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The Ocular Microbiome Is Altered by Sampling Modality and Age

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Background: Studies of the ocular microbiome have used a variety of sampling techniques, but no study has directly compared different sampling methods applied to the same eyes to one another or to a reference standard of corneal epithelial biopsy. We addressed this lack by comparing the microbiome from three conjunctival swabs with those of corneal epithelial biopsy.

Methods: Twelve eyes (11 patients) were swabbed by calcium alginate swab, cotton-tipped applicator, and Weck-Cel cellulose sponge before a corneal epithelial biopsy (48 samples). We then performed 16S rRNA gene sequencing and universal 16S rRNA gene real-time polymerase chain reaction. Negative/blank controls were used to eliminate contaminants. An analysis was performed to examine the concordance of the three swab types to corneal epithelial biopsy. The effect of patient age on the ocular microbiome as determined by epithelial biopsy was also examined.

Results: The ocular microbiome from corneal epithelial biopsies consisted of 31 genera with a relative abundance of 1% or more, including *Weisella*, *Corynebacterium*, and *Pseudomonas*. Of the three swab types, Weck-Cel differed the most from corneal biopsies based on beta-diversity analysis. Cotton swabs were unable to capture the *Bacteroides* population seen on epithelial biopsy. Therefore, calcium alginate swabs seemed to be the closest to epithelial biopsies. Older patients ($\geq$65 years old) had higher alpha diversity ($P < 0.05$) than younger patients. Differential abundance testing showed that there were 18 genera that were differentially abundant between the two age groups, including *Streptococcus* and eight members of the Proteobacteria phylum.

Conclusions: We demonstrate that ocular sampling method and patient age can greatly affect the outcome of sequencing-based analysis of the ocular microbiome.

Translational Relevance: By understanding the impact of different sampling methods on the results obtained from the ocular surface microbiome, future research on the topic will be more reproducible, leading to a better understanding of ocular surface microbiome in health and disease.

Introduction

The human microbiome functions as a complex organ composed of trillions of microbes and millions of microbial genes that affect the regulation of immune tolerance, metabolism, and epithelial barrier function.[1] The microbiome has been shown to be integral to physiologic processes in the gut and most membrane surfaces, including the vagina, skin, and

mouth. Furthermore, diseases have been associated with microbiota alterations, including perturbation in individual bacterial populations and overall microbial community structure. These diseases have a formidable range depending on the surface and cause of microbial change. For example, changes in vaginal microbiota have been associated with bacterial vaginosis and in the skin with psoriasis, acne, and rosacea.[2,3]

The eye can be the site of numerous chronic inflammatory diseases, some of which are presumed infectious, whereas others are autoimmune. Because the ocular surface is the most superficial structure in the eye and therefore environmentally exposed, it is intuitive that disease processes affecting this structure could be influenced by the local microbiome. The ocular surface has been shown to be colonized with thousands of bacterial species and is dominated by Proteobacteria, Actinobacteria, and Firmicutes. The most common taxa at the genus level were *Pseudomonas, Propionibacterium, Bradyrhizobium, Corynebacterium, Acinetobacter, Brevundimonas, Staphylococci, Aquabacterium, Sphingomonas, Streptococcus, Streptophyta,* and *Methylobacterium.*[4,5] It is known that diseases such as Stevens–Johnson syndrome or fungal keratitis can be associated with changes in diversity and species in the normal conjunctival microbiota.[6,7]

There is a relatively significant void in our current knowledge of the ocular surface microbiome for both healthy and diseased states. Although the interior compartment of the eye is considered a sterile environment that is physically separated from the immune system by a blood–retinal barrier, the external portion (conjunctiva, cornea, sclera, and tear film) is exposed to micro-organisms in the external environment.[8] Although the ocular surface lends itself to bacteria as a potentially viable habitat, it has also been shown to be a low biomass paucibacterial environment owing to the antimicrobial properties of tears and the mechanical actions of the eyelid that are inimical to bacterial colonization.[14] In an attempt to increase the biomass obtained from the ocular surface, tissue biopsy specimens from the conjunctiva removed at the time of pterygium surgery have been used, analyzed, and reported.[9] Along with the technical issues arising from use of low biomass samples, another potential cofounder in our knowledge of the ocular surface microbiome is the effects of different sampling and tissue processing methods, which can lead to varied results from study to study.

The most commonly used methods of ocular surface microbiome characterization consist of traditional microbiological culturing and more recently genomic-based methods of detection and identification. Culture-based methods have proven to be limited in their reliability by the percentage of fast-growing micro-organisms that rapidly cultivate on standard media and subsequently dominate the culture composition.[5,10,11] Thus far, 16S rRNA gene sequencing has yielded the most extensive and diverse characterization of the ocular surface microbiome, although the optimal method of sample collection that will yield the most representative results remains unclear. Using contact lenses has yielded a more diverse composition than culturing, although the presence of the contact lens itself has been shown to produce alterations in the surface microbiota, and therefore is a less reliable representation of the true surface microbiome.[12] Deep swabbing using heavy pressure and a cotton swab on the ocular surface has been shown to obtain more accurate and comprehensive results than light swabs owing to opportunistic and environmental species residing on the surface, while bacteria that may be more integrated in ocular function are localized in the mucosal layer and conjunctival epithelium.[5] Although there has been great progress made in the ideal techniques for ocular surface microbiome characterization, there has been no study to date examining the different methods of sampling head to head. Additionally, the majority of studies dealing with the ocular microbiome have been limited to 16S rRNA gene sequencing and relative abundances. Although comparing relative abundances can provide significant compositional data, it may falsely represent data that only absolute abundances can elucidate more clearly. As such, there is a movement in the microbiome field to transition from relative abundances to absolute abundances.[13] Furthermore, to the best of our knowledge, the cornea epithelium as a significant component of the ocular surface has not been studied specifically for its microbiota through biopsy. In the present study, we provide an initial characterization of the microbiome of the cornea epithelial layer through biopsy specimens and describe the concordance between the cornea epithelial biopsy and the corresponding conjunctival swab of the same eye. Additionally, we also compare our findings with those existing in the literature.

