Article

Composition and Comparison of the Ocular Surface Microbiome in Infants and Older Children

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Purpose: Unlike other microbiomes of the body, the composition of the ocular surface microbiome (OSM) in children has yet to be thoroughly explored. Our goal was to evaluate the OSM in young infants and compare its composition to older children using both culture dependent and independent methodologies to assess for differences with age.

Methods: Prospective, observational, cross-sectional study of children <18 years of age at a university-based institution. The mucosal surfaces of both eyes, nose and throat were swabbed with a forensic-quality swab. Half of the swab was plated for culture and the other half underwent 16S sequencing. Culture results and microbiome diversity were analyzed.

Results: Fifty patients (mean age 37 months, range 1–168 months) were enrolled. Forty-seven eyes of 30 patients had positive cultures; four eyes grew >1 species. Culture positive patients were older (43 vs. 29 months, $P = 0.19$). Additionally, older children had greater diversity than children under 6 months of age by 16S sequencing ($P = 0.05$). *Staphylococcus* species were predominant by culture (35/52 isolates) and by 16S sequencing. The OSM was fairly similar to the nose microbiome, whereas the throat microbiome differed significantly and had a higher abundance of *Streptococcaceae* ($P = 0.001$).

Conclusions: The OSM is predominantly composed of *Staphylococcus* species in children, as demonstrated by both culture dependent and culture independent methods. Older children were more likely to have growth on culture and have more a complex bacterial milieu with 16S sequencing.

Translational Relevance: 16S sequencing provides more robust information regarding the composition of the microbiomes than culture dependent methods.

Introduction

Bacteria play an important role in maintaining health throughout the human body. These microorganisms outnumber human cells by a ratio of 10 to 1 and contribute more essential genes for survival than human cells. Whereas the human genome carries only approximately 20,000 genes, the microbiome contributes approximately 8 million genes.[1] In the gastrointestinal tract alone, there are 100 trillion organisms with a genome more than 100 times the size of the human genome.[2]

The microbiome, however, is not uniform throughout the body. The intrinsic factors at each site, such as the oxygen levels and pH, influence the bacterial community.[3] Examples of these niches include the vaginal mucosa in which *Lactobacillus* is the dominant species and the oral mucosa in which *Streptococcus* is the most prevalent species.[1] Microbial interactions further contribute to the development of unique communities via mutualism or competition.[3] Finally, external environmental factors such as aging, surgery, and antibiotics have the potential to shape the microbiome, as these factors could potentially select for or inhibit the growth of certain species.[4]

While studies have examined the composition of the microbiomes in gut, mouth, and vaginal muco-

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sae,[1,5] these concepts have not yet been applied to the study of the ocular surface microbiome (OSM). Early investigations of the OSM relied on culture media; however, traditional culture reveals bacterial growth in only approximately 50% of cases.[6–12] The reason for this low yield may be attributable to the paucibacterial nature of the ocular surface that is more tightly controlled compared to other mucosa, such as the gastrointestinal tract.[13] The difficulty in revealing a culturable microbiome may also be due to an inability of culture techniques to provide a suitable substrate or a lack of a complex network of bacterial messaging to facilitate growth.[14]

When cultures of the ocular surface do reveal bacterial growth, studies found that coagulase-negative *Staphylococci* are the predominant bacteria of the conjunctiva, with contributions from *Cornyebacteria* and *Propionibacteria*.[15] However cultures often fail to demonstrate a complex network of bacterial species on the ocular surface, as they rarely capture more than one organism.[16] Studies that examined bacterial genetic material, such as 16S sequencing, reveal that the ocular surface bacterial flora is more diverse than was recovered with culture techniques.[15–17] Using culture independent methods in healthy adult volunteers, bacteria can be identified in every specimen.[16] Additionally, the technology detected DNA corresponding to more than 50 genera with at least three times higher diversity in the number of bacterial genera than culture-dependent methods.[16,18] As with culture dependent methods, 16S sequencing has found that *Staphylococcus* species are the most abundant organisms on the ocular surface.[16,18,19] As 16S sequencing allows improved detection of microbial species, the technique reveals a relatively consistent "core" of a certain bacterial genera. This core consists primarily of *Staphylococcus* spp., *Propionibacterium* spp., *Corynebacterium* spp., *Streptococcus* spp., and *Haemophilus* spp.[19] Other studies suggest there are smaller and/or variable contributions from genera such as *Bradyrhizobium*, *Pseudomonas*, *Acinetobacter*, and *Ralstonia*.[16,19–21]

