Alterations in the Ocular Surface Microbiome in Traumatic Corneal Ulcer Patients

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Purpose. Corneal ulcers are a common eye inflammatory disease that can cause visual impairment or even blindness if not treated promptly. Ocular trauma is a major risk factor for corneal ulcers, and corneal trauma in agricultural work can rapidly progress to corneal ulcers. This study aims to evaluate the changes in the ocular surface (OS) microbiome of patients with traumatic corneal ulcer (TCU).

Methods. Among 20 healthy control (HC) subjects and 22 patients with TCU, 42 eyes were examined to investigate the OS microbial flora using metagenomic shotgun sequencing.

Results. At the taxonomic composition level, our findings showed that dysbiosis (alterations in richness and community structure) occurs in the OS microbiome of patients with TCU. Notably, Pseudomonas was present at a greater than 30% relative abundance in all individuals in the TCU group. At the species level, the abundance of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* was significantly elevated in the TCU group compared to the HC group. At the functional level, we identified significant differences in the HC and TCU groups. We observed that inflammation-related pathways involved in bacterial chemotaxis, flagellar assembly, and biofilm formation were significantly more abundant in the TCU group. Besides, the pathways related to biosynthesis, degradation, and metabolism were also increased significantly in the TCU group.

Conclusions. These findings indicate an altered OS microbiome in the affected eyes of patients with TCU. Further research is needed to determine whether these alterations contribute to the pathogenesis of TCU or impact disease progression.

Keywords: traumatic corneal ulcer, ocular surface microbiome, shotgun metagenomics

The microbiota of the OS is an emerging field of research. The characteristics of the eye include an external surface composed of mucosal tissues, including the palpebral conjunctiva, the bulbar conjunctiva, and the fornix conjunctiva.[1] The human OS harbors a variety of bacteria, fungi, and viruses due to continuous exposure to the environment.[2] The ocular commensal organisms play a key role in defending against pathogens and maintaining immune homeostasis. Nevertheless, once the integrity of the OS is destroyed, protection is lost.[3] In the setting of corneal injuries, local environmental changes and contamination may exacerbate the growth and invasion of pathogenic and opportunistically pathogenic organisms. Living microorganisms and their products can activate potential adaptive immune responses and lead to disease.[4–6] Various complications will be induced if corneal injury cannot be treated effectively and in a timely manner, such as corneal ulcers, recurrent erosion, and loss of vision.[7] In some developing countries, corneal trauma occurring during agricultural work was shown to be the main factor predisposing individuals to corneal ulceration.[8,9] Compared with some industrialized countries, the incidence of ocular trauma may be higher in China.[10] The purpose of this research was to evaluate changes in the OS microbiome of patients with traumatic corneal ulcer (TCU), providing valuable information for clinical diagnosis and treatment.

Although the composition of the ocular microbiome is still under dispute, data have become available to indicate the distribution characteristics of OS microbial communities in health and disease states. Recent studies have demonstrated the potential relationship between changes in the OS microbiome and some conditions, such as trachoma,[11]

fungal keratitis,[12,13] ulcerative bacterial keratitis,[14] conjunctivitis,[15] dry eye,[16] mesangial gland dysfunction,[17,18] blepharitis,[19] and contact lens wearing.[20,21] However, all of these studies were based on 16S rRNA gene sequencing, which, despite contributing to understanding the potential diversity of the OS bacterial flora, has limited ability to characterize nonbacterial components and functional profiles of the OS microbiome. Shotgun metagenomics allows the simultaneous study of the compositional and functional profiles of the microbiome.[22] Hence, we performed a shotgun metagenomics survey on the OS microbiome of 22 patients with TCU and 20 HC subjects to characterize the compositional and functional changes correlated with TCU.

Materials and Methods

Ethical Permission

The study protocol was approved by the Ethics Committee of the Eye Hospital of Wenzhou Medical University. This study adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects at the time of sample collection.

Study Participants

This study enrolled 22 patients with TCU (age 42—68 years) and 20 HC subjects (age 43–68 years). Participants were enrolled from a combination of patients presenting at the outpatient department at the Eye Hospital of Wenzhou Medical University and middle-aged and elderly persons living in communities across Wenzhou, China. All patients in the TCU group met the criteria for the clinical diagnosis of corneal ulcers: the presence of a corneal epithelial defect with associated suppurative infiltrate, with or without hypopyon, and were accordingly diagnosed with TCU. The agents responsible for corneal trauma were mainly agricultural products (Supplementary Table S1). The healthy volunteers had no history of systemic or ocular diseases or contact lens wear. All samples from the HC and TCU groups were free of topical or systemic antibiotics or steroids from treatment within 6 months.

Sample Collection and Processing

Sample collection was performed in an ophthalmic treatment room sterilized by ultraviolet irradiation. Samples were collected from ocular surface mucosal tissues (upper and lower palpebral, caruncle, and conjunctival fornix) using flocked swabs of the Copan ESwab transport system (Copan Diagnostics Inc., Murrieta, CA, USA) after the instillation of sterile topical proparacaine. All patients with TCU developed unilateral eye disease. A randomly chosen eye from each HC subject was sampled as a control. To avoid contamination, another environmental "air swab" containing the topical anesthetic was prepared as a negative control. Collected swabs were immediately placed on ice and transferred to the laboratory to be frozen at -80 deg Celsius (°C) until processing. Genomic DNA was extracted using pathogen lysis tubes L (QIAGEN, Hilden, Germany) and the QIAamp UCP Pathogen Mini Kit (QIAGEN) in strict accordance with the manufacturer's instructions and was assessed using a Qubit 2.0 Fluorometer and 1% agarose gel electrophoresis. Pathogen lysis tubes L (QIAGEN) with bead beating can effectively lyse fungi and gram-positive bacteria. The extracted DNA was eluted with EB buffer and, after the concentration was determined, placed in short-term storage at -20°C until sequencing. No DNA was detected in the "air swabs" using a Qubit 2.0 Fluorometer, and no DNA bands were found by performing 1% agarose gel electrophoresis on the amplified product of the 16S rRNA gene V3 to V4 region (30 cycles).