Methods

Patient Selection

Patients undergoing superficial keratectomy and/or removal of their cornea epithelium as part of standard treatments were enrolled in this study. The study complied with the Health Insurance Portability and

Accountability Act and adhered to the tenets of the Declaration of Helsinki. The study was additionally approved by the Institutional Review Board at the David Geffen School of Medicine at the University of California Los Angeles.

Biopsy and Swab Collection

All samples were collected by one of the authors (H.Y.H.). Control swabs were included with each eye sampled, which entailed opening the swab in the presence of the patient and waving it around the patient's head. Sterile cotton-tipped applicators were used in all cases in this fashion to serve as the control. The conjunctiva (inferior fornix and palpebral conjunctival surface) were swabbed after a topical anesthetic (tetracaine hydrochloride 0.5% Alcon Laboratories, Inc., Ft. Worth, TX) was applied. An identical sequence of applicators was used to swab the conjunctiva in all cases: sterile cotton-tipped applicators, sterile calcium alginate swabstick (calgi), and Weck-Cel (weck) cellulose sponge (BVI Medical, Waltham, MA). All applicators swabbed the same conjunctival area. All swabs were immediately placed in a cryotube with DNA/RNA Shield (Zymo Research, Irvine, CA). After the swabs, the eyes were prepped for an aseptic procedure with the application of a topical antibiotic (ofloxacin 0.3% Akorn Inc., Lake Forest, IL) and povidone-iodine (betadine) 5% (Alcon Laboratories, Inc.) before the corneal epithelial removal. Manual superficial keratectomy techniques were used to remove the corneal epithelium. For patients undergoing cornea cross-linking for keratoconus ($n = 3$), the epithelium was first disrupted via direct application of 20% ethanol before it was removed. The addition of this 20% ethanol did not alter the amount of recovered microbial DNA as determined by quantitative 16S rRNA gene polymerase chain reaction (PCR) analysis of these three participants as compared with the rest of the group across the various sample types (cotton $P = 0.81$; calgi $P = 0.84$; weck $P = 0.42$; and biopsy $P = 0.80$). The sheets of removed epithelium were transferred to a Weck-Cel sponge before being placed in a cryotube with DNA/RNA shield (Zymo Research, Irvine, CA). At the conclusion of the procedure, the samples were stored in a −80 °C freezer before they were transferred on dry ice to the laboratory for further processing.

16S rRNA Gene Sequencing

DNA was extracted from swabs and biopsies using the DNA Miniprep kit as per the manufacturer's instructions (Zymo Research). The V4 region of 16S ribosomal RNA gene was amplified and underwent pair-end sequencing (2 × 250) on an Illumina MiSeq (San Diego, CA) as previously described.[14] The sequence reads were processed using DADA2 with default parameters to generate amplicon sequence variants (ASV), as previously described.[15] The average sequence depth per sample was 2496. ASV were assigned taxonomy based on the Silva 132 database. ASV that were present in less than 15% of all samples were removed.

Quantitative 16S rRNA Gene PCR

To quantify the amount of bacterial DNA collected from each sampling vehicle, we performed universal 16S rRNA gene quantitative PCR using a TaqMan-based qPCR approach. Previously published universal primers designed to detect total bacterial 16S rRNA gene were used.[16] The set included the forward primer, 5′-TCCTACGGGAGGCAGCAGT-3′ (T m, 59.4 °C), the reverse primer, 5′-GGACTACCAGGGTATCTAATCCTGTT-3′ (T m, 58.1 °C) and the probe, (6-FAM)-5′-CGTATTACCGCGGCTGCTGGCAC-3′-(TAMRA) (T m, 69.9 °C). Each reaction well contained 5 µL of an iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA), 0.2 µM of forward and reverse primers, 0.25 µM of probe, 1 µL of sample DNA, and PCR-grade water to reach a total volume of 10 µL. Samples were run in triplicates. As a reference, stock *Escherichia coli* sample of a known quantity (1.7×10^{10} colony-forming units) was used to create a standard dilution curve. The PCR reaction was performed using a Bio-Rad CFX384 Touch Real-Time PCR Detection System. The following PCR condition was used: 50 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Data are presented as 16S rRNA gene copy number.

