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Methodologic Considerations for Studying the Ocular Surface Microbiome

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The ocular surface microbiome, unlike that of the skin or gut, has not been well characterized. Culture experiments historically suggested a nearly sterile ocular surface, but initial application of molecular methods such as 16S ribosomal RNA and high-throughput sequencing demonstrated a surprisingly rich ocular surface microbiome. However, a major limitation in studying such a low-biomass niche is the potential for artifactual results when amplification-based techniques such as ribosomal polymerase chain reaction and shotgun sequencing are used. It will be essential to establish standards across the field for sample collection, positive and negative controls, and limitation of contamination in both the laboratory setting and computational analysis. New developments in ocular microbiome research, including the generation of reference reagents and fluoroscopic imaging techniques, provide improved means to validate sequencing results and to visualize complex interactions between host cells and bacteria. Through more thorough characterization of the ocular surface microbiome, the connections between a dysregulated surface and ophthalmic disease may be better understood.

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Defining the Microbiome

The microbiome has been defined as an "ecological community of commensal, symbiotic and pathogenic microorganisms within a body space."[1,2] The microbiota comprises all its cellular members, including prokaryotes such as bacteria and archaea, and eukaryotes such as fungi, algae, and protists.[2] The inclusion of phages, viruses, and plasmids is more controversial.[2] The microbiome attributed to a particular host niche should be self-propagating and resident rather than transient. The existence of a resident microbiome has been very well documented in human sites such as the gut and skin.[3,4] There has been much discussion regarding the existence of such a community on the ocular surface. Specifically, it is unclear whether there is truly a living microbiome associated with the ocular mucosa, or whether organisms isolated from this site are simply derived from periodic contamination by the surrounding periocular environment, such as the adjacent skin or nasal mucosa.[5,6] The clinical implications are significant. Just as gut dysbiosis plays a role in inflammatory bowel disease and diabetes mellitus,[7] a dysregulated ocular surface microbiome may predispose to ophthalmic disease such as infectious keratitis or blepharitis.

Current Knowledge of the Ocular Microbiome from Culture Experiments

The ocular surface microbiome was initially characterized by standard culture techniques. Studies from the 1960s and 1970s concluded that the conjunctival surface was sterile. McNatt et al[8] found that 60.9% of conjunctival cultures grew ≥ 1 microorganism, the most common of which were *Propionibacterium acnes* (now *Cutibacterium acnes*) and *Staphylococcus epidermidis*. Nolan found that 61.9% of cultures were sterile, although the remainder predominantly reflected normal upper respiratory tract flora.[9] Whether the failure to grow organisms from the ocular surface represented challenges in culturing organisms found in this site, or actual sterility of the ocular surface, was unclear.

The Unique Antimicrobial Environment of the Ocular Surface

The concept that the ocular surface may be nearly sterile and devoid of a true microbiome is worthy of discussion, as the conjunctiva possesses unique antimicrobial defense mechanisms. The mucosal surface of the eye is constantly bathed in tears, which contain antimicrobial components such as lactoferrin, lysozyme, secretory immunoglobulin A, secretory phospholipase A_2, and complement.[10] The presence of certain resident commensal organisms has also been shown to stimulate a protective immune pathway which prevents the growth of pathologic organisms. In mice, for instance, St Leger et al[5] identified one such organism, *Corynebacterium mastitidis*, which was found to colonize the ocular surface and prompt the production of interleukin 17 by γδ T cells within the ocular mucosa. This led to the recruitment of neutrophils and the resultant inhibition of pathogens such as *Candida albicans* and *Pseudomonas aeruginosa*.[5]

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The Role of Biofilms in the Ocular Surface Microbiome

A key consideration in characterization of the ocular surface microbiome is the role of biofilms. "Biofilm" refers to a mode of microbial growth. Biofilms are contributory to pathogenic colonization of the ocular surface, leading to disease.[11,12] Clinical samples from the Steroids for Corneal Ulcers Trial demonstrated that *P. aeruginosa* keratitis associated with isolates capable of forming *in vitro* biofilms was associated with worse visual acuity both at presentation and at 3 months posttreatment.[13]

Commensal organisms such as *S. epidermidis* carry genes that promote biofilm formation. Suzuki et al[14] found that 21 of 46 conjunctival *S. epidermidis* isolates demonstrated the qualitative presence of biofilms by Congo red agar assays. These strains all carried the icaA gene. The presence of abiotic surfaces such as contact lenses or punctal plugs also lends to biofilm formation.[15–17]

Biofilms increase the difficulty of eradication of an organism. They can also lead to errors in sampling that result in microniches of overrepresented or underrepresented organisms within the conjunctiva secondary to biofilm-mediated dysbiosis. Thus, methods that more broadly sample the conjunctiva provide a more accurate estimate of resident microbiomes.

