The Human Ocular Surface Fungal Microbiome

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Purpose. To enumerate the ocular surface fungal microbiome of healthy human eyes by using next-generation sequencing (NGS).

Methods. Tarsal and fornix conjunctiva from the lower and upper lids of both eyes of healthy individuals were swabbed in duplicate separately. A total of 34 samples were collected from both the eyes of 17 individuals, which were used for the generation of ocular surface fungal microbiomes by NGS. Twenty-four swabs were used for the detection of culturable fungi by the conventional cultivable method. Microbiome generation involved DNA extraction, internal transcribed spacer 2 (ITS2) amplification, library preparation, amplicon sequencing, taxonomic assignment of sequences, diversity analyses, and identification of genera.

Results. The cultivable method detected fungi in 3 out of 24 (12.5 %) ocular surface swabs, whereas NGS identified fungi in 25 of the 34 (73.5 %) swabs. In the cultivable method *Aspergillus* was the only genus detected, whereas NGS detected 65 distinct genera with 12 to 24 genera per microbiome. Genera *Aspergillus*, *Setosphaeria*, *Malassezia*, and *Haematonectria* were present in the 25 eyes in which fungi were detected. Alpha diversity in the two eyes was similar and sex had no effect, but Chao1 and Simpson indices were altered by age.

Conclusions. This study explored the ocular surface fungal microbiome of healthy individuals using NGS and identified a greater degree of diversity of fungi than with the conventional cultivable method. It was observed that several fungal genera were associated with the healthy conjunctiva.

Keywords: ocular microbiome, fungal microbiome, conjunctiva, human

Several bacteria, fungi, viruses, and parasites reside on the ocular surface and are likely to play a role in ocular surface physiology. Several species of these microbes have been implicated in ocular diseases, such as keratitis, uveitis, blepharitis, conjunctivitis, and retinitis.[1] Keilty in 1930[2] used conventional bacterial culture methods and was among the first to enumerate the bacterial flora of the normal conjunctiva, and subsequent studies confirmed these findings.[3–6] These studies established that coagulase-negative *Staphylococcus epidermidis*, *Propionibacterium* sp., *Corynebacterium* sp., *Staphylococcus aureus*, *Micrococcus* sp., *Pseudomonas* sp., and *Escherichia coli* are associated with the ocular surface. It was also demonstrated that a good proportion of the conjunctiva (10%–43%) did not yield growth[6,7] and the diversity on the conjunctiva did not differ between the eyes by using culturing techniques.[8,9] Subsequent studies based on 16S ribosomal RNA (rRNA) gene amplification, cloning, and sequencing[10,11] or amplicon sequencing of the V3-V4 region of the 16S rRNA gene sequence by the use of next-generation sequencing (NGS) revealed a greater degree of diversity and abundance in the bacterial microbiome of the ocular surface[6,10,12,13] than with the culture-dependent method. Based on NGS of the ocular surface bacterial microbiome, it was demonstrated that age did not seem to alter the richness (number of taxa) and diversity (number of taxa and evenness of their distribution) of bacteria,[6,14] but data on sex were not consistent with one study demonstrating significant variation between sexes (with males having greater Shannon diversity than females).[6,14] Furthermore, it was observed that Proteobacteria (64.4%), Firmicutes (15.5%), and Actinobacteria (15.0%) constituted the core phyla[6,12] and the *Corynebacterium* operational taxonomic unit (OTU) was the most abundant on the ocular surface.[14–16]

Compared with the bacterial microbiome, little is known about the ocular surface fungal microbiome. In a recent study[17] on the influence of age and sex on ocular surface microbiota in healthy adults, it was observed that the majority of the reads (98.15%) were bacterial, whereas the remaining were of fungal (0.94%) and viral origins (0.91%). Furthermore, they did not attempt to identify the fungal diversity. Conventional culture-based methods have revealed that the common fungi associated with the ocular surface include *Alternaria* sp., *Fusarium* sp., *Aspergillus niger*, *Aspergillus flavus*, *Curvularia* sp., *Penicillium* sp., *Helminthosporium* sp., *Candida albicans*, *C. guilliermondii*, *C. parapsilosis*, *Saccharomyces cerevisiae*, *Hormodendrum* sp., and *Rhodotorula rubra*.[18–23] Many a times, the culture-based and the PCR-based methods did not yield any fungi.[6,24,25] Considering that fungi are the causative agents of several ocular diseases, such as keratitis, endophthalmitis, blepharitis, and conjunctivitis,[26] it is important to establish the ocular surface fungal microbiome that in the diseased state could be predicted to be different[27] and also shed light on idiopathic ocular surface disorders.[6] With this in view, the present study was undertaken to acquire data on the ocular surface fungal microbiome of healthy individuals by NGS by using internal transcribed spacer 2 (ITS2) sequencing as a proxy for fungi.

Materials and Methods

Recruitment of Subjects

The healthy individuals ($n = 17$) recruited in this study included males ($n = 8$) and females ($n = 9$) in the age range of 23 to 77 years, and the mean age was 42.88 ± 14.67 years. Samples were collected separately from both eyes for all subjects. All the individuals were from Telangana, a state in the southern part of India (Supplementary Table S1). Participants who had undergone any surgery or having any systemic disease, such as diabetes, tuberculosis, syphilis, HIV infection, sarcoidosis, obesity, any form of malignancy, any ocular disease, or had undergone ocular transplantation or was wearing contact lenses and who had taken topical or systemic antibiotics, antifungals, corticosteroids, and nonsteroidal anti-inflammatory drugs 3 months prior to sample collection, were excluded from the study. Informed consent was obtained from all the study subjects prior to sample collection. The study protocols were approved by the Institutional Review Board of L. V. Prasad Eye Institute, Hyderabad (ethics reference number LEC 06-14-060), and adhered to the tenets of the Declaration of Helsinki.

Sample Collection from Conjunctiva

A total of 34 samples were collected from both eyes of 17 individuals. The number of individuals to be sampled was decided based on the population proportion method with 80% confidence intervals. The tarsal and fornix conjunctiva from the lower and upper lids were swabbed two or three times with slight pressure to obtain a pooled sample by using a sterile Isohelix swab (SK-1S; Isohelix, Harrietsham, Kent, United Kingdom) moistened with sterile PBS. After one eye was swabbed, the same procedure was repeated for the other eye with a fresh Isohelix swab. These swabs were processed separately (left eye swab and right eye swab) for DNA extraction, PCR amplification, and sequencing.

