REVIEW

Investigating the Ocular Surface Microbiome: What Can It Tell Us?

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Abstract: While pathogens of the eye have been studied for a very long time, the existence of resident microbes on the surface of healthy eyes has gained interest only recently. It appears that commensal microbes are a normal feature of the healthy eye, whose role and properties are currently the subject of extensive research. This review provides an overview of studies that have used 16s rRNA gene sequencing and whole metagenome shotgun sequencing to characterize microbial communities associated with the healthy ocular surface from kingdom to genus level. Bacteria are the primary colonizers of the healthy ocular surface, with three predominant phyla: *Proteobacteria, Actinobacteria*, and *Firmicutes*, regardless of the host, environment, and method used. Refining the microbial classification to the genus level reveals a highly variable distribution from one individual and study to another. Factors accounting for this variability are intriguing - it is currently unknown to what extent this is attributable to the individuals and their environment and how much is artifactual. Clearly, it is technically challenging to accurately describe the microorganisms of the ocular surface because their abundance is relatively low, thus, permitting substantial contaminations. More research is needed, including better experimental standards to prevent biases, and the exploration of the ocular surface microbiome's role in a spectrum of healthy to pathological states. Outcomes from such research include the opportunity for therapeutic interventions targeting the microbiome.

Keywords: ocular surface, ocular surface microbiome, 16s rRNA gene sequencing, whole metagenome shotgun sequencing

Introduction

The role of the ocular surface microbiome (OSM) in health and disease of the underlying tissue is increasingly receiving attention, and knowledge in the field is expanding rapidly.[1] Among others, following features were identified that warrant further attention. (1) In humans and selected model organisms, the ocular surface (OS) microbiota may play a role in shaping local and systemic immune responses.[2,3] (2) There seems to be a resident OSM.[4] (3) The OS microbiota participates in the defense against pathogenic invaders through nutrient and niche competition.[5] (4) The OSM composition could be influenced by host, environmental and iatrogenic factors; OSM disbalance, called dysbiosis, seems to be associated with a variety of OS and systemic disorders.[6] (5) This offers the opportunity to modulate the OSM homeostasis to improve ocular health.[7–9] Today, we are still at an early stage in OSM research and these questions are subject to active investigation. This review provides an up-to-date picture of the knowledge on OSM composition, with emphasis on bacterial, viral, and fungal diversity at the conjunctiva as well as encountered technical challenges. Microbiome research has made impressive advances in recent years. It was in the 1670s that bacteria living in humans were observed and described as "little living animalcules" for the first time. Compared to other human microbiomes of the gut, skin, vagina, oral and nasal cavities, the OSM is unique due to its sparse microbial colonization, which makes it particularly challenging to analyze. The first reports about the presence of an OSM are based on culture-dependent methods.[10–13] Technological advances, especially modern sequencing technologies have enabled a high-throughput and broad characterization of microbiomes. The term microbiome refers to all microbes, including bacteria, viruses, archaea and eukaryotes, and their genetic material in a defined environment. This term should be distinguished from microbiota, which describes the microbes present in a defined niche.

The OS as a Microbial Niche and Its Microhabitats

The OS is continuously exposed to the environment, to microbes and microparticles with potential infectious, toxic or allergenic effects. Yet it hosts an unusually low microbial load compared to other barrier surfaces and mucosae. This highlights the presence of efficient protection mechanisms at the OS in homeostasis, with balanced immune responses and autoimmunity. Initial mechanical protective mechanisms include blinking and the tear reflex, as well as chemical defense provided by antimicrobial molecules in the tear film.[14] These antimicrobial tear proteins, including lysozyme, lactoferrin, mucins, defensins, interleukins and other antimicrobial peptides, act through various mechanisms such as lethal or growth inhibitory antimicrobial activity and immunomodulatory effects. An additional line of defense is mediated by the conjunctiva-associated lymphoid tissue (CALT). CALT harbors a diverse repertoire of immune cells including immune competent cells, secretory Immunoglobulin A and lymphocytes.[15,16] Additionally, recent findings indicate that resident bacteria on the OS could inhibit the growth of invading pathogens through mutual regulation, as has been observed in other mucosal niches as well.[5,17,18] As previously reviewed, there are dedicated anatomical structures and mechanisms to prevent excessive immune reactions.[6] A key feature seems to be the physical barrier separating microbes from the host's exposed surface, the epithelium, which itself is a pivotal activator of innate and adaptive immune responses. Structures involved in this barrier include the tear film mucus, antimicrobial peptides, secreted immunoglobulins and immune cells. At the OS, different microhabitats with specific anatomical and physiological properties can be distinguished, which may also harbor different flora: the cornea; the limbal, bulbar, tarsal, and fornical conjunctiva; and the lid margin.[19,20] In summary, the OS composed of separate microhabitats, has evolved specific protective mechanisms that exert significant selection pressure on the resident microbiota. This raises the question of whether the OS microbiota have specific properties to survive and outcompete other invading microbes. Consequently, the conjunctiva cannot be simplified as a sparse version of other mucosae; instead, an inherent microbial population of OS is to be expected.

