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Article

Ocular Surface Microbiome Alterations Are Found in Both Eyes of Individuals With Unilateral Infectious Keratitis

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Purpose: To analyze the ocular surface microbiome (OSM) profile in both eyes of individuals with unilateral keratitis.

Methods: In this prospective, cross-sectional study, the conjunctival OSM of adults (>18 years old) presenting to an ophthalmic emergency department with acute unilateral keratitis and controls without an acute infectious process was sampled. Samples underwent DNA amplification and 16S sequencing using Illumina MiSeq 250 and were analyzed using Qiime. Statistical analysis was performed using a two-sided Student *t*-test, diversity indices, and principal coordinate analysis. The main outcome measures included relative abundance and α and β diversity.

Results: Bacterial DNA was recovered from all 34 eyes of 17 individuals with keratitis (mean age, 49.3 $\pm$ 17.5 years) and 16 eyes of controls (mean age, 56.6 $\pm$ 17.0 years). In the two culture-positive eyes, 16S aligned with culture results. Significant differences in α diversities were noted when comparing both eyes of individuals with keratitis to control eyes (all $P < 0.05$), but no significant differences between the eyes of an individual with keratitis. Principal coordinate analysis plots confirmed this finding, demonstrating separation between either eye of patients with keratitis and controls (both $P < 0.01$), however not between eyes in patients with unilateral keratitis. Both eyes of individuals with keratitis had greater abundance of *Pseudomonas* compared with controls both on compositional analysis and linear discriminant analysis.

Conclusions: Alterations in the OSM profile are detected in both eyes of individuals with unilateral keratitis compared with controls. Beyond the causative organism, a greater abundance of potential pathogens and lesser abundance of commensal organisms were found.

Translational Relevance: The OSM profile is altered in both eyes of individuals with unilateral keratitis, which may lend insight into the role of the microbiome in the pathophysiology of disease.

Introduction

Keratitis is a significant cause of ocular morbidity in both adults and children, resulting in permanent visual impairment despite treatment. It results in more than $200 million in health care expenditures annually, including outpatient clinic and emergency department visits, medication costs, and clinician time.[1] Risk factors for bacterial keratitis include contact lens wear, trauma, and immunosuppression, the most common of which is contact lens wear.[2] There are more than 70,000 cases of keratitis per year,[3] with the highest incidence in contact lens wearers.[4] The burden of this disease is likely to increase with increasing use of contact lenses and evolving antibiotic resistance. These factors make the pathophysiology of keratitis especially important to understand.

Interestingly, animal studies have shown that it is not only the causative organism that influences

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infection, but also the bacteria that compose the complex network of microorganisms that reside on the ocular surface referred to as the ocular surface microbiome (OSM). These bacteria are important for physiologic processes like balancing the immune system and defending against pathogens. Disruption of the OSM, such as with contact lens wear, compromises the conjunctival defense systems and decreases immune mechanisms, thereby predisposing toward infection.[5–8] When the OSM in germ-free mice was disrupted with topical gentamicin, there was a decrease in the overall number of OSM bacteria and an increased susceptibility to infection with *Pseudomonas* compared with age- and gender-matched control mice.[8,9] Despite the finding that disruption of the OSM affects susceptibility to ocular infections in mice, these findings have yet to be confirmed in human studies. Therefore, the goal of this study was to evaluate the OSM profile in both eyes of individuals with unilateral keratitis to evaluate whether the noninfected eye had evidence of OSM disruption compared with controls without keratitis.

Methods

The study was conducted with the approval of the University of Miami and the Miami Veterans Affairs Hospital Institutional Review Boards and adhered to the Health Insurance Portability and Accountability Act of 1996 and the tenets of the Declaration of Helsinki. All individuals signed informed consent before participating in the study. Patients with keratitis were recruited from the Bascom Palmer Eye Institute ophthalmic emergency department. All adults (>18 years old) presenting with acute unilateral keratitis were invited to participate. Patients were excluded if they had bilateral keratitis or had undergone ocular surgery within the prior 90 days. Demographic data including age, gender, contact lens wear, and topical antibiotic use was collected. If cultures were obtained, the results were recorded. Controls included individuals of similar age who presented for a routine clinic evaluation, such as for refractive error or cataracts, and did not have evidence of keratitis.

Specimen Collection

The methodology for specimen collection, 16S sequencing, and statistical and bioinformatics analysis was performed in accordance with previously published studies by our group[10–12] as detailed elsewhere in this article. All specimens were collected by study personnel in a standardized fashion using sterile gloves. A dry calcium alginate swab was passed four times along the inferior conjunctival fornix of both eyes without anesthetic. The swab was then placed in an individual tube and labelled with the unique study identifier. The collected specimens were then immediately transported by study personnel directly to the microbiology laboratory and stored in a −80°C freezer.