16S sequencing identifies not only a core microbial community, but also differences in OSM in pathologic states including dry eye syndrome. Individuals with Sjögren's syndrome have been found to have higher bacterial loads on the ocular surface compared to controls.[22] In addition, studies have found an inverse correlation between disease severity and microbial diversity in Sjögren's syndrome.[23] These findings suggest that ocular disease is associated with dysbiosis and that disease severity is correlated with changes in the microbiome. Specifically, more severe ocular disease may be characterized by a relatively lower abundance of commensal bacteria and higher relative abundance of potentially pathogenic bacterial genera.[23]

Despite prior work in adults, few studies have looked at the OSM in children. In the limited studies that have been performed, cultures have been used most often to characterize the microbiome. These studies found that gram-positive organisms such as *Staphylococcus epidermidis* and *Propionibacterium* spp. are the predominant microorganisms.[12] Contrary to the thought that environmental factors alter the microbiome, daily contact lens wear did not appear to change the microbial community. However, these studies only focused on older children (>8 years) and did not include infants or toddlers. In one study using 16S sequencing, authors differentiated the patients into age groups ≤10 years and >10 years. They found increased richness (number of taxa) and diversity (relative abundance) in younger patients, who had an average of 20 more genera than older participants. They also found the abundances of five genera (*Streptococcus, Kocuria, Staphylococcus, Micrococcus,* and *Brachybacterium*) were significantly higher in the ≤10-year age group.[21] Despite these findings, the study did not specify an age range of the participants to clarify if infants and toddlers were included, or do a subgroup analysis in the ≤10-year group.

This demonstrates there is a gap in the existing literature regarding how the OSM develops in early childhood. Therefore, we sought to evaluate if the ocular surface of infants differs from that of older children and if environmental influences in childhood (e.g., ocular surgery) disrupt the OSM. In this paper, we examine these questions and test our hypothesis that the bacterial abundance and diversity of the OSM increases with age, similar to other mucosal surfaces of the body.[24–29] Furthermore, we evaluate whether individuals with a history of ocular surgery have differences in their microbiome compared to surgery naïve children. Finally, to study the uniqueness of the ocular microbiome, we compare the microbiome profile of the eye with the nose and pharynx.

Methods

A prospective, cross-sectional, observational study of children <18 years old at a university-based institution was conducted with approval by the University of Miami Institutional Review Board

(Protocol ID 20140717). The study was conducted at Bascom Palmer Eye Institute in accordance with the principles of the Declaration of Helsinki and was Health Insurance Portability and Accountability Act compliant.

During a 6-month period, all children (<18 years of age) scheduled for a visit in the pediatric ophthalmology clinic at Bascom Palmer Eye Institute were invited to participate. After recruitment, a written consent was obtained from patient or parent/guardian and a written assent was obtained from children 7 to 17 years old after explanation of the nature and possible consequences of the study. A review of medical records was performed to record pertinent background information including age, sex, birth history, ocular history, past medical history, and surgical history. Any use of ocular and/or systemic medication use was documented, particularly the use of antibiotics, corticosteroids, or immunomodulatory drugs. Exclusion criteria included any signs of obvious ocular surface disease/irritation, active infection, skin disease, concurrent contact lens use, or administration of oral or topical antibiotics or topical immunosuppressants within the prior 90 days.

All specimens were collected by the study personnel in a standardized fashion using sterile gloves. During collection, a forensic-quality cotton applicator was passed four times along the surface. The collection process was performed in the following sites: right inferior conjunctival fornix, left inferior conjunctival fornix, right nasal mucosa, and pharyngeal mucosa. No anesthetic was used prior to collection. The applicator was subsequently placed in an individual tube containing thioglycollate broth and labelled with a unique study identifier. Special care was taken to avoid touching other ocular structures to avoid contamination. This process was repeated until collection from all sites was completed. The collected specimens were then immediately transported by study personnel directly to the microbiology laboratory and stored in a −80°C refrigerator.

