The ocular microbiome and microbiota and their effects on ocular surface pathophysiology and disorders

Pasquale Aragona, MD, PhD[a,*], *Christophe Baudouin, MD, PhD, FARVO*[b], *Jose M. Benitez del Castillo, MD, PhD*[c], *Elisabeth Messmer, MD, FEBO*[d], *Stefano Barabino, MD, PhD*[e], *Jesus Merayo-Lloves, MD, PhD*[f], *Francoise Brignole-Baudouin, MD, PhD*[g,h,i], *Leandro Inferrera, MD*[a], *Maurizio Rolando, MD*[j], *Rita Mencucci, MD*[k], *Maria Rescigno, PhD*[l], *Stefano Bonini, MD*[m], *Marc Labetoulle, MD, PhD*[n]

[a] *Department of Biomedical Sciences, Ophthalmology Clinic, University of Messina, Messina, Italy*
[b] *Quinze-Vingts National Eye Hospital, IHU ForeSight, Paris Saclay University, Paris, France*
[c] *Departamento de Oftalmología, Hospital Clínico San Carlos, Clínica Rementeria, Instituto Investigaciones Oftalmologicas Ramon Castroviejo, Universidad Complutense, Madrid, Spain*
[d] *Department of Ophthalmology, Ludwig-Maximilians-University, Munich, Germany*
[e] *Ocular Surface and Dry Eye Center, Ospedale L. Sacco, University of Milan, Milan, Italy*
[f] *Instituto Universitario Fernández-Vega, Universidad de Oviedo, Oviedo, Spain*
[g] *Sorbonne Université, INSERM UMR_S968, CNRS UMR7210, Institut de la Vision, Paris, France*
[h] *CHNO des Quinze-Vingts, INSERM-DGOS CIC 1423, Laboratoire de Biologie Médicale, Paris, France*
[i] *Université de Paris, Faculté de Pharmacie de Paris, Département de Chimie-Toxicologie Analytique et Cellulaire, Paris, France*
[j] *Ocular Surface and Dry Eye Center, ISPRE Ophthalmics, Genoa, Italy*
[k] *Department of Neuroscience, Psychology, Pharmacology and Child Health (NEUROFARBA), Eye Clinic, University of Florence, Florence, Italy*
[l] *Humanitas Clinical and Research Center – IRCCS, Humanitas University Department of Biomedical Sciences, Milan, Italy*
[m] *Department of Ophthalmology, University of Rome Campus Biomedico, Rome, Italy*
[n] *Ophthalmology Département, Hôpitaux Universitaires Paris-Sud, APHP, Université Paris-Saclay, IDMIT Infrastructure, Fontenay-aux-Roses Cedex, France*

[*] Corresponding author: Pasquale Aragona, MD, PhD, Department of Biomedical Sciences, Ophthalmology Clinic, University Hospital "G. Martino", University of Messina, Messina, Italy.
E-mail address: paragona@unime.it (P. Aragona).

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ABSTRACT

The ocular surface flora perform an important role in the defense mechanisms of the ocular surface system. Its regulation of the immunological activity and the barrier effect against pathogen invasion are remarkable. Composition of the flora differs according to the methods of investigation, because the microbiome, composed of the genetic material of bacteria, fungi, viruses, protozoa, and eukaryotes on the ocular surface, differs from the microbiota, which are the community of microorganisms that colonize the ocular surface. The observed composition of the ocular surface flora depends on harvesting and examining methods, whether with traditional culture or with more refined genetic analysis based on rRNA and DNA sequencing. Environment, diet, sex, and age influence the microbial flora composition, thus complicating the analysis of the baseline status. Moreover, potentially pathogenic organisms can affect its composition, as do various disorders, including chronic inflammation, and therapies applied to the ocular surface.

A better understanding of the composition and function of microbial communities at the ocular surface could bring new insights and clarify the epidemiology and pathology of ocular surface dynamics in health and disease. The purpose of this review is to provide an up-to-date overview of knowledge about this topic.

1. Introduction

Mounting evidence indicates that the microbiome/microbiota plays a role in regulating the immunologic status of the human body. The microbiome consists of the genetic material of bacteria, fungi, viruses, protozoa, and eukaryotes present on a specific tissue and differs from the microbiota, which refers to the community of microorganisms that colonize that tissue.