Technical Challenges in Characterizing the Low Abundant OSM

Culture-based methods represent a long established and well-described procedure for the identification of commensal bacteria and fungi.[10–13,21] Cultures have the advantage of isolating living microbes only.[22] In contrast, they have the major drawback of being time-consuming and unfit to describe and quantify the vast composition of complex microbiomes.[23] Indeed, bacterial cultures were initially developed to grow specific pathogens and generate pure cultures for further investigations. A majority of environmental bacteria, though, are not easily cultured, making this method inadequate for the characterization of novel microbiomes being continuously exposed to the environment.[24–27] The advent of modern sequencing technologies has enabled more extensive microbiome characterization. 16s rRNA gene sequencing has been the most used sequencing technology in microbiome research so far. With regard to low abundance microbiomes, this method has the advantage of selecting and amplifying the input material by PCR before sequencing, namely the 16s rRNA gene found in bacteria. This amplification step has the drawback of introducing biases to an extent that depends on the initial template concentration, DNA extraction method and number of PCR cycles used.[28–33] Sequencing of the 16s rRNA gene is aimed to target only bacteria, thus omitting other kingdoms, which represents another limitation. Whole metagenome shotgun sequencing is an emerging sequencing technology. Compared to 16s rRNA gene sequencing, it provides a more comprehensive picture of the taxonomic composition and offers the possibility to study functional profiles of microbiomes. Whole metagenome shotgun sequencing consists of parallel sequencing of a sample's fragmented DNA, whose sequences are then assembled into longer reads and mapped to a database of reference sequences. This method bypasses some of the issues of 16s rRNA gene sequencing since there is no target gene amplification, although a global amplification step may be included in cases of low input DNA content. Its major benefit is the sequencing of DNA regardless of its origin, thus enabling detection of viruses, archaea and eukaryotes, in addition to bacteria. Furthermore, whole metagenome shotgun sequencing increases taxonomic resolution and provides deeper genomic information.[34,35] The broader distribution of marker sequences in target genomes has the drawback of increasing the sensitivity of this method to short random DNA contaminations. This might result in artifactual identification of microbial species, especially in low abundance microbiomes.[36] Both 16s rRNA gene sequencing and whole metagenome shotgun sequencing do not provide absolute quantification of microbial DNA. This can instead be obtained by qPCR of unspecific 16s rRNA genes, enabling an absolute quantification of bacterial load.[37,38] Meta-analyses are essential for

understanding the highly variable results from studies on low abundant microbiomes, but the lack of standardized procedures in such studies make these analyses very difficult.[33] Well-designed controls are necessary to eliminate false positive results and to detect biases introduced during the sampling, DNA extraction and sequencing steps. Negative controls should be included to control sterile working conditions. It seems that contamination originating from the extraction kits represents, among others, an important source of false positive results, especially when working with DNA at low concentrations. It was shown that DNA extraction kits contain a "kitome"-A term describing the genetic material originating from DNA extraction kits, library preparation kits and their solutions, which differs from one production lot to another.[36] To account for systematic biases, positive controls with known microbial composition would be beneficial. Such controls recently became commercially available and include among others a standard from ZymoBiomics comprising fungal and bacterial DNA from a range of gram-positive and gram-negative bacteria with varying GC content (ZymoBiomics Microbial Community Standard, Zymo Research Corp., Irvine, CA, United States). To our knowledge, there is no microbial community standard available containing bacterial, fungal, as well as viral DNA. However, the latter would be relevant since viruses have been detected on the OS as well.[4,39,40] In samples with a small amount of target DNA, host DNA depletion or microbiome enrichment should be considered.[41,42] Host DNA depletion aims to increase the relative concentration of target DNA as well as to eliminate free DNA originating from dead cells. One study addressed the efficiency of different target DNA enrichment methods and found an up to ten-fold increase in relative bacterial DNA content by selective host cell lysis.[43] An increase in target DNA compared to host DNA improves sequencing read quality and decreases sequencing cost by reducing the total amount of reads needed. In summary, research on low-abundance microbiomes progresses with the introduction of modern sequencing technologies but lacks experience and standardization. Well-designed experiments including positive and negative controls, host DNA depletion and absolute quantification of microbial DNA are needed in future studies to improve reproducibility and comparability.

