

Changes in Conjunctival Microbiota Associated With HIV Infection and Antiretroviral Therapy

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PURPOSE. HIV infection is associated with a variety of ocular surface diseases. Understanding the difference of the ocular microbiota between HIV-infected and healthy individuals as well as the influence of antiretroviral therapy will help to investigate the pathogenesis of these conditions.

METHODS. A cross-sectional study was conducted on subjects including HIV-negative individuals, untreated HIV-infected individuals, and HIV-infected individuals with antiretroviral therapy. Conjunctival microbiota was assessed by bacterial 16S rRNA sequencing of the samples obtained from the conjunctival swab.

RESULTS. The microbial richness in ocular surface was similar in HIV-negative, untreated HIV-positive, and highly active antiretroviral therapy (HAART) subjects. The bacterial compositions were similar in the two HIV infection groups but were significantly different from the HIV-negative group. HAART changed the beta diversity of bacterial community as determined by Shannon index. CD4+ T cell count had no significant influence on the diversity of ocular microbiota in HIV-infected individuals.

CONCLUSIONS. The data revealed the compositional and structural difference in conjunctival microbial community in subjects with and without HIV infection, indicating that HIV infection or its treatment, may contribute to ocular surface dysbiosis.

Keywords: conjunctiva microbiota, HIV infection, antiretroviral therapy, 16S rRNA sequencing, dysbiosis

It is estimated that 40 million people all over the world were living with HIV, with more and more people receiving antiretroviral therapy. Although antiretroviral therapy dramatically reduced the AIDS-related mortality, the long-term HIV-related or treatment-related manifestations remain great challenges to HIV-infected individuals. Impaired systemic defense mechanism and local immunity in condition of HIV infection result in imbalance in the host-microbiota interaction. HIV infection has been found to be associated with the alterations in gut, lungs, and oral and vaginal microbiota, which are implicated in the immune status and the occurrence of local complications.¹⁻⁷ HIV infection is associated with a variety of ocular surface lesions, such as ocular surface squamous neoplasia, allergic conjunctivitis, etc.⁸⁻¹² Previous studies have focused on the role of immune dysfunction in HIV-related ocular manifestations; however, it is poorly understood whether the alteration in local microbiota and their interaction with the local immune system also contribute to the diseases.

Although relative few microorganisms inhabit the ocular surface compared with other organs, such as the intestinal tract, oral cavity, and skin, the presence of ocular bacterial communities have been identified with traditional culture-based method. Due to the limited sensitivity and specificity of the culture method, only a small fraction of bacteria in the ocular surface can be identified. In recent years, many DNA-based techniques, such as PCR and DNA sequencing have been applied for microbial pathogen identification. Bacterial 16S rRNA sequencing emerged as a powerful tool in research of ocular microbiota of various ocular surface diseases or conditions.¹³⁻¹⁵

Understanding the change of microbial composition of the ocular surface in response to HIV infection and antiretroviral therapy is of paramount importance to elucidate the pathogenesis of HIV-related ocular surface manifestations. Herein, we used 16S rRNA sequencing to characterize the conjunctival microbiome of HIV-infected and HIV uninfected individuals, and also analyze the effects of immune status and antiretroviral therapy.

METHODS

This cross-sectional observational study was carried out in the Guangzhou Eighth People's Hospital. We enrolled chronic HIV-infected patients and classified them into two groups based on the following criteria: (1) untreated patients with HIV who were infected with HIV and antiretroviral therapy (ART) drug naive (hereafter referred to as the untreated group); (2) patients with HIV with highly active antiretroviral therapy (HAART) for >6 months (hereafter referred to as the HAART group). We also included HIV seronegative subjects seeking routine eye care in the department of ophthalmology as the normal control. Exclusion criteria for all groups included under the age of 18 years, pregnancy, topical or systemic administration of antibiotics, antifungal agents, anti-inflammatory drugs, corticosteroids or immunosuppressive drugs within a month, history of ocular trauma or surgery, active inflammation in the ocular surface, and contact lens use. This study was approved by the Institutional Review Board of the Guangzhou Eighth People's Hospital (AF/SC-02/01.3) and followed the Helsinki Declaration. Written informed consent was obtained from all participants.

Demographic and Clinical Data Collection

The demographic and clinical data were gathered with a standardized table. The personal information was kept confidential. Medical history was retrospectively collected from hospital medical records. In this study, some items of laboratory test results, including CD4+ T cell count and viral load, were also collected for analysis.

Conjunctival Swab Sampling

Sampling was performed by gently wiping the lower bulbar conjunctiva for three times with a commercial swab (Forensic swab, REF: 80.629, Sarstedt AG & CO., Germany). The swabs were placed into the providing sterile tubes and transported to the laboratory on ice. The samples were stored at -80°C until further processing.

DNA Isolation and 16s rRNA Sequencing

Total DNA was extracted using Power Soil Kit (MoBio, REF: 12888-100) according to the protocol provided by the manufacturer. The DNA concentration was determined with Nanodrop and the quality was evaluated with agarose gel electrophoresis. The extracted DNA was PCR amplified with the universal primers 343F (5'-TACGGRAGGCAGCAG-3') and 798R (5'-AGGGTATCTAATCCT-3') targeting the V3-V4 regions of the bacterial 16S rRNA genes and Takara Ex Taq (Takara, REF: RR001Q). The PCR products were purified by AMPure XP beads (Beckman Coulter, UK), and then quantified using Qubit 2.0 (Life Technologies, USA). Normalized amplicons were pooled at equal concentrations for sequencing. Gene sequencing was performed on an Illumina MiSeq platform (Illumina, USA).