Changes of the microbiome/microbiota can derive from several factors, including environment, diet, age, and sex.[81,111] Although these factors have been addressed in the gut and other mucosae, less is known about their role in the ocular surface.

Because the ocular surface mucosa is directly exposed to the environment, it serves as a defense against potentially pathogenic microorganisms. Three mechanisms are involved in this process: mechanical (clearance of the ocular surface through blinking and tear secretion), chemical (antimicrobial elements in the tears, including lysozyme, lactoferrin, and defensins) and immunological (including resident immune competent cells, such as neutrophils, secretory IgA, lymphocytes, etc.).

The ocular surface microbiota is characterized by commensal bacteria that normally do not cause infection or inflammation.[40,52,60,98,120] In fact they contribute to ocular surface homeostasis, together with a mechanism of immunologic tolerance by the ocular surface structures. Bacterial species composing the normal microbiota inhibit the growth of pathogenic bacteria through a mechanism of competition.[74] When some bacteria, like *coagulase-negative staphylococci* (CoNS), overgrow, they can induce infection that causes severe damage to the ocular surface. The exact composition of the normal ocular microbiota is difficult to study, because it can be modified by the factors mentioned above. Furthermore, viruses can affect mucosal health and immunity,[27] likely by stimulating long-term immune tolerance (adaptive immunity) and rapid anti-infectious defenses (innate immunity) when acute disorders arise, as has been suggested for gut mucosa.[8,107]

Knowing the composition of the ocular surface microbiota and the interactions among its components and between the microbiota and the ocular surface structures will contribute to a better understanding of the pathogenic mechanisms underlying ocular surface diseases.

1.1. *Microbiologic terminology*

This paragraph is intended for those who are not familiar with microbiologic terminology and provides an explanation of the terms used in this review.

We start describing the hierarchical organization, namely the taxonomic rank, of the group of organisms involved: domain, kingdom, phylum, class, order, family, genus, species. In this review, as is normally used in papers about microbiome and microbiota, we deal with phyla, genera and species. To give an example, *E. coli* is a species, *Escherichia* the genus and *Proteobacterium* the phylum.

Another important terminology refers to diversity, indicating the range of different kinds of species of unicellular organisms, such as bacteria, archaea, protozoa, and fungi, present in a specific environment. The diversity in microbial population refers to the different metabolic, physiologic and morphologic characteristics that may influence, combined with genomic structure, their activity and behavior. In fact it is known that the microbial diversity on earth is high, but is thought to be even much higher than known. A diversity index is a mathematical measure of species diversity in a specific habitat.

Alpha diversity (*α-diversity*) refers to the number of species present in sites or habitats at a local scale. Beta diversity (*β-diversity*) refers to the mean species diversity living in different habitats in the same ecosystem. Gamma diversity (*γ-diversity*) is the number of species diversity in the entire ecosystem considered (Fig. 1).