The conjunctiva was swabbed by the same person using separate swabs for each eye and care was taken not to touch the lid margin. Swabs required for the detection of culturable fungi were streaked immediately. The other swabs required for DNA extraction were transferred to sterile tubes containing PBS and were frozen at −80°C until DNA extraction.

Culturing of Fungi

Conjunctival swabs were inoculated onto Sabouraud dextrose agar medium (40 g dextrose and 10 g peptone in 1 liter distilled water and final pH adjusted to 5.6 ± 0.2), incubated at 25°C, and observed after 7 days and 4 weeks. The fungi were identified on the basis of colony morphology and microscopic characteristics. To assess the purity of cotton swabs, thioglycollate broth was inoculated with an unused swab moistened with sterile PBS to confirm no growth.

DNA Extraction

Genomic DNA was extracted from conjunctival swab samples following the buccal swab spin protocol of the QIAamp DNA minikit (Qiagen, Hilden, North Rhine-Westphalia, Germany). In the final step, DNA was eluted with 30 µl of AE buffer provided by Qiagen. The quality of the genomic DNA was checked on a 0.8% agarose gel and stored at −80°C until further analysis. Unused Isohelix swabs moistened with sterile PBS were processed as sample blanks for DNA extraction to confirm the absence of contaminating DNA.

PCR Amplification, Illumina Library Preparation, and Amplicon Sequencing

Using the extracted genomic DNA from the conjunctival swabs, we amplified ITS2, a region of the fungal ribosomal small subunit RNA, with primers ITS3 (5′-GCATCGATGAA GAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[28] All PCR reagents were prepared using sterile nuclease-free water. In addition, for each PCR, a negative control (PCR reagents without template DNA) PCR amplification was carried out to exclude the possibility of false-positive PCR results by contamination. Extracts from swabs, reagents of DNA extraction kits, reagents used for PCR, such as water, PCR primers, and PCR reagents, were run with every PCR reaction to be able to detect contaminating DNA. Our results were consistently negative for DNA from Isohelix swabs and all other reagents, including DNA extraction kits and PCR reagents. Also, DNA extraction, PCR, and gel electrophoresis were carried out in three separate rooms to avoid contamination.

The standard Illumina protocol was used to generate the fungal microbiome libraries as described by Dehingia et al.[29] The libraries were sequenced using Illumina MiSeq 2 × 250 base pair (bp) chemistry with a paired-end protocol at Xcelris Genomics Pvt. Ltd., Ahmedabad, India.

Taxonomic Classification of Sequenced Reads

Paired-end reads corresponding to individual samples were merged into single reads by using the Fast Length Adjustment of Short Reads (FLASH) tool.[30] The removal of low-quality reads (mean Phred score, <25) and chimeric sequences, OTU picking using Quantitative Insights into Microbial Ecology (QIIME) software, and assignment of taxonomic lineages were performed as described previously.[31,32] Sparse OTUs containing <0.001% of the total number of high-quality reads sequenced were removed.

Diversity Analyses of Microbiome Samples

R-Vegan 2.4.2 package[33] was used for generating the rarefaction curves to ascertain whether the libraries were representative of the samples analyzed. In addition, alpha diversity indices (Shannon diversity, Simpson index, observed number of OTUs, and Chao1) were obtained for the fungal microbiomes to ascertain the degree of variation within the group. Age and sex were considered while calculating the alpha diversity indices, and a *t*-test was done to test for significance. Microbial beta-diversity was compared across age, sex, and eye (left/right) by using nonmetric multidimensional scaling (NMDS) plots with Bray-Curtis dissimilarity of OTUs and permutational multivariate ANOVA (PERMANOVA) to ascertain whether community structure was dependent on age, sex, and the particular eye (right/left) that was sampled.

Statistical analysis of culture-dependent and culture-independent results was performed using R statistical software, version 3.3.2 (http://cran.r-project.org/, in the public domain).

Results

Fungi Detected by Culturable Approach

Only 3 out of 24 ocular surface swabs (12.50 %) yielded fungi, and they predominantly belonged to the genus *Aspergillus* and were identified as *Aspergillus flavus* (OD001 and OD028) and *Aspergillus niger* (OS002).

Table 1. High-Quality Reads and Percentage of Reads Assigned to OTUs Per Ocular Surface Fungal Microbiome from the 25 Eyes (OD = 12 and OS = 13) of HCs

Serial Number	Sample Identifier*	Number of High-Quality Reads (Q, ≥25)	Number of Reads Assigned to OTUs	Reads Assigned to OTUs (%)
1	OD002	312,286	312,010	99.91
2	OD005	291,130	290,833	99.90
3	OD006	290,802	290,464	99.88
4	OD012	675,351	674,790	99.92
5	OD013	482,754	482,218	99.89
6	OD014	551,690	551,055	99.88
7	OD021	1,371,325	1,370,255	99.92
8	OD028	540,732	540,163	99.89
9	OD033	536,619	536,033	99.89
10	OD034	420,565	420,169	99.91
11	OD035	426,195	425,641	99.87
12	OD036	499,999	499,611	99.92
13	OS001	540,379	539,906	99.91
14	OS002	428,874	428,269	99.86
15	OS005	580,380	579,499	99.85
16	OS012	706,131	705,547	99.92
17	OS013	681,419	680,368	99.85
18	OS014	301,751	301,341	99.86
19	OS017	543,453	542,585	99.84
20	OS021	229,179	228,828	99.85
21	OS026	572,712	571,924	99.86
22	OS028	1,141,634	1,140,134	99.87
23	OS033	471,915	470,852	99.77
24	OS034	300,101	299,461	99.79
25	OS036	470,624	469,938	99.85
		Total 13.37 million reads Average 534,720 reads per microbiome		

* OD and OS refer to the oculus dexter (right eye) and oculus sinister (left eye) of the HCs, respectively.

NGS Analysis of the Ocular Fungal Microbiomes

Out of 34 conjunctival swab samples (17 OD and 17 OS) that were processed (Supplementary Table S1), only 25 samples (12 OD and 13 OS) yielded ITS2 amplicons and were used for Illumina sequencing. The remaining 9 samples were negative for the ITS2 amplicon following PCR, which may be attributed to the low fungal load in the sample or the presence of PCR inhibitors. Illumina sequencing of all the 25 samples together yielded 13.37 million high-quality reads (after removal of chimeric sequences and reads with mean Phred score less than 25). The number of high-quality reads per microbiome ranged from 229,179 to 1,371,325, and the average was 534,720 reads per microbiome (Table 1).