Sequence Data Processing and Bioinformatic Analysis

The paired-end reads in raw sequences data of FASTQ format were processed with Trimmomatic software to identify and cut off ambiguous bases. After trimming of the low-

quality sequences of quality score <20 (Q20), the paired-end reads were assembled with FLASH software. Parameters of assembly included 10 bp of minimal overlapping, 200 bp of maximum overlapping, and 20% of maximum mismatch rate. Further de-noising of the sequences was performed by removing reads with ambiguous or homologous sequences or those that were <200 bp. After primer sequence removal and clustering for clean reads, operational taxonomic unit (OTUs) were generated using Usearch software with a 97% similarity cut off. Representative read of each OTU was selected using QIIME package.¹⁶ All representative reads were annotated and blasted against the Silva database using RDP classifier (confidence threshold was 70%).^{17,18} Distance calculation, operational taxonomic units cluster, rarefaction analysis, and estimator calculation (α -diversity and β -diversity) were performed by the MOTHUR program. Linear discriminant analysis effect size (LEfSe) was used to detect unique biomarkers by determinations of the relative abundances of the members of the bacterial taxonomies.

Statistical Analysis

Values presented are expressed as mean \pm SD. Statistical analysis were performed using SPSS version 18.0 (SPSS Inc., Armonk, NY, USA), GraphPad Prism version 7.0 (GraphPad Software, USA) and the R programming environment. Data were analyzed with ANOVA followed by post hoc Tukey's test or unpaired *t*-test. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

Demographic Characteristics

A total of 75 subjects were included in our study, of which 27 were HIV-negative, 22 were HIV-positive without treatment, and 26 were HIV-positive with HAART. All the subjects were Han ethnicity. There was no significant difference in sex among the three groups. The age of the HIV-negative group was significantly lower than the HAART group. The body mass index (BMI) in the untreated group was significantly lower than the HIV negative group. Just as we have expected, the virus load was significantly lower in the HAART group than that in the untreated group. There was no significant difference in CD4+ T cell count between the HAART group and the untreated group (Table 1).

Sequencing Data

For all the 75 samples, including 27 HIV-negative subjects, 22 untreated HIV-positive subjects, and 26 HAART subjects, 16S rRNA gene were successfully amplified and sequenced. After quality control, a total of 2,430,925 valid tags were generated from the raw sequencing data, ranging from 13,280 to 46,440 for each sample. Then the valid tags were clustered into OTUs, resulting a total of 5539 OTUs, which covered 34 phyla and 648 genera.

Conjunctival Bacterial Diversity Estimates

We applied two indicators, observed species and Shannon index, for the analysis of alpha diversity. There was no significant difference in observed species in the three groups by multiple comparison (Fig. 1A). The Shannon index was

TABLE 1. Demographic Characteristics of Included Subjects

	HIV Negative (N)	HIV Untreated (U)	HIV/HAART (H)	P Value
Sex, n (%)				[†] P = 0.389
Male	19	19	22	
Female	8	3	4	
Age, mean ± SD, year	32.22 ± 12.55	38.18 ± 14.24	39.46 ± 11.71	*P _{NvsU} = 0.109 P _{NvsH} = 0.043 P _{UvsH} = 0.731
Plasma HIV RNA copies/mL, median (min-max)	N.A.	157,000 (1,060-6,640,000)	166 (<20-17,700)	*P _{UvsH} <0.001
CD4 count/μL, median (min-max)	N.A.	49 (5-373)	131.5 (4-462)	*P _{UvsH} = 0.082
BMI, mean ± SD	22.24 ± 2.78	20.03 ± 2.43	20.92 ± 2.83	*P _{NvsU} = 0.006 P _{NvsH} = 0.081 P _{UvsH} = 0.255

* One way ANOVA followed by LSD test.

[†] Fisher's exact test.

[‡] Mann-Whitney U test.

significantly lower in the HAART group compared with the untreated group and the negative group, but there was no significant difference between the untreated group and the negative group (see Fig. 1B). These results suggested the bacterial richness was similar in the three groups and HAART was associated with a lower bacterial diversity within the conjunctival sample.

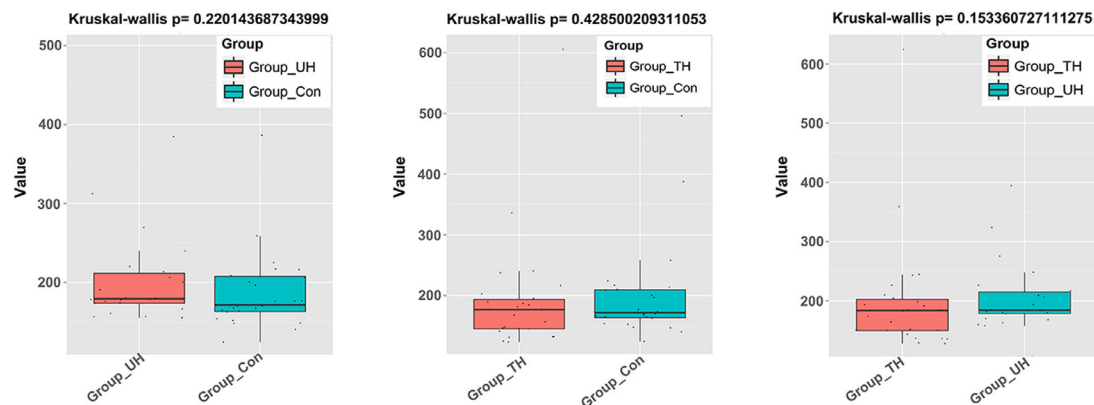
In the principle coordinates analysis (PCoA) plot based on unweighted UniFrac distances, an indicator of beta diversity, we found that the dots of the untreated group and the

HAART group were very closely distributed, whereas they were both far away from the HIV negative group, indicating the untreated group share similar sample microbial community composition with the HAART group but that of the HIV-negative group was quite distinct (Fig. 2).