Metagenomic Shotgun Sequencing

Genomic DNA was sequenced on an Illumina HiSeq X10 platform (Novogene Co., Ltd., Beijing, China) using the metagenomic shotgun sequencing method (2 × 150 cycle runs). After quality inspection by FastQC, the adapter was trimmed by Cutadapt, and low-quality reads were filtered out using Trim Galore.[23] High-quality readings were visualized by the tool SplicingViewer.[24] Trimmed reads were mapped to the human reference genome (hg19) using Bowtie2 (version 2.3.4.3).[25] Using SamTools (version: 0.1.19), aligned reads were removed to obtain clean nonhuman sequences.[26] Then, the remaining reads were assembled by Megahit (version 1.1.3),[27] and the contigs were submitted to MetaGeneMark (version 3.38) for prediction.[28] Redundant amino acid sequences were excluded using CD-HIT (version 4.6) and a threshold of ≥90% sequence identity.[29] We mapped the nonredundant amino acid catalogs to an integrated National Register (NR) database (including bacteria, archaebacteria, fungi, and viruses) with Diamond (version 0.8.22.84).[30] The hit results were submitted to Megan (version 6) with the weighted lowest common ancestor (LCA) algorithm to assess the taxonomic compositions.[31] Functional analysis was carried out via DIAMOND search against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Group (COG) databases.

Statistical Analysis

Statistical analysis was performed using R 3.6.0. Rarefaction curves of the microbiomes were plotted using the Vegan package in R. Alpha diversity was evaluated by the Shannon index and Simpson index. A *t*-test was used to test the differences in the Shannon diversity and Simpson index between the two groups. Principal coordinate analysis (PCOA) with the Bray-Curtis dissimilarities and Jaccard index was used to explore microbial community structure. Permutational multivariate ANOVA (PERMANOVA) was performed for beta diversity analysis. The Kruskal–Wallis test was applied to identify the phylotypes and functional pathways with significantly different relative abundances between HC subjects and patients with TCU; phylotypes and functional pathways with a $Q < 0.01$ were regarded as significantly enriched. To identify environment-associated biomarker taxa, linear discriminant analysis (LDA) effect size (LefSe) was used. An LDA score > 5 was considered to indicate a significant biomarker. The R package "RandomForest" was used to obtain the taxonomic contributions of microbial communities at the species level. The SparCC algorithm was used to obtain the correlations and *P* values between genus abundances (from at least three subjects) in each group.[32] The interaction networks were visualized with Cytoscape3.6.0.[33]

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Table. Features of Subjects and Summary of the Metagenomic Sequencing Data

Feature	TCU (n = 22)	HC (n = 20)
No. of samples	22	20
Age, y	56.7 ± 7.8	56.4 ± 8.2
Male/female	14/8	13/7
Average no. of total reads	162, 231, 216	81, 168, 170
Average no. of nonhuman reads passed filter	4, 483, 517	2, 163, 406

TCU, traumatic corneal ulcer; HC, healthy control; data are the mean ± SD.

Results

Metagenomic Data Analysis

A total of 42 subjects were included with matched age (Mann–Whitney U test, P = 0.97) and sex (Fisher's exact test, P = 1.0) between the TCU (n = 22) and HC (n = 20) groups (Table, Supplementary Table S1). In total, > 5.19 billion reads were obtained, and an average of 3.38 million reads per sample were used for further analysis (Table). After trimming and filtering, the low percentage of the remaining reads might have been due to the dominance of human genomes, as human saliva, nasal cavity, skin, and vaginal specimens routinely have > 90% human content.[34]

Alpha Diversity and Beta Diversity of the Ocular Microbiome

Rarefaction analysis showed that the species richness in each group approached saturation, implying that the current sequencing depth covered the largest species diversity (Supplementary Fig. S1). The Shannon's diversity index and Simpson's diversity index based on the genus profiles in the TCU group were significantly lower than those in the HC subjects (Kruskal–Wallis test; P = 0.0059 for Shannon index and P = 0.0035 for Simpson index; Fig. 1C, D). The alpha diversity results indicated that the richness and evenness of the OS microbial communities in the TCU group were also significantly lower. PCOA and PERMANOVA analysis showed that the OS microbiome community structure of patients with TCU was significantly different from that of controls based on Bray–Curtis dissimilarities and the Jaccard index (P = 0.007992 for Bray–Curtis dissimilarities and P = 0.001998 for Jaccard index; Fig. 1E, F).

Taxonomic Changes in the OS Microbiome

Overall, the detected taxa compositions included four microbial kingdoms, including bacteria, fungi, archaea, and viruses (Supplementary Figure S2). Bacteria were the most abundant kingdom, representing > 90% of the relative abundance in each individual. Bacteria and fungi were shared by all samples, yet archaea and viruses were not found on all OSs. No archaea were found in patients with TCU. Compared to the HC group, the mean relative abundance of bacteria was higher in the TCU group, whereas the mean relative abundance of fungi was lower (Fig. 1A, B).

All kingdoms were classified into 19 phyla and 163 genera. At the phylum level, the OS microbiome was dominated by three phyla: *Proteobacteria* (TCU 75.61%; HC 28.35%), *Actinobacteria* (TCU 2.37%; HC 29.61%), and *Firmicutes* (TCU 13.0%; HC 35.72%). Other phyla with > 1% average relative abundance in each group included *Bacteroidetes* (TCU 3.05%; HC 1.35%), *Chlamydiae* (TCU 0.58%; HC 1.23%), *Deinococcus-Thermus* (TCU 3.55%; HC 0.15%), and Mucoromycota (TCU 0.19%; HC 1.0%) (Fig. 1G). The LEfSe analysis identified three biomarkers at the phylum level. *Actinobacteria* and *Firmicutes* were potential biomarkers for the HC group, whereas *Proteobacteria* was overrepresented in the TCU group (Fig. 1H). Compared with the HC group, the TCU group contained significantly lower levels of *Actinobacteria*, *Firmicutes*, *Mucoromycota*, and *Chlamydiae* and markedly higher levels of *Proteobacteria* (Fig. 1I).