Microbial Flora at the Ocular Surface

A core microbiome is generally described as a set of microbial taxa that are characteristic of a specific host or environment, although its definition and quantification is still subject to debate.[6,44] The exact composition of the ocular microbiota of the healthy eye is difficult to characterize due to the technical and analytical challenges mentioned earlier, leading to varying results depending on the methods and protocols used. In addition, host and environmental factors can influence the composition of the microbial flora, complicating the standardization of studies in humans. Here we attempt to highlight some constant and variable features of the OSM from recent studies published in the scientific literature. First, the scientific community tends to agree that the OS contains fewer microbes than other human microbial niches. The term "paucimicrobial" has been used to describe this sparse microbial colonization, although it lacks a precise definition. Traditional culture techniques originally identified few bacteria and fungi on the OS.[45–47] More recently, a study measured the bacterial mass at the conjunctiva, facial skin and buccal mucosa in healthy adults using 16s gene quantitative PCR. It was reported that 0.05 bacteria were detectable per conjunctival cell, corresponding to less than 40 bacteria per conjunctival swab. In comparison, there were approximately 150-fold more bacteria on the facial skin or buccal mucosa than at the conjunctiva. Furthermore, there were approximately 2 times more bacteria present at the lower than at the upper conjunctiva. In terms of bacterial load, there were no statistically significant differences between men and women, right and left eye, and subjects younger than 30 years and older than 60 years old.[4] To get an overview of the major kingdoms detected on the OS, we searched the literature using PubMed for studies that performed whole metagenome shotgun sequencing of the OSM in which numerical data were available on the relative abundance of major kingdoms at the OSM of healthy subjects. Five studies matching these criteria show that bacteria account for the great majority of the OSM (average 91%, median 93%, range [78–98%]), followed by viruses (average 5%, median 1%, range [1–7%]), and fungi or other eukaryotes (average and median 4%, range [0.02–20%]) (Figure 1).[39,48–51] Of note, bacterial DNA was detected in all participants in these studies, while fungi and viruses were not. For instance, in one study, fungi and viruses were detected in 35% and 41% of subjects, respectively.[49] For comparison, the skin is mainly colonized by bacteria, followed by viruses (< 1/4) and eukaryotes (< 1/10), with frequencies varying by anatomical location and individual.[52]
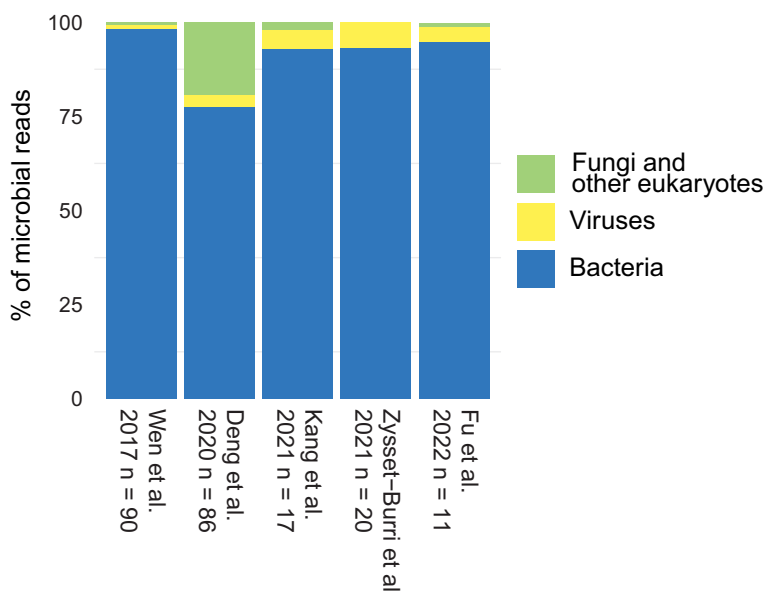
Figure 1 Relative composition of major kingdoms on human conjunctiva (% of microbial reads), based on 5 studies using the metagenomic shotgun technique, showing an abundant number of bacterial reads (average 91%, median 93%, range [78–98%]), less viral reads (average 5%, median 1%, range [0–20%]), and minimal fungal or other eukaryotic reads (average 4%, median 4%, range [1–7%]). References: Fu et al 2022;[51] Zysset–Burri et al 2021;[39] Kang et al 2021;[49] Deng et al 2020;[48] Wen et al.[50]