16S Sequencing

16S sequencing was done using previously published methods.[10,12] Briefly, swab heads were aseptically transferred into PowerSoil sample collection tubes and lysed using a MagnaLyser tissue disruptor (Roche, Indianapolis, IN). Total DNA was isolated using a PowerSoil/Fecal DNA isolation kit (Mo-Bio, Germantown, MD) as per manufacturer's specifications. All samples were quantified via the Qubit Quant-iT dsDNA Broad- Range Kit (Invitrogen, Life Technologies, Grand Island, NY) to ensure that the minimum mass of DNA was met. Samples were sent to the University of Minnesota Genomic Center for microbiome analysis as follows: to enrich the sample for the bacterial 16S V5-V6 rDNA region, DNA was amplified using fusion primers designed against the surrounding conserved regions that are tailed with sequences to incorporate Illumina (San Diego, CA) flow cell adapters and indexing barcodes. Each sample was polymerase chain reaction amplified with two differently bar coded V5–V6 fusion primers and were advanced for pooling and sequencing. Amplified products were concentrated using a solid-phase reversible immobilization method and quantified by electrophoresis using an Agilent 2100 Bioanalyzer. The pooled 16S V5–V6 enriched, amplified, barcoded samples were loaded into the MiSeq reagent cartridge and then onto the instrument along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 300 cycles with custom primers designed for paired-end sequencing.

Statistical Analysis

Using QIIME 2.0 (Quantitative Insights into Microbial Ecology, version 2.0), sequences were quality filtered and demultiplexed using exact matches to the supplied DNA barcodes and primers. Resulting sequences were then searched against the SILVA database (v 123) of 16S sequences, clustered at 97% by uclust (closed-reference OTU picking) to obtain phylogenetic identities. Blank calcium alginate swabs were used as negative controls and carried along with the ocular samples through the same pipeline of DNA extraction to QIIME analysis. To discount the possibil-

Table 1. Demographic Data of Individuals with Unilateral Keratitis and Controls

	Keratitis ($n = 17$)	Controls ($n = 16$)	P Value
Age, years	49.3 ± 17.5	56.6 ± 17.0	.12
Range	20–74	29–82	
Gender, % male (n)	41 (7)	75 (12)	.0514
Race, % (n)			.8337
White	65 (11)	75 (12)	
Black	35 (6)	18.7 (3)	
Other	0 (0)	6.3 (1)	
Ethnicity % (n)			.3399
Non-Hispanic	59 (10)	75 (12)	
Hispanic	41 (7)	25 (4)	
Contact lens wear, % (n)	65 (11)	0 (0)	.02

ity of implement-induced artifacts, OTUs with representation in the blank samples were eliminated. OTU tables were rarefied to the sample containing the lowest number of sequences and QIIME 2.0 was used to summarize taxa at the phylum and genera levels.

Several different statistical analyses were performed to analyze the OSM. Shannon, Chao1, and observed α diversity measures were used to compare the biodiversity of the control to keratitis, control to noninfected, and the keratitis to contralateral eyes. Principal coordinate analysis plots were used to evaluate β diversity. Genus-level heat maps with hierarchical clustering were used to show compositional similarities and differences between the eyes. Heat map trees of hierarchy were used to evaluate differences in abundance between the control and keratitis and control and nonkeratitis eyes. Linear discriminant analysis was used to examine the effect size of particular organisms between groups.[13]

Results

A total of 33 patients were included, 17 with keratitis (mean age, 49.3 ± 17.5 years; range, 20–74 years) and 16 controls (mean age, 56.6 ± 17.0 years; range, 29–82 years), for a total of 50 eyes. Demographic data are detailed in Table 1. Bacterial DNA was recovered from all 34 eyes of 17 patients with keratitis and all left eyes of 16 controls. In the two culture-positive eyes, 16S aligned with culture results.

The α diversity matrices are demonstrated in Figure 1. Regardless of the α-diversity measured (Shannon, Chao1, observed), there were significant differences when comparing either eye of individuals with keratitis to control eyes (all $P < .05$), but no significant differences between the infected and noninfected eyes of an individual with keratitis. This observation implies that irrespective of the matrix used, numbers of distinct and identifiable bacterial OTUs were significantly different in both eyes of keratitis patients, compared with controls.

Aligning with the α-diversity findings, principal coordinate analysis plots for β diversity demonstrated separation between keratitis and control eyes (Fig. 2A; $P < 0.01$) and nonkeratitis and control eyes (Fig. 2B; $P < 0.01$), but no significant separation between the two eyes of an individual with keratitis (Supplementary Fig. S1A). Taken together, these findings suggests that both eyes of an individual with unilateral keratitis have OSM alterations as compared with controls. Taken together with the earlier α diversity results, it was clear that changes in β diversity was a sum of overall change in bacterial diversity, as well as changes in bacterial numbers between the groups. Additionally, it was interesting to see that keratitis mediated OSM changes in the affected eyes were significantly different from the control eyes, as evident from the phyla-level dendrograms shown in Supplementary Figure S1B.