Culture

In the microbiology laboratory, one-half of the swab was plated for culture onto chocolate agar, 5% sheep blood agar, and enriched thioglycollate broth. Plates were incubated at 35°C in a CO_2 incubator and observed for growth for a period of 7 days. Isolates were identified using standard microbiological protocols and in vitro susceptibility was determined using a combination of breakpoint minimal inhibitory concentrations, E tests (Vitek; BioMerieux, Durham, NC) and disk diffusion (BD Diagnostics, Franklin, NJ) in accordance with Clinical Laboratory Standards Institute protocols.

16S Sequencing

The other half of the swab underwent DNA extraction and 16S sequencing. The specimens were sent to an outside service, Second Genome, Inc. (San Francisco, CA). Nucleic acid isolation was performed with the MoBio PowerMag Microbiome kit (Carlsbad, CA) according to manufacturer's guidelines and optimized for high-throughput processing. All samples were quantified via the Qubit Quant-iT dsDNA High Sensitivity Kit (Invitrogen, Life Technologies, Grand Island, NY) to ensure that they meet minimum concentration and mass of DNA. To enrich the sample for bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primers designed against the surrounding conserved regions that are tailed with sequences to incorporate Illumina (San Diego, CA) adapters and indexing barcodes. A pool containing 16S V4 enriched, amplified, barcoded samples was loaded into a MiSeq reagent cartridge (Illumina, San Diego, CA) and then onto the instrument along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing. Sequenced paired-end reads were merged using USEARCH and the resulting sequences compared to an in-house strains database using USEARCH (usearch_global). All sequences hitting a unique strain with an identity ≥99% were assigned a strain Operation Taxonomic Unit (OTU). To ensure specificity of the strain hits, a difference of ≥0.25% between the identity of the best hit and the second-best hit was required (e.g., 99.75 vs. 99.5). For each strain OTU, one of the matching reads was selected as representative and all sequences will be mapped by USEARCH (usearch_global) against the strain OTU representatives to calculate strain abundances. Representative OTU sequences were assigned taxonomic classification via mothur's Bayesian classifier, trained against the Greengenes reference database of 16S rRNA gene sequences clustered at 99%.

All samples were processed in a Good Laboratory Practice-compliant laboratory to ensure the quality and integrity of the data. Quality control was ensured by including negative controls. Negative controls consisted of a DNA-free DNase-free water blank. The controls were carried through polymerase chain reaction amplification, amplicon quality control,

library pooling, and sequencing. The sequences were processed only if there was no amplification detected in the negative control. Finally, data quality filters on the raw sequences were done with Illumina software. Any OTUs found in the negative control were filtered out of the data analysis.

Statistical and Bioinformatics Analysis

Microbiome analysis was performed by Second Genome, Inc. using Adonis, a nonparametric multivariate analysis of variance.[30] Additional statistical analysis utilized the Statistical Package for the Social Sciences software (SPSS, Inc., Chicago, IL). *T*-tests and analysis of variance were used to compare between groups. Categorical variables were compared with Fisher's exact test. A Pearson correlation was used to determine the relationship between variables such as age and prior antibiotic use. A *P*-value of ≤0.05 was considered statistically significant.

Results

Study Population

Fifty children (62% male) were enrolled. The mean age was 37 months (SD ± 36 months, range 1–168 months). Approximately one-third of the patients (30%) had no prior surgery, whereas 32% had unilateral and 38% had bilateral surgery. There was no statistically significant difference in the age of the surgical groups (32.3 ± 30.2 months in the nonsurgical group versus 39.5 ± 39.2 months in the surgical group, $P = 0.52$).