Bacterial Composition of the OSM

To get an overall picture of the most abundant bacterial taxa on the OS, we performed a literature search in PubMed that included studies on OSM using sequencing (16s rRNA gene or whole metagenome shotgun sequencing) and in which numerical data on the relative abundance of bacterial taxa were available. Studies including fewer than 11 individuals were excluded. 17 publications featuring 18 cohorts of healthy individuals, including 2 pediatric, met these criteria.[4,39,49,51,53–65] Of the available data, relative abundances were reported in at least 2 different cohorts for 20 genera, as shown in Figure 2A. Data from conjunctival samples in one study were provided to us by one of the present co-authors.[39] Note that in most papers but not all, data were available if bacterial taxa were detected with a relative frequency of > 1%; for convenience, we excluded those with a relative frequency of < 1%. To give a measure to the most abundant bacterial taxa, we calculated weighted average and median values considering the frequency with which different taxa were reported, thus accounting for possible "outliers" like bacterial taxa that are very abundant in a minimal number of studies.[1] In the following, the weighted average and median (WA and WM) therefore refer to average and median values multiplied by the proportion of datasets in which a given taxon is reported with an abundance of > 1% among the 18 cohorts considered. Globally, the most abundant genera were *Corynebacterium* (data availability 17/18, average and WA 11%, median and WM 9%) and *Pseudomonas* (data availability 9/18, average 19%, WA 10%, median 18%, WM 9%), followed by *Staphylococcus* (data availability 13/18, average 6%, WA 5%, median 6%, WM 4%), *Streptococcus* (data availability 9/18, average 7%, WA 4%, median 4%, VM 2%), *Acinetobacter* (data availability 10/18, average 6%, WA 3%, median 6%, WM 3%), *Propionibacterium* (data availability 7/18, average 8%, WA 3%, median 5%, WM 2%), *Bacillus* (data availability 5/18, average 10%, WA 3%, median 8%, WM 2%), *Agrobacterium* (data availability 2/18, average 17%, WA 2%, median 17%, WM 2%), *Sphingomonas* (data availability 5/18, average 4%, WA 1%, median 3%, WM 1%), *Cutibacterium* (data availability 3/18, average 7%, WA 1%, median 5%, WM 1%), and *Enhydrobacter* (data availability 3/18, average 6%, WA 1%, median 4%, WM 1%). Less abundant genera that have been reported in at least two cohorts include, by order of abundance, *Chryseobacterium, Rothia, Massilia, Moraxella, Neisseria, Paracoccus, Ralstonia, Pedobacter*, and *Prevotella*. At the phylum level, *Proteobacteria* (data availability 15/15, average 45%, median 52%, range [15–65%]), *Actinobacteria* (data availability 15/15, average 23%, median 17%, range [5–60%]), and *Firmicutes* (data availability 15/15, average 19%, median 17%, range [2–45%]) were the most prevalent phyla identified in all studies regardless of the sequencing method (Figure 2B). Other common phyla are *Bacteroidetes* (data availability 12/15, average 6%, median 5%), *Cyanobacteria* (data
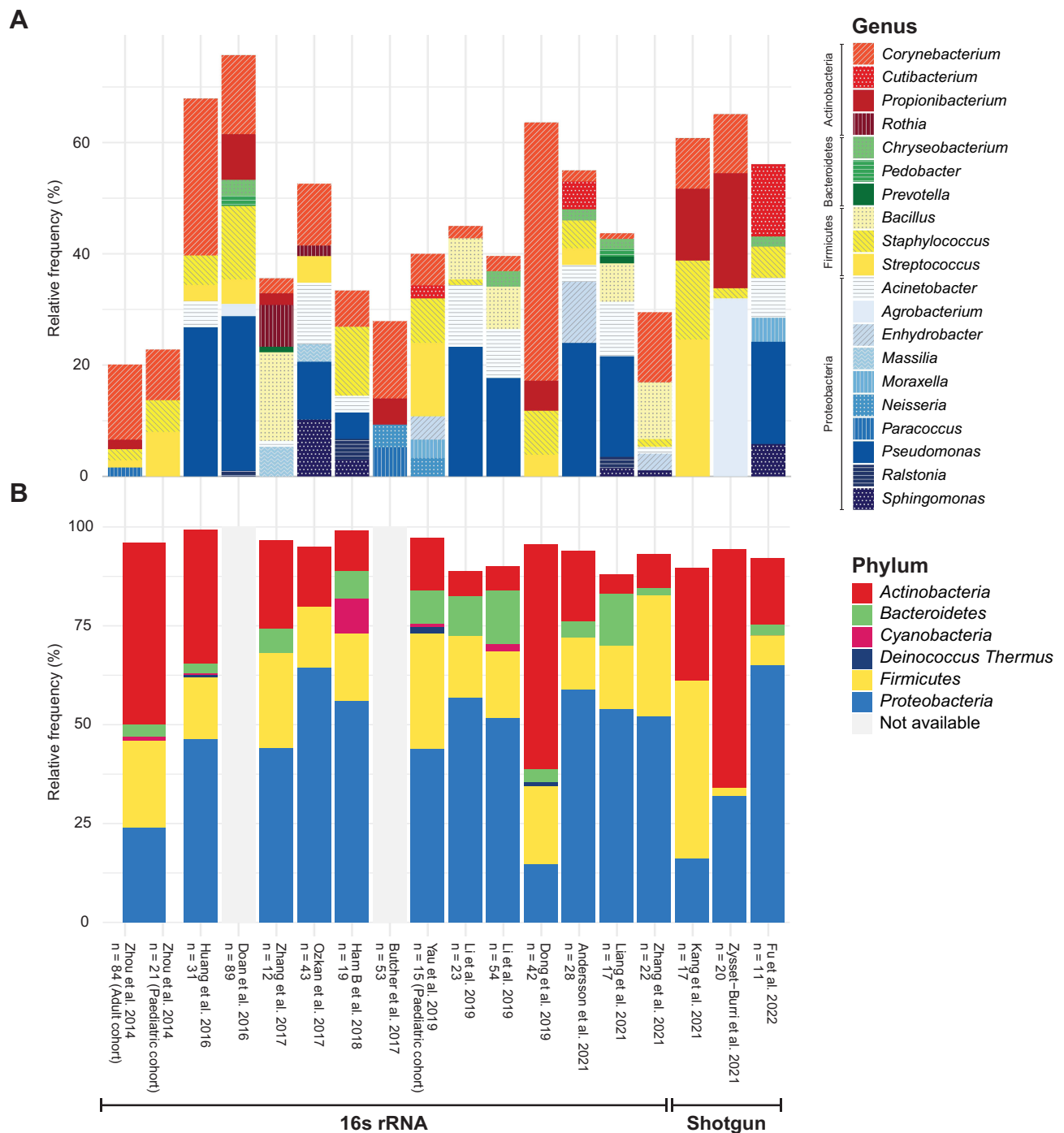
Figure 2 The relative abundance of ocular surface bacteria in healthy individuals at (**A**) Genus and (**B**) Phylum-level, as reported in 18 datasets from 17 publications. The classification of bacterial taxa is indicated with different colors. Two pediatric cohorts are included. At Genus level, *Corynebacterium* was most frequently reported with a relative frequency of > 1% (17/18), followed by *Staphylococcus* (13/18), *Acinetobacter* (10/18), *Pseudomonas* (9/18), and *Streptococcus* (9/18). At the Phylum level, the relative abundance of *Actinobacteria* (15/15), *Proteobacteria* (15/15), and *Firmicutes* were available in all datasets; those of *Bacteroidetes* (12/15), *Cyanobacteria* (5/15), and *Deinococcus Thermus* (4/15) were only available in a subset of the datasets. References: Fu et al 2022;[51] Zysset–Burri et al 2021;[39] Kang et al 2021;[49] Zhang et al 2021;[64] Liang et al 2021;[60] Andersson et al 2021;[53] Dong et al 2019;[55] Li et al 2019 (n= 54);[59] Li et al 2019 (n= 23);[58] Yau et al 2019;[62] Butcher et al 2017;[54] Ham B et al 2018;[56] Ozkan et al 2017;[61] Zhang et al 2017;[63] Doan et al 2016;[4] Huang et al 2016;[57] Zhou et al 2014 (adult and paediatric cohorts).[65]