A genus-level heat map with hierarchical clustering demonstrated that there were compositional differences when comparing the control eyes with the eyes with keratitis and with the contralateral eye of patients with keratitis (Fig. 3A and B). As evident from the heat maps, the bacterial genera contributing to bacterial dysbiosis in either eyes are similar, albeit clustering separately owing to a dissimilar relative abundance between the affected and contralateral eye. No significant differences with antibiotic use, contact lens wear, or gender were found. These compositional differences were supported by the heat tree, which is a hierarchical structure of taxonomic classifications to quantitatively (median abundance) and statistically (nonparameter Wilcoxon rank-sum test) depict

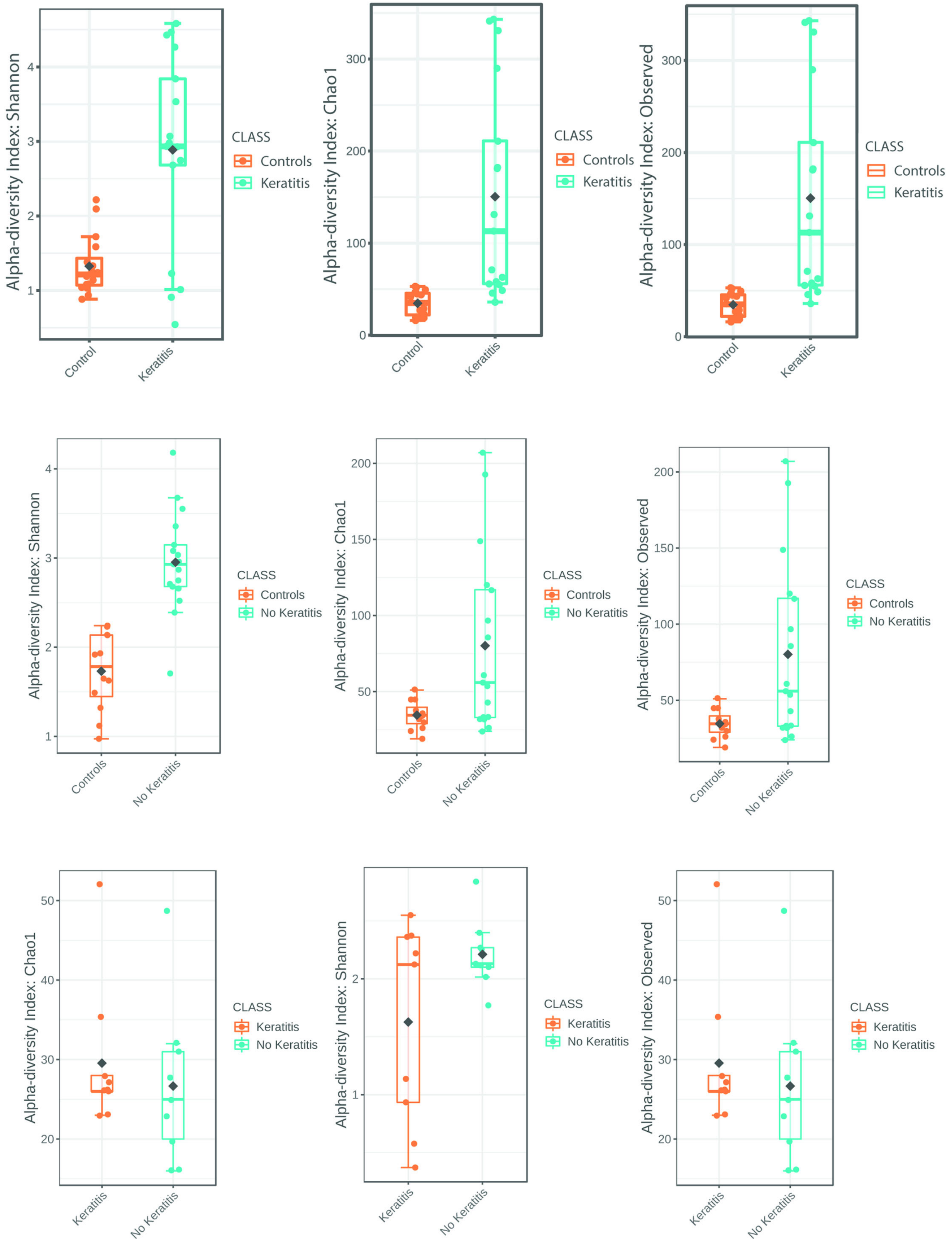

Figure 1. Diversity matrices comparing the Shannon, Chao1, and observed α diversities between the control and keratitis eyes (A), control and noninfected eyes of patients with keratitis (B), and keratitis to contralateral eyes (C). Test of significance was Mann–Whitney *U* test and *P* values were absolute.

