The Influence of Age and Sex on Ocular Surface Microbiota in Healthy Adults

Xiaofeng Wen,[1] Li Miao,[1] Yuhua Deng,[1] Paul W. Bible,[1] Xiao Hu,[1] Yanli Zou,[1] Yu Liu,[1] Shixin Guo,[1] Juanran Liang,[1] Tingting Chen,[1] Guang-Hua Peng,[2,3] Wei Chen,[4,5] Lingyi Liang,[1] and Lai Wei[1,6]

[1]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China
[2]Department of Pathophysiology, Basic Medical College of Zhengzhou University, Zhengzhou, He'nan, China
[3]Department of Ophthalmology, General Hospital of Chinese People's Liberation Army, Beijing, China
[4]Department of Biostatistics, University of Pittsburgh, Pittsburgh, Pennsylvania, United States
[5]Division of Pulmonary Medicine, Allergy and Immunology, Department of Pediatrics, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania, United States
[6]Guangdong Province Key Laboratory of Brain Function and Disease, Sun Yat-sen University, Guangzhou, China

Correspondence: Lai Wei, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China; weil9@mail.sysu.edu.cn.
Lingyi Liang, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China; lianglingyi@gzzoc.com.

XW, LM, and YD contributed equally to the work presented here and should therefore be regarded as equivalent authors.

PURPOSE. A growing body of evidence suggests that the microbiome of the ocular surface confers potent immunoregulatory functions and has a key role in the physiologic maintenance of healthy eyes and in the pathogenesis of ocular diseases. Although the microbiome is known to be affected by age and sex, the influence of these factors on ocular surface microbiota in healthy adults remains largely unknown.

METHODS. Ocular surface microbiome samples were obtained from the inferior bulbar conjunctiva of 48 young and 42 old adults at Zhongshan Ophthalmic Center. Using metagenomic shotgun sequencing, we characterized the sex- and age-differences in conjunctival microbiome profiles of healthy adults.

RESULTS. Male and female groups differed only in the β diversity of bacterial communities, while there were significant differences in bacterial composition, metabolic functions, and the abundance of antibiotic resistance genes between young and old adult groups.

CONCLUSIONS. Our findings suggest that age and sex collectively shape the conjunctival microbiome, and may change the immune homeostasis of the ocular surface through alterations of its commensal microbiome.

Keywords: conjunctival microbiome, metagenomic shotgun sequencing, age and sex

Increasing evidence indicates that sex hormones influence ocular surface health.[1] Immune associated diseases, such as ocular allergy and dry eye, often are caused by alterations in steroid hormone levels.[2,3] The aging process also significantly impacts the immune system and eye health, especially with regard to the T cell compartment.[4] Moreover, it is well known that the risk of ocular surface diseases increases with age and women are more affected than men,[1,4] making it clear that there is a strong relationship among age and sex in immune homeostasis of the ocular surface. Additionally, mounting evidence suggests that diverse commensal microorganisms inhabiting the ocular surface have potent immunoregulatory functions and have an important role in the ocular health and disease pathogenesis.[5–8] Although the microbiome is known to be affected by age and sex,[9] the influence of these factors on ocular surface microbiota in healthy individuals remains controversial. Using 16S rRNA sequencing, Ozkan et al.[10] found no effect of age on the microbial α diversity and a higher Shannon index in males, while a study by Zhou et al.[11] indicated no effect of sex on the microbial diversity and a significantly higher richness and Shannon index in children. Moreover, Shin et al.[12] reported an effect of sex on the ocular surface microbial community structure.

We characterized the sex- and age-differences in conjunctival microbiome profiles of healthy adults using a metagenomic shotgun sequencing approach. We found that male and female groups differed only in the β diversity of bacterial communities. Differences between young and old adult groups were more dramatic. Age groups showed significant differences in bacterial composition and metabolic functions. Moreover, several antibiotic resistance genes were enriched in the conjunctival microbiome of old adults. Our findings suggested that age and sex collectively shape the conjunctival microbiome and that aging appears to be a stronger factor in reshaping the ocular surface microbiome, especially with regard to antibiotic resistance. Based on these findings, we concluded that the aging process is a risk factor that changes the immune homeostasis of the ocular surface through alterations of its commensal microbiome.

Investigative Ophthalmology & Visual Science

TABLE. Demographic Characteristics of the Healthy Volunteers and Summary of the Metagenomic Sequencing Data

	Young	Old
No. of sample (subject)	96 (48)	84 (42)
Average age	27.9	67.1
Male/female	23/25	19/23
Average No. of total reads	51,147,732	33,111,046
Average No. of nonhuman reads passed filter	1,476,867	1,341,236
Average % of nonhuman reads passed filter	2.9	4.1

MATERIALS AND METHODS

Subject Recruitment

This study was approved by the institutional review board of Zhongshan Ophthalmic Center, Sun Yat-sen University (protocol #2015MEKY011) and was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects. Nonsmoking healthy volunteers with no signs of systemic disease or ocular disease and no history of antibiotics treatment or contact lens wear in the past 6 months were recruited at Zhongshan Ophthalmic Center. The basic demographic information is listed in the Table.

Sampling of Conjunctival Microbiome

Ocular surface microbiome was sampled by applying a sterile semicircle MF Membrane filter (REF: HAWP01300, 0.45 μm in diameter; Merck Millipore, Burlington, MA, USA) against the inferior bulbar conjunctiva for 10 to 15 seconds. The conjunctival impression cytology samples were placed into sterile Eppendorf tubes, containing 300 μL Tissue and Cell Lysis Solution with 1 μL Proteinase K, provided in the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Ambleside, UK) and immediately frozen at −80°C.