availability 5/15, average 3%, median 1%), and *Deinococcus Thermus* (data availability 4/15, average 1%, median 1%). Although there is currently no consensus regarding the presence of a core OSM, it appears from these data that the genus *Corynebacterium* is systematically detected in the studies and is quite abundant on average, although it is not ubiquitously detected when individual subjects are considered. In a study that included negative environmental controls,

Corynebacterium was also the taxon that showed the highest enrichment in the conjunctiva when compared with the environmental controls, followed by *Propionibacteria, Staphylococcus*, and *Streptococcus* among others.[4] Among the 18 datasets, *Pseudomonas* appeared to be very abundant only in a subset of the studies (55%). When compared to environmental negative controls, *Pseudomonas* displayed a poor conjunctival enrichment in one study.[4] Previous culturing studies commonly isolated Coagulase-negative *Staphylococci* species (predominantly *S. epidermidis* and *S. aureus*), as well as *Propionibacterium, Corynebacterium*, and *Streptococcus*.[12,45,47,66–70] These genera were also detected by sequencing, although the results obtained by culturing differed in that a vast majority of coagulase-negative *Staphylococci* were isolated, which does not appear to be the case from sequencing studies. This difference highlights one of the drawbacks of culturing, that facilitates the growth of bacteria adapted to the given culture conditions. However, data from culturing provide an important additional layer of evidence for those bacteria whose growth was previously demonstrated, as culturing has the advantage of isolating viable bacteria. To compare the OSM depending on its sampling location, two studies compared the bacterial composition of the conjunctiva with that of the lid margin and periocular skin, which revealed significant differences in microbial community structure. Indeed, *Proteobacteria* were present at all sites and predominated at the conjunctiva, whereas *Firmicutes* were more prevalent and predominant on the periocular skin (45%) and at the lid margin (53%).[19,71] Moreover, the Shannon diversity index, a metric used to characterize the overall species diversity, differed significantly between sites and was highest at the skin and lowest at the conjunctiva. One of these studies further addressed the difference between two conjunctival sites: limbus and fornix, showing no significant differences in bacterial community.[19] In summary, based on the studies characterizing the composition of the OSM so far in up to 89 healthy subjects, there is currently no definition of the core ocular microbiota. Further studies with large sample sizes under standardized conditions are required to validate the current findings and account for this high variability. Furthermore, the OSM from healthy individuals may be compared to the OSM from patients with eye diseases. However, this topic lies outside the scope of this review and the data are still limited.