Molecular Methods for Characterizing the Ocular Surface Microbiome

Traditional culture methods remain the gold standard for identification of microorganisms. However, some organisms are invisible to this technique because they are uncultivable, such as *Treponema pallidum*. Certain microbes also require very specific conditions for culture, such as *Mycobacterium* spp.[18]

16S ribosomal RNA (rRNA) sequencing was developed in the 1980s and is a cornerstone technology for bacterial identification (Fig 1). The 16S rRNA genes are defined by both highly conserved sequences as well as hypervariable regions. Polymerase chain reaction (PCR) using universal primers targeting the conserved regions then allows for amplification while subsequent analysis (typically DNA sequencing) of the amplicon identifies the variable sequences, which are species-specific. Bioinformatic analysis is then applied to align the results with sequence databases to make taxonomic assignments.[19] The limitations of this technology include that it identifies bacteria only (although very similar analysis of 18S/28S/ITS DNA can be utilized for fungi), provides incomplete taxonomic resolution (often to the genus rather than the species level), and is prone to amplification errors of PCR. In addition, although the regions targeted by PCR primers are highly conserved, they are not 100% conserved, which may introduce bias into amplification.[20] In contrast, in metagenomic sequencing, all genomes (microbial or host) present in a sample are sequenced. "Shotgun" metagenomics refers to the untargeted nature of this sequencing (Fig 2).[21]

Challenges of Characterizing a Low-biomass Niche

16S rRNA gene sequencing has demonstrated a surprisingly rich ocular surface microbiome. In a 2011 study, Dong et al[22] conducted 16S rRNA sequencing of conjunctival samples from 4 subjects.[23] An average of 221 species-level phylotypes were generated per subject. In total, the combined bacterial community reflected 5 phyla and 59 genera. Twelve genera were thought to comprise the "core" of the ocular surface microbiome given that they were present in all subjects. Of note, approximately one-third of the total identified reads reflected as yet-unclassified organisms. Similarly, a 2007 study utilizing 16S ribosomal DNA PCR similarly found atypical organisms on the ocular surface of patients with dry eye syndrome, namely *Rhodococcus erythropolis*, *Klebsiella* sp., and *Erwinia* sp.[24]

Doan et al[23] performed 16S ribosomal DNA gene deep sequencing on 356 conjunctival samples collected from 89 healthy subjects. Quantitative 16S PCR revealed that the conjunctiva harbored ~0.02 bacterial genome per human genome, which the authors termed "paucibacterial," compared with ~20 bacterial genomes per human genome on the skin.[23] The finding of several hundred taxonomic classifications of bacteria from conjunctival samples in earlier studies is at odds with the total bacterial DNA load on a swab being on the order of ~100 bacteria by these calculations. Doan et al[23] found that nearly all DNA sequences were derived from *Staphylococcus, Corynebacteria, Propionibacteria,* and *Streptococcus*, consistent with findings from culture experiments. These results suggest that earlier studies had overestimated the diversity of ocular surface bacteria, possibly through artifactual amplification or contamination of reagents. Additional work by Ozkan et al evaluated conjunctival samples in 45 subjects over time using both bacterial culture and 16S rRNA sequencing. An average of 16 operational taxonomic units (OTUs) were identified at each time point in each ocular surface sample using 16S rRNA sequencing, significantly less than in preceding studies. However, greater diversity was found using high-throughput sequencing; in total, 56 genera were identified, as compared with 9 genera in the culture arm of the experiment.[25] Use of metagenomic deep sequencing, specifically Biome Representational In Silico Karyotyping,[26] has additionally demonstrated the presence of several viruses including Torque Teno Virus and Merkel Cell Polyoma Virus in many subjects' conjunctiva, suggesting the presence of a resident virome that is invisible to 16S analysis.[20,27] Thus, 16S ribosomal profiling, which is widely used to characterize other niches, seems to overestimate bacterial diversity on the ocular surface while also being unable to characterize the resident virome.

The work of Ozkan et al also called into question the idea of a "core" microbiome shared between humans[25], such as is found in the skin, gut, and other niches.[3,4] This harkens back to the question of whether conjunctival microbes are transient passengers from the adjacent skin or nasal mucosa, or a self-propagating community exhibiting temporal stability. Longitudinal sampling of subjects over 3 months found that no OTU was common to all subjects at any particular time point. However, at the phylum level, there was a higher degree of commonality noted; the majority of OTUs (94.9%) identified belonged to *Proteobacteria, Firmicutes*, and *Actinobacteria.* Genera present across all time points included *Corynebacterium, Sphingomonas,*