In addition, 11 blank tubes with a membrane that did not contact the conjunctiva were prepared as negative control.

DNA Extraction and Metagenomic Sequencing

DNA was extracted from conjunctival impression cytology samples using the MasterPure Complete DNA and RNA Purification Kit (Epicentre) according to the manufacturer's instructions.

A total of 100 ng DNA was sonicated into fragments of 300 to 400 base pairs (bp) using Bioruptor (Diagenode, Belgium) and then subjected to sequencing library preparation using KAPA LTP Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA) following manufacture's protocol. DNA libraries were sequenced on the Illumina HiSeq2500 sequencer using HiSeq PE Cluster Kit v4 and HiSeq SBS V4 250 cycle kit (Illumina, San Diego, CA, USA) and the initial processing was performed by CASAVA (v1.8.2, Illumina).
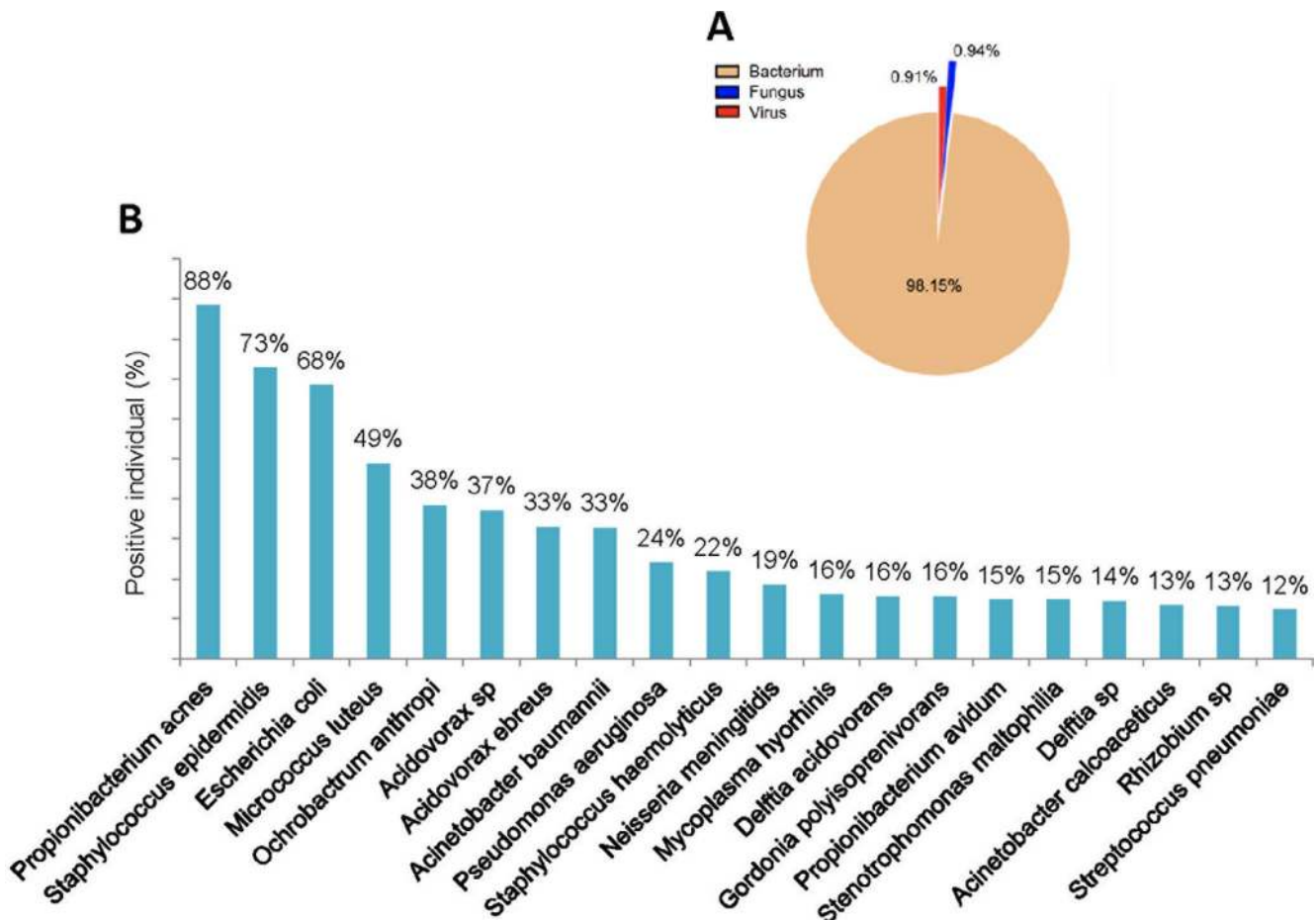
FIGURE 1. Relative composition of conjunctival microbiome (A) and positive rate of bacterial species in healthy individuals detected by metagenomic sequencing. (B) Top 20 bacterial species with the most frequent appearance are shown.
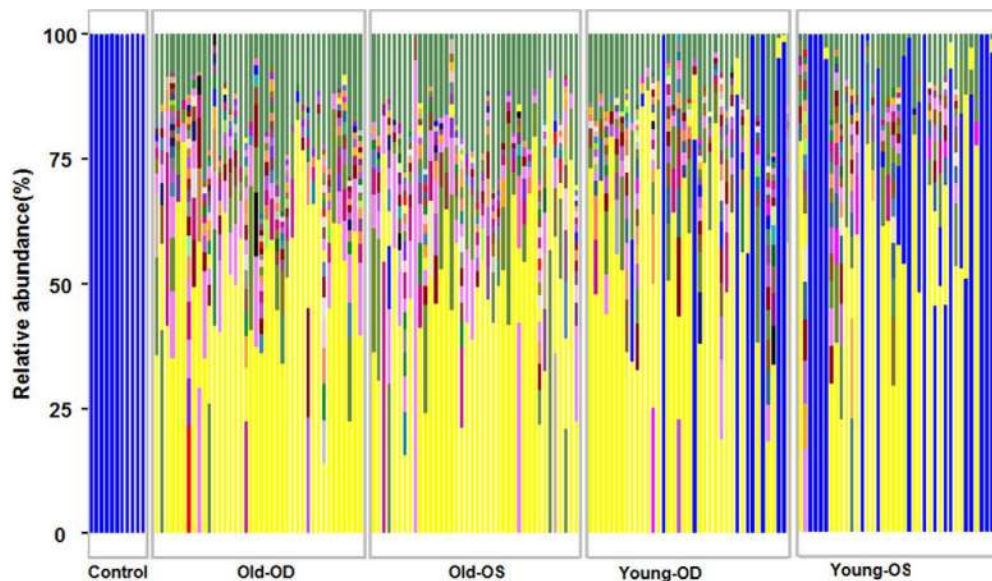
FIGURE 2. Relative composition of bacterial community on healthy human conjunctiva. Eleven blank tubes with a membrane that did not contact the conjunctiva were prepared as negative control. The data show almost 100% of the reads are duplications or of low quality.
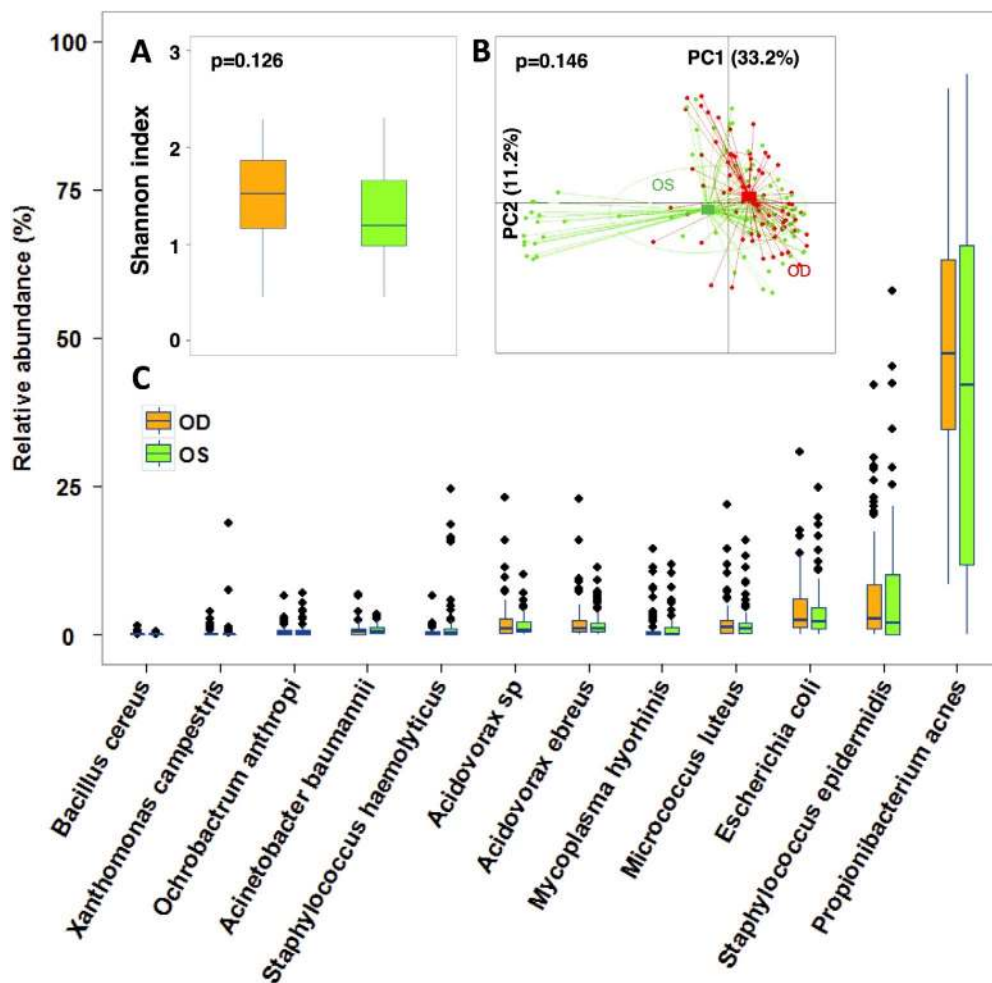