Contamination Filtering

Owing to the low biomass nature of the ocular microbiome, 16S rRNA gene sequencing can potentially lead to false-positive identification of contaminated taxa. To correct for this, contamination filtering was performed similar to a previously published study.[17] ASV abundances were standardized by changing all counts to relative abundance. The relative abundance was then multiplied by total 16S rRNA gene copy number.[17] Negative controls included blank swab controls and nuclease-free water. ASVs that were only present in the samples but not in the negative controls were kept. ASVs that were present only in the negative control and not in the samples were excluded. For

ASVs that were present in both negative controls and patient samples, linear regression was performed. The filter threshold was calculated by using the standard error of the linear regression, multiplying it by 5, and then calculating the exponent.[17] ASVs that did not meet the threshold were excluded from analysis (See Supplementary Fig. S1).

Statistical Analysis

For demographic data, means are expressed in conjunction with their standard deviations. Significance of differences between means was determined using the Student *t*-test. Categorical data were compared using the Fisher exact test. Age was dichotomized into a binary variable using 65 years old as a cutoff.

For 16S rRNA gene sequencing data, samples were rarefied to the lowest read of 572. Alpha diversity metrics including Faith's phylogenetic diversity, Chao1 (a metric of species richness), and the Shannon Index (a metric of both species richness and evenness) were computed using QIIME.[18] Analysis of variance was used to assess significance of differences in alpha diversity measures. A metric of differences between samples (beta diversity) was measured using DEICODE in QIIME2, which implements a centered log ratio transformation and robust Aitchison distance.[19] The DEICODE distance matrix was visualized subsequently by principal coordinate analysis in R.[20] Univariate Adonis, a permutational analysis of variance, was executed using 10,000 permutations to test for differences in beta diversity. Differential abundance testing was performed using DESeq2 in R, which uses an empirical Bayesian approach to minimize dispersion and fit nonrarified count data to a negative binomial model.[21] *P* values for differential abundance were converted to q values to correct for multiple hypothesis testing (0.05 for significance).

Results

Patients

Twelve eyes of 11 patients consisting of 6 females and 5 males with a mean age of 61.5 years were recruited with varying preexisting conditions including band keratopathy (16.7%), Salzman's nodular degeneration of the cornea (16.7%), epithelial basement membrane dystrophy (33.3%), limbal stem cell deficiency (8.3%), and keratoconus (25.0%) (Table). None of the patients were wearing contact lenses at the time of the epithelial removal nor were habitual contact lens users, including the cohort of keratoconus patients. The two patients with band keratopathy both had corneal edema and glaucoma and had undergone prior cataract surgeries. They were both on topical glaucoma medications at the time of epithelial removal. The patient with Salzman's degeneration had no prior ophthalmic surgical history and was not on ophthalmic medications or artificial tears. The sole limbal stem cell deficiency patient had limited, segmental limbal stem cell deficiency from her past

Table. Patient Information

Participant	Eye ID	Eye	Sex	Age (y)	Diagnosis
1	P1	L	M	75	Band keratopathy*
2	P2	R	F	83	Band keratopathy*
3	P3	L	F	70	Salzman's degeneration
3	P4	R	F	70	Salzman's degeneration
4	P5	R	F	82	EBMD
5	P6	R	F	88	LSCD
6	P7	R	M	25	Keratoconus
7	P8	R	M	27	Keratoconus
8	P9	L	F	21	Keratoconus
9	P10	R	M	48	EBMD
10	P11	L	F	80	EBMD
11	P12	L	F	78	EBMD

EBMD, epithelial basement membrane dystrophy;
LSCD, limbal stem cell deficiency.
*Note that for patients undergoing chelation removal of calcium (band keratopathy), the epithelium was removed before exposure to ethylenediaminetetra-acetic acid.

history of multiple glaucoma surgeries. Additionally, she has had an endothelial keratoplasty nearly 4 years prior. At the time of the epithelial removal, she was on glaucoma medications and artificial tears. She had stopped topical steroid 2 years before the epithelial removal procedure. All patients with epithelial basement membrane dystrophy had their epithelium removed to improve their vision owing to irregular astigmatism. None of them had recurrent corneal erosions. Only one of the four patients (participant 4) was not using artificial tears at the time of their procedure. Last, the three keratoconus patients were not on any ophthalmic medications including artificial tears and had no prior ophthalmic histories.

Microbiome of Epithelial Biopsy

When examining the epithelial biopsy microbiome of the 12 eyes, we saw 46 different genera that had at least 1% relative abundance in at least one sample (Fig. 1).The six identifiable genera that had the highest relative abundance across all samples were *Aquabacterium, Weissella, Corynebacterium, FCS020 group, Methylobacterium,* and *Bacteroides*. These 46 genera belonged to 4 phyla: Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes. There was no statistical difference by beta diversity or alpha diversity of the epithelial biopsy by condition.