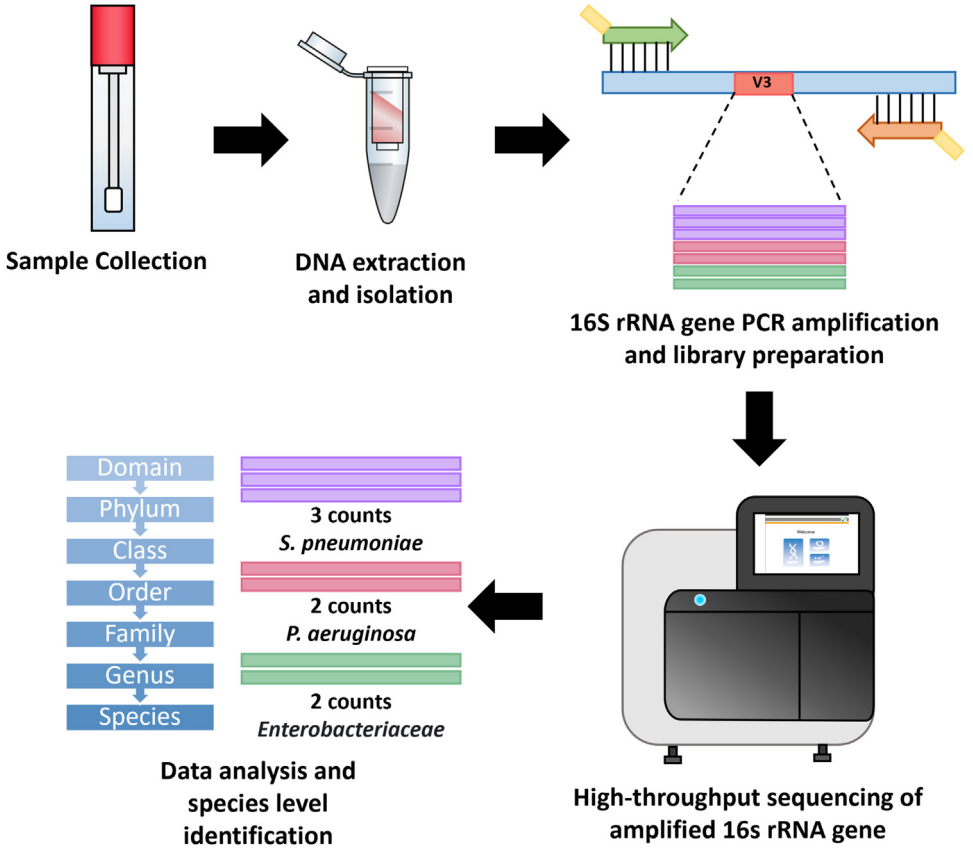

Figure 1. A visual description of 16S ribosomal RNA (rRNA) gene sequencing. Sample collection is followed by DNA extraction and isolation. Polymerase chain reaction (PCR) is then used to amplify a targeted region of the 16S rRNA gene. High-throughput sequencing of the amplified gene allows for species-level identification of the microbial organisms within the sample.

Streptococcus, Acinetobacter, and *Anaerococcus*. There was also some degree of stability within individuals over time, suggesting the potential for a unique barcode of organisms particular to each subject.[25]

Further work by Ozkan et al[28] evaluated a variety of human anatomic sites, studying both tissue samples and surface swabs. Samples included eyelid margin tissue, conjunctival tissue, skin swabs, and conjunctival surface swabs. Sterile, nylon-flocked swabs were used to obtain the surface samples while tissue samples were collected at the time of routine ocular surgery. There was a statistically significant difference in absolute number of taxa identified among all 4 groups ($P < 0.001$). Of these, conjunctival tissue demonstrated the least diversity and richness of organisms.[28] The genera *Staphylococcus* and *Corynebacterium* were found to be most prevalent on the skin and eyelid margin. In particular, *Corynebacterium* was found most consistently on skin swabs, and its relative abundance decreased linearly from the skin, to the lid margin, to the ocular surface, to the conjunctival tissue. This may be consistent with routine bacterial transfer from skin to ocular surface, rather than persistent colonization of the ocular surface with *Corynebacteria*.[28] There were some OTUs, such as *Acinetobacter* and *Aeribacillus*, found most commonly on the ocular surface and in low levels at the remaining 3 sites. The findings suggest that ocular and periocular niches may be distinct in their resident communities.[28] A similar conclusion based on principal coordinate analysis of mucosal, conjunctival, and skin metagenomics was reached by Doan et al.[23]

Work in other paucibacterial sites of the body has yielded similar concerns with respect to potentially artifactual characterizations of the microbiome. A 2014 study which used 16S rRNA and entire genome sequencing to identify the placental microbiome concluded that the placenta was physiologically colonized by bacteria, and that its bacterial profile differed in those with a history of antenatal urinary tract infection or sexually transmitted infection, as well as those with preterm birth < 37 weeks.[29] One of the phyla noted to have been present was *Cyanobacteria*, which rely on photosynthesis for generation of energy, raising the question of its true presence in placenta. *Cyanobacteria* was similarly detected by 16S rRNA and ribosomal DNA sequencing in autopsied brain tissue samples of patients with and without multiple sclerosis.[30] These findings were later hypothesized to be related to contamination by reagents used in sample processing.[31] Follow-up work by de Goffau et al[32] analyzed tissue samples from 80 placental biopsies acquired during prelabor Cesarian section deliveries. These were spiked with a positive control (*Salmonella bongori*). Metagenomic deep sequencing of total DNA was performed, as was 16S rRNA gene