Rarefaction analysis of the reads of the individual microbiomes exhibited a tendency to plateau, implying that all the representative species have been recovered. Plateauing of the curves also indicated reasonable sequencing depth and coverage of the sequenced samples (Fig. 1A).

More than 99% of the reads from each of the fungal microbiomes could be assigned to an OTU (at 97% sequence identity) (Table 1). A total of 554 OTUs were identified from the 25 ocular fungal microbiome libraries that were analyzed, and it included 189 reference OTUs and 365 de novo OTUs respectively (Supplementary Table S2). The observed increase in the number of de novo OTUs compared with the reference OTUs would imply a limited number of available sequenced fungal organisms.

Fungal Community Composition on the Conjunctival Surface

Attempts were made to assign the OTUs to the phylum and genus level. Ascomycota (mean abundance, 47.74%) and Basidiomycota (mean abundance, 26.87%) were the two dominant phyla and were detected in all 25 eyes, whereas Zygomycota was a minor phylum (mean abundance, 0.08%) and was detected only in the ocular surface microbiome of 2 eyes. The remaining reads could not be assigned to a known phylum (mean abundance, 25%) and were included as unclassified (15.33%), unidentified (9.49%), and others (0.48%, which included singletons, unassigned reads, and

Table 2. Abundance of Fungal Phyla in the Ocular Surface Fungal Microbiomes from the 25 eyes (OD = 12 and OS = 13) of HCs

Serial Number	Phylum	Abundance		Present Out of 25 Eyes Sampled
		Mean	Range	
1	Ascomycota	47.743	5.247–98.737	25
2	Basidiomycota	26.874	0.344–76.158	25
5	Zygomycota	0.077	0.000–0.983	2
3	Unclassified	15.332	0.57–74.308	25
4	Unidentified	9.493	0.007–58.39	25
6	*Others	0.482	0.187–0.985	25

* Includes singletons, unassigned reads, and sparse OTUs (with <0.001 % of total high-quality reads).

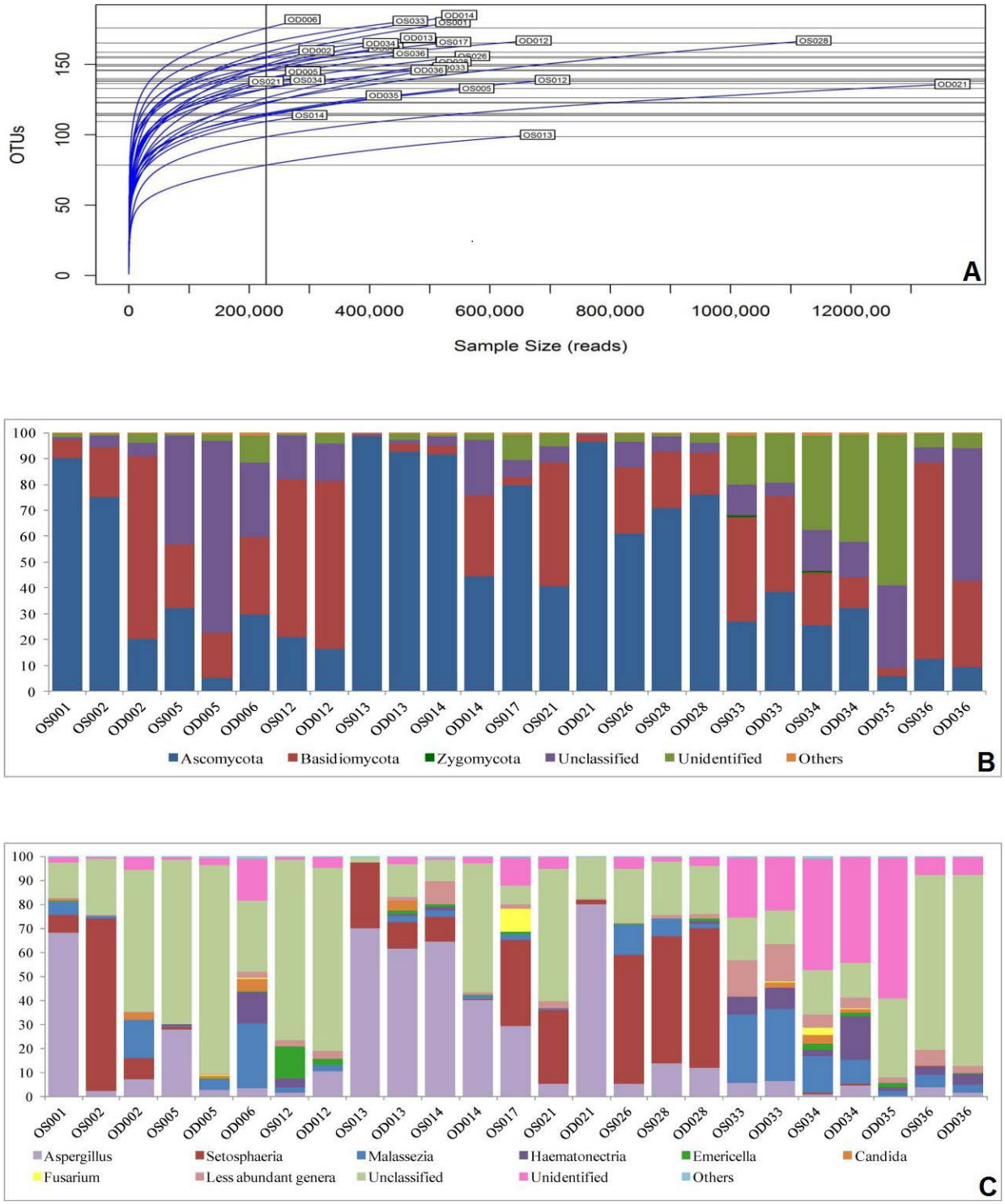

Figure 1. Abundance of fungal phyla and genera in the microbiomes of healthy conjunctiva. (**A**) Rarefaction curves of the ocular surface fungal microbiomes of the 25 eyes (OD = 12 and OS = 13) of healthy controls (HCs). (**B**) Abundance of fungal phyla in the 25 conjunctival microbiomes. "Others" includes singletons, unassigned reads, and sparse OTUs (with <0.001% of total high-quality reads). (**C**) Abundance of the 7 dominant fungal genera (*Aspergillus*, *Setosphaeria*, *Malassezia*, *Haematonectria*, *Emericella*, *Candida*, and *Fusarium*) with >0.50% abundance. All the other genera with <0.50% abundance in the 25 conjunctival microbiomes (OD = 12 and OS = 13) from HCs were included as less abundant genera. Singletons, unassigned reads, and sparse OTUs (with <0.001% of total high-quality reads) are shown as "Others".