Conjunctival Bacterial Composition

Taxonomic analysis revealed the conjunctival bacterial composition in different groups. At phylum level, the

A. Observed OTUs



B. Shannon index

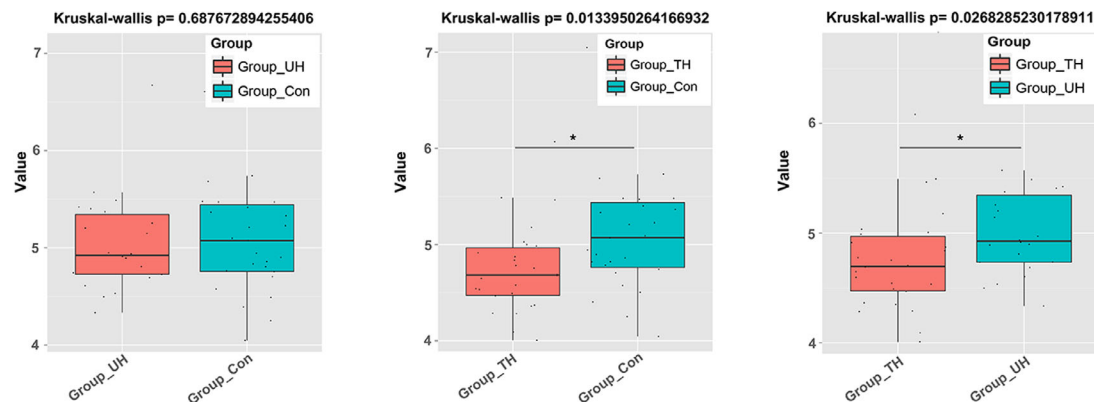


FIGURE 1. Comparison of alpha diversity among in HIV negative subjects (CON), untreated HIV-positive subjects (UH), and HIV-positive with HAART treatment (TH). Alpha diversity was measured by observed species and the Shannon diversity index.

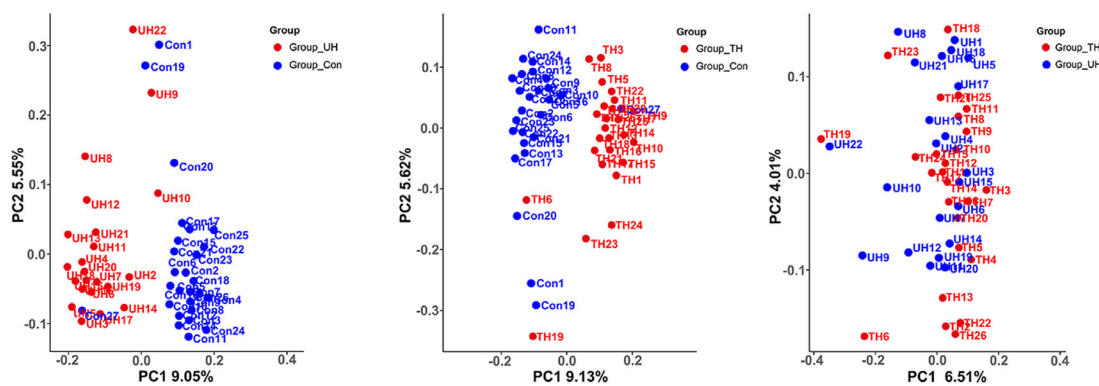


FIGURE 2. Comparison of beta diversity among in HIV negative subjects (CON), untreated HIV-positive subjects (UH), and HIV-positive with HAART treatment (TH). Beta diversity was measured by PCoA plot based on unweighted UniFrac distances.

average relative abundances of 5 main phyla were above 1%, including Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Gemmatimonadetes. Proteobacteria was the predominant phylum in all the three groups. At genus level, the average relative abundance of the 10 main genera reached 1% or above, Pseudomonas, Bacillus, Bacteroides, Escherichia_Shigella, Prevotella_9, Faecalibacterium, Lactococcus, Blautia, Streptococcus, and Lachnoclostridium. Pseudomonas was the predominant genus in all groups. The relative bacterial abundances in the individual sample were shown at phylum level in Figure 3A and at genus level in Figure 3B. The average relative abundance of bacterial taxa in different groups are shown in Table 2.