As shown in Figure 2A, 15 genera with an average relative abundance > 1% were found, including Pseudomonas, Streptococcus, Corynebacterium, Cronobacter, Staphylococcus, Escherichia, Meiothermus, Vibrio, Mycobacterium, Chlamydia, Clostridioides, Mycobacteroides, Paenibacillus, Pseudoalteromonas, and Alistipes. Of these, the top five most abundant genera were Pseudomonas (TCU 57.49%; HC 0.47%), Streptococcus (TCU 4.24%; HC 22.93%), Corynebacterium (TCU 0.53%; HC 19.42%), Cronobacter (TCU 2.88%; HC 8.03%), and Staphylococcus (TCU 2.25%; HC 3.83%). LEfSe analysis identified Pseudomonas and Corynebacterium as biomarkers for the TCU and HC groups, respectively (Fig. 2B). Notably, Pseudomonas was present at a relative abundance of > 30% in all patients with TCU. Interestingly, subjects HC5, HC7, HC16, and HC18 showed high domination by Corynebacterium, accounting for 73.36%, 78.48%, 72.05%, and 53.95%, respectively (Fig. 2C). Our previous research has demonstrated that some Corynebacterium spp. could produce amino acids in large quantities.[35] Twelve genera were found to be differentially abundant between the groups. Pseudomonas, Meiothermus, and Alistipes were significantly enriched in the TCU group, and Chlamydia, Clostridioides, Corynebacterium, Cronobacter, Mycobacterium, Mycobacteroides, Paenibacillus, Staphylococcus, and Streptococcus were enriched in the HC group (Fig. 2D).

At the species level, the OS microbiome in the HC and TCU groups was categorized into 274 species, with 36.29 ± 9.37 (range, 18-64) species detected in the samples from each individual. Of note, unclassified species accounted for a high proportion (range, 13.26%-86.42%). Although some individuals showed a dominance of *Corynebacterium* and *Pseudomonas* at the genus level, unclassified *Pseudomonas* species and *Corynebacterium* species accounted for the majority. Figure 3A shows the top 20 species in the TCU and HC groups. Among these species, 14 were found to differ significantly between the HC group and the TCU group. Compared with the HC subjects, the OS microbiome of the TCU group had higher abundances of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Meiothermus silvanus* and lower abundances of *Rhizophagus irregularis*, *Streptococcus pneumoniae*, *Mycobacteroides abscessus*, *Clostridioides difficile*, *Strepto-*

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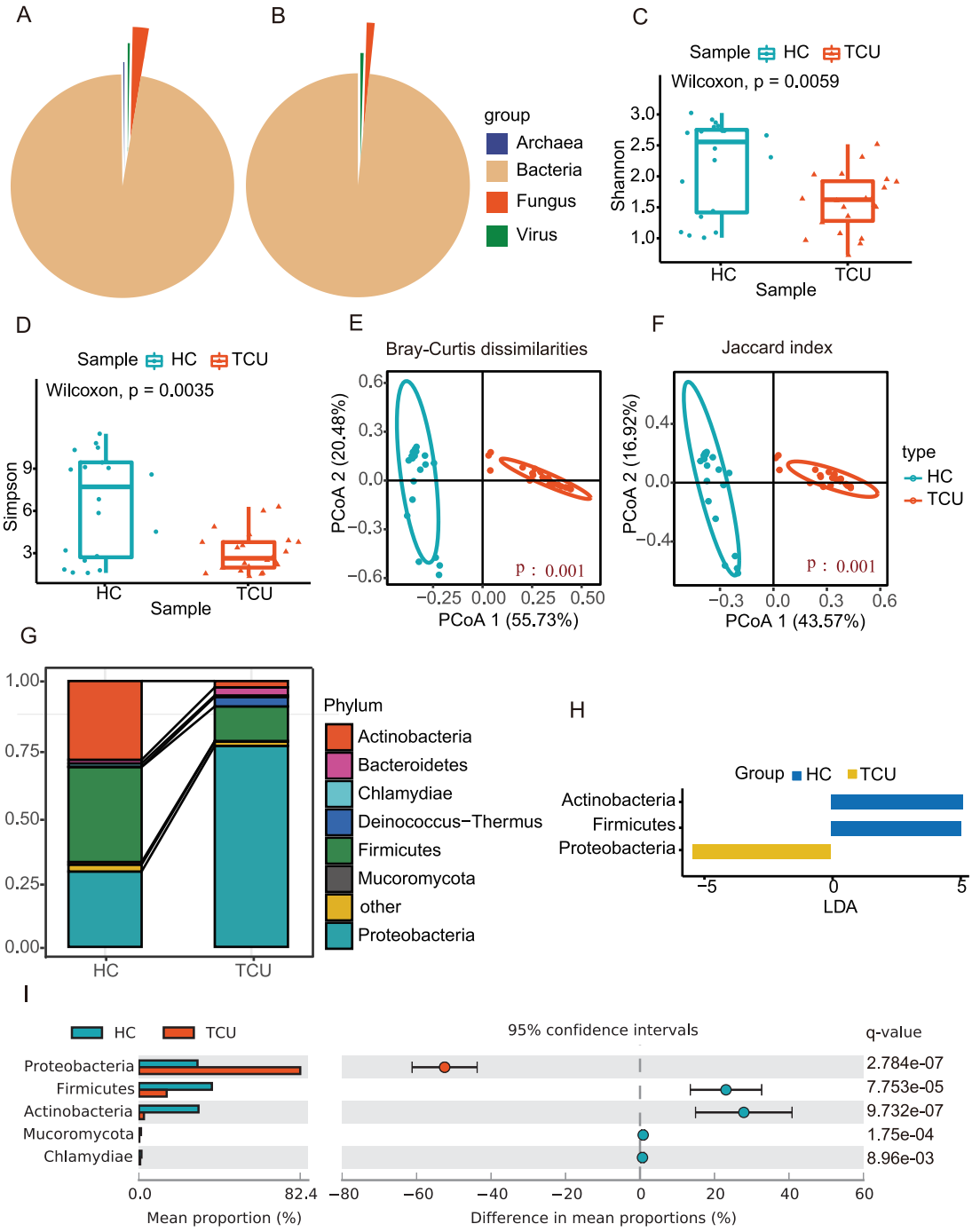