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Table 3. Abundance of Fungal Genera in the Ocular Surface Fungal Microbiomes from the 25 Eyes (OD = 12 and OS = 13) of HCs

Serial Number	Genus	Abundance		Present Out of 25 Eyes Sampled
		Mean	Range	
1	*Aspergillus*	21.050	0.085–79.946	25
2	*Setosphaeria*	14.974	0.001–71.83	25
3	*Malassezia*	7.374	0.003–29.792	25
4	*Haematonectria*	2.735	0.0001–18.026	25
5	*Emericella*	1.146	0–13.176	24
6	*Fusarium*	0.520	0–9.372	21
7	*Cladosporium*	0.073	0–0.642	20
8	*Choiromyces*	0.003	0–0.028	20
9	*Candida*	0.892	0–5.494	17
10	*Neosartorya*	0.043	0–0.259	17
11	*Penicillium*	0.225	0–1.613	14
12	*Nectria*	0.003	0–0.016	14
13	*Pichia*	0.238	0–1.537	12
14	*Xeromyces*	0.388	0–8.069	12
15	*Leptosphaerulina*	0.244	0–4.271	9
16	*Arthrinium*	0.253	0–6.319	9
17	*Eurotium*	0.169	0–2.295	7
18	*Aureobasidium*	0.131	0–1.914	7
19	*Cryptococcus*	0.088	0–1.327	6
20	*Cercospora*	0.101	0–1.861	5
21	*Podospora*	0.019	0–0.226	5
22	*Saccharomyces*	0.069	0–0.833	5
23	*Colletotrichum*	0.106	0–2.656	5
24	*Hanseniaspora*	0.052	0–0.857	4
25	*Trichoderma*	0.040	0–0.309	4
26	*Phoma*	0.005	0–0.076	4
27	*Pestalotiopsis*	0.037	0–0.663	4
28	*Passalora*	0.080	0–1.998	4
29	*Acremonium*	0.048	0–0.635	3
30	*Clavispora*	0.010	0–0.211	3
31	*Khuskia*	0.002	0–0.024	3
32	*Myrothecium*	0.108	0–2.644	3
33	*Nigrospora*	0.010	0–0.176	3
34	*Alternaria*	0.119	0–2.966	3
35	*Ramichloridium*	0.013	0–0.326	3
36	*Metschnikowia*	0.004	0–0.061	2
37	*Rhizopus*	0.077	0–0.983	2
38	*Sagenomella*	0.024	0–0.564	2
39	*Wallemia*	0.002	0–0.036	2
40	*Ceratocystis*	0.001	0–0.036	2
41	*Chrysosporium*	0.018	0–0.443	2
42	*Mycosphaerella*	0.032	0–0.791	2
43	*Paecilomyces*	0.018	0–0.44	2
44	*Pseudozyma*	0.003	0–0.065	2
45	*Stachybotrys*	0.007	0–0.18	2
46	*Ustilago*	0.002	0–0.046	2
47	*Arxula*	0.024	0–0.602	1
48	*Bullera*	0.022	0–0.551	1
49	*Calcarisporiella*	0.007	0–0.166	1
50	*Dactylella*	0.042	0–1.059	1
51	*Galactomyces*	0.006	0–0.141	1
52	*Hypoxylon*	0.015	0–0.38	1
53	*Ochrocladosporium*	0.003	0–0.082	1
54	*Phaeosphaeriopsis*	0.031	0–0.78	1
55	*Phanerochaete*	0.025	0–0.62	1
56	*Phialophora*	0.015	0–0.383	1
57	*Pyrenochaeta*	0.002	0–0.06	1
58	*Quambalaria*	0.159	0–3.963	1
59	*Rhodotorula*	0.015	0–0.369	1
60	*Simplicillium*	0.005	0–0.125	1
61	*Strelitziana*	0.014	0–0.337	1
62	*Sympodiomycopsis*	0.001	0–0.036	1
63	*Torulaspora*	0.060	0–1.492	1
64	*Villosiclava*	0.009	0–0.232	1
65	*Zygoascus*	0.026	0–0.644	1
66	Unclassified	36.374	2.099–87.168	25
67	Unidentified	11.109	0.007–58.637	25
68	*Others	0.482	0.187–0.985	25

* Include singletons, unassigned reads, and sparse OTUs (with <0.001 % of total high-quality reads).

sparse OTUs with <0.001% of total high-quality reads) (Fig. 1B; Table 2, Supplementary Table S3). The reads in the unclassified, unidentified, and others categories varied from 0.007% to 74.31% across all the eyes sampled (Table 2, Supplementary Table S3).

At the genus level, only 56.18% of the reads (7,510,145/13,368,000) could be assigned to 65 different genera. Only four genera, namely, *Aspergillus* (21.05%), *Setosphaeria* (14.97%), *Malassezia* (7.37%), and *Hematonectria* (2.73%) were present in all the 25 eyes sampled with a mean abundance of >1%. Four other genera, namely, *Emericella*, *Fusarium*, *Cladosporium*, and *Choiromyces* were present in more than 20 of the 25 eyes. The remaining 57 genera were present in 1 to 17 eyes and their abundance was <1%. A number of reads were assigned as unclassified (36.37%) and unidentified (11.11%) genera and were present in all the 25 eyes sampled (Fig. 1C; Table 3, Supplementary Table S4). The inability to assign a significant number of reads both at the phylum and genus level indicate the presence of several hitherto unknown fungal taxonomic groups on the conjunctival surface and reiterates the limitations of the existing fungal sequence databases.