Culture Dependent Methods

Thirty (60%) patients had a positive culture in at least one eye; 18 (36%) of patients were culture-positive in both eyes. Cultures from four eyes from different patients grew >1 species, with the culture from the contralateral eye growing either one ($n = 2$) or no ($n = 2$) species. Of the 52 total isolates, *Staphylococcus* spp. were the most common (35/52) followed by *Streptococcus viridans* (10/52). Although individuals with positive cultures were older than individuals with negative cultures (43 months SD 41.8 months versus 29 SD 25.6 months), the difference was not statistically significant ($P = 0.19$). Prior surgery did not correlate with culture growth ($P = 0.71$). Neither gender nor ethnicity correlated with culture growth ($P = 0.75$ and $P = 0.58$, respectively).

16S Sequencing

Bacterial DNA was recovered from all eyes, nasal mucosa, and pharyngeal mucosa of all patients (100 eyes of 50 patients, 50 nasal mucosa, 50 pharyngeal mucosa) using 16S sequencing. The maximum total number of de novo raw OTUs was 776, which was decreased to 745 after filtering. The total number of reads was 70,060,846, with a combined number of sequences (total filtered reads + strain hit reads) of 32,515,209 and 31,356,744 sequences after removal of OTUs unclassified at the kingdom level. Filtering reduced the final number of OTUs to 551. OTUs from the sequences that passed sample quality check were used for downstream analyses. One hundred percent of sequences were classified at the kingdom level, 99.97% at the phylum and class levels, 99.95% of sequences were classified at order, 99.45% at family, 92.28% at genus, 1.413% at species, and 1.664% at strain levels (Supplementary Fig. S1). Rarefaction curves reached saturation, which is indicative that samples were sequenced to sufficient depth and composition from samples from all conditions were likely captured. The top five most abundant phyla (in order of abundance) were: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, and *Bacteroidetes*. The top seven most abundant families (in order of abundance) were: *Staphylococcaceae, Streptococcaceae, Corynebacteriaceae, Moraxellaceae, Enterobacteriaceae, Oceanospirillaceae*, and *Bacillaceae*.

No differences were noted between right and left eyes (Supplementary Fig. S2). The OTU richness for the right eye was 80.6 (SD 30.8) alpha diversity units/condition and the left eye was 79 (SD 26.2) ($P = 0.49$); similarly, the Shannon diversity was 0.632 (SD 0.877) in the right eye compared to 0.585 (SD 0.722) in the left eye. The proportional abundance for the top three bacterial classes at the family level was similar between the eyes (right versus left eye): *Staphylococcaceae* (57.8 vs. 55.1), *Streptococcaceae* (12.7 vs. 21), and *Corynebacteriaceae* (8.76 vs. 3.73).

Similar to culture dependent methods, older infants and children (>6 months old) tended to have greater diversity of the OSM than young infants (<6 months old). Of the 264 OTUs tested, 75 were significantly different. Forty-two of the 75 significantly different OTU were enriched in children older than 6 months. The phylum breakdown for these significantly different OTUs in the older children included eight enriched in the *Actinobacteria* phylum, 18 enriched the *Firmicutes* phylum, and 16 enriched in the *Proteobacteria* phylum. Of note, older children

also had 21 reduced OTUs in the *Firmicutes* phylum and six reduced OTUs in the *Proteobacteria* phylum when compared to young infants. Of the 75 significant OTUs, 18 were able to be identified at the strain level of which 14 were enriched in the over 6-month age class. They included three strains from *Corynebacterium* (*C. tuberculostearicum, C. bovis,* and *C. mastitidis*) and two strains from *Psychromonas* (*P. arctica* and *P. ingrahamii*). The observed alpha-diversity estimate was lower in children >6 months old compared to young infants (84 ± 19.4 vs. 93.1 ± 15.3); however, the Shannon diversity was higher in older children (0.986 ± 0.903 vs. 0.458 ± 0.59). The percent proportional abundance of the top three families also differed between older (>6 months) and younger (<6 months) groups: *Staphylococcaceae* (44.1 ± 45.9 vs. 58.2 ± 45.5), *Streptococcaceae* (18.7 ± 29.5 vs. 23.5 ± 40.7), and *Moraxellaceae* (3.49 ± 7.19 vs. 12.5 ± 26.6); however, these differences were not statistically significant. Older children did have statistically significant differences in the relative abundance of the *Oceanospirillaceae* (7.32 vs. 0), *Listeriaceae* (4.42 vs. 0), *Psychomonadaceae* (2.57 vs. 0.002), and *Leuconostocaceae* (2.07 vs. 0) ($P < 0.001$ for all, Kruskal-Wallis rank sum test). There were significant beta diversity differences between the two age classes ($P = 0.05$), regardless of factors such as sex or race.