Figure 1. Alpha and beta diversity of microbiota. The distribution of kingdom and major phylum. Average abundance (%) of kingdom from microbiomes of HC group (**A**) and TCU group (**B**). Alpha diversity measured by the Shannon diversity index (**C**) and Simpson index (**D**), Student's *t*-test. Nonmetric multidimensional scaling (NMDS) plots of beta diversity based on Bray–Curtis dissimilarities (**E**) and the Jaccard index (**F**) according to disease status. The *P* values were generated by the PERMANOVA test with 999 permutations. (**G**) Major phyla, less abundant (< 1%) and unclassified taxa are grouped together as "other." Biomarker phyla (**H**) and differential phyla (**I**) in each group are depicted. HC, healthy control; TCU, traumatic corneal ulcer.

coccus pyogenes, *Corynebacterium accolens*, *Paenibacillus odorifer*, *Cronobacter sakazakii*, *Mycobacterium tuberculosis*, *Thalassospira xiamenensis*, and *Pseudoalteromonas luteoviolacea* (Fig. 3B). Because LEfSe analysis found no biomarker taxa in the species, the random forest algorithm was selected as an alternative selection method to determine the variable importance. The MeanDecreaseAccuracy and MeanDecreaseGini values of all different species were > 0.1. Among these, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* had the strongest classification contributions (Fig. 3C, D). *Pseudomonas fluorescens* was found in all patients with TCU. Except for subject TCU3, *Pseudomonas aeruginosa* was present in the OS microbial community of all patients with TCU (Supplementary Fig. S3).

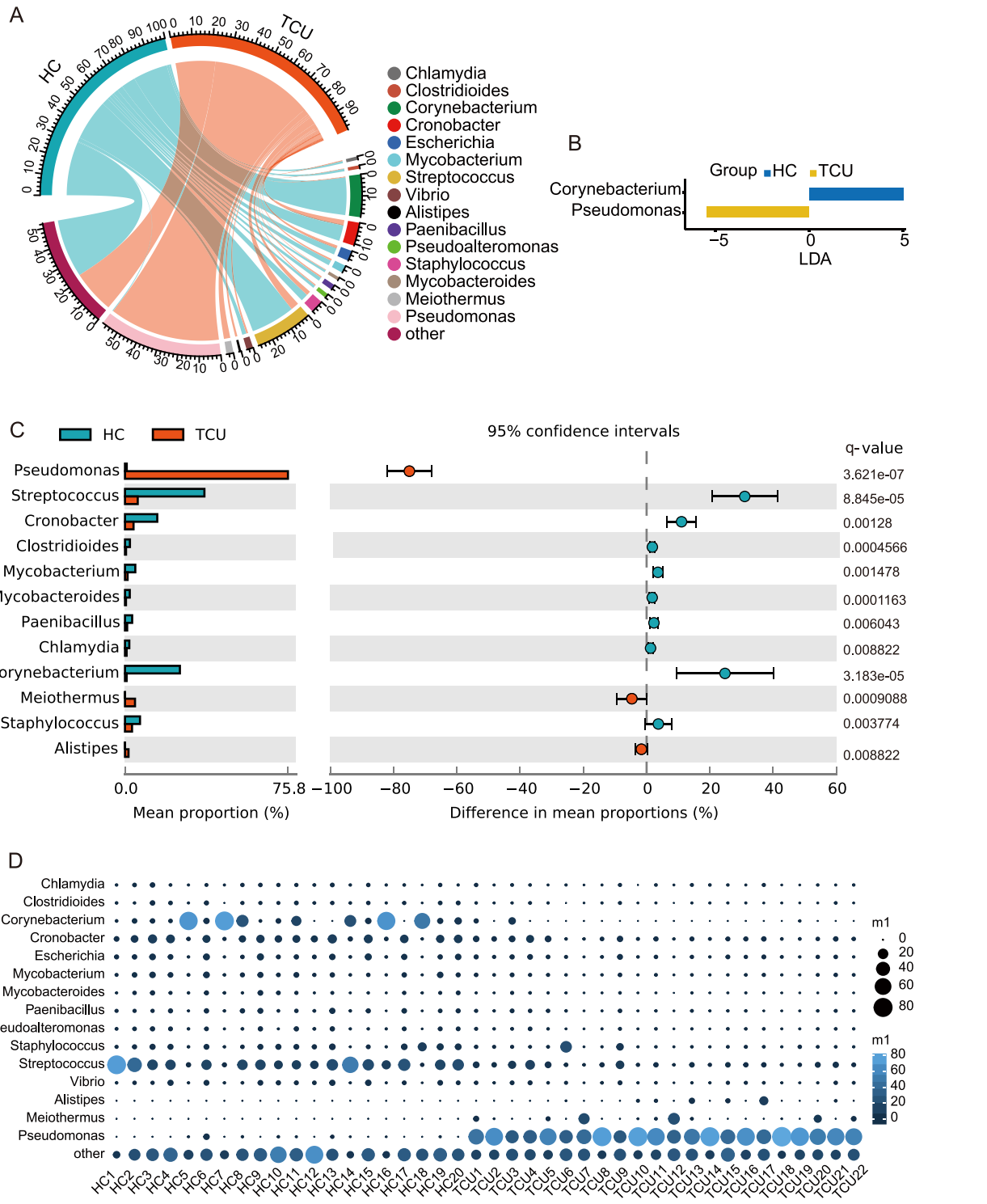

Figure 2. Major genera in ocular microbiota of patients with TCU and healthy subjects. (**A**) Major genera, less abundant (< 1%) and unclassified taxa are grouped together as "other." Biomarker genera (**B**) and differential genera (**C**) are depicted. (**D**) Bubble chart showing the relative abundances of major genera (> 1%) in the ocular microbiomes of HC subjects and patients with TCU.