Alpha Diversity of the Fungal Community on the Conjunctival Surface

The alpha diversity of the conjunctival surface fungal communities in the right and left eyes of the healthy controls was monitored and the data indicated that the Shannon diversity index ($P = 0.503$), observed number of OTUs (richness) ($P = 0.346$), Simpson index for evenness ($P = 0.558$), and Chao1 index for richness ($P = 0.224$) were not statistically significant, indicating that the alpha diversity in the two eyes was similar (Figs. 2a–d).

We then compared the alpha diversity indices of the ocular conjunctival fungal flora for differences between age and sex of the individuals. Our results indicated that sex did not alter Shannon diversity ($P = 0.463$), OTU number ($P = 0.725$), Simpson diversity ($P = 0.129$), and Chao1 indices ($P = 0.521$); age also did not alter the Shannon diversity index ($P = 0.917$) and OTU number ($P = 0.763$), but age significantly altered the Simpson diversity index ($P = 0.019$) and Chao1 index ($P = 0.022$) (Figs. 2e–l).

Beta Diversity of the Fungal Community on the Conjunctival Surface

We further analyzed the data for beta diversity by using the NMDS plots using Bray-Curtis dissimilarity of OTUs. It was observed that the fungal community structure was not dependent on the eye (left/right) that was sampled (PERMANOVA, $P = 0.533$) nor was it influenced by age (PERMANOVA,

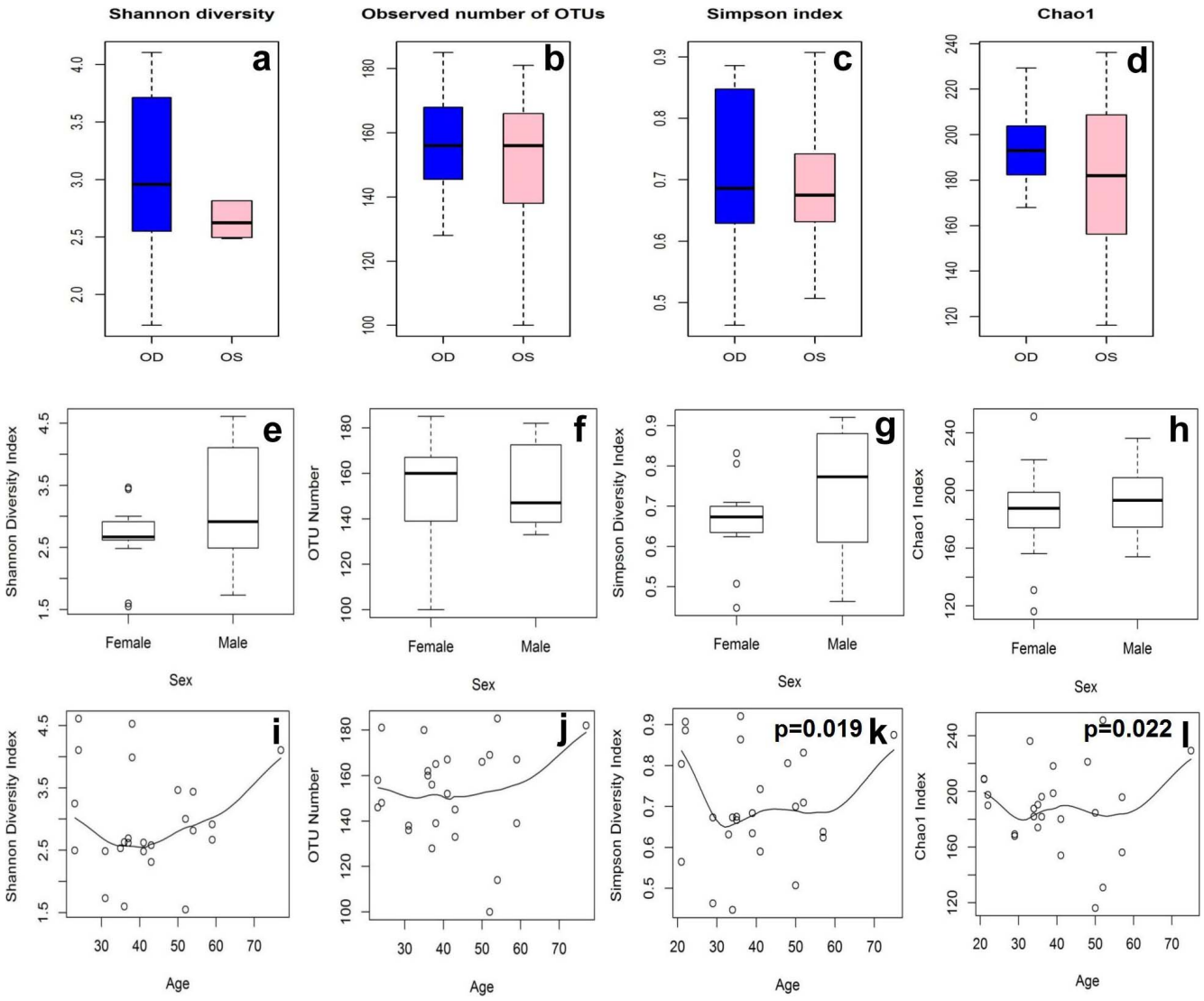

Figure 2. Alpha diversity indices with respect to type of eye, sex, and age. (**a–d**) Comparison of the alpha diversity indices in the ocular surface fungal microbiomes of the 25 eyes (OD = 12 and OS = 13) of HCs. The *P* values between OD versus OS for Shannon diversity ($P = 0.503$) (**a**), Observed number of OTUs ($P = 0.346$) (**b**), Simpson index ($P = 0.558$) (**c**), and Chao1 index ($P = 0.224$) (**d**) were not statistically significant. (**e–h**) Illustrate that sex did not alter Shannon diversity ($P = 0.463$) (**e**), OTU number ($P = 0.725$) (**f**), Simpson diversity ($P = 0.129$) (**g**), and Chao1 index ($P = 0.521$) (**h**). (**i–l**) Illustrate that age also did not alter Shannon diversity index ($P = 0.917$) (**i**) and OTU number ($P = 0.763$) (**j**), but age significantly altered the Simpson diversity index ($P = 0.019$) (**k**) and Chao1 index ($P = 0.022$) (**l**). Median values (*horizontal line*) and interquartile ranges are indicated in the plots **a–h**.