Effects of Swab Types and Biopsy Collection Methods on Corneal Microbial Diversity and Abundance

There were 461 ASVs present in both the patient sample and negative controls. After contamination filtering, 323 ASVs remained. In the first set of analyses, we assess the beta diversity across the three swab types along with corneal biopsy. There was no statistical significance in beta diversity by sample type, however when one examines the 95% confidence interval ellipse in Figure 2A, the calgi swab and the cotton are similar to the epithelial biopsy in regard to beta diversity. Weck-Cel showed a very different dispersion as compared with the other samples, however this difference did not reach significance. Similar to beta diversity, there was no statistically significant difference in alpha diversity metrics by sample type as

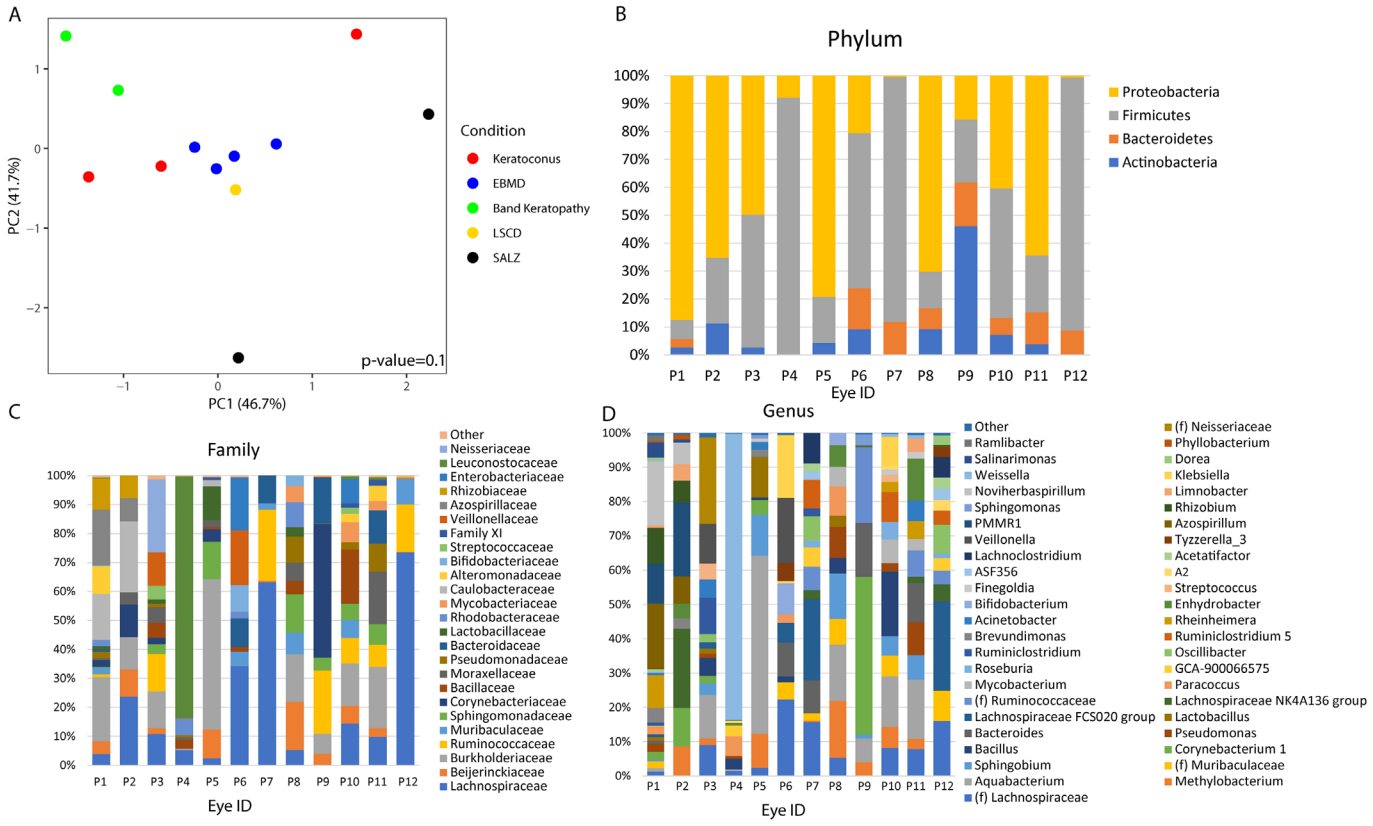

Figure 1. Microbiome of the epithelial biopsy. (A) Principal coordinate analysis plot colored by condition. Taxonomic summary of the epithelial biopsy by each patient on a (B) phylum, (C) family, and (D) genus level.

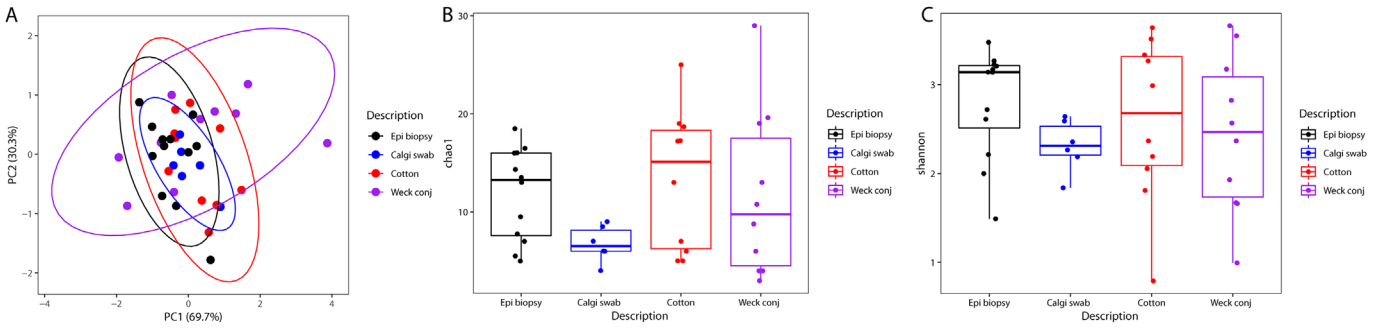

Figure 2. Beta and alpha diversity differences by sampling methodology. (A) Principal coordinate analysis plot colored by sample type with 95% confidence interval ellipse. (B) Boxplot of chao1 (species richness) by sample type. (C) Boxplot of Shannon index (species evenness) by sample type.

shown in Figures 2B and 2C. In both of these analyses, the Calgi swab more closely approximated the epithelial biopsy result as compared with cotton and weck.