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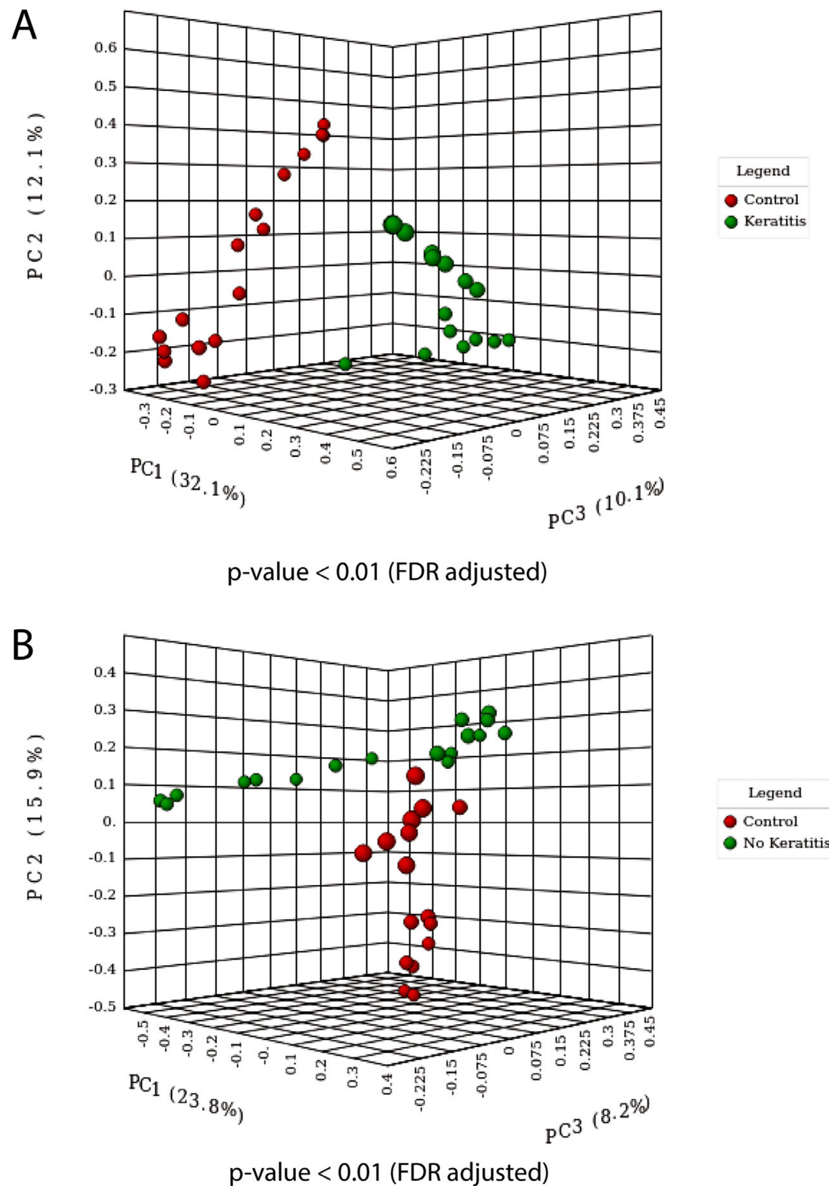

Figure 2. Principal coordinate analysis plots (unweighted UniFrac) demonstrating separation keratitis and control eyes (A) and nonkeratitis and control eyes (B). Test of significance was Monte-Carlo permutation with Bonferroni's correction (FDR).

taxon differences between controls and affected keratitis eye (Fig. 4A) as well as controls and contralateral eye (Fig. 4B). As evident from other analyses, the relative perturbation in significantly changing bacterial populations (up or down) is quite similar in both eyes in keratitis patients, compared with controls. In particular, several genera of Proteobacteria were higher whereas others were lower in infected individuals. Figure 5 provides a detailed analysis of the classes that significantly differed between the control and keratitis eyes (Fig. 5). A stricter measure, namely linear discriminant analysis was used on the dataset, which integrates statistical significance with biological consistency (effect size) estimation. The statistics is based on nonparametric factorial Kruskal–Wallis sum-rank test to detect features with significant differential abundance, followed by a linear discriminant analysis to estimate the effect size of each differentially abundant features. Upon comparing the control to keratitis and control to nonkeratitis eyes, *Pseudomonas* again emerged with a high linear discriminant analysis effect size in both eyes of infected individuals (Fig. 6), implying that it has a major contribution to the observed OSM dysbiosis in keratitis patients.

With regard to composition, phyla Proteobacteria, Actinobacteria, and Firmicutes were all more than 10-