$P = 0.859$) (Figs. 3a, 3b). However, the fungal community appeared to vary significantly (PERMANOVA, $P = 0.009$) depending on sex (Fig. 3c).

Identification of Fungal Genera on the Conjunctival Surface

It was observed that 4 of the 65 identified genera were present in all the 25 eyes in which fungi were detected and included the genera *Aspergillus*, *Setosphaeria*, *Malassezia*, and *Haematonectria*. The mean abundance of these genera ranged from 2.73% to 21.05% and accounted for 46.12% of the total abundance. It is of interest to also note that in some conjunctiva there was a dominance of a single genus, such as *Aspergillus* in ODO21 (79.95%) and OS001 (68.36%) samples, *Setosphaeria* species (71.83%) in OS002, and *Malassezia* (29.79%) in OD033. (Table 3, Supplementary Table S4).

Ocular Pathogens on the Healthy Conjunctival Surface

A number of well-known ocular pathogens were also identified on the conjunctival surface of the normal eye, such as those affiliated to the genera *Acremonium*, *Candida*, *Rhodotorula*, *Aspergillus*, *Penicillium*, *Paecilomyces*, *Saccharomyces*, *Trichoderma*, *Malassezia*, *Fusarium*, *Cryptococcus*, *Ustilago*, *Pichia*, and *Arthrinium* (for details refer to discussion). Some of these genera, such as *Candida*, *Aspergillus*, *Malassezia*, and *Fusarium* were also found to be the dominant genera (abundance, >0.50%) in the normal conjunctival microbiome (Fig. 1C)

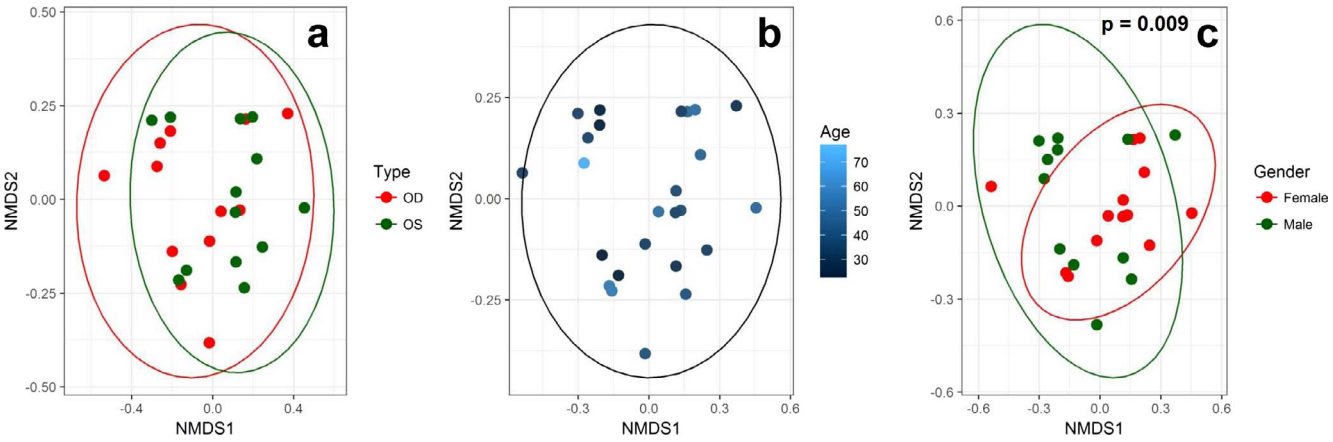

Figure 3. Beta diversity measurements with respect to type of eye, age, and sex. (**a**–**c**) Comparison of the beta diversity in the ocular surface fungal microbiomes of the 25 eyes (OD = 12 and OS = 13) of HCs. Beta diversity was analyzed using the NMDS plots using Bray-Curtis dissimilarity of OTUs. The fungal community structure was not dependent on the eye (left/right) that was sampled (PERMANOVA, $P = 0.533$) (**a**) nor was it influenced by age (**b**; PERMANOVA, $P = 0.859$) (**b**), but the fungal community appeared to vary significantly (PERMANOVA, $P = 0.009$) (**c**) depending on the sex.

Discussion

Conventional culture-based methods,[2] more refined culture methods,[7] PCR-based 16S rRNA gene amplification, and NGS based on specific regions of 16S rRNA gene[10,12] have confirmed the presence of bacteria on the ocular surface.[27]

Compared with bacteria, the detection of fungi on the ocular surface has been more difficult and many a times the culture-based and the PCR-based methods did not yield any fungi.[6,24,25] One way of improving the chances of detection and identification of ocular surface fungi of healthy individuals would be to use conjunctival swabs of the eye as the source of DNA and undertake NGS using ITS2 sequencing as a proxy for fungi. This is the first study using the above approach to characterize the ocular fungal microbiome of healthy individuals. We were also conscious of the fact that we need to discuss our results in the light of possible contaminating DNA from swabs, reagents of DNA extraction kits, reagents used for PCR, such as water, PCR primers, and PCR reagents. Appropriate reagent controls were run with every PCR reaction to be able to detect contaminating DNA. Extracts of the Isohelix swabs and the above reagents, including DNA extraction kits and PCR reagents, did not yield any DNA or any amplicons on PCR using ITS2 primers. Sequencing also did not yield any fungal or bacterial reads. We did not use whole-genome multiple displacement amplification to avoid any PCR bias.[34]

Using the culturable approach, we found that only 3 out of 24 ocular surface swabs (12.50%) yielded fungi and were identified as *Aspergillus flavus* (OD001 and OD028) and *Aspergillus niger* (OS002). It was also observed that the frequency of positive cultures of fungi ranged from 0% to 32% and most swabs yielded only one culture.[24,25,35] It was also observed that filamentous fungi, such as *Aspergillus* sp. and *Fusarium* sp., were the most common fungi.[35,36] The other genera that were identified in earlier studies included *Candida* sp., *Rhodotorula* sp., *Stachybotrys* sp., *Botrytis* sp., *Cladosporium* sp., *Fusarium* sp., *Rhizoctonia* sp., *Alternaria* sp., *Scopulariopsis* sp., *Isaria* sp., *Geotrichum* sp., *Papulospora* sp., *Gliocladium* sp., *Hormodendron* sp., *Saccharomyces* sp., *Rhizopus* sp., and *Nigrospora* sp.[18–22] These studies also indicated that *Penicillium* predominates in temperate climates, whereas *Aspergillus* in the tropics. The current study and earlier studies[23,37] also confirm that *Aspergillus* was indeed the most common isolate from the healthy conjunctival swabs from India. Compared with these conventional culture-based methods, the NGS approach yielded 189 reference OTUs and 365 de novo OTUs, which were affiliated to 3 phyla and 65 genera. This in itself is indicative of a significant increase in fungal diversity on the ocular surface compared with the conventional culturable approach, although the NGS approach does not differentiate between viable and nonviable microorganisms.[6]