In the HC group, only three viruses, namely, *Torque teno virus* (TTV), *Gammapapillomavirus 8, and Ateline gammaherpesvirus 3,* were detected. In the study by Doan et al., TTV was found on 65% of the OSs of HC subjects. Previous work found that herpes virus was present in the tears of HC subjects.[36] Although the richness of the OS microbiome of the TCU group was decreased, the number of virus types in the TCU group was obviously increased compared to that in the HC group. The viruses included *Betapapillomavirus 1*, *Betapapillomavirus 2*, *Betapapillomavirus 3*, *Eel River basin pequenovirus*, *Human alphaherpesvirus 1*, *Human polyomavirus 5*, *Microviridae Fen7918_21*, and *TTV* (Supplementary Fig. S4). The increased number of virus types may be related to the introduction of contamination in cases of

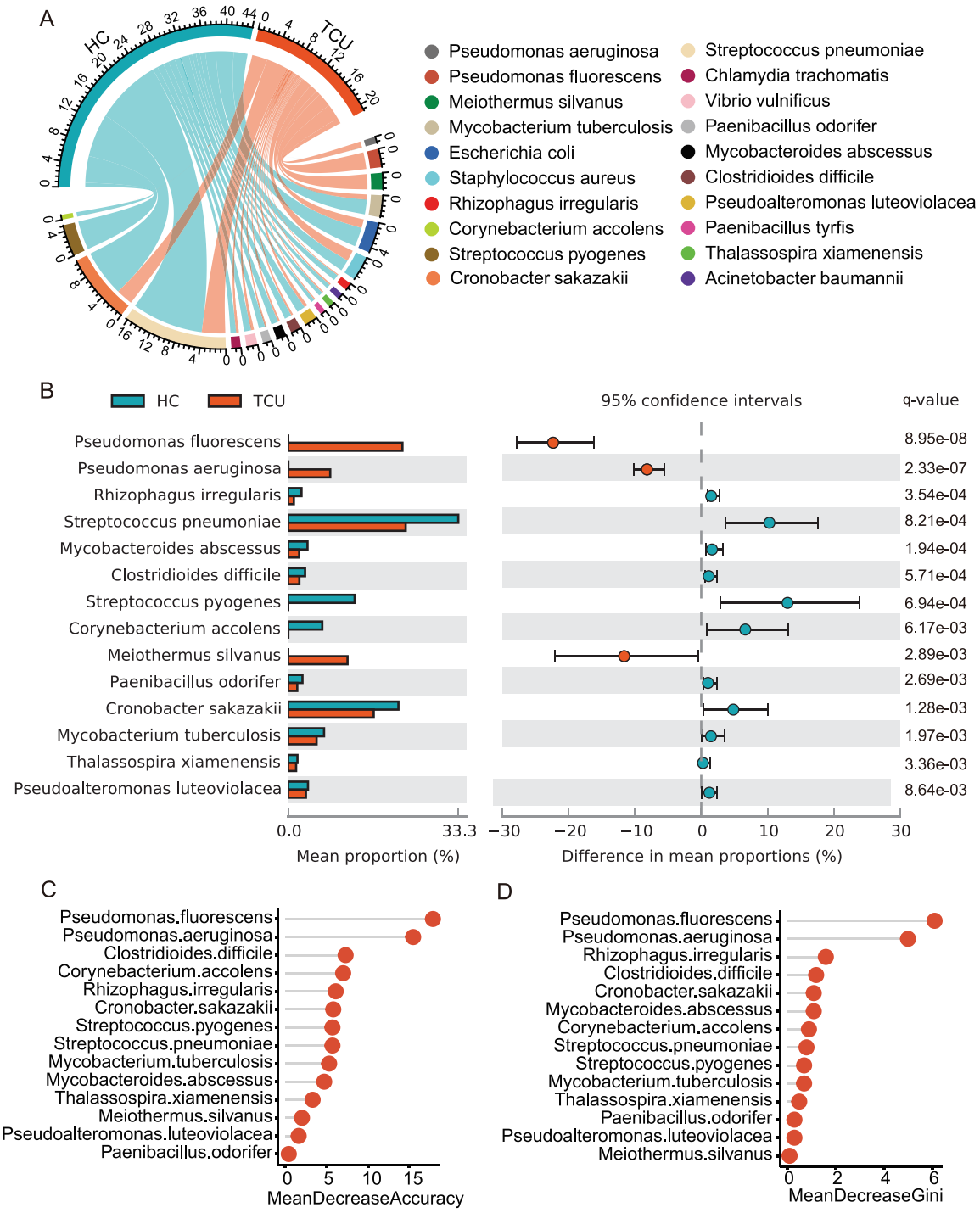

Figure 3. The distribution and differences of the top 20 species between the HC group and TCU group. (**A**) Chordal graph of the top 20 species between the HC group and TCU group. (**B**) Histogram of unique differential species in each group. (**C**) The MeanDecreaseAccuracy and MeanDecreaseGini of all differential species were calculated by the random forest algorithm.

eye injury and to the reduced ability to remove pathogens after OS homeostasis has developed an imbalance.