Difference in Relative Abundance in Conjunctival Microbiota Among the HIV-negative, Untreated, and HAART Groups

Comparison of the relative abundance across the three groups with statistical analysis revealed the significantly

different taxa associated with HIV infection and HAART. At phylum level, Proteobacteria and Bacteroidetes were significantly more abundant in the untreated HIV-positive and the HAART groups compared with HIV-negative control, whereas Actinobacteria and Firmicutes were significantly less abundant in the two HIV-positive groups. At genus level, Bacillus, Escherichia_Shigella, Lactococcus, Prevotella_9, and Pseudomonas were more abundant, whereas Bacteroides, Blautia, Faecalibacterium, Lachnoclostridium, and Parabacteroides were less abundant in the two HIV-positive groups. At both phylum and genus levels, the relative abundance of the main taxa seemed to be similar between the two HIV-positive groups (Fig. 4).

Bacteria Associate With HIV Infection Status and HAART

We used LEfSe to identify bacterial taxonomies that differentiate conjunctival microbiota in the HIV-negative, untreated, and HAART groups. Based on the threshold of linear

TABLE 2. Relative Abundance of Bacterial Taxa in HIV Negative Subjects (CON), Untreated HIV-Positive Subjects (UH) and HIV-Positive With HAART Treatment (TH) at Phylum and Genus Levels

	CON Group	UT Group	TH Group	P Value		
				P _{UvsC}	P _{TvsC}	P _{TvsU}
Phylum Level						
Proteobacteria	55.02 ± 6.84%	60.07 ± 5.46%	62.17 ± 9.76%	0.004	<0.001	0.141
Firmicutes	25.54 ± 5.44%	17.85 ± 3.78%	17.10 ± 10.11%	<0.001	<0.001	0.336
Bacteroidetes	11.18 ± 2.12%	13.94 ± 1.85%	13.78 ± 2.99%	<0.001	<0.001	0.406
Actinobacteria	4.74 ± 1.71%	4.04 ± 1.63%	3.43 ± 1.41%	0.077	0.001	0.072
Gemmatimonadetes	1.52 ± 0.82%	1.27 ± 0.78%	1.11 ± 0.83%	0.146	0.026	0.218
Genus level						
Pseudomonas	41.51 ± 10.13%	45.35 ± 7.51%	47.97 ± 9.50%	0.073	0.010	0.151
Bacillus	3.18 ± 2.42%	4.20 ± 1.73%	4.94 ± 3.85%	0.05	0.025	0.204
Bacteroides	4.73 ± 1.39%	3.66 ± 0.96%	3.34 ± 1.21%	0.002	<0.001	0.157
Escherichia_Shigella	2.93 ± 1.67%	3.48 ± 1.05%	3.74 ± 1.36%	0.095	0.029	0.228
Prevotella_9	2.46 ± 0.67%	3.10 ± 0.90%	2.85 ± 0.96%	0.003	0.047	0.18
Faecalibacterium	5.26 ± 1.76%	1.28 ± 1.38%	0.75 ± 1.11%	<0.001	<0.001	0.076
Lactococcus	1.77 ± 0.92%	2.45 ± 1.03%	2.82 ± 1.91%	0.009	0.006	0.206
Blautia	1.42 ± 0.65%	0.52 ± 0.45%	0.41 ± 0.49%	<0.001	<0.001	0.222
Streptococcus	0.61 ± 0.36%	1.00 ± 0.72%	0.83 ± 0.52%	0.009	0.039	0.181
Lachnoclostridium	1.37 ± 0.67%	0.38 ± 0.41%	0.31 ± 0.35%	<0.001	<0.001	0.27

The bacterial taxa with relative abundance >1% were listed in the table, and the values were expressed as mean ± SD. Statistical analysis were performed with Student's *t*-test.