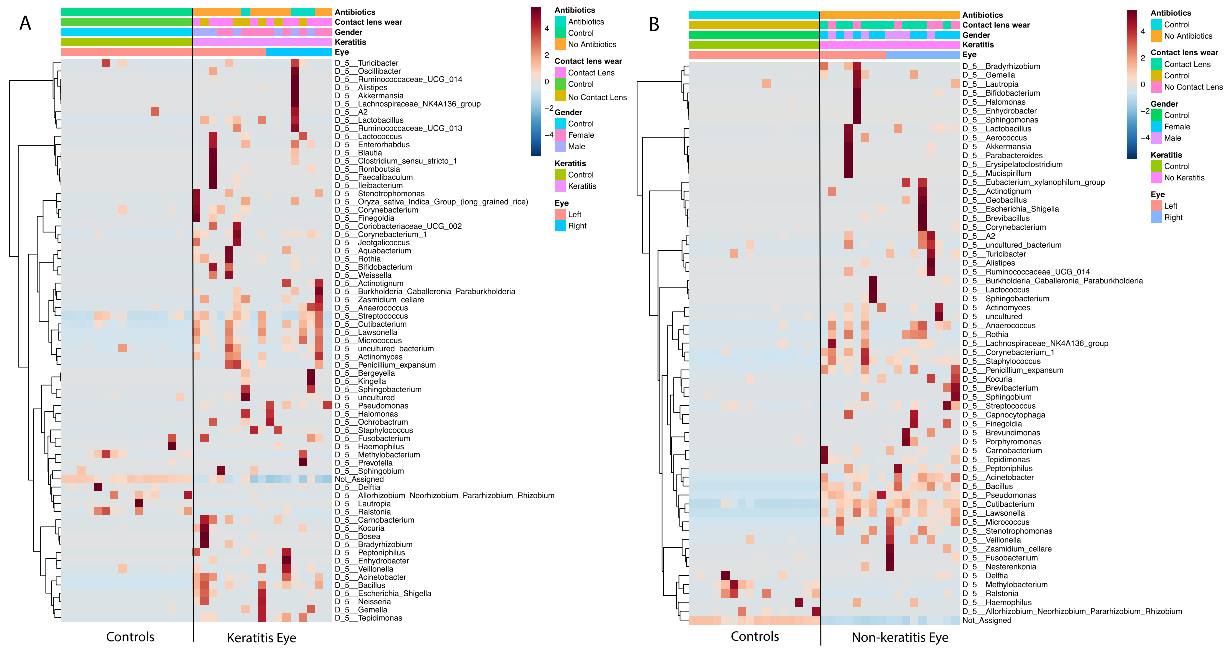

Figure 3. Genus-level heat map with hierarchical clustering showing that there were compositional differences when comparing (A) control eyes to the eyes with keratitis and (B) control eyes to the noninfected eye of patients with unilateral keratitis.

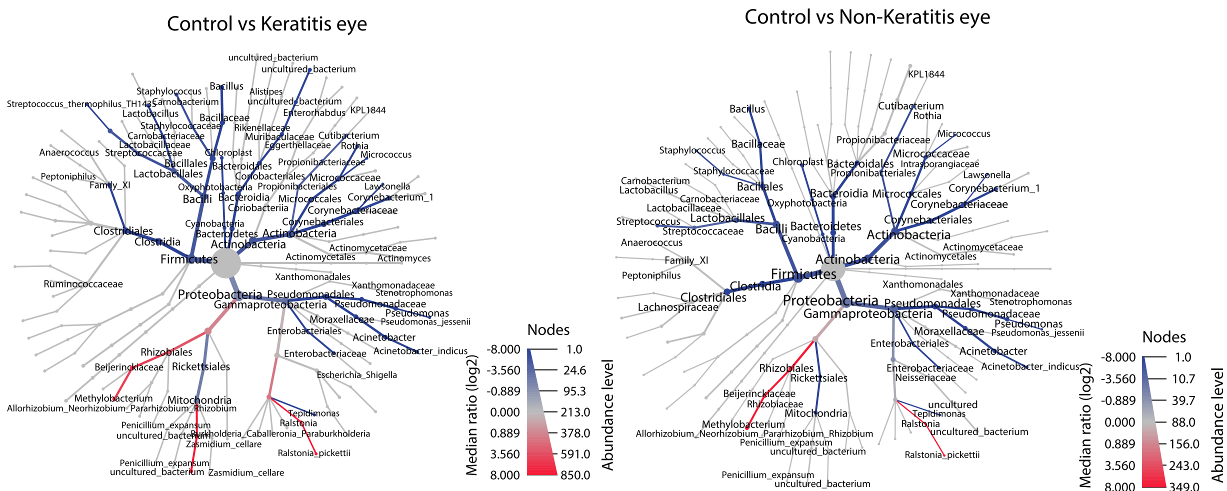

Figure 4. Heat map tree of entire hierarchy from phylum to genus displaying increases in abundance for certain phyla when comparing controls to the keratitis (A) and nonkeratitis (B) eyes.

fold greater in the eyes with keratitis and nonkeratitis compared with controls. The main genera contributing to this difference in composition are shown in Supplementary Figure S2. For example, *Pseudomonas* was more than 700-fold higher in the eyes with keratitis compared with controls. Other genera contributing to the difference included the Actinobacteria genera *Micrococcus*, which was 100-fold greater, and *Cutibacterium*, which was 10-fold greater in eyes with keratitis compared with controls (both $P < 0.0001$), whereas Proteobacteria *Ralstonia* was 5.7-fold lower ($P = 0.0009$). When comparing the nonkeratitis eye to controls, the phyla Proteobacteria, Actinobacteria, and Firmicutes were all more than five-fold greater in the noninfected eyes of patients with keratitis compared with controls. The genera contributing to this difference were similar, with *Micrococcus* and *Cutibacterium* demonstrating a 100-fold and 13-fold higher