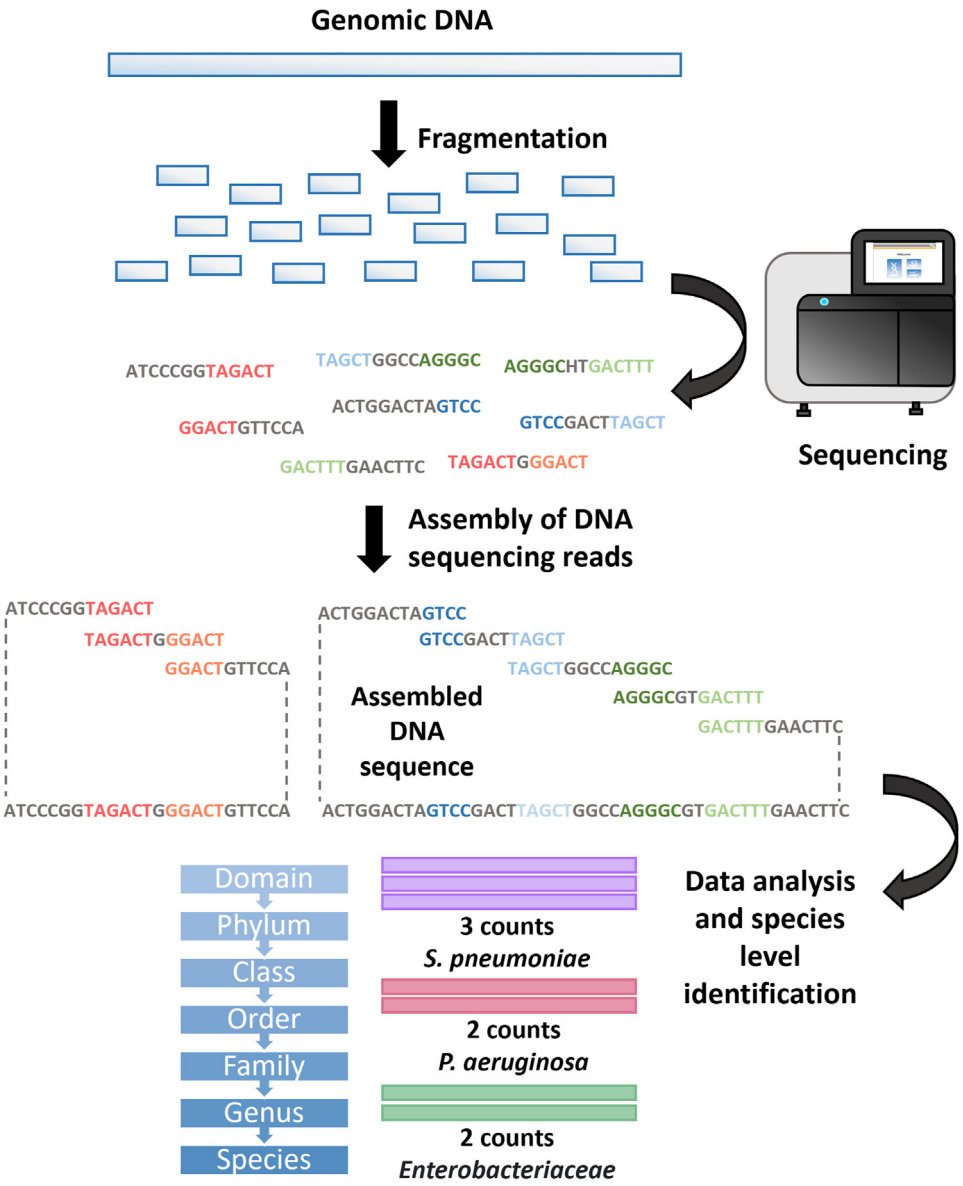

Figure 2. A visual description of shotgun metagenomics, a technique which involves random fragmentation of genomic DNA. DNA is extracted from the sample, and polymerase chain reaction amplification is performed. Samples are pooled together in equal proportions and library quantification is performed, followed by sequencing. Bioinformatic pipelines are used to assemble sequencing data into partial or full microbial genomes or align the sequences to databases of microbial marker genes. As depicted, shotgun sequencing can read all genomic DNA in a sample, rather than a specific targeted region as in 16S ribosomal RNA sequencing.

amplicon sequencing. The only organism consistently detected across all samples using both sequencing methods was *S. bongori*. The only true bacterial signal detected, with statistical significance across both methods, was *Streptococcus agalactiae*, a known colonizer of the genital tract and a well-established cause of neonatal sepsis.[32] Thus it was concluded that there is no clear evidence for a placental microbiome; if one does exist, it does so below the limit of detection of current technology. These and similar studies strongly suggest that low-biomass samples have potential to generate artifactual results when subjected to amplification-based techniques such as ribosomal PCR and shotgun metagenomic sequencing.

Considerations for Sample Collection in Microbiome Research for Low-biomass and Paucibacterial Samples

Variation in sampling and processing methods in microbiome research, as well as a lack of consensus on the optimal experimental controls to be used, remain obstacles for validating results across multiple studies.[33,34] For sampling of the ocular microbiome, swab material, pressure applied to swabs, and duration of tissue contact must be taken into consideration.[22,35] Deep swabbing using a cotton swab and exerting heavy pressure on the

ocular surface have been proven optimal when compared with light swabbing.[22] Katzka et al[35] acknowledged the lack of studies examining and comparing different methods of sampling of the ocular microbiome, and attempted to address this. They found that cotton and calcium alginate swabs were inferior to Weck-Cel sponges in terms of amount of bacterial DNA collected. However, among the 3, results from the calcium alginate swabs were most representative of the microbiome seen in epithelial biopsies. Further studies are required as differential recovery of organisms from these 3 methods may have been influenced by the sequence in which each swab was performed.[35]