The alpha diversity indices (Shannon diversity index, Simpson index for evenness, observed number of OTUs, and Chao1 index for richness) of the ocular fungal microbiome in both OD and OS eyes were similar. The above alpha diversity indices were also not altered by sex, thus confirming the results of Wu et al.[25] who observed that sex did not influence the detection of *C. albicans* in children and adolescents of 21 years of age or less. But in the present study it was observed that two of the indices, namely, Chao1 index for richness and Simpson index for evenness were significantly altered by age in contrast to the observations of Wu et al.[25] In this study, the beta diversity of the ocular fungal community was not dependent on the eye (left/right) nor was it influenced by age but appeared to vary significantly depending on the sex.

Defining the core ocular fungal microbiome would help to define a healthy ocular microbiome.[27] According to Turnbaugh et al.,[38] certain microbial types that are always present constitute the core microbiome, whereas others that are transient depending on factors, such as the environment, lifestyle, and physiological differences, constitute the variable microbiome. Such a definition would be all the more meaningful if all the fungi in the microbiome are identified. Unfortunately, in the ocular fungal microbiome, a significant number of OTUs both at the phylum and genera level are unidentified, implying that the available ITS2 fungal database needs to be strengthened.

To the best of our knowledge, studies on fungi associated with the healthy conjunctiva using NGS are scarce, but reviews exist that enumerate all fungi associated with the conjunctiva of keratitis individuals by using the conventional culturable approach.[36,39,40] Karsten et al.[36] studied all publications reporting microorganisms implicated in keratitis between 1950 and 2012 and identified 92 genera, and subsequently, Thomas and Kaliamurthy[39] reported 65 genera of fungi as associated with mycotic keratitis. In most of these publications, only single fungal genera or at the most three genera have been reported. In comparison, the present study identified 65 genera out of which only 18 and 21 genera were

common with the study of Thomas and Kaliamurthy[39] and Karsten et al.,[36] respectively. The three studies shared 26 genera that included *Acremonium*, *Candida*, *Rhodotorula*, *Aspergillus*, *Penicillium*, *Paecilomyces*, *Phoma*, *Cladosporium*, *Aureobasidium*, *Phialophora*, *Colletotrichum*, *Myrothecium*, *Alternaria*, *Saccharomyces*, *Pyrenochaeta*, *Torula*, *Trichoderma*, *Rhizopus*, *Cercospora*, *Stachybotrys*, *Chrysosporium*, *Malassezia*, *Fusarium*, *Cryptococcus*, *Ustilago*, and *Phaeosphaeriopsis*, which were common to this study and one or two of the above studies.[36,39] These findings may imply that the above 26 genera are potential opportunistic pathogens. The reason why the remaining 39 genera in this study did not match the findings of Karsten et al.[36] and Thomas and Kaliamurthy[39] is surprising because some of them are known to cause keratitis, such as fungi affiliated to the genera *Pichia*, *Nectria*, *Arthrinium*, *Pestalotiopsis*, *Khuskia*, and *Nigrospora*. In a recent review, Maharana et al.[40] reported that fungi such as *Fonsecaea pedrosoi*, *Lasiodiplodia theobromae*, *Cylindrocarpon* species, *Scedosporium prolificans*, *Metarhizium anisopliae*, *Paecilomyces* species, and *Pythium insidiosum* are rare in mycotic keratitis. In addition, the observed variation in the above studies may also be attributed to the fact that the age of the individuals sampled, the region of their origin, and the pathology were not identical.[24,41–43] It is also possible that some of them are not pathogenic and are associated only with healthy conjunctiva. In addition, the discrepancy in the detection of microorganisms by the culturable method versus the NGS method could be attributed to several other factors. For instance, organisms present in low numbers (even one) if amenable to culture would get enriched following culture but getting sufficient DNA from a single organism may not be possible. Other factors that contribute to the discrepancy include ease in DNA extraction, sequencing artifacts, taxonomic misidentification, choice of reagents and kits, and PCR bias.[44,45] Nevertheless, the culture-independent method reveals a greater degree of diversity, for instance 12 to 24 different genera per sample as observed in this study versus 1 to 2 culturable fungi.[20,46]

In summary, fungi affiliated to 3 phyla and 65 genera have been detected in the ocular surface fungal microbiome of healthy individuals without any ocular infection. The alpha diversity in the two eyes was not significantly altered and sex had no effect, but the Chao1 index for richness and Simpson index for evenness were significantly altered by age. The phyla Ascomycota and Basidiomycota and the genera *Aspergillus*, *Setosphaeria*, *Malassezia*, and *Haematonectria* were present in all the 25 eyes in which fungi were detected.