When comparing OSM of the eye to that of the nose and the throat, the eye microbiome was more similar to the nose than the throat. Throat samples had higher OTU richness than eye samples and differed significantly (Fig. 1 and Supplementary Fig. S3). Sixty-six of the 137 significantly different OTUs were enriched, with greater *Bacteroidetes, Firmicutes, Fusobacteria,* and *Proteobacteria* in the throat. Thirty-three of the 137 significant OTUs were able to be identified at the strain level. There were three strains from *Staphylococcus* (*S. condimenti, S. devriesei,* and *S. aureus*) and three strains from *Corynebacterium* (*C. tuberculostearicum, C. propinquum,* and *C. bovis*) enriched in the eye samples. There were three strains from *Streptococcus* (*S. HKU30, S. uberis,* and *S. infantis*) and five strains from *Prevotella* (*P. veroralis, P. nanceiensis, P. pallens, P. sp. oral taxon 299,* and *P. salivae*) enriched in throat samples. Weighted ordination using abundance reveals most samples separated according to mucosal type ($P = 0.001$). Wilcoxon signed rank test performed on alpha diversity measure of the sampled mucosa and revealed no significant differences between the OTU richness of the right versus left eye ($P = 0.36$) or the eye versus nose ($P =$

0.28); however, there were differences between the eyes and throat ($P = 0.03$) and the nose and throat ($P = 0.004$). Similar findings were elicited when analyzing Shannon diversity (right eye versus left eye $P = 0.94$, eye versus nose $P = 0.14$, eye versus throat $P = 0.03$, and nose versus throat $P = 0.032$). The throat had significantly higher relative abundance of *Streptococcaceae* than eye samples (throat 71.6 ± 35.2 versus eye 19.9 ± 33.1) and nose samples (throat 71.6 ± 35.2 versus nose 30.2 ± 36.8). Eye samples had significantly higher relative abundance of *Staphylococcaceae, Moraxellaceae, Corynebacteriaceae, Oceanospirillaceae,* and *Listeriaceae* than throat samples. Nasal samples had significantly higher relative abundance of *Staphylococcaceae, Corynebacteriaceae,* and *Veillonellaceae* than throat samples.

Discussion

We found that all children had recoverable bacterial from 16S sequencing of their ocular surface. Prior studies have found the OSM consists of the same four predominant species as detected by culture: *Corynebacteria, Propionibacteria, Staphylococcus,* and *Streptococcus*.[18] Our study agrees with these findings, with regard to both culture and 16S sequencing. Additionally, we found that *Pseudomonas, Bradyrhizobium,* and *Acinetobacter* do not contribute greatly to the OSM.[16,21] We also found smaller contributions from the families of *Moraxellaceae, Enterobacteriaceae, Oceanospirillaceae,* and *Bacillaceae,* which overlap with other studies,[21] although there are differences in cases such as *Ralstonia,* which was not detected in our population.