The context of the sampling must also be taken into account, including consideration of the ocular site sampled, recent manipulations to the ocular site before sample collection, and the examination room environment.[25,35,36] Different ocular sites have been shown to have unique microhabitats and varying diversity profiles.[25] Kong et al[36] demonstrated statistically significant shifts in the bacterial community on the skin of atopic dermatitis patients across different timepoints compared with healthy controls. In this study, time to sample collection from bathing and application of topical medications were considered carefully so as to not affect results. It was found that in those subjects who had received even intermittent treatment, such as oral antimicrobials up to 4 weeks before sample collection, skin microbial diversity expanded and levels of *Staphylococcus aureus* declined, corresponding to symptomatic improvement in disease flares.[36] Parallel confounders for the ocular surface might include contact lens wear, topical drop or medication administration, or presence of hardware such as scleral buckle, keratoprosthesis, or glaucoma drainage device.

Considerations for Limiting Environmental and Reagent Contamination

The ocular surface is a low-biomass niche, and as a result is more prone to increased contaminating reads in sequencing data when compared with regions with higher biomass.[37] There is risk for contamination at every step of processing, from sample collection to library preparation and sequencing.[25,37] Studies on ocular microbiome research have emphasized the need for dedicated laboratory benches, reagents, and personnel for processing of low-biomass samples separate from high-biomass samples.[25,35–39] Special consideration must be taken in recognition of the reagent microbiome and potential for reagent contamination.[31,32,38,39] Contaminating DNA—the "kitome"—is ubiquitous in extraction kits and PCR reagents, and can have a significant impact on 16S rRNA gene surveys and shotgun metagenomics.[31,33,38–40] For example, in a study investigating the human skin microbiome, a relatively low-biomass niche, the use of different DNA extraction kits resulted in changes in the profile of bacterial communities captured.[41] The kitome has been shown to vary between extraction kits sourced from different manufacturers, and even between distinct lots of the same kit.[39] Best practices include diligent recording of the kit and batch used to process each sample, and treatment of each kit batch as a factor in the statistical analysis.[42]

Given the pervasive effects of contamination in microbiome research, extensive work on the utility of various techniques to identify and remove contamination has been performed.[5,37,38,43] Salter et al[38] used a generalized approach of removing sequences identified as contaminants in published databases or reference lists; however, this method does not identify study-specific contaminants and risks removing true sequences. Other studies removed sequences present in negative-control samples, an approach that risks eliminating real sequences that may have been present in the negative control due to multiplexing artifacts.[39,44]

Karstens et al[37] investigated 4 computational approaches to identifying contaminants in a dilutional series of a mock microbial community. These included filtering sequences based on relative abundance or sequences present in a negative control, predicting sequence proportion arising from defined contaminant sources, and identifying sequences that have an inverse correlation with DNA concentration.[37] Decontam is an open-source R package that removes external contaminants in metagenomic sequencing data and has shown success in identification and removal of contaminant DNA sequences.[35,43] A second method, Source Tracker, was superior to Decontam in a well-defined experimental environment, but performed poorly when the environment under investigation was unknown, which is the case in most low microbial biomass research.[37] As a result, Decontam is currently the preferred computational method for identifying and eliminating contaminants, although it is agreed that a single approach to contamination removal may not work well across all microbiome studies. It is imperative that researchers continue to report methods for removing contaminants from samples to gather further data and optimize this process.

Considerations for Appropriate Controls for Ocular Surface Microbiome Characterization

The need for inclusion of negative controls in microbiome research has been widely agreed upon, with recommendations requiring that a single type of negative control be included during sampling, extraction, and amplification given that bias can be introduced at each of these steps.[35,45] Potential negative sampling controls include a swab taken from the contralateral (nondiseased) eye, blank swabs, and nuclease free water.[35] The selection of an appropriate negative control for sampling is dependent on the nature of the study being conducted; however, the material should be collected in the same room and at the same time as the biological samples, and should undergo the same laboratory treatment.[35] Extraction controls monitor for contamination within DNA extraction kits, and amplification controls monitor for contaminant DNA present in reagents and the laboratory environment during library preparation and sequencing.[45,46] The work of Karstens et al[37] did raise concerns regarding the use of negative controls, however. Removal of amplicon sequence variants present in the

negative control led to misclassification of mock community amplicon sequence variants as contaminants, and vice versa.