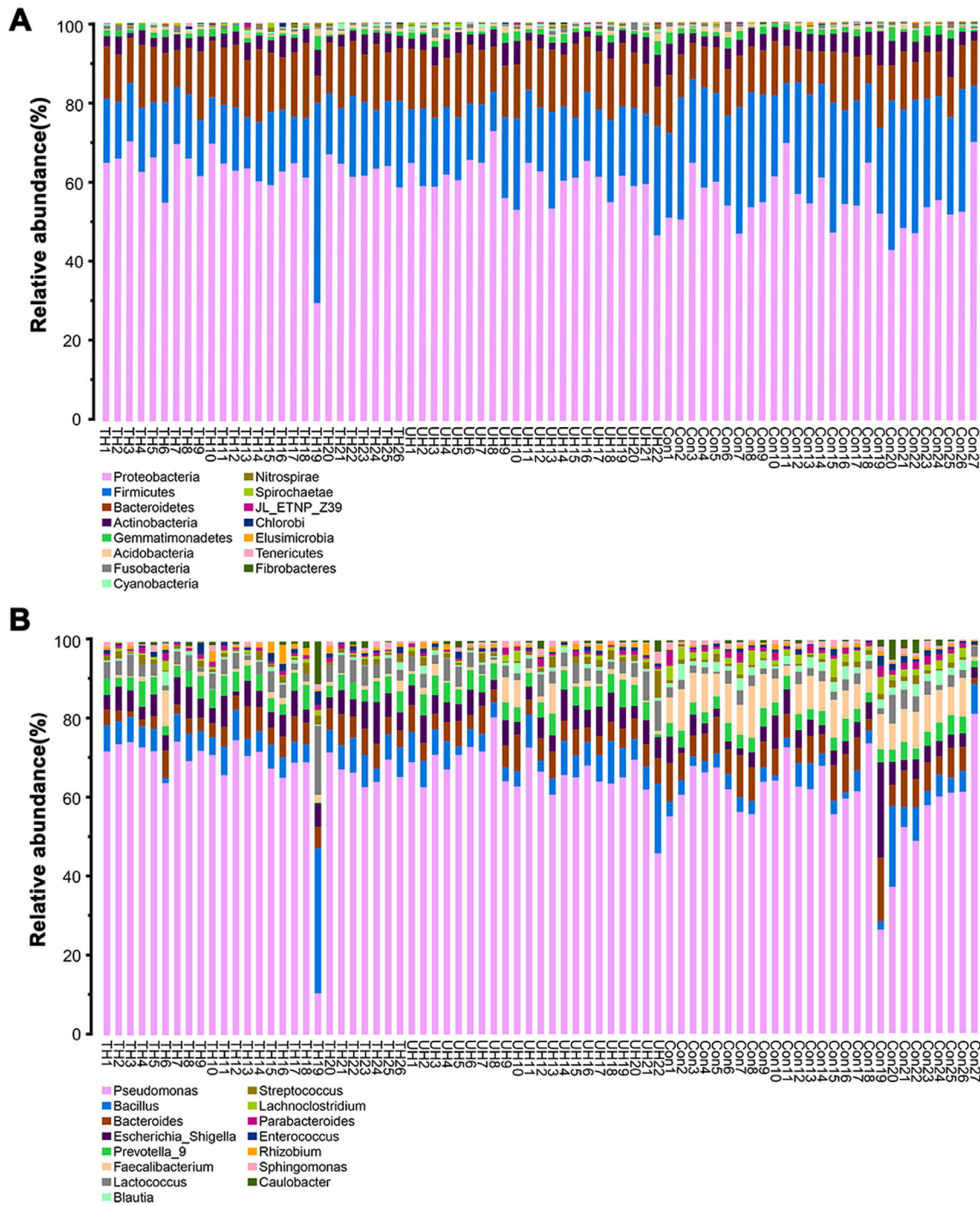


FIGURE 3. Conjunctival microbiota composition in HIV negative subjects (CON), untreated HIV-positive subjects (UH), and HIV-positive with HAART treatment (TH). Top 15 abundant taxa at phylum (A) and genus (B) levels were showed in the plot.

discriminative analysis LDA score >3, we identified several bacterial taxa at genus level are associated with the different HIV status and HAART. The top three bacterial taxa associated with untreated group were Bacteroidetes, Prevotellaceae, and Bacteroidales. Gammaproteobacteria, Proteobacteria, and Pseudomonadales were the top three bacterial taxa in the significant association with the HAART group. Clostridiales, Clostridia, and Firmicutes were most strongly associated with the HIV-negative group (Fig. 5).

Effect of CD4+ T Cell Count on Microbial Community in Conjunctiva

In order to investigate whether CD4+ T cell count influenced the conjunctival microbiota, we divided the subjects into the untreated group and the HAART group into two subgroups based on CD4+ T cell count with a cutoff value of 200/mL. We found there were no evident difference in alpha diversity and beta diversity between the high and low CD4+ T cell count subgroup in both the untreated and the HAART