We successfully recovered 551 OTUs from the ocular surface in children. This is slightly higher than prior studies in adults, which recovered approximately 460 OTUs from normal conjunctiva.[31] A more recent publication, however, argued that contamination is a risk in ocular microbiome studies, and that the number of OTUs on the adult ocular surface is actually much lower. Ozkan et al.[32] recovered approximately 183 OTUs from healthy adult individual, consistent with the thought that the antimicrobial environment makes the ocular surface less diverse than other mucosal sites. There are several reasons why our data may differ from Ozkan et al.[32] First, our methodologies were not identical. Our sequencing depth was an average of two times deeper than Ozkan et al.,[32] which may partially explain the greater abundance of OTUs recovered. Additionally, our OTU filtering strategy differed. Ozkan et al.[32] reduced
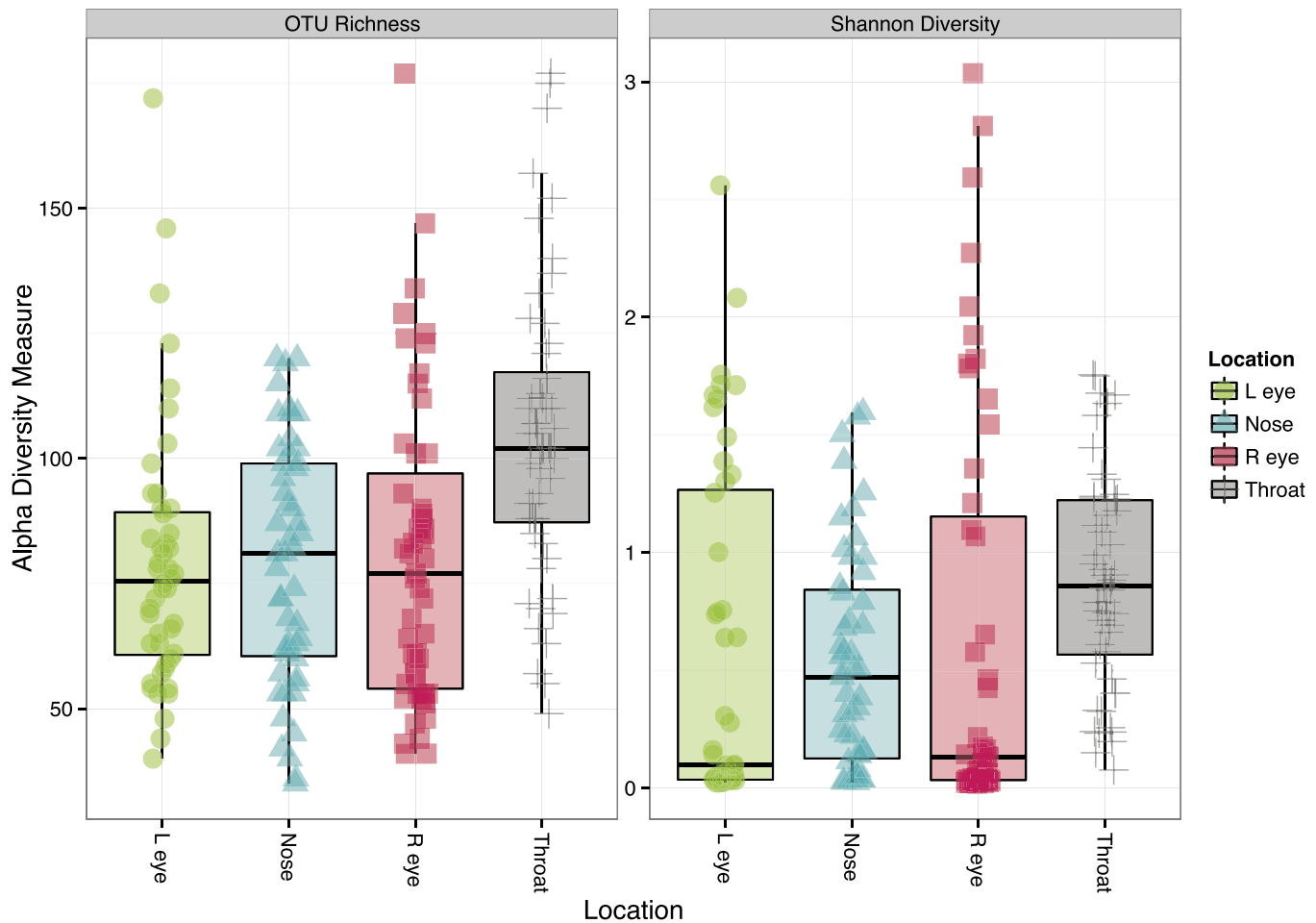
Figure 1. Differences in the microbiome in the mucosa of the head and neck by location demonstrated by alpha diversity measures of the eyes, nose, and throat, showing the similar OTU richness between the left and right eyes with greater richness in the throat.