Correlation and Co-Occurrence Analyses of the OS Microbiome

To investigate the co-occurrence of OS microorganisms, we constructed interaction networks based on pairwise correlations between the relative abundances of the different genera (Fig. 4A, B). Overall, the strength of the microbial co-occurrence for the TCU group was weaker than that for the HC group, suggesting possible ecological disturbance of the ocular microbiome in patients with corneal ulcers. In the HC group, 10 genera (with > 10 interactions), namely, *Clostridioides*, *Cronobacter*, *Escherichia*, *Mycobacterium*, *Mycobacteroides*, *Paenibacillus*, *Pseudoalteromonas*, *Rhizophagus (fungus)*, *Thalassospira*, and *Vibrio*, were identified, indicat-

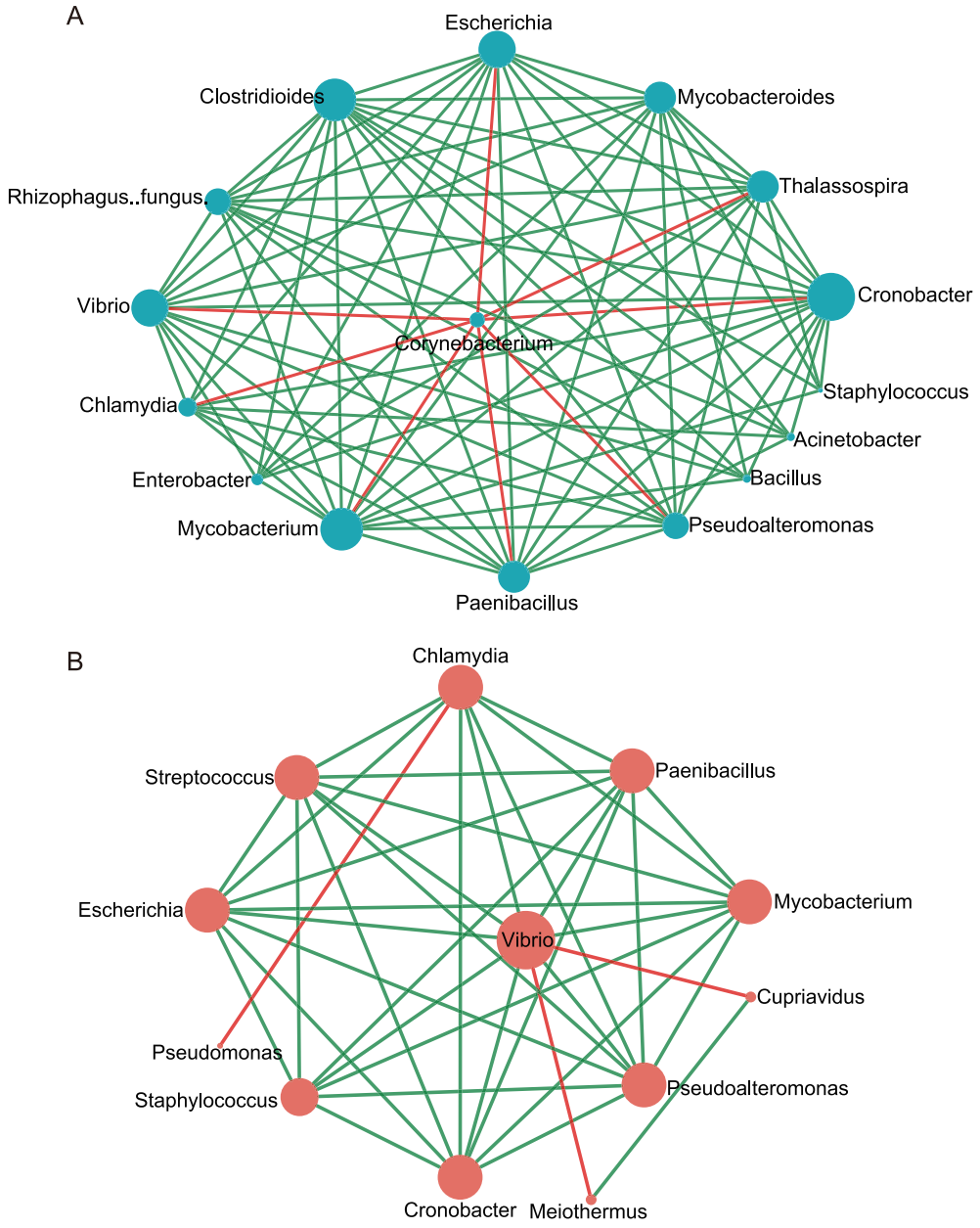

Figure 4. Microbial correlation based on relative abundance. Interaction network in the OS microbiome of HC subjects (**A**) and patients with TCU (**B**) (Spearman correlation magnitude > 0.4 and q < 0.05 are shown). Each node represents a genus (relative abundance > 0.5% in at least one group), and the size of the nodes is proportional to their degree of interaction. The co-abundance (positive correlation) and co-exclusion (negative correlation) are indicated by green and red connections, respectively.

ing their possible key roles in the network. In the TCU group, no genera had connectivity > 10. Interestingly, in the interaction network of the HC group, only *Corynebacterium* was negatively related to other genera, and *Corynebacterium* exhibited only negative interactions.

Functional Alterations in the OS Microbiome

At present, the functional profiles of the OS microbiome are still poorly understood. In this study, we investigated the differences in functional pathways between the HC group and TCU group based on a substantial amount of metagenomic shotgun sequencing data. The metagenomic genes of all samples were mapped onto KEGG orthologous groups and the COG database. The overall functional profiles were significantly different between the HC group and the TCU group (Fig. 5A, B; Supplementary Fig. S5B, C). Among bacterial pathways using the KEGG orthologous group annotation, a total of 53 differential pathways were found, of which 52 were significantly enriched in the TCU group, and only the synthesis of tyrosine was enriched in the HC group. Genes related to metabolism, degradation, and biosynthesis were significantly increased in the TCU group. All subjects had a higher abundance of genes related to ABC transporters, the two-component system, glyoxylate and dicarboxylate metabolism, fatty acid metabolism, fatty acid degradation, and folate biosynthesis.