Concerningly, a 2019 review of standards of controls in microbiome research found that the use of controls is not necessarily commonplace. Hornung et al[45] reviewed all 2018 issues of 2 journals—Microbiome and the International Society of Microbial Ecology journal—and found that of 265 relevant publications, a negative control was used in only 30% and a positive control was used in only 10%. Continued advocacy for the use of both positive and negative controls for sampling, extractions, and library preparation, as well as the use of a universal DNA extraction workflow, have unfortunately not yet led to a consensus.[45,47–49] In an attempt to introduce standardization into the microbiome research space, Sergaki et al[33] attempted to produce and validate a DNA reference reagent for gut microbiome research. DNA reference reagents are a collection of defined mock bacterial communities specific to a selected microbiome which can serve as a positive control in investigations of that niche. The utility of positive controls in microbiome research has been shown in various studies, particularly in calibration of sequencing methods and guiding of sample exclusion.[32,45,46]

Sergaki et al[33] used 4 reporting measures to evaluate kits' ability to extract DNA from the mock community of the reference reagent: sensitivity, false-positive relative abundance, diversity, and similarity. These were calculated in accordance with guidelines set forth by Amos et al.[34] The study analyzed 8 DNA extraction kits, multiple bioinformatics pipelines, and 2 next-generation sequencing approaches to demonstrate the ability of the gut reference reagent to detect biases in the DNA extraction step using the aforementioned measures.[33] The effectiveness of this reagent, as well as the introduction of 4 reporting measures to evaluate extraction protocols, serves as an exciting prospect for the development of an ocular site-specific reference reagent that would allow for standardization of microbiome research and reduction of bias introduced in various steps of the processing pipeline.

Other models for further study and testing of the ocular surface microbiome are in development. Ozkan et al found that mice had a similar tissue-related ocular surface microbiome to humans, which could be useful in studying the effects of therapeutic drops, inflammation, and aging on the ocular surface.[50] It is possible that microbiome transfer experiments might allow validation of the presence of specific constituents in an animal model.

Imaging for Validation of the Ocular Microbiome

Imaging also provides another means of validating the presence of organisms on the ocular surface. Two methods which have proven valuable for this purpose include fluorescence *in situ* hybridization (FISH) and click chemistry detection. Described by DeLong et al,[51] FISH is a method involving the hybridization of intact cells with fluorescently labeled DNA oligonucleotides complementary to 16S rRNA. These cells are then subsequently viewed using fluorescence microscopy.[51] Studies have shown that FISH labeling is effective for visualization of microbes on contact lenses.[52] However, despite deliberate inoculation of murine corneas with *P. aeruginosa* and *Staph aureus,* these organisms could not be subsequently identified from tear fluid samples.[53] This held true despite variations in the strength of the inoculum (10^4 versus 10^8 colony forming units [CFU]).[53] A recent development in this technique was the addition of Combinatorial Labeling and Spectral Imaging, which allows for the identification of up to hundreds of taxa from a single microscope image. Spectroscopy is performed on each pixel in a digital fluorescence image.[54] This allows operators to distinguish up to 16 different fluorophores by the shape of the emission spectrum, even if the fluorophores overlap in spectrum.[55,56] Combinatorial labeling allows for the use of multiple fluorophores to create a spectral signature for each taxon, leading to discrimination of hundreds of different taxa simultaneously.[55] Combinatorial Labeling and Spectral Imaging-FISH allows for 3-dimensional visualization of functional relationships among taxa, and researchers have used this technique to demonstrate the relation of bacterial to host epithelial cells on the tongue biofilm.[56]

Click chemistry with specific bacterial probes also allows for visualization of microbes. This method utilizes an alkyne-functionalized D-alanine probe that detects only live bacteria; bacteria are fed a reagent which is then expressed in the peptidoglycan cell wall. These bacteria are subsequently detected using addition of an azide-fluorophore via a click chemistry reaction resulting in cell surface fluorescence. Imaging is then performed by fluorescence microscopy.[53,54] This method has shown utility in the detection of live bacteria deliberately inoculated onto corneal epithelial cells and the mouse ocular surface.[53] Alkyne-functionalized D-alanine labeling of the healthy mouse ocular surface illustrated that no metabolically active bacteria was detectable on the cornea; however, bacteria was visualizable on the conjunctiva and appeared filamentous in morphology.[53] An alternative to alkyne-functionalized D-alanine is the addition of the reagent 4-N,N-Dimethylamino-1,8-naphthalimide conjugate of trehalose (DMN-Tre), a solvatochromic trehalose probe.[53,57] The advantage of using DMN-Tre is that it involves a more succinct workflow and is more selective for detecting actinobacteria which encode Ag85 mycolylate trehalose. This class includes *Corynebacteria* and *Propionibacteria*, 2 species which in previous studies have been shown to colonize the conjunctiva.[23] DMN-Tre successfully allowed for visualization of filamentous bacteria on the conjuctiva.[53] However, the presence of other microbes cannot be ruled out given that these imaging methods exclude labeling of these organisms. Regardless, the information obtained by these imaging modalities remains helpful in visualizing complex interactions between host cells and bacteria, as well as validating sequencing results by direct visualization of bacteria on the ocular surface.

Areas of Future Study

It will be important to characterize whether individuals carry a particular microbial "signature," and whether there is variability in the microbiome over time. The effects of external variables such as contact lens use, topical or systemic medication use, or surgery is also of interest, as are the ways in which changes in this community of microbes mediates disease. In terms of validation methods, aforementioned imaging techniques are best suited to highly metabolically active bacteria; development of methods of visualization for latent organisms should also be explored.