Viruses at the OS

Virus is the second most detected kingdom on the healthy OS so far.[39,48–51] Yet there is little information on the composition and function of the OS virome, a term referring to the set of all viruses in a community and their genetic material. An important increase of interest on this topic has occurred during the SARS-CoV-2 pandemic as conjunctivitis was the most diagnosed ophthalmic disease among COVID-19 patients.[72] Studies searching for the presence of SARS-CoV-2 virus in tears have yielded widely variable results, with positive rates ranging from 0% to 72%, and the question whether ocular transmission of SARS-CoV-2 is possible is still debated.[73,74] Additionally, it has been shown that resident viruses of the OS were involved in the pathogenesis of ocular adnexal neoplasia. The *Human papilloma virus (HPV)* associated with conjunctival papilloma and squamous cell carcinoma is a typical example; another is *Merkel cell polyomavirus (MCV)* associated with Merkel cell carcinoma.[75,76] It appears that the OS virome may be important not only in disease but also in maintaining OSM homeostasis. In the studies summarized below, the three methods used for viral detection include whole metagenome shotgun sequencing, Biome representational in silico karyotyping (BRiSK) and viral-directed PCR. It is important to note that none of these three techniques can detect RNA viruses; this requires RNA sequencing, which has not been addressed in studies to date. Microbial interactions are crucial for the sustainability of various ecosystems, including the interplay between species not only within the same kingdom but also between different kingdoms.[77] For instance, interactions between viruses and bacteria lead to the modulation of the antiviral immune response and thus influence the viral infectivity.[78] On the other hand, bacteria-infecting viruses, called bacteriophages (phages), are one of the main regulators of bacterial population density and distribution. A review states that every 48 hours 50% of all bacteria on earth are killed by phages.[79] This efficiency to control bacterial population by phages highlights their relevance in ecosystems and makes phages attractive for therapy, for instance as alternative to antibiotics.[80] Recent results in gut microbiome research suggests that the relationship between phages and their bacterial hosts might be rather mutualistic than competitive.[79] As phages depend on the survival of their bacterial host, they can contribute to bacterial fitness through various mechanisms through phage "morons". Morons are independent DNA elements within the phage genomes introduced through horizontal gene transfer.[81,82] There is evidence that phages are present at the OS as well. Indeed, in one study applying whole metagenome shotgun sequencing to healthy conjunctival

samples from 17 individuals, phages of the genera *Siphoviridae* were among the 15 microbial genera detected with a relative abundance of more than 1%.[49] In this study, the relative abundance of viruses was on average 5% and the positive rate for viruses 41%. Two other viral genera were *Anelloviridae* and *Alphatorquevirus*. The family *Anelloviridae* include the genus *Alphatorquevirus*, to which the *Torque teno virus (TTV)* genotypes belong.[49] Interestingly, occurrence of *TTV* in vitreous fluid has been associated with seasonal hyper acute pan uveitis, and another study indicates that *TTV* triggers an adaptive immune response.[83,84] In one study, *TTV* was also identified in all seven culture-negative endophthalmitis samples versus 57.1% of the fourteen culture-positive samples, whereas no *TTV* was found in the control group of seven subjects with non-inflamed eyes.[40] Another study also detected *TTV* in 86.3% of 80 healthy conjunctival samples using BRiSK and confirmed for 65% of the samples by directed quantitative PCR. As *TTV* was the predominant virus found in this study, it was hypothesized that *TTV* may be an overlooked or unknown resident virus of the OS. Two other viruses, namely the *Multiple Sclerosis–associated retrovirus* and *Human Endogenous Retrovirus K* were found in 86.3% of all samples with BRiSK.[4] The clinical significance of this result is unclear, as these represent known human endogenous retroviruses and thus likely originated from sequencing of host DNA.[85] Following three viruses were identified with a very low positive rate (2/80 samples) and their significance is unclear: *MCV, HPV,* and *Abelson murine leukemia virus*. Another study detected elevated levels of *MCV, TTV,* and *HPV* in healthy anophthalmic conjunctiva compared to the conjunctiva in healthy eyes.[86] According to the researchers, these results suggest that enucleation and prosthesis placement may affect the OS viral microbiome. Due to the oncogenic nature of *MCV*, understanding the mechanisms by which the OS regulates this virus may have clinical importance. In general, deciphering the viral and fungal as well as bacterial constituents of the healthy OS may be crucial for understanding OSM homeostasis. The pathogenic and oncogenic characteristics of certain microbes found on the healthy OS underline the clinical relevance of this research.

Fungi at the OS

Only a minor proportion of the OSM is attributed to eukaryotes, most of which are fungi.[39,48–51] Since fungi are pathogens in ocular diseases like blepharitis, conjunctivitis, keratitis and endophthalmitis,[87] several studies about fungi at the ocular surface exist. However, only a limited number of studies have attempted to investigate the fungal OSM in healthy human eyes. Attention has been brought to this matter by the discovery that individuals developing fungal keratitis have an altered bacterial composition not only at the surface of the affected eye but also at the fellow non-affected eye when compared to healthy subjects' eyes. This observation suggests that bacterial composition influences susceptibility to fungal infection.[88] Here we focus on fungi possibly associated with the healthy conjunctiva and review the first attempts to define a core fungal microbiome. Although other eukaryotes are known to cause OS disease such as *Acanthamoeba*, studies in this regard are very limited.[89] Traditional culture techniques and sequencing methods (whole metagenome shotgun sequencing and amplicon sequencing) have led to discordant results about the composition and function of the fungal microbiome. For fungal amplicon sequencing, the internal transcribed spacer (ITS) region is used as a sequencing target.[52] When compared to sequencing, the culture-dependent method identifies a reduced spectrum of fungi, as many fungi are not cultivable.[90] Cultivation also shows a lower positivity rate (proportion of samples in which fungi are detected) than sequencing. Two studies compared the results obtained from cultivation to ITS2 amplicon sequencing in the same healthy individuals. In these studies, cultivation detected only 1 to 4 genera with a positivity rate of 8.90% to 12.50%, whereas amplicon sequencing detected 65 to 94 genera with a positivity rate of 40% to 73.50%.[91,92] Overall, the positivity rate for fungi at the OS based on the culture technique varies between 3% to 28% in different studies.[92] The identification of fungi by the sequencing method also has its limitations as many fungi are under-represented in the databases and therefore remain unclassifiable.[93] Secondly, confirming that a fungus identified by sequencing is not artifactual at the investigated site is difficult. Furthermore, discordant results are obtained from the two different sequencing methods (whole metagenome shotgun sequencing and amplicon sequencing), implying that an accurate representation of the true fungal composition at the OS is not attained.[49] A study using whole metagenome shotgun sequencing showed a lower positivity rate for fungi compared to amplicon sequencing (35% vs 40% to 73.50%) and detected only two phyla (*Ascomycota* and *Basidiomycota*) out of four that had been found on the OS with ITS2 so far.[49] It has been hypothesized that this difference is due to the short read lengths generated by whole metagenome