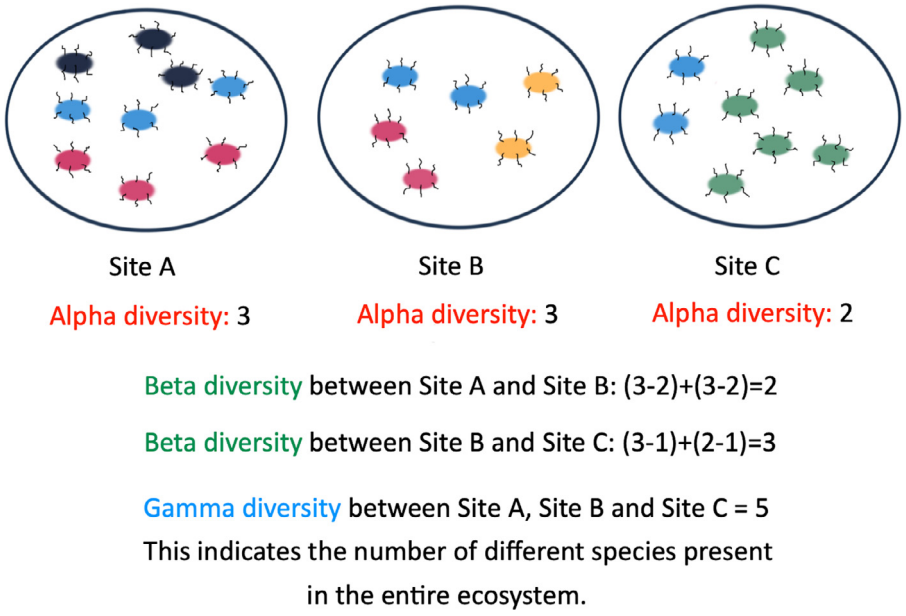

Fig. 1 – Schematic representation of three different habitats in an ecosystem. The different colors indicate different bacterial genera. The alpha diversity indicates the number of microorganism species in a specific habitat; the beta diversity between two different habitats represents the number of different microorganism species present in those habitats; gamma diversity represents the number of different microorganism species present in an ecosystem.

The Shannon diversity index is commonly used to characterize species diversity. It accounts for both abundance and evenness of the species present in a community.

The Shannon index is more informative than simply the number of species present in an environment. Therefore, it is a good tool to give a quantification of diversity in a community.

New microbiological tools based on genomic identification help to improve the recognition of the microorganisms present in a defined habitat.

To explain how changes in diversity may affect the development of ocular surface disorders, it was shown that changes in *α-diversity* may influence the development of ocular surface alterations in patients with graft versus host disease (GVHD) by the activation of the immune response toward the host tissue.[94] In their paper Shimizu and coworkers showed the presence of *Staphylococcus epidermidis*, followed by *Alpha-haemo Streptococcus, Corynebacterium* sp., *Propionibacterium acnes*, aerobic Gram-positive cocci, *Staphylococcus aureus*, *Haemophilus influenzae*, and aerobic Gram-positive rod were the species detected in the GVHD group. On the other hand, *Staphylococcus epidermidis*, *Propionibacterium acnes*, and *Propionibacterium* sp. were the most represented in the control group. An explanation of the higher *α-diversity* demonstrated in the conjunctival tissue of GVHD patients may be the conditioning regimen that is faced by these patients before hematopoietic stem cells transplantation, undergoing a series of immunosuppressing treatments that may allow the uncontrolled development of ocular surface microorganisms. The modified ocular surface flora may be the trigger for an unbalance of the donor/recipient T cells that may result in autoimmune aggression.

Another example is that Dong and coworkers demonstrated a modification of bacterial population among patients with different degrees of meibomian gland dysfunction (MGD) compared with a control group.[30] They failed to showed a difference in *α-diversity* between the groups, although they demonstrated in moderate/severe MGD a higher abundance of *Corynebacterium*, while the severe MGD showed an abundance of *Staphylococcus* higher than the other groups considered. The control non-MGD group showed a lower abundance of *Sphingomonas* in comparison with all the MGD groups. This indicates that the *α-diversity* gives a measure of the number of species present in an environment without addressing their abundance.

2. The ocular surface microbiome and microbiota

Microbial colonization of the conjunctiva probably starts soon after birth, although it has been proposed that colonization can start *in utero*.[109] At birth there is a dramatic change in abundance and types of ocular surface flora, and infants born vaginally have a greater number of different bacterial species than those delivered by Caesarean section.[31] Two days after birt, the swabs become more constantly positive, regardless of the method of delivery, indicating that colonization is a progressive process.[64] Geographic location, diet, and environment can influence the ocular surface microbial flora in the following weeks and months.

Gram-positive organisms, such as *Staphylococcus, Corynebacterium, Streptococcus,* and *Propionibacterium* sp., although not abundant, could play a role as commensals on

Table 1 – Microbiome composition, at the phylum level in healthy children aged less than 18 years old.[19]

Conjunctiva (%)	Lid margin (%)	Periocular skin (%)
Proteobacteria (57)	Firmicutes (53)	Firmicutes (45)
Firmicutes (17)	Bacteroidetes (19)	Proteobacteria (35)
Bacteroidetes (13)	Proteobacteria (17)	Bacteroidetes (13)
Actinobacteria (11)	Tenericutes (7)	Actinobacteria (5)
	Actinobacteria (3)	Fusobacteria (1.7)

structures like lids, cornea, conjunctiva, and tear film. The composition of the microbiota may be quite stable throughout life, but significant changes can be induced by external factors like contact lens (CL) wear, cosmetics, use of preservatives or antibiotics, ocular surgery, infections, or other ocular or systemic disorders.