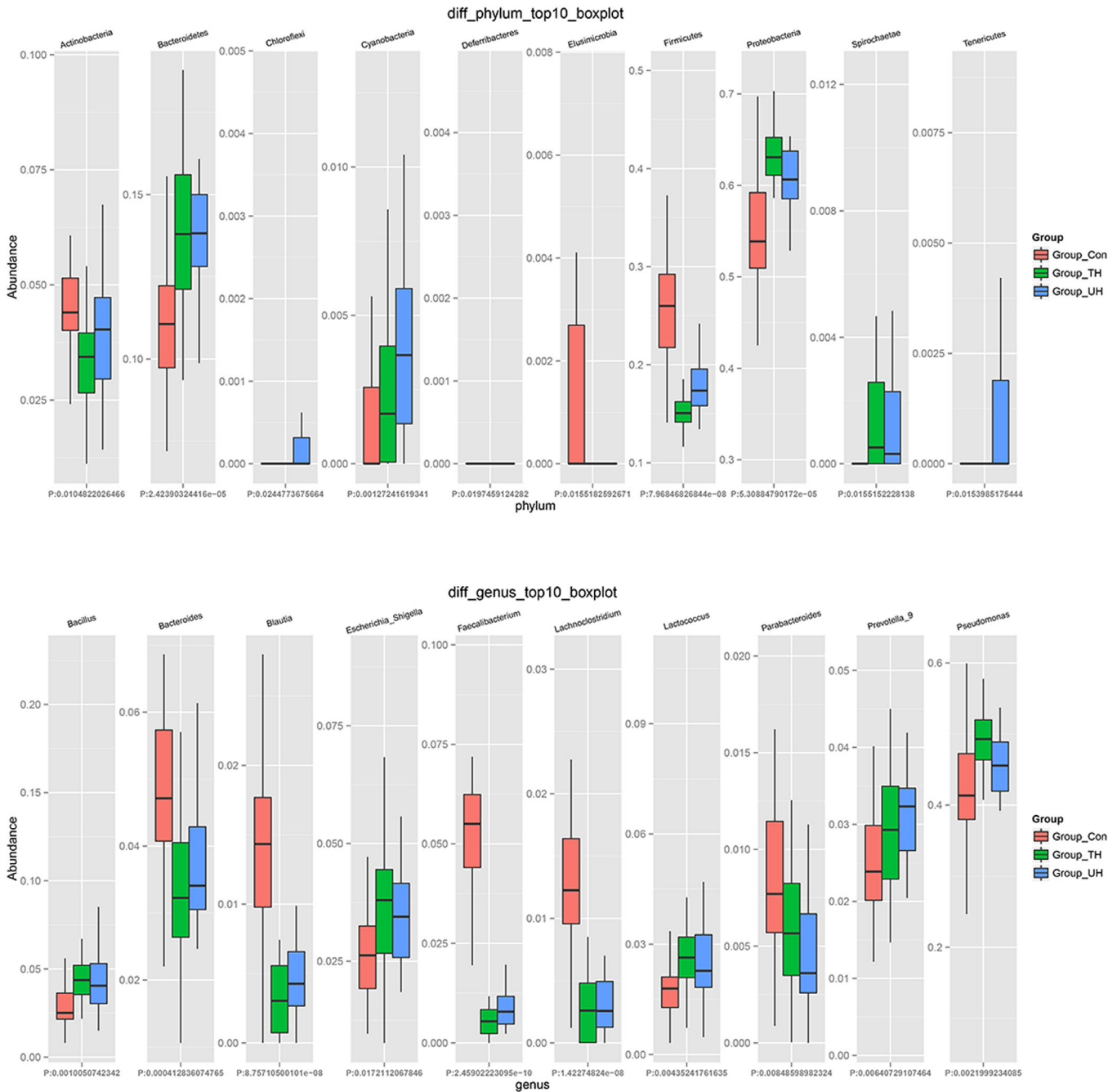


FIGURE 4. Different bacterial taxa in HIV negative subjects (CON), untreated HIV-positive subjects (UH), and HIV-positive with HAART treatment (TH) at phylum and genus levels.

groups, as shown by observed species, Shannon index, and PCoA in Figure 6.

DISCUSSION

Despite the relative low degree of abundance and diversity of the microbial community in the ocular surface, the influence of ocular microbiota on ocular innate immune function and autoimmunity as well as its association with ocular diseases has attracted much more attention in recent years. Wen et al. found that age and sex collectively shaped the conjunctival microbiome and thus influences

the immune homeostasis.¹⁹ Shin et al. found that contact lens wearing altered the microbial composition of the ocular conjunctiva, which was similar to skin microbiota.²⁰ In subjects with trichiasis, decreased diversity and an increased abundance of *Corynebacterium* and *Streptococcus* were observed.²¹ Conjunctival microbiota analysis based on 16S rRNA sequencing is considered to be a promising approach for identifying the causative pathogens of conjunctivitis.²²

Many non-AIDS related diseases which contribute to the reduced life expectancy and compromised life quality of HIV-infected individuals, is considered to be driven by persistent inflammation and immune response.^{23,24} The

