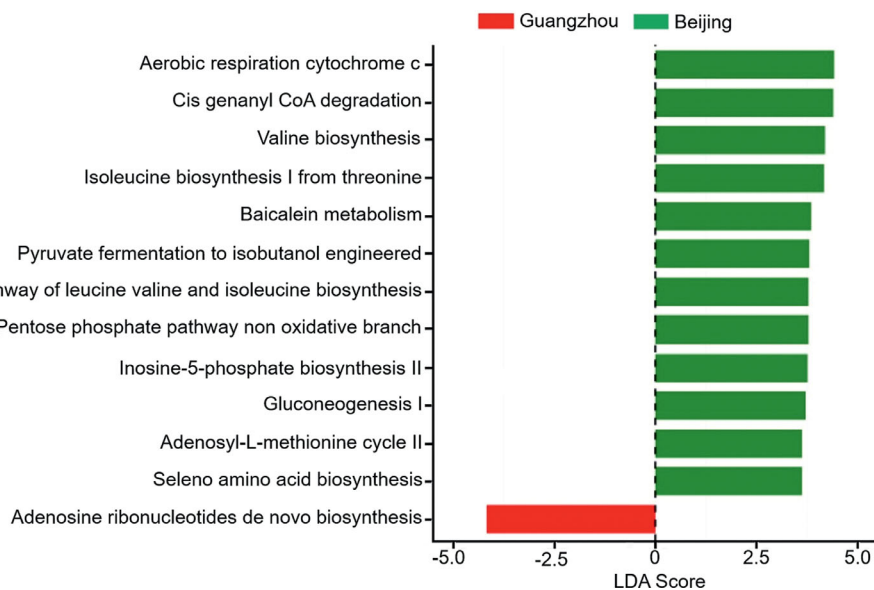

FIGURE 5. KEGG pathways differentially identified metagenomes from Beijing (*green*) and Guangzhou (*red*) samples. CoA, coenzyme A.

DISCUSSION

The microbiota inhabiting the human body maintains physiologic conditions and contributes to pathogenesis of many diseases.[23] However, the local microbial community of the eye is largely unknown. It is important to define the commensal microbiota of the healthy ocular surface to understand its role in ocular health. Toward that goal, our current work defines the metagenome of healthy conjunctiva and provides a foundation for future studies of infectious and allergic diseases of the ocular surface. Our results suggest that although bacterial species account for the

majority of the ocular surface microbiota (Fig. 1A), the fungal and viral presence on healthy conjunctiva warrants additional attention for future study.