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References

1. Willcox MDP. Characterization of the normal microbiota of the ocular surface. *Exp Eye Res*. 2013;117:99–105.
2. Keilty RA. The bacterial flora of the normal conjunctiva with comparative nasal culture study. *Am J Ophthalmol*. 1930;13:876–879.
3. Hori Y, Maeda N, Sakamoto M, Koh S, Inoue T, Tano Y. Bacteriologic profile of the conjunctiva in the patients with dry eye. *Am J Ophthalmol*. 2008;146:729–734.
4. Sankaridurg PR, Markoulli M, de la Jara PL, et al. Lid and conjunctival micro biota during contact lens wear in children. *Optom Vis Sci*. 2009;86:312–317.
5. Fernández-Rubio ME, Rebolledo-Lara L, Martinez-García M, Alarcón-Tomás M, Cortés-Valdés C. The conjunctival bacterial pattern of diabetics undergoing cataract surgery. *Eye* 2009;24:825.
6. 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.
7. Perkins RE, Kundsin RB, Pratt MV, Abrahamsen I, Leibowitz HM. Bacteriology of normal and infected conjunctiva. *J Clin Microbiol*. 1975;1:147–149.
8. Stapleton F, Willcox MD, Fleming CM, Hickson S, Sweeney DF, Holden BA. Changes to the ocular biota with time in extended- and daily-wear disposable contact lens use. *Infect Immun*. 1995;63:4501–4505.
9. Sthapit PR, Tuladhar NR. Conjunctival flora of normal human eye. *JSM Ophthalmol*. 2014;2:1021.
10. Graham JE, Moore JE, Jiru X, et al. ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. *Invest Ophthalmol Vis Sci*. 2007;48:5616–5623.
11. Deepthi KG, Jayasudha R, Girish RN, et al. Polybacterial community analysis in human conjunctiva through 16S rRNA gene libraries. *Exp Eye Res*. 2018;174:1–12.
12. Dong Q, Brulc JM, Iovieno A, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci*. 2011;52:5408–5413.
13. Schabereiter-Gurtner C, Maca S, Rölleke S, et al. 16S rDNA-based identification of bacteria from conjunctival swabs by PCR and DGGE fingerprinting. *Invest Ophthalmol Vis Sci*. 2001;42:1164–1171.
14. 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.
15. 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.
16. 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.
17. Wen X, Miao L, Deng Y, et al. The influence of age and sex on ocular surface microbiota in healthy adults. *Invest Ophthalmol Vis Sci*. 2017;58:6030–6037.
18. Mitsui Y, Hanabusa J. Corneal infections after cortisone therapy. *Br J Ophthalmol*. 1955;39:244–250.
19. Hammeke JC, Ellis PP. Mycotic flora of the conjunctiva. *Am J Ophthalmol*. 1960;49:1174–1178.
20. Ainley R, Smith B. Fungal flora of the conjunctival sac in healthy and diseased eyes. *Br J Ophthalmol*. 1965;49:505–515.
21. Williamson J, Gordon AM, Wood R, Dyer AM, Yahya OA. Fungal flora of the conjunctival sac in health and disease. Influence of topical and systemic steroids. *Br J Ophthalmol*. 1968;52:127–137.
22. Ando N, Takatori K. Fungal flora of the conjunctival sac. *Am J Ophthalmol*. 1982;94:67–74.
23. Sehgal SC, Dhawan S, Chhiber S, Sharma M, Talwar P. Frequency and significance of fungal isolations from conjunctival sac and their role in ocular infections. *Mycopathologia*. 1981;73:17–19.
24. Segal E, Romano A, Eylan E, Stein R. Fungal flora of the normal conjunctival sac. *Mycoses*. 1977;20:9–14.
25. Wu TG, Mitchell BM, Carothers TS, et al. Molecular analysis of the pediatric ocular surface for fungi. *Curr Eye Res*. 2003;26:33–36.

26. Klotz SA, Penn CC, Negvesky GJ, Butrus SI. Fungal and parasitic infections of the eye. *Clin Microbiol Rev*. 2000;13:662–685.
27. Zegans ME, Van Gelder RN. Considerations in understanding the ocular surface microbiome. *Am J Ophthalmol*. 2014;158:420–422.
28. Vilgalys R, Hibbett DS, John S, Hopple J. Phylogenetic implications of generic concepts in fungal taxonomy: the impact of molecular systematic studies. *Mycol Helv*. 1994;6:73–91.
29. Dehingia M, Thangjam devi K, Talukdar NC, et al. Gut bacterial diversity of the tribes of India and comparison with the worldwide data. *Sci Rep*. 2015;5:18563.
30. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. 2011;27:2957–2963.
31. Kalyana Chakravarthy S, Jayasudha R, Ranjith K, et al. Alterations in the gut bacterial microbiome in fungal keratitis patients. *PLoS One*. 2018;13:e0199640.
32. Jayasudha R, Kalyana Chakravarthy S, Sai Prashanthi G, et al. Alterations in the gut bacterial and fungal microbiomes are associated with bacterial keratitis, an inflammatory disease of the human eye. *J Biosci*. 2018;43:835–856.
33. Oksanen FJ, Blanchet G, Friendly M, et al. Vegan: community ecology package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. R-package. Version 2.4-2.
34. Yilmaz S, Allgaier M, Hugenholtz P. Multiple displacement amplification compromises quantitative analysis of metagenomes. *Nat Methods*. 2010;7:943.
35. Gopinathan U, Sharma S, Garg P, Rao GN. Review of epidemiological features, microbiological diagnosis and treatment outcome of microbial keratitis: experience of over a decade. *Indian J Ophthalmol*. 2009;57:273–279.
36. Karsten E, Watson SL, Foster LJR. Diversity of microbial species implicated in keratitis: a review. *Open Ophthalmol J*. 2012;6:110–124.
37. Sharma S. Diagnosis of fungal keratitis: current options. *Expert Opin Med Diagn*. 2012;6:449–455.
38. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project: exploring the microbial part of ourselves in a changing world. *Nature*. 2007;449:804–810.
39. Thomas PA, Kaliamurthy J. Mycotic keratitis: epidemiology, diagnosis and management. *Clin Microbiol Infect*. 2013;19:210–220.
40. Maharana P, Sharma N, Nagpal R, Jhanji V, Das S, Vajpayee R. Recent advances in diagnosis and management of mycotic keratitis. *Indian J Ophthalmol*. 2016;64:346–357.
41. Rosa RH Jr, Miller D, Alfonso EC. The changing spectrum of fungal keratitis in South Florida. *Ophthalmology*. 1994;101:1005–1013.
42. Basak S, Basak S, Mohanta A, Bhowmick A. Epidemiological and microbiological diagnosis of suppurative keratitis in Gangetic West Bengal, Eastern India. *Indian J Ophthalmol*. 2005;53:17–22.
43. Houang E, Lam D, Fan D, Seal D. Microbial keratitis in Hong Kong: relationship to climate, environment and contact-lens disinfection. *Trans R Soc Trop Med Hyg*. 2001;95:361–367.
44. Brooks JP, Edwards DJ, Harwich MD, et al. The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol*. 2015;15:66.
45. Huang Y, Yang B, Li W. Defining the normal core microbiome of conjunctival microbial communities. *Clin Microbiol Infect*. 2016;22:643.
46. Srinivasan M, Gonzales CA, George C, et al. Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, south India. *Br J Ophthalmol*. 1997;81:965–971.

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