Gram-negative bacteria (*Haemophilus* spp., *Neisseria* spp., *Pseudomonas* spp.), fungi and viruses, less common or not yet identified, can also be found on the ocular surface microbiome, even with minimal or no signs of inflammation or infection. Even less frequently, pathogens like *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, as well as other potentially pathogens like CoNS, can be found in the microbiota of a noninflamed ocular surface. These bacteria can be responsible for serious infections, such as post-cataract endophthalmitis and CL-related keratitis.[51,75,105]

2.1. *The ocular microbiome and microbiota in health*

In a study carried out in children aged below 18 years, Cavuoto and coworkers, using 16S rRNA sequencing, demonstrated that the phyla found in the conjunctival microbiome were different from those found in the lid margin and periocular skin (Table 1).[19] Although qualitative differences were found among the flora composition of the diverse sites, no differences were found when the beta diversity of microbial composition was taken into account, indicating that the overall number of species was similar in all the sites considered. The Shannon index, representing the richness and evenness of the species present, was the lowest in the conjunctiva, while no differences were found between lid margin and skin. This indicates that the protective measures existing on the ocular surface can influence the composition of the conjunctival microbiome.

Zhou and coworkers addressed the effect of age on microbiota composition.[125] In a case-control trial, carried out in West Africa and including 105 normal subjects and 115 patients with trachoma, conjunctival samples were examined with 16S rRNA sequencing. The conjunctiva of healthy subjects demonstrated the presence of three dominant phyla: *Actinobacteria* (46%), *Proteobacteria* (24%), and *Firmicutes* (22%). Furthermore, 13 genera were identified and, of these, 6 were present in 80% of samples with an abundance >1%: *Corynebacterium* (16%), *Streptococcus* (5%), *Propionibacterium* (4%), *Bacillus* (3%), *Staphylococcus* (3%), and *Ralsontia* (2%) (Table 2a). Comparing a group of healthy children 10 years or younger to a group over 10 years old, *Corynebacterium, Propionibacterium, Myceligenerans,* and *Paracoccus* were more abundant in the older group, although richness and Shannon diversity index appeared to be higher in young subjects. In the younger group, *Streptococcus, Kocuria, Staphylococcus, Micrococcus*, and *Brachybacterium* were most abundant. In the older group, seasonality was also studied, and it was found that *Bacillus* and *Tumebacillus* genera were more abundant in the dry season.

Doan and coworkers studied the composition of conjunctival bacteria by using 16S rDNA gene quantitative PCR to evaluate the bacterial load in adult subjects.[27] Specimens were obtained from upper and lower conjunctiva, periocular skin, and oral mucosae. Bacteria were characterized at each site using metagenomics data, and biome representational *in silico* karyotyping (BRiSK) data were used to investigate the presence of fungi and viruses. The core cluster microbiome, identified by deep DNA sequencing, consisted of the same four predominant species present in 86.3% of all subjects, as detected by culture: *Corynebacterium* (14.2%), *Propionibacterium* (8.2%), *Staphylococcus* (4.4%), and *Streptococcus* (13.2%), likely with sporadic or very low amounts of several other species (Table 2b); moreover, the cultures were positive for *Streptococcus viridans*, *Micrococcus, Bacillus*, unidentified Gram-positive rods, *Neisseria*, unidentified Gram-positive cocci, and *Haemophilus*. The lower conjunctiva contained more bacterial DNA than the upper conjunctiva. There were no statistically significant differences between women and men, but there was a significantly higher bacterial load in subjects older than 60 years compared to those younger than 30 years old. *Torque teno virus* (*TTV*), a single-strand circular DNA anellovirus, was present in healthy subjects with at least one copy per 100 epithelial cells. This virus was also associated with seasonal hyperacute panuveitis and culture-negative endophthalmitis.[63,99]