Understanding whether the ocular surface microbiota changes in response to continuous exposure to varying environment is an important question. Our data provides the first evidence that the diverse environments in different cities may continuously shape the ocular surface microbiome of the local population (Fig. 3). The age and sex of volunteer groups from Guangzhou, Wenzhou, and Beijing were matched, and their genetic background was all mixed Han Chinese and indistinguishable. The area and the size
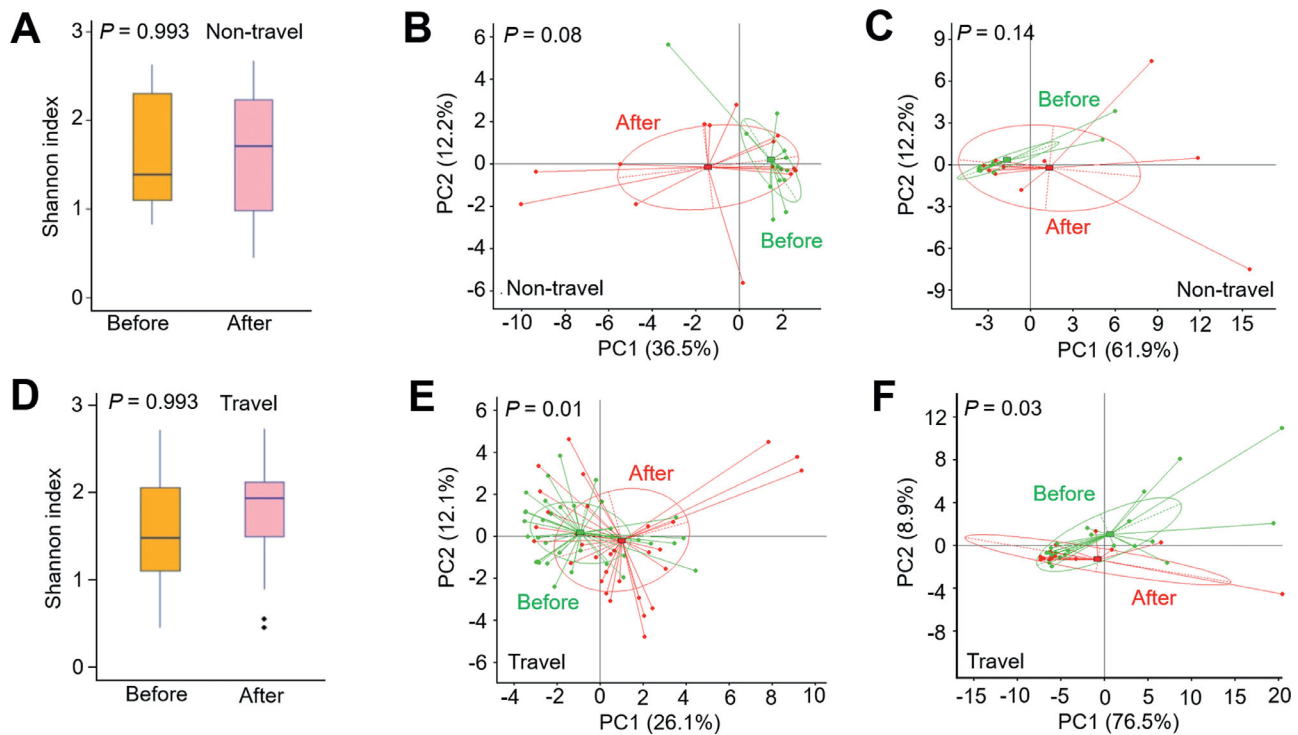
FIGURE 6. Environmental changes cause divergence in the conjunctival microbiome. The comparison of conjunctival microbial diversity between samples collected within three weeks from volunteers who did not travel showed no significant differences as measured by alpha diversity (t = 0.009; P = 0.993) (**A**), principal component analysis of bacterial abundance (Z = −1.792; P = 0.08) (**B**), and functional pathway abundance (Z = −1.517; P = 0.14) (**C**). The comparison of conjunctival microbial diversity between samples collected within three weeks from volunteers who traveled to other cities showed significant changes as measured by alpha diversity (t = 2.964; P = 0.003) (**D**), principal component analysis of bacterial abundance (Z = −2.589; P = 0.01) (**E**) and functional pathway abundance (Z = −2.188; P = 0.03) (**F**). P values associated with principal component plots represent a Wilcoxson rank-sum test on samples' projection onto PC1.

of population in these three cities differ significantly. Additionally, some environmental factors, such as temperature, humidity and air quality, of these three cities were distinct (data not shown). Thus, it resulted in the significant difference in sunlight exposure, life style and local dietary habits among three cities. Especially, for example, the weather in the north of China, such as Beijing, is dry and cold, and wheat is a primary daily food. While in south of China, such as Guangzhou, the weather is wet and warm, so that the rice is a primary daily food. The different were actually affected by or resulted from environment factor, which would also impact on ocular surface microbiome. Further studies are needed to explore these environmental factors that how their influencing ocular surface microbiota.

In addition, traveling to a different city may reshape the ocular surface microbiome (Fig. 6). The short ship traveling to nearby cities around Guangzhou within the same province showed no difference in taxonomic composition and metabolic functions. It might be explained that these tourists shared the same environmental factors. However, the long trip traveling to other provinces reshaped the ocular surface microbiome, as well as the metabolic functions. The comparison between long and short trip have further explained that environmental transformation could influence in microbial composition. However, more efforts are needed to define how quickly the microbiota can be changed or restored.

Previous studies using traditional culture methods and 16s rRNA sequencing detected many core members of the ocular surface microbiota.[2,3] Our study in princi-

ple found a similar commensal repertoire of bacteria on the ocular surface. Some differences between traditional culture methods and metagenomics sequencing were found (Figs. 1, 2), because of the sensitivity, selective pressure and other factors. However, the microbiota defined by the Miami,[8] Gambia,[9] New York,[10] Seattle,[11] and our cohorts differs in both composition and abundance of key commensals. Because our results from different cities also showed the potential geographical diversification of ocular surface microbiota, it is understandable that different cohorts from all over the world present unique profiles of bacterial composition among their conjunctival commensals.

In this study, we only sampled the ocular surface microbiota from the inferior bulbar conjunctiva. Although some unexposed sites which indirectly contacting with environment through nictation, such as superior tarsal, orbital and superior bulbar conjunctiva, it was reasonable of us to infer the most similar microbial composition in these physiologic sites. Some limitations were that we did not further investigate whether or how life style and local dietary habits influence in ocular surface microbiome in the same environment. Thus, the results described would promote us to conduct further research.

CONCLUSIONS

Healthy conjunctiva harbors a unique microbiota, and environmental changes may lead to the alteration of the ocular surface microbiome, especially the dominate species. Our data provide a rich resource for future efforts to identify

the pathological microbiota that may reshape ocular surface immunity.

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