FIGURE 3. The commensal communities inhabiting the left and right ocular surfaces are statistically indistinguishable. No significant difference is detected in the α diversity (measured by Shannon index, Mann-Whitney U test [A]), β diversity (measured by PCA analysis of bacterial relative abundance, P value calculated from a Wilcoxson rank-sum test on samples' projection onto PC1 [B]), and the relative abundance of core conjunctival bacterial species (Student's t-test, [C]) between the left and right eyes of the same volunteer.
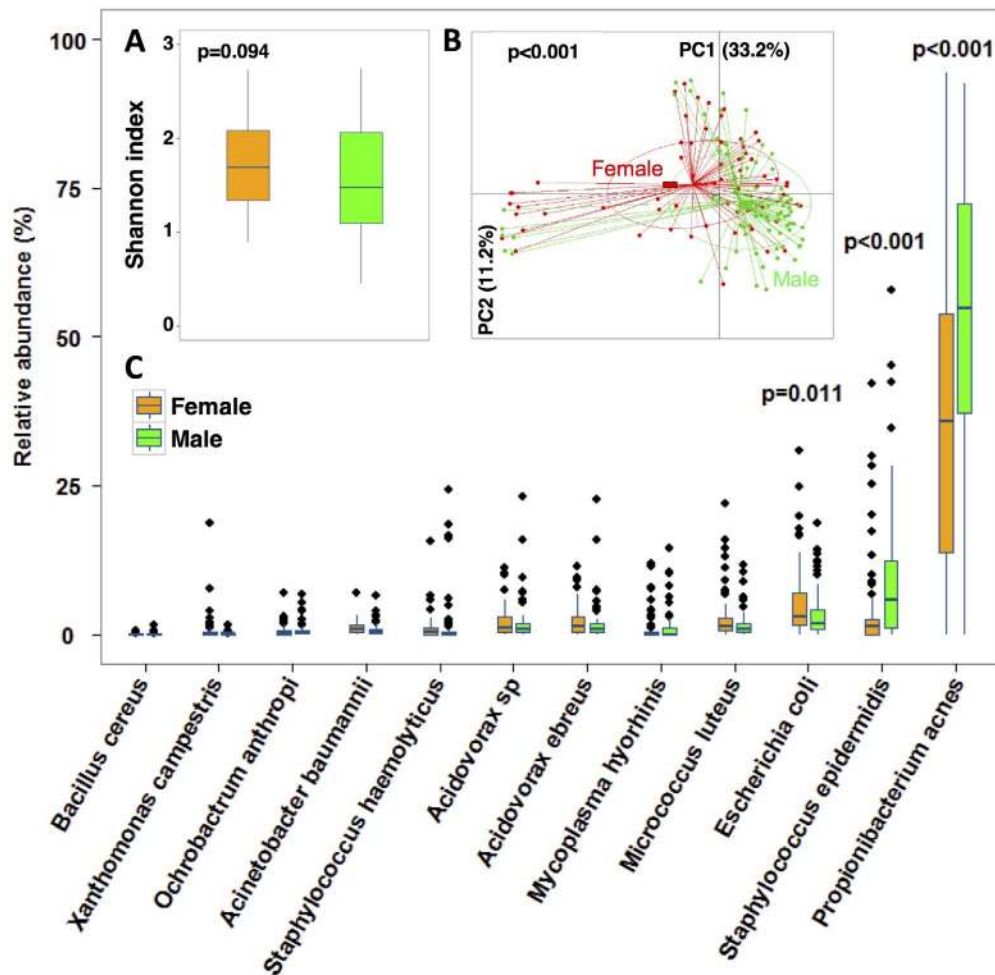
FIGURE 4. Sex-differences in conjunctival microbiome of healthy adults. Male and female groups differ only in the β diversity of bacterial communities (B), while no significant difference is observed in Shannon diversity index (A). (C) The differences in relative abundance of core conjunctival bacterial species between male and female groups are summarized.