Dong and coworkers, in a 16S rRNA metagenomics sequencing study carried out on four healthy subjects, found that five bacterial phyla were the most represented, three of which—*Proteobacteria* (64%), *Actinobacteria* (19.6%), and *Firmicutes* (3.9%)—accounted for 87.9% of all sequences.[29] At the genus level, twelve genera showed a ubiquitous distribution among all subjects, accounting for 96% of known bacterial sequence reads (Table 2c). Remarkably, results obtained with culture-based methods differed from those obtained with 16S rRNA gene sequencing. While the former method, in accordance with other reports, revealed the highest prevalence of *Staphylococcus, Propionibacterium*, and *Corynebacterium* species, the latter method showed a higher prevalence of *Pseudomonas, Propionibacterium*, and *Bradyrhizobium*, with only 4% of *Staphylococcus* spp. and a three-times higher number of different bacteria species than culture methods. This discrepancy reflects the drawback of each method: cultures favor bacteria that best fit with the provided medium of culture (most of them having been developed with the aim of isolating specific pathogens, even if their abundance is low), while sequencing methods favor the most abundant species in the sample based on the genome identification. Furthermore, a difference was found when the bacterial harvest was performed with soft versus strong pressure of the swab, determining a different sampling depth. With strong pressure, were obtained a significantly higher abundance of reads classified as *Proteobacteria* (*Bradyrhizobium, Delftia*, and *Sphingomonas*), while with soft pressure the most relevant findings were *Firmicutes* (*Staphylococci*) and *Actinobacteria* (*Corynebacterium* spp.) and a major

Table 2 – Genus-level representation of ocular surface microbiome, detected by sequencing methods, in healthy subjects. In brackets are reported the percentages of positive sample presenting the specific taxa.

Paper	(a) Zhou et al, 2014	(b) Doan et al, 2016	(c) Dong et al, 2011	(d) Ozkan et al, 2017	(e) Wen et al, 2017
Setting	Western Africa	United States	United States	Australia	China
Population	105	107	4	45	90
Taxa	*Corynebacterium* (16) *Streptococcus* (5) *Propionibacterium* (4) *Bacillus* (3) *Staphylococcus* (3) *Ralsontia* (2)	*Corynebacterium* (14) *Propionibacterium* (8) *Staphylococcus* (13) *Streptococcus* (4)	*Pseudomonas* (20) *Propionibacterium* (20) *Bradyrhizobium* (16) *Corynebacteria* (15) *Acinetobacter* (12) *Brevundimonas* (5) *Staphylococcus* (4) *Aquabacterium* (2) *Sphingomonas* (1) *Streptococcus* (1)	*Corynebacterium* (39) *Sphingomonas* (32) *Streptococcus* (16) *Acinetobacter* (7) *Anaerococcus* (7)	*Propionibacterium* (88) *Staphylococcus* (73) *Escherichia* (68) *Micrococcus* (49) *Ochrobactrum* (38) *Acidovorax* (37) *Acinetobacter* (33) *Pseudomonas* (24)

reduction in *Proteobacteria*. This highlights that the normal microbiota are not randomly organized on the ocular surface; probably some species are more likely to be closer to the epithelium, and/or more adhesive, due to the biofilm they develop with time.[29]

Also Ozkan and coworkers demonstrated the presence of differences in the flora composition identified by two different techniques of analysis, namely conventional swabs and culture vs rRNA sequencing.[80] With the former technique, *Staphylococcus* (46.5%), *Propionibacterium* (34.9%), *Micrococcus* (24.8%), and *Corynebacterium* (6.2%) were the most abundant microorganisms detected among positives samples, while the latter technique identified the majority of phyla as *Proteobacteria* (74.4%), *Actinobacteria* (48%), and *Firmicutes* (34.9%); the genera present, included *Corynebacterium* (39.5%), *Sphingomonas* (32.6%), *Streptococcus* (16.3%), *Acinetobacter,* and *Anaerococcus* (7.0%) expressed as percentages of subjects presenting the specific operational taxonomic unit (OUT), further demonstrating that the two methods produce different results. Some longitudinal stability of taxa at the individual level was also shown. They concluded that there is a low diversity of microorganisms on the ocular surface, not confirming the presence of a core microbiome, but rather individual-specific core microbiomes (Table 2d).