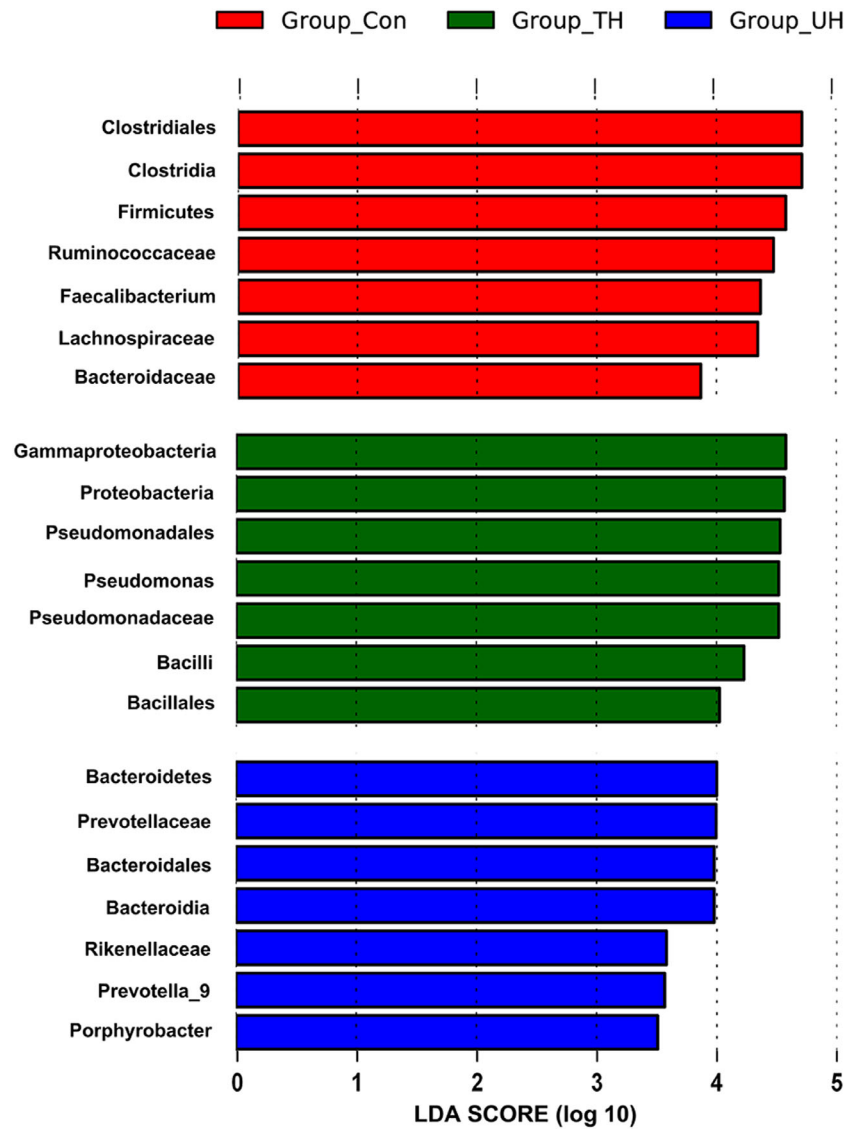


FIGURE 5. Microbial biomarker discovered by linear discriminative analysis (LDA) effect size (LefSe). Histogram of biomarker bacteria in HIV negative subjects (CON), untreated HIV-positive subjects (UH), and HIV-positive with HAART treatment (TH). The LDA effect size (>3.0-fold) was used to detect the biomarkers.

alteration of local microbiota has been found to be associated with HIV infection and contributed to the inflammation in diverse tissues. Lozupone et al. reported that HIV infection induced highly characteristic changes in gut microbiota, which were not consistently restored to an uninfected state by HAART.² The compositional and functional alteration of gut microbiota was implicated in “multi-factorial” disorders, including metabolic, infectious, neoplastic, and autoimmune disorders.²⁵ Beck et al. found the oral and lung microbiota in HIV-infected individuals who were treatment naive differed from the uninfected group and the HAART group in the abundance of Veillonella, Rothia, and Granulicatella despite the overall similarity of the microbiomes among the three groups.⁴ Significantly higher bacterial richness was observed in the cervical microbiota of HIV-positive patients, and Mycoplasmatales, Pseudomonadales, and Staphylococcus were associated with high-grade squamous intraepithelial lesions.³ Rectal microbiota is not significantly altered in

untread HIV-positive patients but prior treatment induces a shift toward a more pathogenic microbiota.¹

The conjunctival flora of HIV-positive patients has been investigated with culture methods in an earlier study by Yamauchi et al., in which bacterial organisms in the conjunctival sac were detected in 48.5% of the eyes of HIV-positive patients without systemic antibiotics treatment and no difference in the conjunctival flora was found between of HIV-negative and HIV-positive patients.²⁶ Another study by Giles et al. found bacteria culture were positive in 62.50% of HIV-positive patients on antiretroviral treatment.²⁷ The study by Fontes et al. found negative cultures were found in 33% of the eyes of HIV-positive patients and in 56.7% of the eyes of HIV-positive patients receiving oral trimethoprim/sulfamethoxazole (TMP/SMZ).²⁸ Compared with these results in the culture-based studies, our study obtained a positive rate of 100% in bacteria detection for all three groups, supporting the notion that sequencing-based