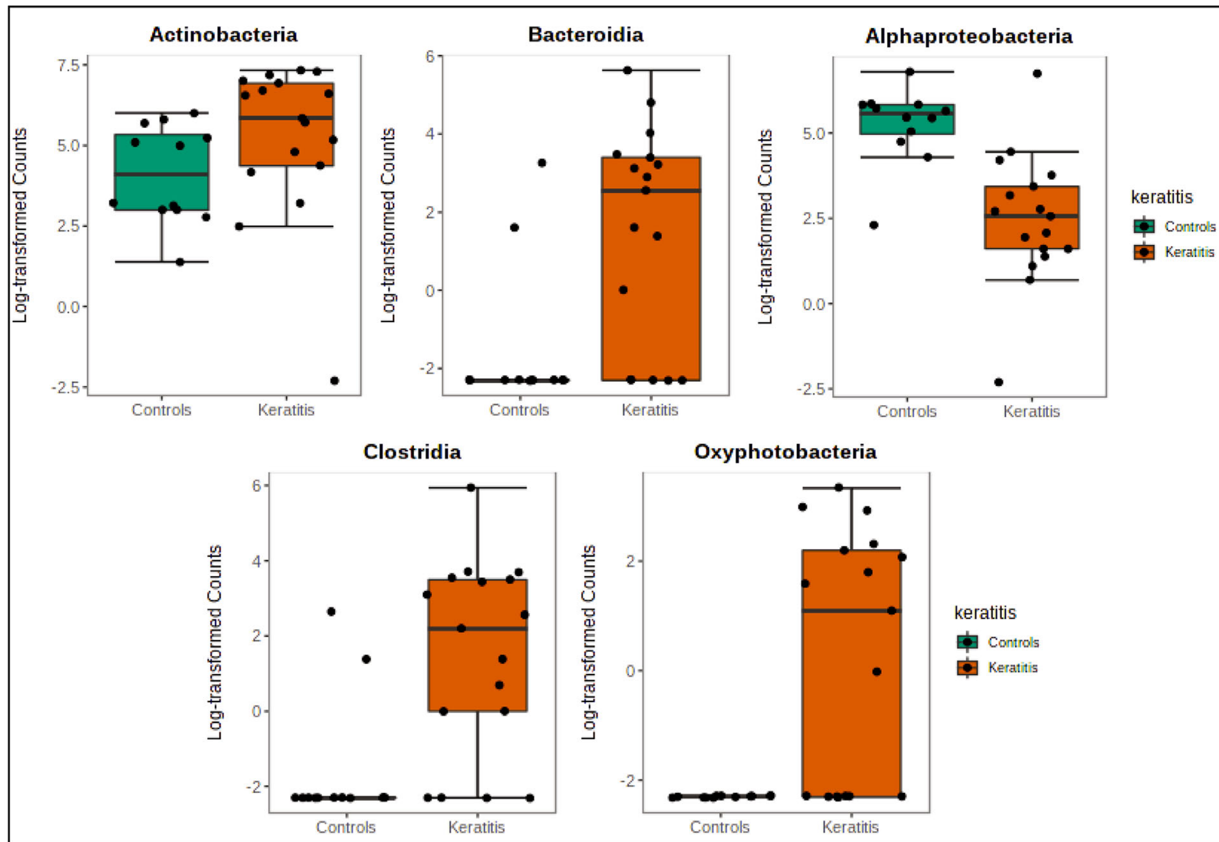

Figure 5. Bacterial classes which significantly differed between the control and keratitis eyes. Test of significance was Mann–Whitney *U* test and *P* values were absolute.

abundance, respectively ($P < 0.001$), whereas *Ralstonia* was 15-fold lower ($P = 0.004$).

Discussion

Our study demonstrated OSM alterations in both eyes (infected and noninfected) of individuals with unilateral keratitis compared with controls. Interestingly, we did not find differences by α diversity metrics or principal coordinate analysis plots between the two eyes of individuals with unilateral keratitis, suggesting that OSM abnormalities are present in both eyes. Overall, we found a greater abundance of potential pathogens and a lower abundance of commensal organisms in both eyes of individuals with unilateral keratitis compared with controls. Additionally, we identified that *Pseudomonas* was more commonly recovered in individuals with unilateral keratitis in contrast to commensal organisms such as *Methylobacterium* and *Ralstonia* in controls.

Our data support the idea that the OSM is an important, yet largely unexplored, contributor to keratitis. Infectious bacterial keratitis occurs when the defense systems of the ocular surface are altered and bacteria can invade. Known risk factors for keratitis include contact lens wear, trauma, prior ocular surface surgery. and immunosuppression, the most common of which is contact lens wear.[2] However, not all patients who have these risk factors develop keratitis. For instance, one individual may develop keratitis after sleeping in his or her contact lenses once, whereas others habitually misuse their contact lenses without negative sequelae. Additionally, when infection occurs, the severity of disease varies between individuals infected with the same organism. The underlying OSM composition may play a vital contributory role in these phenotypic differences.