Data Analysis

After quality control by FastQC, the sequence reads were preprocessed with removal of human reads by HiSAT2 (v2.0.1)[13] and DeconSeq[14] to obtain clean nonhuman sequences. The nonhuman sequences were aligned to our custom microbial genome collection using Burrows-Wheeler Aligner (BWA 0.7.5a)[15] and grouped into distinct taxonomic units according to their species level classification. The ratio of the total mapped reads of each species, normalized by the genome size and the total mapped microbial reads within each sample was used to represent the relative abundance of each species. Community diversity (Shannon Index) was calculated according to the method described in the Mothur program.[16] The abundance of microbial KEGG pathways was analyzed through the use of the HMP Unified Metabolic Analysis Network (HUMAnN2).[17] Based on the relative abundance of bacterial species and microbial KEGG pathways, principal component analysis (PCA) was performed using Ade4 package in R statistical software (v3.1.1). The nonhuman reads also were mapped to the Antibiotic Resistance Gene Database (ARDB)[18] using DIAMOND (v0.7.11)[19] to identify the antibiotic resistance genes in each sample. The differences in bacterial species and functional pathways characterizing the groups were evaluated by linear discriminant analysis (LDA) Effect Size (LefSe).[20]

Statistical Analysis

All statistical analyses were implemented in the SPSS software (v17.0). *P* values were calculated as indicated in Figure legends using the parametric (Student's *t*-test) or nonparametric (Mann-Whitney *U* and Wilcoxon rank-sum) tests. For all boxplots, center lines represent the median and the box edges represent the first and the third quartiles.