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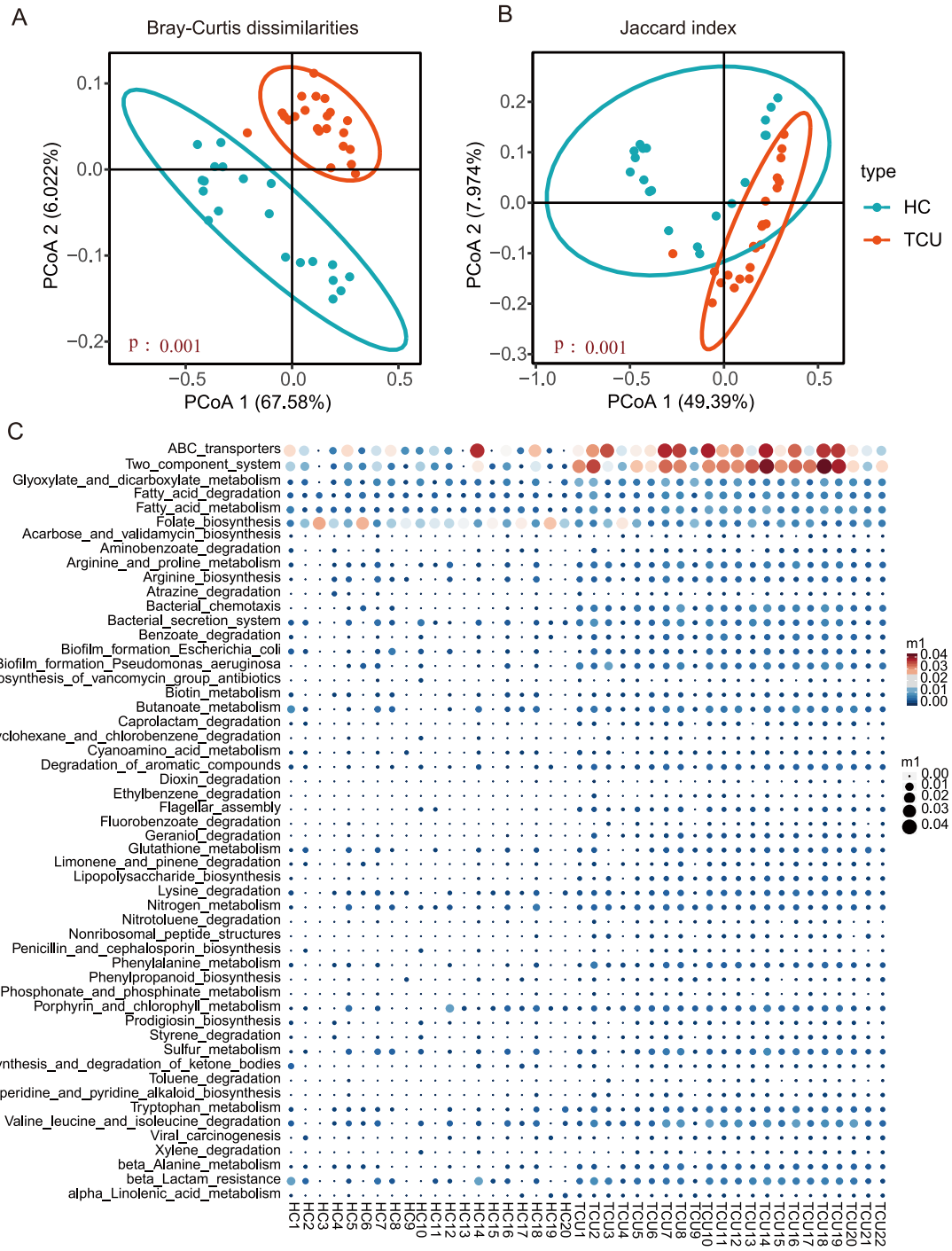

Figure 5. KEGG functional pathways of the OS microbiome. PCOA plots of Bray–Curtis dissimilarities (**A**) and Jaccard index (**B**) in which samples were colored based on grouping. (**C**) The relative abundances of 53 KEGG functional pathways were significantly different in the TCU group and in the HC group.

A total of 23 functions of COG categories were annotated. Among them, seven COG categories related to metabolism were found, including amino acid transport and metabolism, carbohydrate transport and metabolism, coenzyme transport and metabolism, inorganic ion transport and metabolism, lipid transport and metabolism, nucleotide transport and metabolism, and secondary metabolite biosynthesis, transport, and catabolism. Whether the metabolic activity of the OS microbiome plays a role in maintaining OS homeostasis is worthy of further research. Compared with the HC group, the genes related to secondary metabolite biosynthesis, transport, and catabolism, cell wall/membrane/envelope biogenesis, energy production and conversion, lipid transport and metabolism, and inorganic ion transport and metabolism were significantly overrepresented in patients with TCU, whereas cell cycle control, cell division, chromo-

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some partitioning, and chromatin structure and dynamics were significantly under-represented.

Discussion

Studies based on 16S RNA sequencing and traditional culture methods have found that human OSs are colonized by a wide variety of microorganisms. Although recent studies have investigated the composition and changes in conjunctival microbiome profiles of individuals with fungal keratitis and bacterial keratitis,[12,13] a comprehensive study of OS microbiome changes associated with keratitis using shotgun metagenomics is lacking. Whether the ocular microbiome is associated with OS infections also remains unknown. In this study, the OS microbial communities of HC subjects and patients with TCU were delineated and compared based on shotgun metagenomics.

Although there is no consensus on the composition and influencing factors of the normal OS microbial community, our results indicated that the OS microbes of normal individuals and patients with TCU were markedly different. The microbial floras of the samples from HC subjects were more enriched than those of patients with TCU. Significant differences in beta diversity were observed between the HC and TCU groups. These results indicated that there were significant changes in the evenness, richness, and community structure of the ocular microbiome of patients with TCU.