As shown in Figure 3, each collection method leads to distinct microbial profiles. There was a total of 42 genera of bacteria with greater than 1% relative abundance across all sample types belonging to 4 different phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. 16S rRNA gene qPCR data shows that epithelial biopsy was able to recover the most amount of bacterial DNA (Fig. 3A). Cotton and calgi swab collected significantly less bacterial DNA than weck ($P = 0.001$). The Weck cel collected a similar amount of bacterial DNA as the epithelial biopsy. Differential abundance testing done by DESeq2 across sample types in relation to epithelial biopsies is represented in Figure 3C–E. There were no differences on a phylum level based on DESeq2 analysis. When comparing the calgi swab with an epithelial biopsy, there were 13 genera of bacteria that were differentially abundant. *Actinomyces* was overrepresented in calgi and 12 other genera were under-represented. When comparing cotton swabs with epithelial biopsy, seven genera were differentially abundant. *Actinomyces* was again overrepresented in the cotton swabs as compared with epithelial biopsy while the other six genera were under-represented. The highest relatively abundant genus was *Bacteroides* and it was under-represented in the cotton swabs as compared with the epithelial biopsies. When comparing weck with epithelial biopsy, there were five total genera that were differentially abundant. *Rheinheimera* was under-represented and four other genera were overrepresented. The highest relatively abundant genus was *Phascolarctobacterium* and it was overly represented in the weck samples as compared with the epithelial biopsy.

Effects of Age on Microbial Diversity and Abundance

In addition to analyzing the microbial composition by sampling methodology, we also examined the effects of age on microbiome diversity and abundance when controlling for sample type. Eight samples were obtained from patients older than 65 years old (78.3 ± 6.3 years old) and four from patients younger than 65 years old (30.3 ± 12.1 years old). To minimize the variability of sampling type by age, we focused our analysis on only epithelial biopsy samples. Although there was no difference by age based on beta diversity, age did have a significant effect on alpha diversity (Fig. 4). There was also no statistical difference between the amount of bacterial DNA recovered in older patients as compared with younger patients by epithelial biopsy ($P = 0.14$). Patients that were older had greater diversity as measured by chao1 and the Shannon index. The taxonomic profile of young and older patients is summarized in Figure 4E. There was no statistical difference by DESEq2 on a phylum level. There were 18 differentially abundant genera between the two age groups, all of which were over-represented in older patients as compared with younger patients. The genera that had a relative abundance of greater than 1% and was statistically over-represented in older patients were *Azospirillum*, *Noviherbaspirillum*, *Phyllobacterium*, *Salinarimonas*, *PMMR1*, and *Lachnospiraceae ND3007 group*, and two unidentified genera belonging to the families Rhizobiaceae and Clostridiales vadinBB60 group. We also analyzed alpha and beta diversity by sex and condition. There was no difference by sex by beta or alpha diversity ($P = 0.30$ and $P = 0.41$, respectively). Similarly, there was no difference by condition by beta or alpha diversity ($P = 0.70$ and $P = 0.48$).