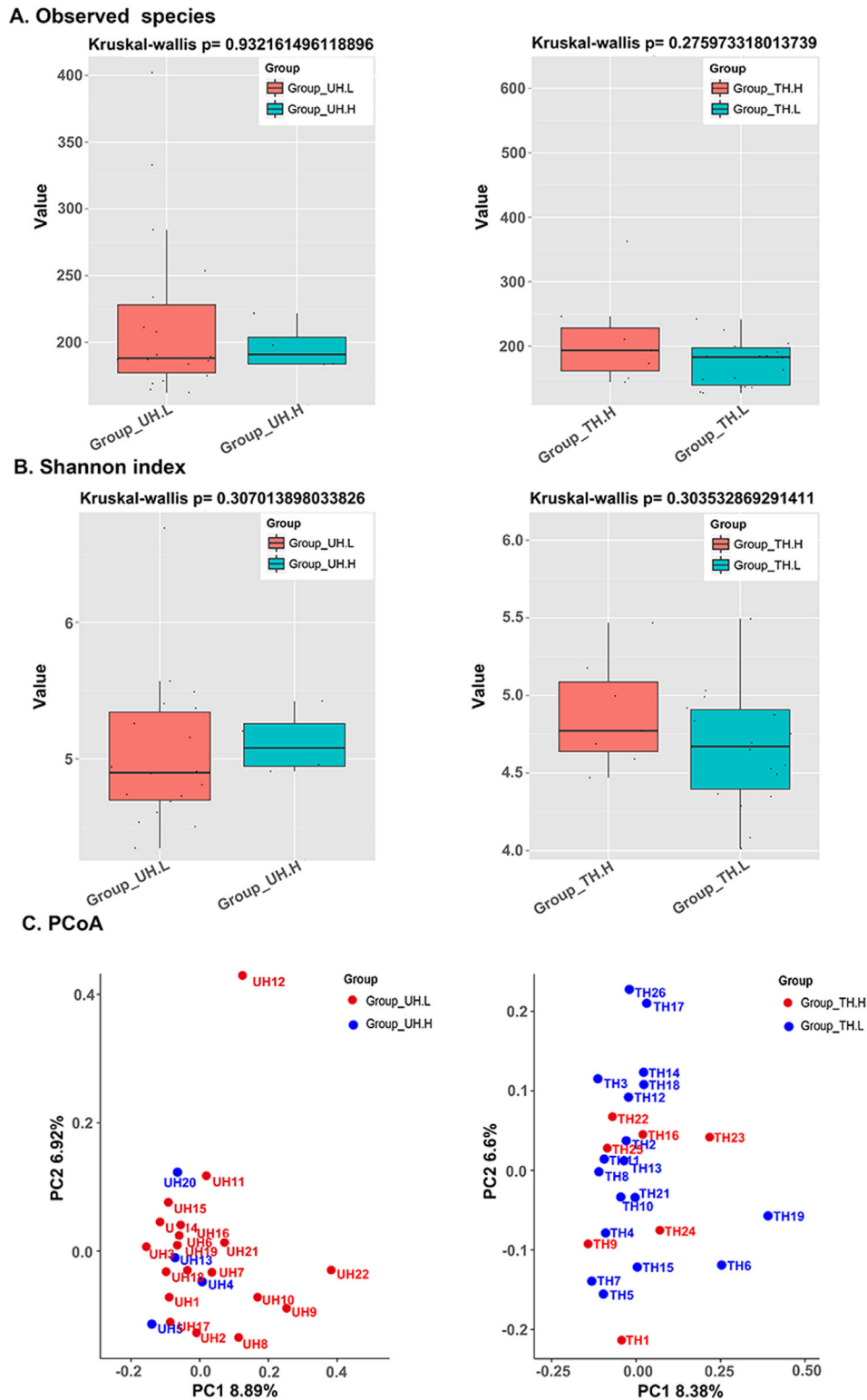


FIGURE 6. Characterization of the diversity of conjunctival microbiota between HIV-positive individuals subgrouped by CD4+ T cell counts at 200/mm³. The alpha-diversity was determined with observed species (A) and the Shannon index (B), and the beta-diversity was determined with PCoA based on unweighted UniFrac distances (C). UH.L and UH.H represent the low CD4+ subgroup and the high CD4+ subgroup in the untreated group, respectively; TH.L and TH.H represent the low CD4+ subgroup and the high CD4+ subgroup in the HAART group, respectively.

detection is a highly sensitive method for investigation of conjunctival microbiota.

The major microbial taxa at phylum and genus level detected in our study were generally consistent with previous reports on conjunctival microbiota based on 16S sequencing.^{13,15,29–31} There was no significant difference in the observed species among the three groups, indicating the bacterial richness was not influenced by the HIV infection status. Our study revealed a distinctive microbiota composition in HIV-positive subjects including untreated subjects and HAART subjects, characterized by increased abundance of *Pseudomonas*, *Bacillus*, *Escherichia*, *Shigella*, *Prevotella*, and *Lactococcus*, and decreased abundance of *Bacteroides*, *Faecalibacterium*, *Blautia*, *Lachnospirillum*, and *Parabacteroides*. Considering the similarity in the bacterial composition between untreated group and HAART group, we speculate this microbial shift might be related with the immune dysfunction in HIV-positive individuals but the causal relationship needs to be investigated by further longitudinal studies.

The fact that the untreated group shared high similarity with the HAART group in bacterial composition but differed from that in the HIV-negative group indicated that the HAART failed to consistently restore the conjunctival microbiota to a HIV-negative state. This finding was consistent with the results reported by other researchers regarding HAART on gut microbiota. We thought the potential explanation for this is that the immune dysfunction in HIV-positive subjects was not evidently changed after HAART, as indicated by the similarity in the CD4+ T cell count between the untreated group and the HAART group in spite of the dramatically reduced virus load. We performed subgroup analysis based on the CD4+ T cell count to explore the impact of immune function on conjunctival microbiota, but the results indicated there was no significant difference both in bacterial richness and diversity between the low and high CD4+ T cell count subgroups. The small sample size in the subgroups and the cross-sectional nature of this study might limit the strength of the results. We expect that future longitudinal studies based on larger sample sizes to investigate the immune function on conjunctival microbiota in HIV-positive individuals. The relationship between CD4+ T cell count with ocular manifestation in HIV-positive individuals remains a topic of debate. Martin-Odoom et al. reported that ocular complications occurred more frequently among those with CD4+ T cell count below 200 cells/ μ ,¹² whereas Singalavanija found that there was no association between CD4+ T cell count and anterior segment disorders.³² Notably, we observed that HAART had evident efficacy on conjunctival microbiota. The Shannon index in the HAART group was significantly different from that in the untreated group, which suggests the bacterial diversity might be altered by HAART. Microbial diversity is considered to be closely associated with the healthy state. Previous studies demonstrated altered bacterial diversity are associated with a variety of diseases in different tissues and organs.^{33–37} The implication of the decrease in the Shannon index in the HAART group remains to be investigated by further studies.

Several limitations in this study should be noted. The subjects in each group were not completely demographically matched and there might be potential confounding factors we did not analyze which possibly influenced the results. The relatively small numbers in each group also limited the statistical power in analysis of the factors related with microbiota composition. Due to the nature of the cross-sectional

study in which we collected the conjunctival sample at a single timepoint, the causal relationship of HIV infection, HAART, and conjunctival microbiota composition was difficult to determine. Moreover, conjunctiva microbiota might vary depending on the subject population, living environment, and even the sampling and detection techniques.

In conclusion, this study indicates that HIV infection and ART have evident influence on conjunctival microbiota. The bacterial communities in conjunctiva differed significantly depending on HIV infection status and HAART. We also identified characteristic signature of taxa in HIV-infected individuals and HAART individuals compared with HIV-negative subjects. These findings provide a basis for further investigation on the potential impact of conjunctival microbiota changes on HIV infection related ocular conditions, such as inflammation, opportunistic infection, immune reaction, and neoplasia.

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