Wen and coworkers studied the microbial flora of healthy subjects in two groups divided by age (48 younger [aged 23–44 years] and 42 older subjects [aged 47–84 years]) with a further subdivision by sex.[111] Using metagenomic shotgun sequencing analysis, they found that on average 98.1% of microbial reads were of bacterial origin, while the presence of fungi and viruses was similar, accounting for 0.94 and 0.91%, respectively. Two main species were represented, with *Propionibacterium acnes* present in the 88% and *Staphylococcus epidermidis* in 73% of the subjects examined (Table 2e). Considering gender differences, a significant beta diversity could be shown, with an increased number of *P. acnes* and *S. epidermidis* in males and an increased number of *Escherichia coli* in females. There was a higher diversity measured by Shannon index in the older adult group compared to the younger, indicating that the abundance and evenness of bacterial flora was higher in the older group. In this group, *Staphylococcus haemolyticus, Micrococcus luteus,* and *E. coli* were more abundant, while *Ochrobactrum anthropi*, *Mycoplasma hyorhinis* and *P. acnes* were more abundant in the younger group. Considering gender, it was found that women in the older group were the only subgroup whose microbial flora composition was distinguishable from the others, having a higher prevalence of *E. coli*. Moreover, significant beta diversity between males and females was found in the older group, indicating a difference in the bacterial composition. According to the authors, this demonstrates that age has a stronger influence than sex on the microbial flora composition, with sex having only a secondary role. Conjunctival microbiome analysis showed that the carbohydrate, lipid, nucleotide, and amino metabolic pathways were more enriched in the older than in the younger group. Therefore, it can be said that the bacterial composition and metabolic function of the conjunctival flora differed between young and old adults, and gender can play a role only if interacting with age.[42,111]

Recently, a study of the conjunctival microbiome of healthy people living in three different Chinese cities–Beijing (20 subjects), Wenzhou (18 subjects), and Guangzhou [48 subjects])–demonstrated regional differences.[26] The three cities are characterized by different environmental settings and eating habits determined by climate and local customs. Beijing is in the north of China, with dry, cold weather and a primary diet of wheat. Guangzhou is in the south of China, with wet, warm weather and a rice-based diet. Wenzhou is a coastal city with a humid subtropical climate and fish as a primary source of nutrition. The study was carried out using a metagenomic shotgun sequencing approach. On average, the results demonstrated that 77.5% of microbial reads were of bacterial origin, whereas 19.5%, and 3% of reads were of fungal and viral origins, respectively. *P. acnes* and *S. epidermidis* were predominant in the conjunctival microbiome of healthy Chinese participants, while *P. acnes* was the most prevalent species in Guangzhou and Wenzhou, *P. aeruginosa* was the prevalent taxa in Beijing. Furthermore, changing the environment can directly influence the microbiota. Two subgroups were identified among 48 young volunteers living in Guangzhou according to their travel habits, with the “nontravel” group taking only short trips to nearby cities and the “travel" group taking longer trips to other Chinese cities in other provinces, where they stayed for at least 15 days. Two conjunctival sample collections were performed with a 3-week interval. While the composition and function of the microbiome did not change significantly in the nontravel group, modifications in the

taxonomic composition and metabolic functions of the microbiome were observed in the travel group. Taken together, these results suggest that environmental changes may lead to the alteration of the ocular surface microbiome.

Deepthi and coworkers used 16S rRNA gene libraries to study the healthy conjunctivae of 45 Indian preoperative cataract patients and described a great number of bacterial communities. These included 211 clones representative of 7 phyla: *Actinobacteria, Firmicutes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Bacteroidetes*, and *Deinococcus–Thermus*. Seventeen species were identified that were never reported in ocular infections. The genera more frequently encountered were *Corynebacterium, Staphylococcus, Cutibacterium, Escherichia*, and *Acinetobacter*. A great number of ubiquitous genera were found in each patient examined.[24]

Hence, the composition of the microbiota residing on the healthy ocular surface changes with many factors, and there is no consensus on whether a core microbiome does exist. Its composition seems to be more dictated by environment and age, and less by sex.

Kugadas and Gadjeva indicated a possible role of microbiota in the regulation of the immune ocular surface response in healthy subjects, illustrating a possible role in regulating the production of secretory IgA from the lacrimal gland and the differentiation of neutrophils on the eye-associated lymphoid tissue.[61]

In conclusion, it is possible to affirm that, from the studies addressing the normal composition of microbiota/microbiome of the healthy ocular surface, it is not possible to find a definite characterization of the microbial species present. Many of these studies however, are based on a limited number of participants, an important limitation.