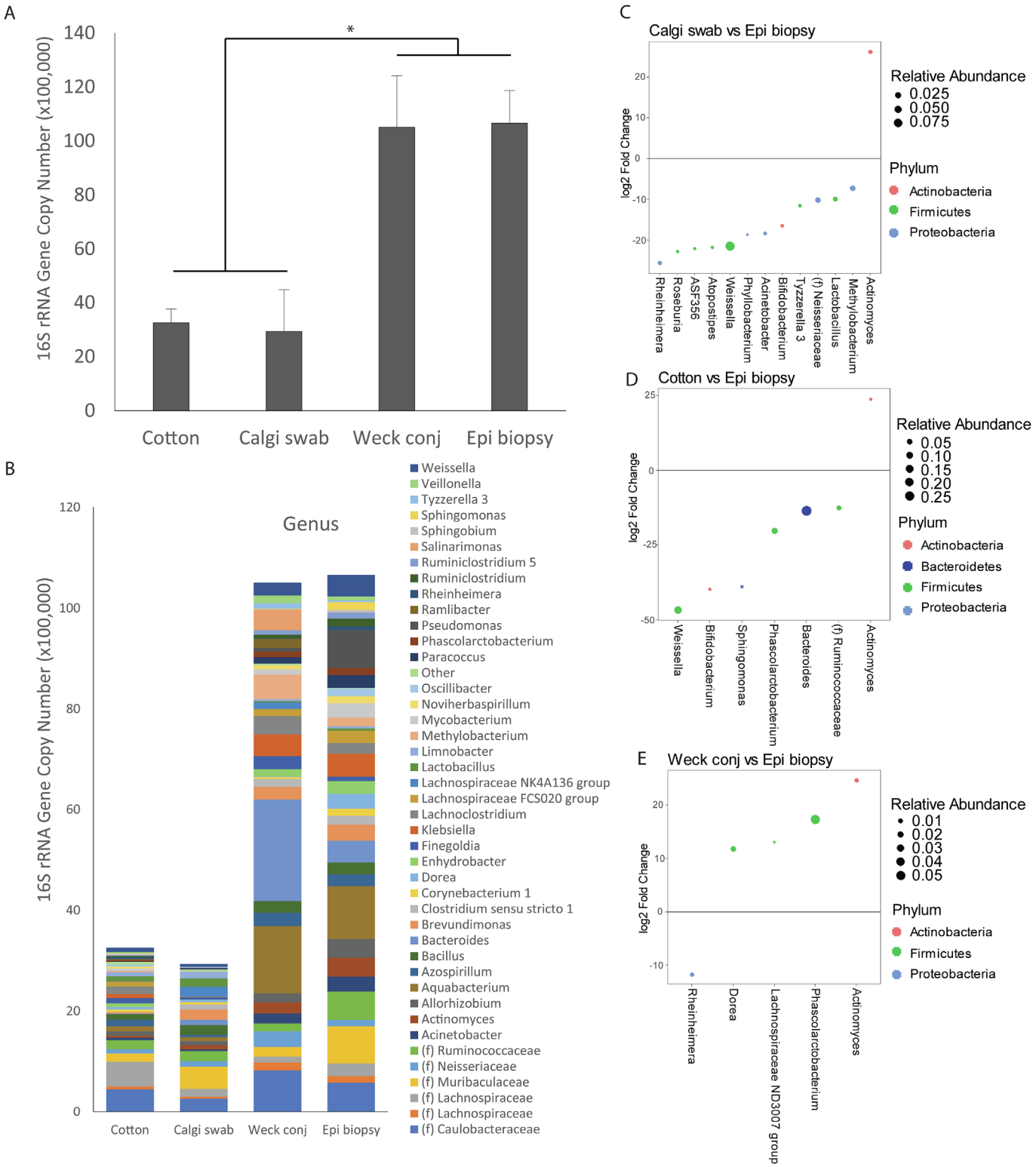

Figure 3. Microbial genera and bacterial DNA recovered differs by sampling methodology. (A) Quantitative 16S rRNA gene copy number by sampling methodology. (B)Taxonomic summary plots by sample type. Only genera with a relative abundance of 1% or more are shown. DESEq2 analysis comparing (C) calgi swabs to with epithelial (Epi) biopsy, (D) cotton swabs to epi biopsy, and (E) Weck-Cel swabs with epi biopsy.