The discovery and characterization of the gut microbiome is one of the great advances in 21st century medical research. It is likely that local microbial and microbiome-related physiology contribute to ocular surface health and disease. Molecular characterization of the ocular surface microbiome is challenging due to its paucibacterial nature. Appropriate methods, use of standardized controls, and confirmation of important results will be essential to work in the field.

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Abbreviations and Acronyms:
DMN-Tre = 4-N,N-Dimethylamino-1,8-naphthalimide conjugate of trehalose; **FISH** = fluorescence *in situ* hybridization; **OTU** = operational taxonomic unit; **PCR** = polymerase chain reaction; **rRNA** = ribosomal RNA.

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References

1. Lederberg J, McCray AT. "Ome sweet" omics — a genealogical treasury of words. *Scientist*. 2001;15:8.
2. Berg G, Rybakova D, Fischer D, et al. Microbiome definition re-visited: old concepts and new challenges. *Microbiome*. 2020;8:103.
3. Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature*. 2009;457:480–484.
4. Grice EA, Kong HH, Conlan S, et al. Topographical and temporal diversity of the human skin microbiome. *Science*. 2009;324:1190–1192.
5. St Leger AJ, Desai JV, Drummond RA, et al. An ocular commensal protects against corneal infection by driving an interleukin-17 response from mucosal γδ T cells. *Immunity*. 2017;47:148–158.e5.
6. Zegans ME, Van Gelder RN. Considerations in understanding the ocular surface microbiome. *Am J Ophthalmol*. 2014;158:420–422.
7. DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current understanding of dysbiosis in disease in human and animal models. *Inflamm Bowel Dis*. 2016;22:1137–1150.
8. McNatt J, Allen SD, Wilson LA, Mizoguchi E. Anaerobic flora of the normal human conjunctival sac. *Arch Ophthalmol*. 1978;96:1448–1450.
9. Nolan J. Evaluation of conjunctival and nasal bacterial cultures before intra-ocular operations. *Br J Ophthalmol*. 1967;51:483–485.
10. McDermott AM. Antimicrobial compounds in tears. *Exp Eye Res*. 2013;117:53–61.
11. Zegans ME, Becker HI, Budzik J, O'Toole G. The role of bacterial biofilms in ocular infections. *DNA Cell Biol*. 2002;21:415–420.
12. Behlau I, Gilmore MS. Microbial biofilms in ophthalmology and infectious disease. *Arch Ophthalmol*. 2008;126:1572–1581.
13. Zegans ME, DiGiandomenico A, Ray K, et al. Association of biofilm formation, Psl exopolysaccharide expression, and clinical outcomes in *Pseudomonas aeruginosa* keratitis: analysis of isolates in the steroids for corneal ulcers trial. *JAMA Ophthalmol*. 2016;134:383–389.
14. Suzuki T, Kawamura Y, Uno T, et al. Prevalence of *Staphylococcus epidermidis* strains with biofilm-forming ability in isolates from conjunctiva and facial skin. *Am J Ophthalmol*. 2005;140:844–850.