shotgun sequencing which makes the eukaryotic genome challenging to assemble, as it is larger, more complex, and richer in repeats compared to prokaryotic genomes.[94] These issues should be considered in future whole metagenome shotgun sequencing studies of the OSM. ITS has been formally proposed for adoption as the primary fungal barcode marker to the Consortium for the Barcode of Life and might currently be the best method to specifically capture the ocular surface fungal core and transient microbiota.[95] Fungi that have been identified at the OS using cultivation include *Alternaria sp., Fusarium sp., Aspergillus niger, Aspergillus flavus, Curvularia sp., Penicillium sp., Helminthosporium sp., Candida albicans, Candida guilliermondii, Candida parapsilosis, Saccharomyces cerevisiae, Hormodendrum sp.*, and *Rhodotorula rubra*.[91] In a study using ITS2 amplicon sequencing to characterize fungi on the eyes of 17 healthy adults living in the southern part of India, 12 to 24 genera per microbiome were identified, making a total of 65 distinct genera. The two dominant phyla were *Ascomycota* (mean abundance (MA): 47.74%) and *Basidiomycota* (MA: 26.87%) followed by *Zygomycota* (MA: 0.08%) (in two eyes). The 4 genera *Aspergillus* (MA: 21.05%), *Setosphaeria* (MA: 14.97%), *Malassezia* (MA: 7.37%) and *Hematonectria* (MA: 2.73%) were present in all eyes in which fungi were detected. There was no difference in alpha diversity between the left and the right eye and sex had no influence, whereas alpha diversity was altered by age.[91] Using the same method, another study on healthy eyes including 45 healthy adults from an urban community in the Shandong Province in China detected 4 phyla, the most abundant being *Basidiomycota* (MA: 78.67%) and *Ascomycota* (MA: 19.54%), followed by *Zygomycota* (MA: 0.13%) and *Neocallimastigomycota* (MA: 0.01%). In total, 94 genera were identified, among which 51 were identified in at least two individuals, while the remaining 43 were detected only once. Overall, only *Malassezia* accounted for a relative abundance >1% in each individual. *Malassezia* is a major component of the skin microbiome.[96] "Core" fungal taxa of the healthy OS, defined by an abundance of at least 0.01% in more than 80% of the participants, included *Basidiomycota* and *Ascomycota* at the phylum level and *Malassezia* (MA: 74.65%), *Rhodotorula* (MA: 1.93%), *Davidiella* (MA: 1.89%), *Aspergillus* (MA: 1.25%) and *Alternaria* (MA: 0.61%) at the genus level.[92] Taken together, *Basidiomycota* and *Ascomycota* were the most abundant phyla on healthy eyes according to studies using sequencing, despite variable abundances. Relative abundance and distribution of the dominant genera varied significantly among individuals within and in between the studies. More studies will be necessary to confirm our knowledge about commensal and transient ocular surface fungal microbiome composition and function, as well as influencing factors such as sex, age, occupation, environment, season and geographic location.

The OS Microbiome in the Context Its Host's Immunity and Tear Proteome

Human microbiomes are diverse and vary from one host to another because of a series of factors. The gut microbiome, for instance, is influenced by host factors such as age, sex, genetics, and immune status, as well as by environmental factors such as diet and drugs. In turn, human microbiomes have an impact on the host's health, including ocular health.[97] Indeed, it was recently pointed out that compositional and functional alterations of the gut microbiome, in interaction with the immune system, were associated with ocular diseases.[98–101] Moreover, it was shown that a healthy gut microbiome strengthens the ocular mucosal immune barrier through increasing immune effectors such as IgA and complement in the tear film of mice. Evidence suggests that the OSM is in a symbiotic relationship with the immune system and plays a role in the education, function, and induction of the immune system.[2,3,102] A recent study demonstrated a causal relationship between the presence of *Corynebacterium mastitidis* at the OS and efficient defense against pathogens through activation of local immunity.[2] Additional evidence was provided in mouse models demonstrating an increased susceptibility to keratitis when the ocular surface was depleted from commensal bacteria through lack of local recruitment of neutrophils usually induced by the resident bacteria.[103] The OSM itself appears to be influenced by various host and environmental factors, whose role and importance are still elusive.[65] A better understanding of the factors determining OSM composition and diversity could therefore substantially broaden the understanding of pathogenesis of OS diseases. One study has investigated the interplay between the healthy OSM and the tear proteome. This was the first investigation that found associations between taxonomical and functional features of the OSM and the tear proteome in humans.[39] Analysis of the tear proteome with chromatography tandem mass spectrometry showed the 5 most abundant proteins to be lactotransferrin, albumin, lipocalin-1, lysozyme, and immunoglobulin A. Apart from albumin,