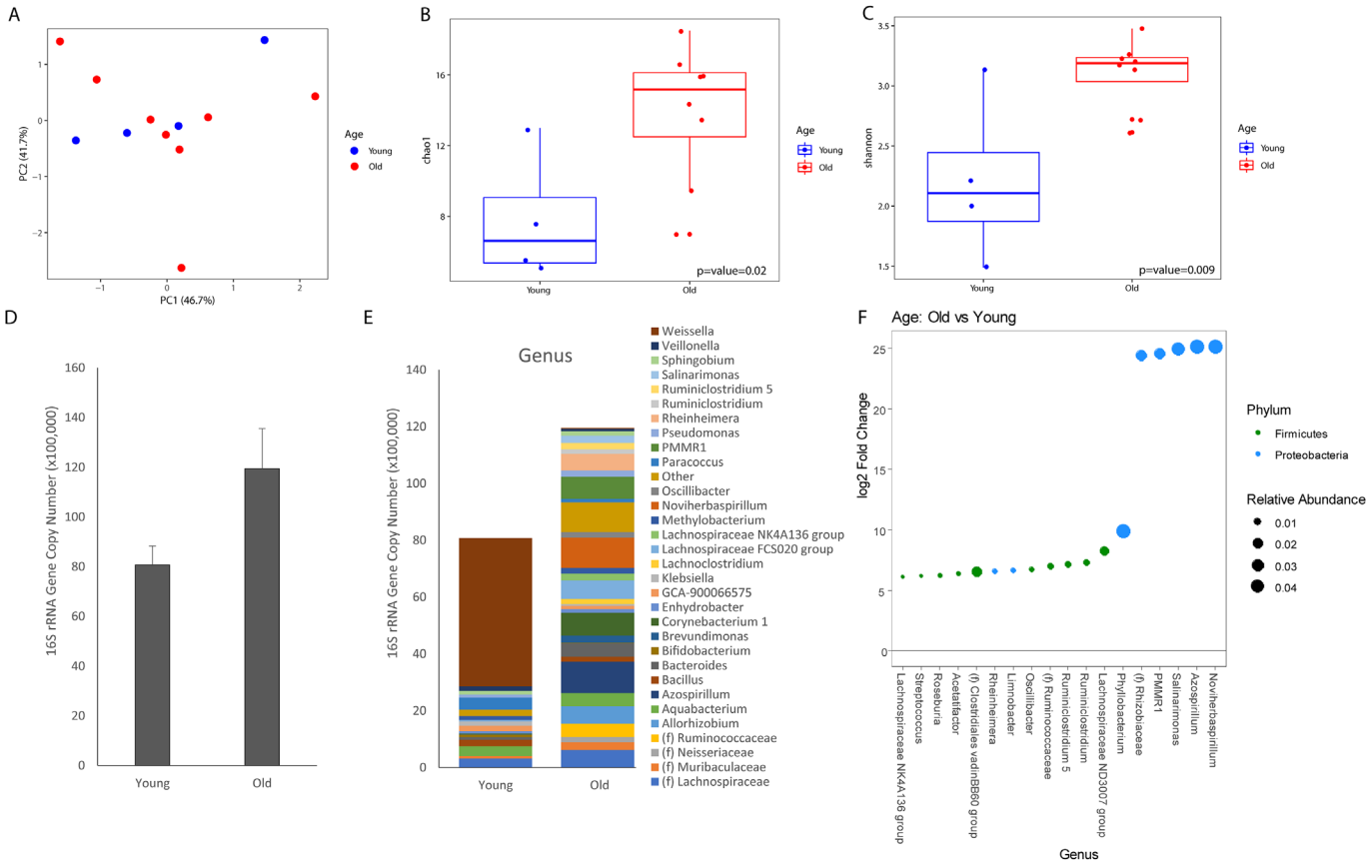

Figure 4. Microbial genera differ by age. (A) Principal coordinate analysis plot colored by age group. (B) Boxplot of chao1 (species richness) by age group. (C) Boxplot of Shannon index (species evenness) by age group. (D) Quantitative 16S rRNA gene copy number by age group. (E) Taxonomic summary plots by age group. Only genera with a relative abundance of 1% or more are shown. (F) DESEq2 analysis comparing old with young samples.

Discussion

We have shown that an analysis of the ocular microbiome is affected by many different factors, such as sampling strategies, age, and the use of proper controls. Unlike the intestinal microbiome, the ocular surface has a very low biomass and so DNA contamination can greatly impact the results. DNA contamination can occur at any point from sample collection to DNA extraction and sequencing library preparation.[22] Contamination can come from personnel, equipment, and even reagents.[17,22] Despite adequate measures to control for possible contamination, sequences derived from contaminating DNA are likely to be present in ocular surface microbiome datasets. Therefore, we believe that it is imperative to use suitable controls throughout the process to control for potential false positives in low biomass samples. In our study, we used nuclease-free water and a cotton swab that was only present in the air around the patient as negative controls. In a similar fashion to Ozkan et al,[17] we removed ASVs that were only present in the negative control and ASVs that did not meet the threshold of our linear regression. Applying this approach, we decreased our number of unique ASVs from 461 to 323. Although other studies have reported higher numbers of unique bacterial sequences or operational taxonomic units, as high as 7300, these studies did not take into account for possible contamination.[23] The low number of ASV is consistent with the idea that the ocular surface is a relatively sterile environment with a restricted set of resident microbes. Our findings and number of ASVs is consistent with previously published works that have included rigorous filtering steps for possible contamination.[17]

Even after controlling and filtering out contaminants, there is still a rigorous debate in the field as to what constitutes the normal ocular microbiome.

Similarly, it is not settled whether the various components of the ocular surface (eyelid margin, conjunctiva, tear film, cornea surface) harbor the same microbiota. The majority of studies examining this area have relied on conjunctival swabs to sample the ocular surface microbiome as an extension of the conventional microbiological protocol. However, there remains a lack of uniformity in how it is done across studies which we feel is relevant in the context of a low biomass environment. As an example, others have noted that the pressure by which patients are swabbed and the type of swabs may affect the outcome.[5] Similar to a study by Ozkan et al, in which they compared fornix and limbus conjunctival biopsy to conjunctival swabs, we showed that corneal epithelial biopsy result is different from swabs.[24] In our corneal epithelial biopsy samples, *Weisella*, *Corynebacterium*, Lachnospiraceae, and *Aquabacterium* made up the genera with the highest relative abundances. In the study performed by Ozkan et al, only eight genera had a relative abundance of 1% or more on fornix and limbus biopsy.[24] Of the eight genera, *Pseudomonas, Acinetobacter, Veillonella,* and *Thermoanaerobacterium* were the most common genera found on fornix and limbus conjunctival biopsy. In our study, we found 31 genera that had a relative abundance of 1% or greater, including six of the eight genera reported by Ozkan et al, such as *Pseudomonas*, *Acinetobacter*, and *Veillonella*. *Thermoanaerobacterium* and *Geobacillus* were the only two genera that were not captured in our analysis. In our study, we implemented different sampling methods and the most up-to-date microbiome analysis using sequence variants instead of operational taxonomic units, which might explain the higher sensitivity in our study as compared with the study by Ozkan et al in 2018. This difference could also be explained by the difference between corneal biopsies and fornix/limbus biopsy. In culture-based methods *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* are the most commonly seen organisms in the healthy eye.[25] Although our finding of *Corynebacterium* is consistent with previous culture-based methods, the likely reason why *Staphylococcus* and *Propionibacterium* were not seen in our cohort is because of the rigorous filtering step that we performed to minimize possible contamination. In our sample, *Staphylococcus* was present at a relative abundance of 3.1% and *Propionibacterium* was present at a relative abundance of 0.003%. However, these genera were also highly prevalent in our control samples and did not meet the cutoff to be ruled out as a possible contaminant. It is possibly that these genera would be present in our analysis if we were less stringent; however, the downside to that would be the introduction of false-positive organisms. We are aware, however, that an epithelial biopsy is not practical in all patients. For that reason, as part of our study, we examined how various sampling transport media and methodologies can affect microbial analysis with epithelial biopsy as a reference.