The concept of the OSM affecting susceptibility to ocular infections has been demonstrated in animal models, because the disruption of ocular surface bacteria enhanced the development of *P aeruginosa* keratitis in mice.[8,9] Despite data from animal models, studies in humans are limited. Similar to

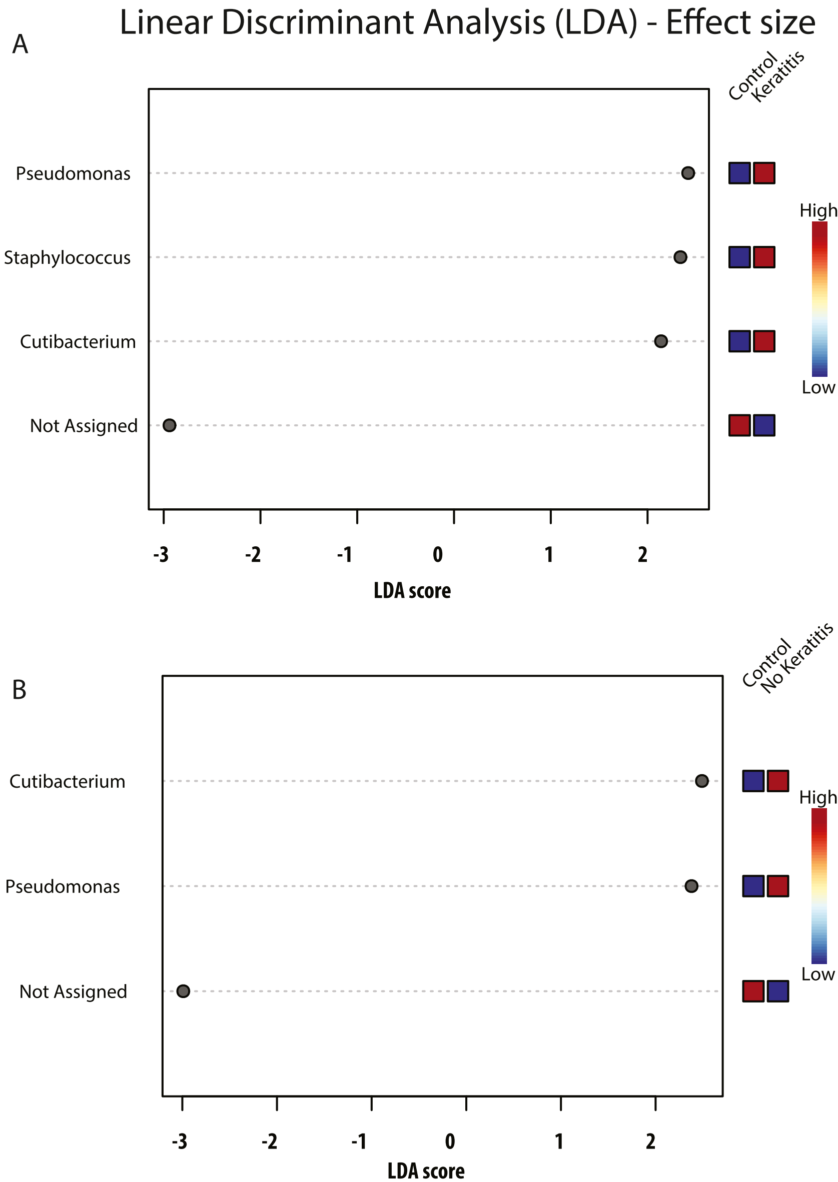

Figure 6. Linear discriminant analysis comparing the control to keratitis (A) and control to nonkeratitis eyes (B), finding a high effect size for *Pseudomonas* in both eyes of infected individuals when each eye was compared with controls.

our findings, a study of eight individuals with unilateral fungal keratitis found that both the infected and fellow eyes showed decreased bacterial diversity with lesser abundances of *Corynebacterium* and *Staphylococcus* and higher abundances of Proteobacteria such as *Pseudomonas*, *Achromobacter*, *Caulobacter*, and *Psychrobacter* compared with controls.[14] The same concept that the relative abundance of commensal organisms plays a role in the pathogenesis of ocular surface infections applied when comparing the OSM in both the infected and noninfected eyes in individuals with keratitis with controls.