these proteins all have antimicrobial activity. Functional classification based on Gene Ontology (GO) categories of the 2172 identified protein groups, revealed that 9% of the human tear proteins are involved in the biological process of antimicrobial humoral response. These results are consistent with previous knowledge that protection against pathogens is among the most important functions of the tear film.[104] It has been hypothesized that antimicrobial components of the tear film may influence the OSM and vice versa. In line with this hypothesis, a disturbed tear film composition could favor dysbiosis of the OSM, lower protection against pathogens and ultimately lead to disease.[39] Another study assessing the association between tear proteome and OSM in healthy eyes showed abundance of amino acid metabolism pathways (with the highest proportion being the tyrosine metabolism).[49] Amino acids are naturally found in tears and have a positive effect on maintaining OS homeostasis, and it has been hypothesized that bacteria at the OS may play a role in the metabolism of tear film amino acids.[105,106] Since *Corynebacterium* sub-species are known to produce amino acids, the *Corynebacterium* found on the OS may also be involved in amino acid metabolism.[107] However, more research is needed to assess whether the healthy OSM contributes to the abundance of amino acids in tears and their potential beneficial effects. Kang et al detected microbial pathways involved in inorganic ion transport and metabolism as well as lipid metabolism and suggest further exploration whether the OSM affects tear film stability through these pathways.[49]

Discussion and Conclusions

The Human Microbiome Project[108] aimed to characterize the microbiomes of the skin, gastrointestinal tract, oral cavity, nasal passages, and the urogenital tract to understand its role in health and disease. While there is evidence that microbes are involved in various host processes, a dysbiosis in the microbiota may be associated with several diseases including eye disease. Although the OS is continuously exposed to the environment including potentially pathogenic microbes, the OSM has not been characterized in the Human Microbiome Project. It has been much debated whether environmental microbes can adhere to and colonize the OS since host factors (mechanical barriers such as blinking and tear reflex as well as chemical defense such as anti-microbial tear proteins) create a harsh environment for microbes.[109] Early traditional culture techniques showed the presence of bacteria at the conjunctiva of healthy subjects.[12] However, these techniques are limited and biased since only cultivable species grow under the provided conditions and represent only a small proportion of the total microbial population in a sample. A more thorough characterization of the OSM has been provided by modern sequencing technologies. Using whole metagenome shotgun sequencing, it has been shown that in addition to bacteria, viruses (including bacteriophages) and eukaryotes (mainly fungi) colonize the human OS. While these techniques have been effective in identifying microbes in large biomass samples such as the gut, data inconsistencies (amplification bias, misidentification of microbial species) pose a problem in low microbial niches such as the OS. For this reason, there is a lack of reproducible data on taxonomic composition including diversity, abundance, and homeostasis of the OSM, thus the definition of a core microbiota is still missing. Nevertheless, it is important to mention that many different definitions of "core microbiota" have been used in microbiome studies so far and no consensus has been reached. After a detailed analysis of these definitions, the following description was proposed:

> The core microbiota is a suite of members shared among microbial consortia from similar habitats, which is important for understanding stability, plasticity, and functioning across complex microbial assemblages.[110]

This may be of interest for further studies focused on determining the core and transient microbiome of the ocular surface. Another consideration with potential public health relevance regards the influence of environmental factors in OSM composition. Indeed, it is known that factors such as hygiene behavior, contact with young children, or seasonal and climatic conditions affect conjunctivitis incidence.[111] In turn, the manner and extent to which external factors shape OSM structure is largely unknown. While few studies attempting to address this question have obtained interesting results, we are still humbled by the complexity of this matter.[48,65]

In conclusion, OSM research is still at an early stage and needs further investigation. It is of upmost importance to define a core microbiota in this niche with low microbial abundance. Indeed, this will provide the basis for understanding how the OSM shapes host physiology in health and disease, and ultimately developing microbial-based therapeutic approaches for the prevention and treatment of ocular diseases.

Abbreviations

OSM, ocular surface microbiome; OS, ocular surface, CALT, conjunctiva-associated lymphoid tissue; WA, weighted average; WM, weighted median; HPV, Human papilloma virus; MCV, Merkel cell polyomavirus; BRiSK, Biome representational in silico karyotyping; TTV, Torque teno virus; ITS, internal transcribed spacer; MA, mean abundance; GO, Gene Ontology.

Disclosure

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