At least for the purposes of the present study, assuming that the corneal epithelium harbors the reference microbiota, when we evaluated the various swabbing transport media and methodologies, we found that no individual transport media was able to perfectly mimic the results from corneal epithelial biopsy. However, we believe that weck results were the most different. Given the low biomass on the conjunctiva, we would expect potentially different recovery rates of bacterial DNA, with Weck-Cel being the least as it was the last in the sequence to be used. However, Weck-Cel had the highest amount of bacterial DNA recovered. This finding would suggest that the sequence by which the collection was done was not as important as the collection material itself. Even though the Weck-Cel recovered as much bacterial DNA as the epithelial biopsy, the composition seemed to be different from that of the epithelial biopsy. Based on principal coordinates analysis, it seems that Weck-Cel swabs had the greatest difference from epithelial biopsy as compared with either calgi or cotton swabs. This outcome was driven potentially by an overabundance of *Bacteroides* and *Phascolarctobacterium* in the Weck-Cel samples as compared with the epithelial biopsy samples. Based on beta-diversity analysis, it seems that cotton swabs or calgi swabs are able to mimic the composition of epithelial biopsy. However, through differential abundance testing, we see that cotton swabs were unable to capture the *Bacteroides* population that was seen in relatively high abundance on the epithelial biopsy. For these reasons, we believe that calgi swabs are the most representative of the microbiome seen from epithelial biopsies. Although the composition of the major genera is present in the calgi swabs, the calgi swabs did, however, have the lowest average species richness as determined by the chao1 index. This factor could mean that calgi swabs may not be an ideal sampling method for rare or low abundant species on the eye. That being said, as a limitation of our study, we cannot absolutely rule out that the differential recovery of organism diversity between different sampling media was influenced by the sequence in which they were performed, as detailed in the Methods section. Even though we are recovering genetic material and not viable organisms for conventional microbiological assays, the sequence as used in this study could potentially predispose the Weck-Cel samples to yield a different spectrum of organisms. Future studies such as randomization of the sampling sequence or compar-

ing the same material used multiple times could address this issue.

In addition to understanding how sampling methodology and contamination can affect the analysis of the ocular microbiome, we also examined how ocular microbiome varies by age. Understanding that our cohort had different underlying corneal conditions, depending on the participant's age, our analysis shows that there were significant age-related differences in the microbiome, especially by alpha diversity metrics. Although this finding is novel for the eye, similar changes have been noted for the microbiome, including the gut. There is some established observed natural maturation process of the gut microbiome and standard changes that happen with time.[26] One study in particular noted that alpha diversity could be changing with age owing to the weakening of the intestinal barrier with age, causing an increase in inflammatory response and accordingly the increased presence of *Blautia* and Lachnospiraceae.[27] Perhaps with age there is a similar weakening of particular tissues in the eye, which could potentially increase the exposure to the environment, reduce the eyes ability to physically decrease the inhabitance of opportunistic and environmental species, and potentially increase inflammatory response. Although we did not see any differences in the ocular microbiome by condition, possibly limited by our small sample size, there are several studies that have shown differences in the conjunctival microbiome of patients with Stevens–Johnson syndrome and Sjogren's syndrome, dry eyes, and contact lens wearers.[28–31] In patients, with Stevens–Johnson syndrome, the conjunctival microbiome showed an increase in pathogenic bacteria such as gram-negative bacilli, *Corynebacterium,* and *S aureus*.[28,31] Additionally, the natural aging process predisposes the patient to be exposed to systemic and topical ophthalmic medications which could further alter our findings and the resident microbiota. Excepting active usage of glaucoma medications in three of our patients and artificial tears in an additional three as detailed previously, none of the patients were actively using nor had been using topical or systemic antibiotics. That being said, historic medication exposures, as well as past surgical history may have an influence on a patient's resident microbiota which we are unable to elucidate in our study.

As the field of the ocular microbiome expands, it will become important for optimal sampling methodologies to be identified and used consistently across studies. Although our study's main limitations are its low number of patients and their heterogeneity, it is the only study we know of that compares different sampling methodologies within a single patient. It is also one of the few studies that also used corneal epithelial biopsy as a way to collect microbiome samples and universal 16S rRNA gene quantitative PCR to determine absolute bacterial values as opposed to relative abundances. Even with its low sample size, we show that the methods by which patients are sampled and the age at which they are sampled can greatly affect the outcome and analysis of the ocular microbiome. Although we did not see differences in beta or alpha diversity by sex or condition, this finding may have been due to our small sample size. Larger studies will be needed to validate these findings. How these microbial populations affect ocular health and disease and how they change over time within an individual should be the subject of future research.

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