RESULTS

Metagenomic Shotgun Sequencing Analysis Reveals Diversity in Ocular Surface Microbiota of Healthy Volunteers

We obtained ocular surface microbiome samples from the inferior bulbar conjunctiva of 48 young (age, 23–44) as well as 42 old (age, 47–84) adults at Zhongshan Ophthalmic Center. A total of 180 samples collected from both eyes of healthy volunteers were subjected to metagenomic shotgun sequencing analysis. Although the majority of all reads were of human origin, an average of approximately 1.4 million nonhuman quality-filtered microbial sequence reads per sample were obtained for subsequent analysis (Table). All 11 blank samples were subjected to the same sequencing library preparation
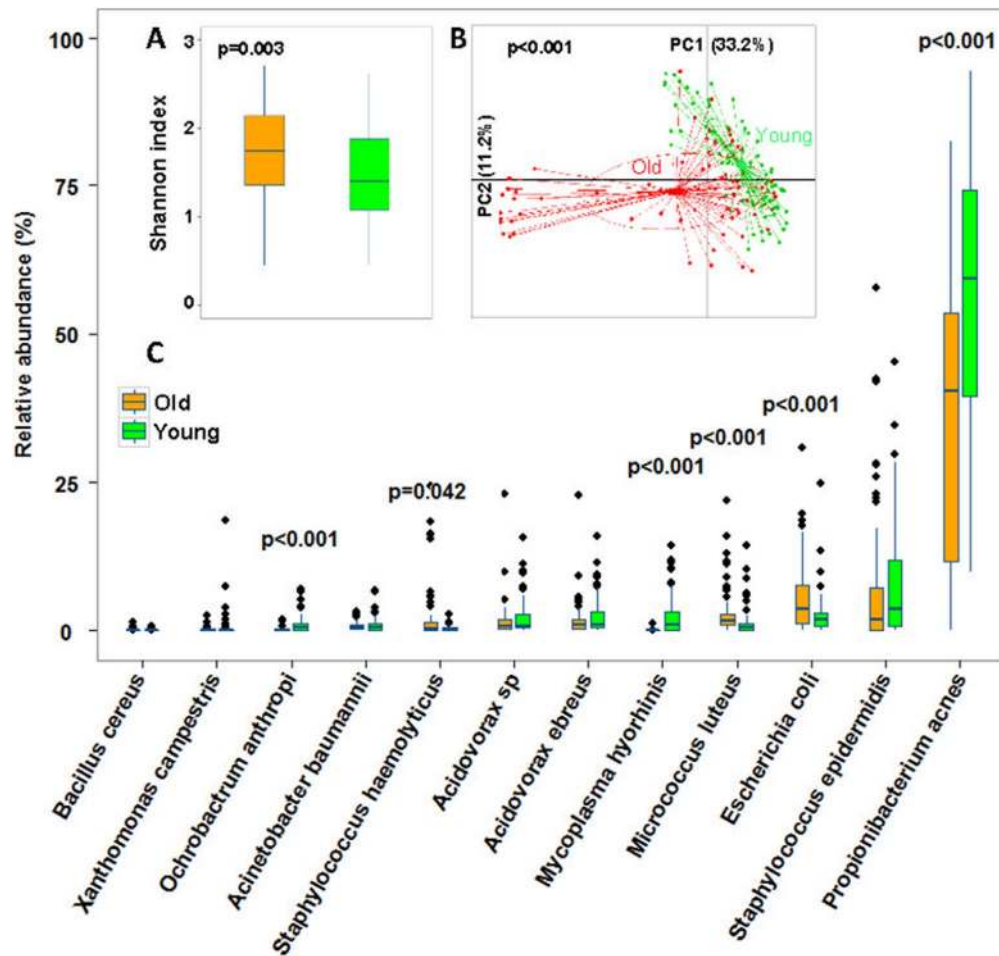
FIGURE 5. Age-differences in conjunctival microbiome of healthy adults. Significant differences are found in community diversity (including α and β diversity) of conjunctival microbiome between young (age, 23–44) and old (age, 47–84) groups (**A**, **B**). (**C**) The differences in relative abundance of core conjunctival bacterial species between young and old groups are shown.

process and data analysis. The results confirmed that almost 100% of the reads were duplications or of low quality.

Our metagenomic data showed that on average 98.15% of microbial reads were of bacterial origin, while 0.94% and 0.91% were of fungal and viral origins, respectively (Fig. 1A). The ocular surface microbiome was predominated by bacterial species; thus, the following analysis focused on the bacterial community. *Propionibacterium acnes* could be detected in 88% of healthy volunteers, while the positive rate for *Staphylococcus epidermidis* was 73% (Fig. 1B). In terms of bacterial community composition, each individual had a unique conjunctival microbiota (Fig. 2). Moreover, we examined whether the bacterial community of the ocular surface differed between the left (OS) and right (OD) eyes of the same volunteer, the results showed that no significant difference was detected in either α (measured by Shannon index, $P = 0.13$, Mann-Whitney U test, Fig. 3A) or β (measured by PCA, $P = 0.15$, Wilcoxson Rank-Sum test, Fig. 3B) diversity of bacterial composition. Also, there was no significant difference in the relative abundance of core bacterial species (Fig. 3C).

Sex-Differences In Conjunctival Microbiome of Healthy Adults

Next, we investigated the differences in bacterial composition and relative abundance of specific species between male and female groups. No significant difference in α diversity was observed ($P = 0.094$, Fig. 4A), but variations in β diversity were significant ($P < 0.001$, Fig. 4B). *P. acnes* and *S. epidermidis* decreased significantly from male to female ($P < 0.001$), while *Escherichia coli* increased significantly in the female group ($P = 0.01$, Fig. 4C). Core bacterial species are summarized in Figure 4C.