the number of OTUs by removing rare OTUs with relative abundances less than 0.0001%, which lowered the OTU diversity by >80%. In our analysis, we did not impose a relative abundance filter as low abundance taxa could be important contributors to the microbial community. Instead, we applied a prevalence filter so that the default threshold was to keep OTUs present in more than 5% of the samples. Second, our population differed. Ozkan et al.[32] evaluated adult patients with a mean age of 38 ± 10 years, while we examined participants under 18 years of age. Higher abundance of OTUs on the ocular surface of children provides support for the theory that immunologic tutoring occurs with age and leads to decreases in bacterial diversity, and lower numbers of OTUs in the adult OSM.

Our study supports the finding that there is an evolution of the microbiome that occurs with age, as posited by other authors.[19] Although there was no change in overall abundance in the bacteria with age, there was a shift in the overall composition of the microbiome, as some phyla expanded while others decreased in abundance. This is seen in change in the OTUs between the age groups, as enrichment occurred in the *Actinobacteria* phylum, a net enrichment occurred in the *Proteobacteria* phylum, and an overall reduction occurred in the *Firmicutes* phylum. Although the significance and clinical implications of this finding are currently unknown, we suspect this change in the landscape may reflect the maturation of the ocular surface as it is exposed to pathogens and/or other challenges such as ocular surgery or topical antibiotics.

Our study also found that there was greater complexity in older children compared to younger children. This appears to conflict with prior studies that indicate the adult microbiome is less complex than the pediatric microbiome.[21] The explanation for

this variability may two-fold. First, there is the definition of "child" that is used in analysis. For instance, we considered the younger group of children to be <6 months of age, as there would not likely be significant environmental influences, as these infants are mostly within the family home, have a limited diet primarily consisting of breast milk or formula, and do not have significant hand-to-eye contact. Also, they are much less likely to have any eye surgery or topical or oral antibiotic exposures prior to this age. We compared this group to children >6 months of age, as these exposures are more likely in older children. Secondly, there are periods of microbiome turbulence early in life, as the microbiome matures and undergoes immunologic tutoring. Once stabilized, the OSM is tightly controlled through both chemical and immunologic means in adults, which may ultimately result in fewer bacterial species and lower overall numbers of bacteria. Interestingly in our study, differences in microbiome composition and diversity were not found with brief interruptions such as ocular surgery, which supports the theory of a tightly controlled homeostatic milieu.

This variation with age is supported by a prior study comparing the OSM in children ≤10 years old to participants >10 years old.[21] The authors found that the abundance of *Streptococcus* was higher in younger participants, similar to our findings in that younger patients had slightly higher proportional abundance of *Streptococcus*; however, this was not statistically significant in our patient population. The difference may be attributable to the environmental conditions, as the children in the study were located in Gambia and are known to have a high prevalence of nasopharyngeal *Streptococcus pneumoniae* carriage. In addition, Zhou et al.[21] found that children aged ≤10 years had greater richness and diversity in the bacterial communities of the conjunctiva than older participants. Although this may initially seem to contradict our results in which the OSM of older children was more diverse, this finding may be attributable to the manner in which children were analyzed. Zhou et al.[21] defined children as ≤10 years and compared these to participants <10 years old, however did not specifically mention or separately analyze infants. As we know from studies of other mucosal surfaces, such as the lung and the gut, there are periods of fluctuation, tutoring, and maturation of the microbiome particularly very early in childhood. The microbiome undergoes periods of turbulence with sinusoidal fluctuations in stability. As this turbulence may extend beyond 10 years old and into adolescence, this may explain the difference in findings.