15. Wiley L, Bridge DR, Wiley LA, et al. Bacterial biofilm diversity in contact lens-related disease: emerging role of Achromobacter, Stenotrophomonas, and Delftia. *Invest Ophthalmol Vis Sci*. 2012;53:3896−3905.
16. Kackar S, Suman E, Kotian MS. Bacterial and fungal biofilm formation on contact lenses and their susceptibility to lens care solutions. *Indian J Med Microbiol*. 2017;35:80−84.
17. Hadjiargyrou M, Donnenfeld ED, Grillo LM, Perry HD. Differential bacterial colonization and biofilm formation on punctal occluders. *Materials (Basel)*. 2019;12:274.
18. Woo PCY, Lau SKP, Teng JLL, et al. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin Microbiol Infect*. 2008;14:908−934.
19. Rosselli R, Romoli O, Vitulo N, et al. Direct 16S rRNA-seq from bacterial communities: a PCR-independent approach to simultaneously assess microbial diversity and functional activity potential of each taxon. *Sci Rep*. 2016;6:32165.
20. Gupta S, Mortensen MS, Schjørring S, et al. Amplicon sequencing provides more accurate microbiome information in healthy children compared to culturing. *Commun Biol*. 2019;2:291.
21. Quince C, Walker AW, Simpson JT, et al. Shotgun metagenomics, from sampling to analysis. *Nat Biotechnol*. 2017;35:833−844.
22. Dong Q, Brulc JM, Iovieno A, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci*. 2011;52:5408−5413.
23. Doan T, Akileswaran L, Andersen D, et al. Paucibacterial microbiome and resident DNA virome of the healthy conjunctiva. *Invest Ophthalmol Vis Sci*. 2016;57:5116−5126.
24. Graham JE, Moore JE, Jiru X, et al. Ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. *Invest Ophthalmol Vis Sci*. 2007;48:5616−5623.
25. Ozkan J, Nielsen S, Diez-Vives C, et al. Temporal stability and composition of the ocular surface microbiome. *Sci Rep*. 2017;7:9880.
26. Muthappan V, Lee AY, Lamprecht TL, et al. Biome representational *in silico* karyotyping. *Genome Res*. 2011;21:626−633.
27. Siegal N, Gutowski M, Akileswaran L, et al. Elevated levels of Merkel cell polyoma virus in the anophthalmic conjunctiva. *Sci Rep*. 2021;11:15366.
28. Ozkan J, Willcox M, Wemheuer B, et al. Biogeography of the human ocular microbiota. *Ocul Surf*. 2019;17:111−118.
29. Aagaard K, Ma J, Antony KM, et al. The placenta harbors a unique microbiome. *Sci Transl Med*. 2014;6:237ra65−237ra65.
30. Branton WG, Lu JQ, Surette MG, et al. Brain microbiota disruption within inflammatory demyelinating lesions in multiple sclerosis. *Sci Rep*. 2016;6:37344.
31. de Goffau MC, Lager S, Salter SJ, et al. Recognizing the reagent microbiome. *Nat Microbiol*. 2018;3:851−853.
32. de Goffau MC, Lager S, Sovio U, et al. Human placenta has no microbiome but can contain potential pathogens. *Nature*. 2019;572(7769):329−334.
33. Sergaki C, Anwar S, Fritzsche M, et al. Developing whole cell standards for the microbiome field. *Microbiome*. 2022;10:123.
34. Amos GCA, Logan A, Anwar S, et al. Developing standards for the microbiome field. *Microbiome*. 2020;8:98.
35. Katzka W, Dong TS, Luu K, et al. The ocular microbiome is altered by sampling modality and age. *Transl Vis Sci Technol*. 2021;10:24.
36. Kong HH, Oh J, Deming C, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res*. 2012;22:850−859.
37. Karstens L, Asquith M, Davin S, et al. Controlling for contaminants in low-biomass 16S rRNA gene sequencing experiments. *mSystems*. 2019;4:e00290-19.
38. Salter SJ, Cox MJ, Turek EM, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol*. 2014;12:87.
39. Glassing A, Dowd SE, Galandiuk S, et al. Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. *Gut Pathog*. 2016;8:24.
40. Mohammadi T, Reesink HW, Vandenbroucke-Grauls CMJE, Savelkoul PH. Removal of contaminating DNA from commercial nucleic acid extraction kit reagents. *J Microbiol Methods*. 2005;61:285−288.
41. Bjerre RD, Hugerth LW, Boulund F, et al. Effects of sampling strategy and DNA extraction on human skin microbiome investigations. *Sci Rep*. 2019;9:17287.
42. Kim D, Hofstaedter CE, Zhao C, et al. Optimizing methods and dodging pitfalls in microbiome research. *Microbiome*. 2017;5:1−14.
43. Davis NM, Proctor DM, Holmes SP, et al. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*. 2018;6:226.
44. D'Amore R, Ijaz UZ, Schirmer M, et al. A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. *BMC Genomics*. 2016;17:55.
45. Hornung BVH, Zwittink RD, Kuijper EJ. Issues and current standards of controls in microbiome research. *FEMS Microbiol Ecol*. 2019;95:fiz045.
46. Eisenhofer R, Minich JJ, Marotz C, et al. Contamination in low microbial biomass microbiome studies: issues and recommendations. *Trends Microbiol*. 2019;27:105−117.
47. Human Microbiome Project Consortium. A framework for human microbiome research. *Nature*. 2012;486:215−221.
48. Becker L, Steglich M, Fuchs S, et al. Comparison of six commercial kits to extract bacterial chromosome and plasmid DNA for MiSeq sequencing. *Sci Rep*. 2016;6:28063.
49. Greathouse KL, Sinha R, Vogtmann E. DNA extraction for human microbiome studies: the issue of standardization. *Genome Biol*. 2019;20:212.
50. Ozkan J, Majzoub ME, Coroneo M, et al. Comparative analysis of ocular surface tissue microbiome in human, mouse, rabbit, and guinea pig. *Exp Eye Res*. 2021;207:108609.
51. DeLong EF, Wickham GS, Pace NR. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science*. 1989;243:1360−1363.
52. Wan S, Metruccio M, Kroken A, et al. Impact of contact lens wear and dry eye on the amicrobiomic status of the murine cornea. *Invest Ophthalmol Vis Sci*. 2018;59:902.
53. Wan SJ, Sullivan AB, Shieh P, et al. IL-1R and MyD88 contribute to the absence of a bacterial microbiome on the healthy murine cornea. *Front Microbiol*. 2018;9:1117.
54. Siegrist MS, Whiteside S, Jewett JC, et al. d-Amino acid chemical reporters reveal peptidoglycan dynamics of an intracellular pathogen. *ACS Chem Biol*. 2013;8:500−505.
55. Mark Welch JL, Rossetti BJ, Rieken CW, et al. Biogeography of a human oral microbiome at the micron scale. *Proc Natl Acad Sci U S A*. 2016;113:E791−E800.
56. Wilbert SA, Mark Welch JL, Borisy GG. Spatial ecology of the human tongue dorsum microbiome. *Cell Rep*. 2020;30:4003−4015.e3.
57. Kamariza M, Shieh P, Ealand CS, et al. Rapid detection of *Mycobacterium tuberculosis* in sputum with a solvatochromic trehalose probe. *Sci Transl Med*. 2018;10:eaam6310.