Age and Sex Collectively Shape the Conjunctival Microbiome

We next addressed the question of whether aging affects the ocular surface microbiome. The healthy volunteers were divided into young and old groups. Our results showed that Shannon diversity index score was significantly increased in the old group compared to the young group ($P = 0.003$, Fig. 5A). Significant difference also was observed in the β diversity group ($P < 0.001$), and the separation clearly is visible in the PCA plot in Figure 5B. We found a significantly greater abundance of *S. haemolyticus* ($P = 0.042$), *Micrococcus luteus*, and *E. coli* in the old compared to the young groups, and a significant decrease in *Ochrobactrum anthropi*, *Mycoplasma hyorhinis*, and *P. acnes* ($P < 0.001$ for all, Fig. 5C) from young to old. The bacterial marker species with significant power to distinguish old and young groups are presented in Figure 6 with linear discriminant analysis scores.

Furthermore, when we divided our study groups into young-male, young-female, old-male, old-female groups, the
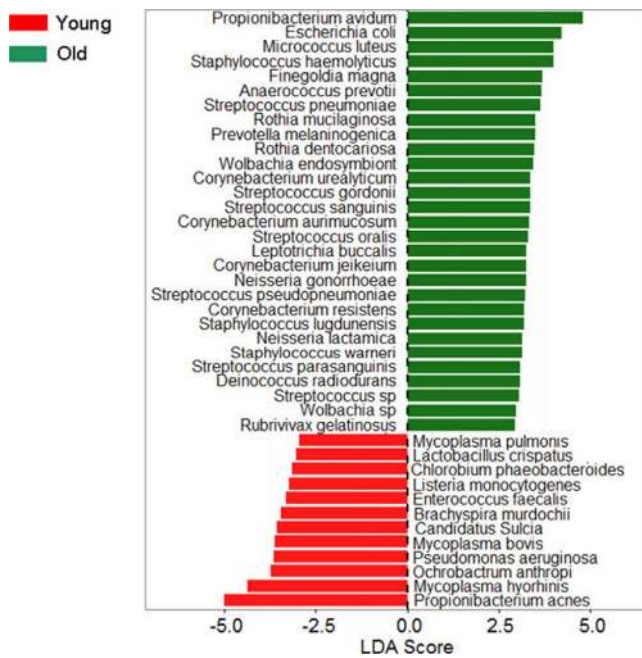
FIGURE 6. The bacterial species with significant differences in relative abundance between old and young groups. The LefSe program is used to find the bacterial species that specifically distinguish the old (*green*) from young (*red*) conjunctival microbiomes (LDA score > 3).

results showed that the old-female group was the most distinguishable from the other three groups, while no significant difference was identified between young-male and young-female groups (Fig. 7A). Importantly, three antibiotic-resistance genes encoding Class A β-lactamase, BL2b_tem,

BL2b_tem1, and BL2b_tem2, were found highly discriminative only in the microbiome of old adults (Fig. 7B).

Our data also showed significant alterations in metabolic pathways of conjunctival microbiome, especially the carbohydrate and lipid metabolism as well as nucleotide and amino metabolism, between old and young groups (Fig. 8). Taken together, the composition and function of ocular surface microbiome, as well as the abundance of antibiotic resistance genes change with age in healthy adults.

DISCUSSION

The human ocular surface is colonized by an expansive, diverse microbial community, as evidenced by previous researches using 16S rRNA sequencing and traditional culture-based methods.[10-12,21-23] The normal microbiota has a protective immunologic role in preventing the proliferation of pathogenic species and alterations in the homeostatic microbiome may be related to ophthalmic pathologies.[7,8,11,24-26] It is well known that aging alters the human immune system and sex-specific differences also lead to the varied prevalence of immunologic diseases in males and females.[4] Thus, it is important to define the influence of age and sex on ocular surface microbiota in healthy adults.

Although the effect of age and sex on the ocular surface microbiome has been investigated by previous studies, the conclusions are controversial: Ozkan et al.[10] found no effect of age on the microbial α diversity and a higher Shannon index in males, while a study by Zhou et al.[11] indicated no effect of sex on the microbial diversity and a significantly higher richness and Shannon index in children younger than 10 years. Moreover, Shin et al.[12] reported an effect of sex on the ocular surface microbial community structure. Our study demonstrated that male and female groups differed only in the β diversity of bacterial communities, while there were
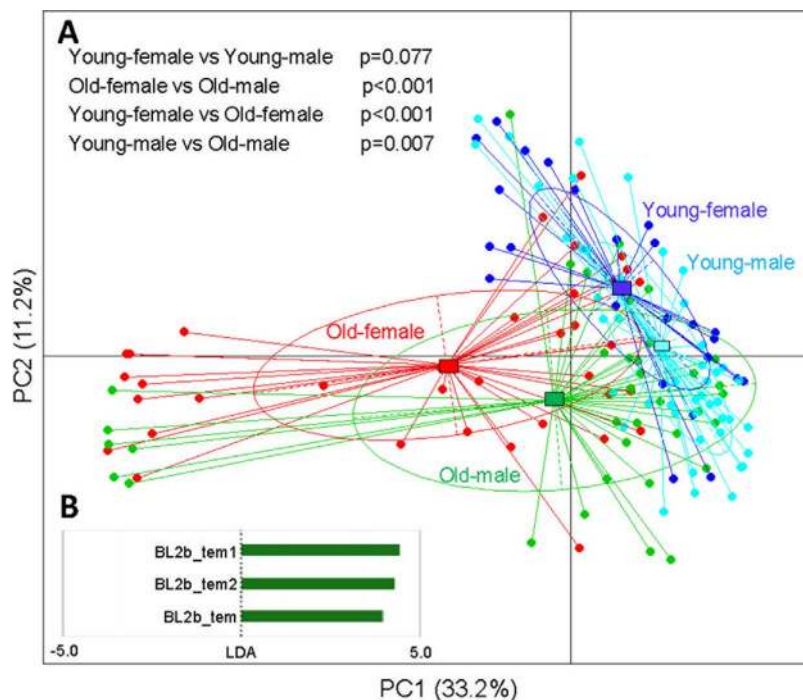