Our findings of increasing complexity in childhood agrees with studies in other organ systems, such as the gastrointestinal tract. Prior studies demonstrate an infant's gut microbiome undergoes rapid maturation over the first year of age and is not fully established in an adult form until approximately 3 years of age.[33] The dynamic nature of the microbiome has important implications, as the microbiome is susceptible to external influences that can dramatically affect the short- and long-term health of the host.[34] Dysbiosis, or shifts in microbial composition, can occur in the setting of dietary changes, antibiotic exposure, or infection.[4] Dysbiotic conditions can disrupt the finely tuned regulatory circuits that maintain a checks and balances and favor invasion and growth of pathogenic species.[34] As such, it is interesting that we did not find differences in OSM between children who had and did not have ocular surgery. This would imply that ocular surgery does not seem to permanently alter the OSM composition despite the use of topical preoperative sterilization such as betadine and postoperative steroid and antibiotic drops. It is important to note, however, that, we excluded children within 90 days of any surgical procedure or who were currently using topical antibiotics or immunosuppressive medications. In doing so, we may have masked a dynamic period during which the OSM is affected by these interventions.

In our study, the overall bacterial abundance was highest in the throat, followed by the nose and then eyes. This is similar to prior studies using cultures, which have demonstrated that there is a marked difference between the ocular and oral mucosa. These studies have shown that as few as 10% of conjunctival specimens will grow more than 64 colony-forming units.[35] By comparison, 100% of swabs taken from the oral mucosa will show growth on culture, with a bacterial growth of up to 10^8 colony-forming units.[36] The apparent difference in abundance found by studies using cultures is supported by our study using 16S sequencing. The number of OTUs from the ocular surface differs from studies of the oral mucosa in which a greater number of OTUs can be identified in the pharynx[37,38] and studies of the gut in which up to thousands of OTUs can be identified.[39] This finding is not unexpected, as there are approximately 100 bacterial OTUs in the gut compared to one human gene, whereas in the eye the ratio is reversed at approximately one bacterial OTU for 100 human genes.

Additionally, we demonstrated that the composition of the ocular and throat mucosa differs, as ocular samples had a significantly higher relative abundance of *Staphylococcus*, *Corynebacterium*, *Marinomonas*, *Brochothrix*, and *Moraxella* than throat samples. In contrast, throat samples had a significantly higher abundance of *Streptococcus* spp., with strains such as *S. HKU30*, *S. uberis*, and *S. infantis*, and *Prevotella* spp., such as *P. veroralis*, *P. pallens*, and *P. salivae* that were enriched. The presence of these bacteria is noted to be common in the oral mucosa, as *Streptococcus* spp. have been found to be in the highest abundance in the gingiva, buccal mucosa, and hard palate, whereas the tonsils and throat contain the highest abundance of *Prevotella*.[40]

The study has several limitations. First, it is important to consider the limitation of culture–dependent and independent methods for sampling the OSM. Due to its paucibacterial nature and other factors, culture-dependent mechanisms of studying the OSM may fail to reveal the diversity of the microbiome. Newer technologies that rely on extraction and amplification of bacterial genetic material, such as 16S sequencing, have the ability to identify a wider diversity of microbes than culture-based techniques but are more susceptible to noise, sampling errors, and contamination. For instance, some authors suggest that there are variations in the microbiome in a vertical dimension, and thus sampling with gentle swabbing versus "deep" swabbing may reveal difference characterizations of diversity.[41] Second, the small number of patients may not accurately encompass the true diversity of the OSM across various ages, ethnicities, environments, and prior exposures. In addition, there is a selection bias in the lack of regional diversity as we only collected samples from individuals presenting for care at our institution. However, prior work has shown no significant differences in the OSM between gender, prior surgery, or use of topical medication.[42] Third, patients presenting for eye care may not be reflective of the general population. Finally, the cross-sectional nature of the study does not allow for following the evolution of an individual microbiome over time. Future studies expanding the number of patients and collecting samples in longitudinal fashion will allow for a more in depth and accurate review of the ocular surface and neighboring microbiomes.

We demonstrated that 16S sequencing can be used to characterize the OSM in children. We explored factors that can influence the OSM such as age and ocular surgery. Additionally, we showed there are similarities and differences between the OSM and nasal and pharyngeal microbiomes. Future studies exploring longitudinal changes in the OSM will be important in providing fundamental knowledge regarding the age-dependent pattern of colonization, as well as the implication of the similarities and differences in health and disease.

Acknowledgments

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