Our findings raise the possibility that the OSM can be used to identify individuals more likely to develop keratitis and in those with disease and identify individuals who may suffer worse visual morbidity. In our study, the abundance of certain members of the phylum Proteobacteria was a marker of keratitis identified in the noninfected eye, namely, an increase in *Pseudomonas* and a decrease in *Ralstonia*. Our theory is that some individuals have ocular surface dysbiosis, which can occur owing to a variety of factors (e.g., contact lens use, environmental factors, genetic predisposition) and that these individuals are

predisposed to developing infectious keratitis. This hypothesis is supported by disease models in other organ systems, such as the gastrointestinal tract. For example, *Clostridium difficile* is typically present in low numbers in the healthy adult human gastrointestinal tract. When a disruption such as antibiotic treatment induces dysbiosis, there are alterations in the gut mucus layer, losses of the epithelial barrier, and decreases in the absorption of nutrients. This in turn predisposes the gastrointestinal tract to infection.[15] Just as *C difficile* is a constituent of the healthy gut microbiome, *Pseudomonas* is a normal constituent of the OSM.[5] Similarly, because oral antibiotics induce alterations in the gut microbiome, it is possible that contact lens wear induces alterations in the OSM, allowing a commensal organism to become pathogenic. This insight can be translated to the clinical management of patients. For example, if an individuals is found to have this profile, he or she may be deemed to be at higher risk of developing bacterial keratitis, and therefore the clinician may counsel the patient to avoid certain behaviors such as contact lens use. Also, if the OSM profile implies a severe disease course, patients can be started on more aggressive treatment earlier in the course of disease.

Furthermore, identifying the OSM's contribution to the pathophysiology of bacterial keratitis may lead to new therapeutic avenues. Unlike other mucosal sites, such as the gut, ophthalmologists have the advantage of having direct access to their target organ, making this concept more feasible clinically. The administration of microbes via transplantation, prebiotics, or probiotics has been shown to restore the balance between healthy and pathogenic bacteria in other organ systems, such as in *C difficile* gastrointestinal infections.[16–20] In the eye, this concept can be extended to the topical delivery of medications, with the goal of aiding or accelerating the restoration of OSM homeostasis. Support for this concept has been established in an animal study that found that colonizing the gut of germ-free mice with strains of coagulase negative *Staphylococcus* provided resistance to corneal infection with *Pseudomonas*.[8] Translating to the concept to humans, one study prescribed a mixture of topical artificial tears and oral administration of capsules containing *Bifidobacterium lactis* and *Bifidobacterium bifido* in individuals with dry eye.[21] *Bifidobacteria* were selected given their role in regulation of intestinal homeostasis and modulation of immune responses and their effectiveness as an adjuvant treatment in conditions, such as ulcerative colitis.[22] Encouragingly, Schirmer scores and tear break up times increased compared with controls who only used artificial tears.[21] Topical probiotics have also been applied to vernal keratoconjunctivitis in a four-week study that prescribed topical eye drops containing *Lactobacillus acidophilus* in a dilute saline solution.[23] *Lactobacilli* are nonpathogenic, gram-positive commensal gut bacteria with demonstrated efficacy as a probiotic in various conditions, including atopic dermatitis via strengthening the mucosal barriers, promoting IgA production, and balancing the T helper ratio.[24] Again, encouraging results were seen with improved photophobia, itching, tearing, and conjunctival hyperemia in six of seven patients compared with baseline.[23] Based on these animal and human studies, there may be a role for the topical administration of bacteria as an alternative and/or adjunctive therapy for keratitis.

Our study findings are preliminary and need to be considered in the light of the study limitations. First, although we recruited individuals with suspected bacterial keratitis, this was based on clinical suspicion. Only two of the cultures were positive and thus we cannot be sure that the remaining individuals all had a bacterial process. Second, our populations were not identical with respect to several variables, such as gender and contact lens wear. Furthermore, our sample size does not allow for an evaluation of possible OSM confounders, such as eye-related (e.g., contact lens) or patient-related (e.g., hypertension, diet, exercise) factors. Third, the study is limited by the inherent issues of 16S sequencing, because it may be susceptible to noise, sampling errors, and contamination. Additionally, there is the possibility of error owing to amplification, which can alter the relative abundance of gene sequences. To minimize these effects, we included blank specimens that went through DNA extraction and QIIME analysis and acted as negative controls, and subsequently eliminated any OTUs found in blank samples from further analysis. Finally, 16S does not provide information on the entire OSM, because its species-level coverage is approximately 10% to 15% and it does not take into account the viruses and fungi that inhabit the ocular surface.

Despite these limitations, this study provides an important preliminary step in understanding the role of the OSM in the pathophysiology of keratitis, noting that the OSM was abnormal in the unaffected eye of individuals with unilateral keratitis.

Future Directions

Future studies should be directed at enrolling larger and more diverse patient populations in both the keratitis and control groups to represent the broad

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spectrum of the OSM in disease and healthy states. With a greater number of patients, the analysis of potential ocular and systemic confounders could be examined. Additional studies are also needed to evaluate whether OSM manipulation, via direct delivery of organisms or administration of prebiotics, can restore a healthy OSM and assist in the treatment of infectious keratitis. This approach has been demonstrated in other organ systems and in other ocular diseases. More studies are needed to clarify the contribution of the OSM to keratitis risk and severity, an important question given the potentially blinding nature of the disease.

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