FIGURE 7. Age and sex collectively shape the conjunctival microbiome. (A) PCA analysis on the relative abundance of bacterial species in young-male (*cyan*), old-male (*green*), young-female (*blue*), and old-female (*red*) groups. *P* values between groups represent a Wilcoxon Rank-sum test of the samples' projections onto the first principle component (PC1). (B) Antibiotic resistance genes enriched in the old group (identified by LefSe, LDA score > 3).
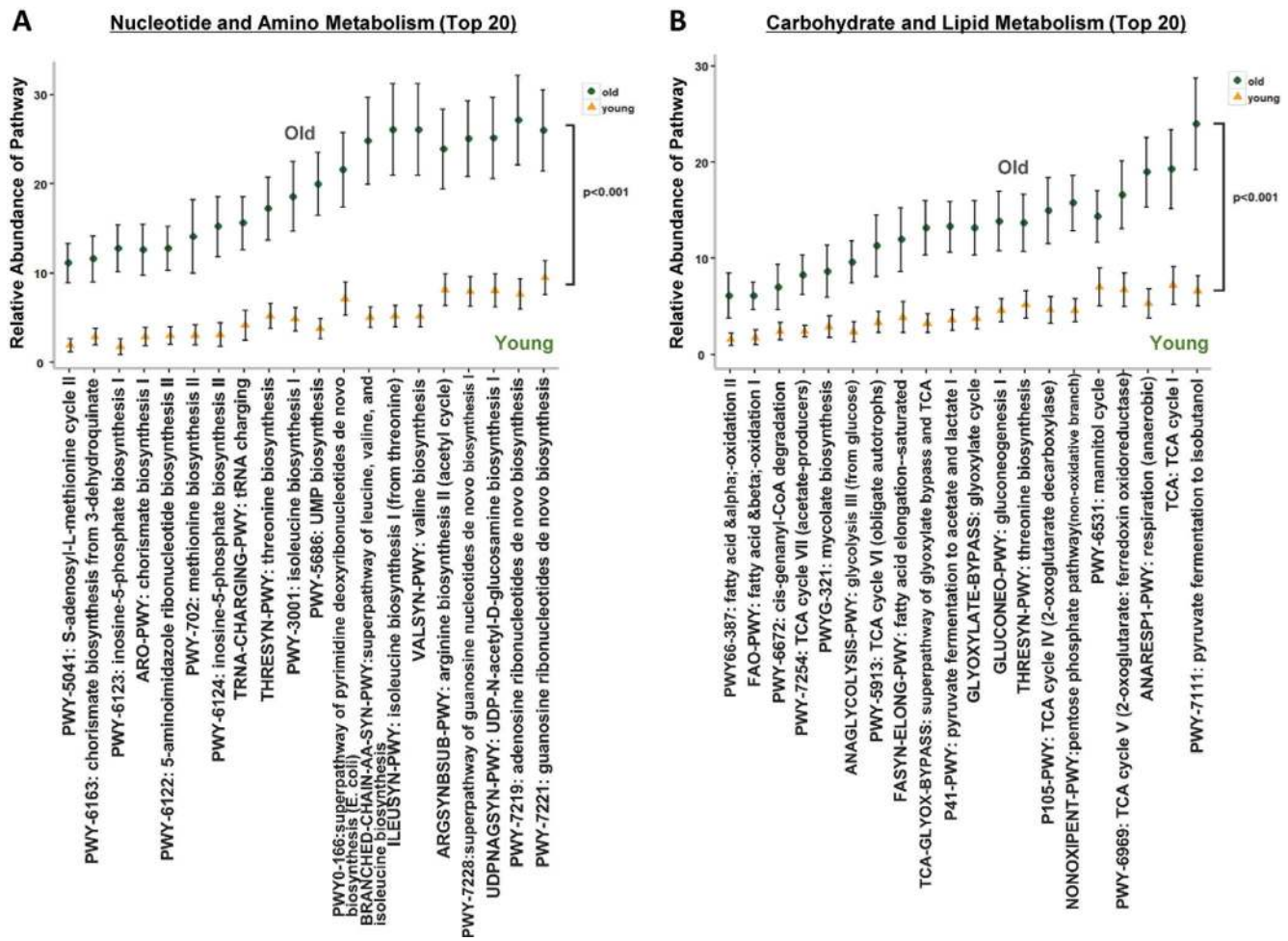
FIGURE 8. Enrichment of "nucleotide and amino metabolism" (**A**) and "carbohydrate and lipid metabolism" (**B**) metabolic pathways related genes annotated by the KEGG database in old volunteers. The *P* values were calculated by Wilcoxon Rank-sum test.

significant differences in bacterial composition and metabolic functions between young and old adult groups, suggesting that age and sex collectively shape the conjunctival microbiome. The inconsistency with previous studies may be explained partly by the use of the metagenomic sequencing approach in our study, which may result in a much broader range of microbes.