Similar to the study by Wen et al.,[2] our results also found that the OS microbial community was dominated by bacteria. At the phylum level, previous studies also consistently indicated that the microbial flora colonizing the OS was dominated by *Proteobacteria, Actinobacteria*, and *Firmicutes*.[11,15,37–39] Among the dominant genera (> 1%), the detection of *Pseudomonas, Corynebacterium, Streptococcus*, and *Staphylococcus* was consistent with the study of Ge et al.[13] In an earlier study, researchers reported that *Pseudomonas, Corynebacterium, Staphylococcus, Streptococcus,* and *Acinetobacter* represented the "core genera" in the healthy conjunctival microbiome.[38] In our study, these genera were shared by at least 50% of HC subjects.

Notably, we found that compared to healthy individuals, the TCU group had significantly increased *Pseudomonas*. Interestingly, at the species level, *Pseudomonas* was categorized into 20 species, and the precise distribution of each *Pseudomonas* species also varied among individuals. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas sp. 22 E 5*, *Pseudomonas sp. Root9*, *Pseudomonas syringae*, and *Pseudomonas syringae group genomo sp. 3* could be detected in over 70% of patients with TCU. *Pseudomonas aeruginosa* is a typical pathogen in keratitis. The research of Tuzhikov et al. showed that the OS microbiome of patients with ulcerative bacterial keratitis was dominated by *Pseudomonas aerugenosa* and a cohort of satellites. In our study, although *Pseudomonas aerugenosa* was not the dominant species, it was found in all patients with TCU except TCU3 and had a strong classification contribution.

In addition, the OS microbiomes of subjects HC5, HC7, HC16, and HC18 were dominated by *Corynebacterium*. Examples of OS microbial communities dominated by a single genus were also reported by previous studies.[37] Recent studies on the healthy OS microbiome showed that OTUs associated with *Corynebacterium* were the most abundant.[1,11,20,39] Moreover, in the interaction network of the HC group, there was only negative correlations between Corynebacterium and other genera. St. Leger et al.[40] reported that *Corynebacterium mastitidis* on the OS of mice can protect eyes against *Candida albicans* and *Pseudomonas aeruginosa* infections by facilitating neutrophil recruitment. Our research did not find *Corynebacterium mastitidis*, and whether other *Coryneform* species have similar effects remains to be further studied.

At the species level, the composition and relative abundance varied markedly among individuals, possibly depending on physiological differences, environment, and lifestyle. Among the top 20 species in the HC group, *Chlamydia trachomatis*, *Staphylococcus aureus, Escherichia coli*, and *Streptococcus pneumoniae* are well-known ocular pathogens, which implies that a healthy OS has powerful mechanisms to suppress pathogens from exerting pathogenic effects.[37] OS infection results from virulence being enhanced by external factors, such as antibiotics, infection, preservatives, surgery, the insertion of removable contact lenses, and other surface disorders.[3]

In this study, we demonstrated changes in the OS microbiome functional spectrum in patients with TCU. All patients with TCU had higher abundances of fatty acid degradation- and metabolism-related genes than the HC individuals. Among the COG categories that were identified, the genes related to lipid transport and metabolism were also obviously increased in the TCU group. Lipids are an important component of the tear film, which can prevent OS dewetting and water evaporation and provide a smooth refractive layer.[41,42] Lipid-based products are effective in the treatment of dry eye.[43] To determine whether OS microbial communities can synthesize lipids and transport them to the OS to participate in OS lubrication, animal-based research is needed.

Using the KEGG orthologous group annotation, we observed that the genes related to flagellar assembly and bacterial chemotaxis were more abundant in the TCU group than the HC group. Bacteria can move toward favorable conditions and away from adverse environments through chemotaxis-guided movements, which play an important role in the onset of post-traumatic corneal infections. Interestingly, the results of alpha diversity analysis indicated that the richness of the OS microbial community in the TCU group was decreased, but the genes related to metabolism, degradation, and biosynthesis were significantly increased at the functional level. The reason may be that OS trauma may destroy the innate immune system of the corneal epithelium, resulting in uncontrolled growth and metabolism of the OS flora.

Notably, genes related to biofilm formation by *Pseudomonas aeruginosa* and *Escherichia coli* were overrepresented in the TCU group. Bacterial biofilm was defined as "sessile bacterial communities growing on a surface." Compared with free-living or planktonic bacteria, bacteria in biofilms are more resistant to antibiotics and to the host immune response.[44] Because the host immune response and antimicrobial therapies have difficulty eliminating bacteria growing in biofilms, a chronic inflammatory response may be produced at the site of the biofilm.[45] Earlier studies have shown that *P. aeruginosa* cannot colonize healthy corneal epithelial cells well, but its adherence is significantly increased when the corneal epithelium is damaged.[46]

In addition, we also found that genes related to virus carcinogenesis were enriched in the TCU group. Compared to the HC group, TCU group had clearly increased numbers of virus types. Possible associations between eye neoplasms and viruses include hepatitis C in ocular adnexal MALT

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lymphoma, herpes virus 8 in Kaposi sarcoma, human immunodeficiency virus in conjunctival squamous cell carcinoma, and human papillomavirus in conjunctival papilloma and squamous cell carcinoma.[3] Further research is needed on whether the imbalance of OS homeostasis increases the risk of virus carcinogenesis.

There are several limitations in our study. First, although our results are statistically supported, the sample size is insufficient. In China, because most eye medicines are sold as nonprescription drugs at a pharmacy, the majority of patients with ocular injuries began to use topical medication before the initial visit to the Eye Hospital of Wenzhou Medical University. Therefore, we recruited only 22 patients with TCU without any history of medication use from February 2018 to September 2019. Our team is also enrolling more subjects, and further studies will compare patients with keratitis with or without corneal ulcers to explore the association between OS microbiome changes and disease severity. Second, most of our subjects are middle-aged and elderly persons, which may lead to a certain age bias in the study results.

Conclusions

Overall, we have clearly described the taxonomic composition, functional profiles and microbial co-occurrence of the OS microbiome in patients with TCU and HC subjects. The results of shotgun metagenomics analysis will provide important references for clinical diagnosis and treatment. The results are also of great importance for the future development of probiotic eye drops for treating corneal ulcers.

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