Interestingly, the conjunctival microbiome of young male and female groups was not distinguishable. However, a significant difference could be found in the composition and function of conjunctival microbiota between old male and female groups, with the most significant difference shown in the old female population. It suggests that during the reshaping of ocular surface microbiome, aging is a stronger factor, while sex only influences microbiome when interacting with age. Previous reports have indicated that a sex–steroid imbalance after menopause initiates profound changes in ocular surface microenvironment and predisposes woman to many autoimmune, inflammatory, and allergic ocular diseases, such as dry eye syndrome.[27] It is unclear whether the alteration of conjunctival microbiota found in the old female population is the consequence of sex-hormone imbalance or one of the causes leading to microenvironmental changes on the ocular surface. In addition, several antibiotic resistance genes were enriched in the conjunctival microbiome of old adults, and these genes have been demonstrated to deactivate penicillin and cephalosporin.[28] It strongly suggests that the aging process

is a risk factor that may change immune homeostasis of the ocular surface through alterations of its commensal microbiome and lead to the acquirement of bacterial strains with antibiotic resistance.

Our study has several limitations. All subjects are living in the south of China. It is conceivable that populations living in different regions or different environments might demonstrate a distinct conjunctival microbiome. Further studies are needed to illustrate how these communities are affected and the contribution of ocular microbiome to disease pathology.

CONCLUSIONS

The healthy ocular surface microbiome is shaped collectively by age and sex, and the aging process may be a stronger factor. They may change the immune homeostasis of the ocular surface through alterations of its commensal microbiome.

Acknowledgments

References

1. Mantelli F, Moretti C, Macchi I, et al. Effects of sex hormones on ocular surface epithelia: lessons learned from polycystic ovary syndrome. *J Cell Physiol*. 2016;231:971–975.

2. Truong S, Cole N, Stapleton F, Golebiowski B. Sex hormones and the dry eye. *Clin Exp Optom*. 2014;97:324–336.

3. Sacchetti M, Lambiase A, Moretti C, Mantelli F, Bonini S. Sex hormones in allergic conjunctivitis: altered levels of circulating androgens and estrogens in children and adolescents with vernal keratoconjunctivitis. *J Immunol Res*. 2015;2015: 945317.

4. Gubbels Bupp MR. Sex, the aging immune system, and chronic disease. *Cell Immunol*. 2015;294:102–110.

5. de Paiva CS, Jones DB, Stern ME, et al. Altered mucosal microbiome diversity and disease severity in Sjögren syndrome. *Sci Rep*. 2016;6:23561.

6. Kugadas A, Gadjeva M. Impact of microbiome on ocular health. *Ocul Surf*. 2016;14:342–349.

7. Kugadas A, Christiansen SH, Sankaranarayanan S, et al. Impact of microbiota on resistance to ocular pseudomonas aeruginosa-induced keratitis. *PLoS Pathog*. 2016;12:e1005855.

8. Kugadas A, Wright Q, Geddes-McAlister J, Gadjeva M. Role of microbiota in strengthening ocular mucosal barrier function through secretory IgA. *Invest Ophthalmol Vis Sci*. 2017;58: 4593–4600.

9. Singh P, Manning SD. Impact of age and sex on the composition and abundance of the intestinal microbiota in individuals with and without enteric infections. *Ann Epidemiol*. 2016;26:380–385.

10. Ozkan J, Nielsen S, Diez-Vives C, Coroneo M, Thomas T, Willcox M. Temporal stability and composition of the ocular surface microbiome. *Sci Rep*. 2017;7:9880.

11. Zhou Y, Holland MJ, Makalo P, et al. The conjunctival microbiome in health and trachomatous disease: a case control study. *Genome Med*. 2014;6:99.

12. Shin H, Price K, Albert L, Dodick J, Park L, Dominguez-Bello MG. Changes in the eye microbiota associated with contact lens wearing. *MBio*. 2016;7:e00198.

13. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 2015;12:357–360.

14. Schmieder R, Edwards R. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. *PLoS One*. 2011;6:e17288.

15. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25:1754–1760.

16. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*. 2009;75:7537–7541.

17. Abubucker S, Segata N, Goll J, et al. Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput Biol*. 2012;8:e1002358.

18. Liu B, Pop M. ARDB–antibiotic resistance genes database. *Nucleic Acids Res*. 2009;37:D443–D447.

19. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods*. 2015;12:59–60.

20. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12:R60.

21. Zegans ME, Van Gelder RN. Considerations in understanding the ocular surface microbiome. *Am J Ophthalmol*. 2014;158: 420–422.

22. Dong Q, Brulc JM, Iovieno A, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci*. 2011; 52:5408–5413.

23. Doan T, Akileswaran L, Andersen D, et al. Paucibacterial microbiome and resident DNA virome of the healthy conjunctiva. *Invest Ophthalmol Vis Sci*. 2016;57:5116–5126.

24. Lu LJ, Liu J. Human microbiota and ophthalmic disease. *Yale J Biol Med*. 2016;89:325–330.

25. 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.

26. St Leger AJ, Desai JV, Drummond RA, et al. An ocular commensal protects against corneal infection by driving an interleukin-17 response from mucosal γδ T cells. *Immunity*. 2017;47:148–158.

27. Versura P, Giannaccare G, Campos EC. Sex-steroid imbalance in females and dry eye. *Curr Eye Res*. 2015;40:162–175.

28. Jeong SH, Bae IK, Lee JH, et al. Molecular characterization of extended-spectrum beta-lactamases produced by clinical isolates of Klebsiella pneumoniae and Escherichia coli from a Korean nationwide survey. *J Clin Microbiol*. 2004;42:2902–2906.