2.2. *Ocular surface immunologic tolerance*

Mucosal immune tolerance is the capacity of the immune system to modulate the response to specific antigens. It is different from mucosal unresponsiveness since it actively involves an immune response.[38]

In normal conditions the ocular surface is in contact with a wide variety of microbial flora, from which it is protected by immunologic tolerance. The basis for this mechanism is the cross-talk among the epithelial cells, the microbiota, and the expression of a particular subset of innate immune-receptors on the epithelial cells that drive both innate and adaptive mechanisms. Therefore, changes of the microbiota can activate the immune response, determining whether or not to induce an inflammatory reaction.

The innate component of the immune tolerance mechanism resides mainly on the epithelium lining the ocular surface through the activity of the glycocalyx and the presence of tight junctions acting as an important barrier. Specific receptors present on the epithelial cells and belonging to a select group, called pathogen-associated molecular pattern receptors (PAMP), are mainly involved. These are the Toll-like receptors and NOD-like receptors.[48] The activation of these receptors produces an immediate response through the downstream stimulation of transcription factors, such as NF-κB and MAPKs, leading to the gene regulation for the production of several cytokines, including IL-1, IL-17, TNF-alpha, and chemokine ligands, among others, that initiate both the innate and adaptive responses.[11,48] The ocular surface epithelium has on its membrane receptors that are able to present antigens to dendritic cells (DCs) and lymphocytes, so modulating their activity.[20,25,96,124] Thus, the epithelium participates in the immune processes, regulating the type of response through the activation of specific cells residing on the ocular surface, such as DCs, macrophages, and lymphocytes.

Lack of inflammation in the normal ocular surface is determined by the action of tolerance mechanisms on the defense immune response, otherwise leading to an inflammatory state. The epithelium is the structure that determines the type of immune response: either immune tolerance or proinflammatory.[103] Goblet cells actively contribute to such modulation of the immune response through cross-talk with tolerogenic DCs, mediated by transforming growth factor-β (TGF-β), while other substances, like interleukin-10 (IL-10), can block the evolution of ocular surface mucosa inflammatory processes.[20,88,126]

The immune tolerance process also involves the epithelial cells that induce an adaptive response by influencing the activity of DCs present on the ocular surface with several subtypes. Some of these cells are involved in the immune tolerance process, others in activating the inflammation.[2,83] DCs initiate the adaptive process, priming both effector T cells *in situ* or by activating T cells residing in the draining lymph node.[36] T cells residing in the epithelium are mainly CD8+, although NK and γδ T cells are also present. CD8+ and CD4+ T cells are similarly distributed in the stroma.[33] DCs are abundant in the ocular surface epithelium, more in the conjunctiva and less in the cornea in normal circumstances.[44,59,78,89] CCR7+ DCs migrate to the regional lymph nodes in order to activate the adaptive process of mucosal tolerance, under the impulse of epithelial cells through the activation of the NF-κB signaling system.[9,46]

The mucosal tolerance toward specific antigens is mediated by regulatory T cells (Treg) that inhibit the inflammatory response by effector B and T cells.[6] Tregs are activated in response to antigen-activated DCs, including microbiota antigens, deriving from the ocular surface epithelium. In normal conditions, ocular surface DCs, imprinted by a tolerogenic profile, migrate to the lymph nodes, where they encounter naïve T cells. Under this stimulus, tolerogenic Tregs move toward the ocular surface epithelium, where they are activated by the corresponding antigen. In this way, Tregs exert their regulatory function and contribute to local homeostasis, inducing a noninflammatory immune activity (Fig. 2).

3. Bacterial organization and its relationship with the ocular surface structures

3.1. *Bacteria and ocular surface interaction: the role of glycosaminoglycans*

Bacteria are attached to the ocular surface or on the tear film, thanks to glycosaminoglycans (GAGs) and extracellular matrix proteins, including collagen, glycoproteins, and proteoglycans.[56,87] GAGs show multi-domain, multimeric assembled, and multi-molecular network, suggesting that they can work as functional receptors. GAGs are composed of

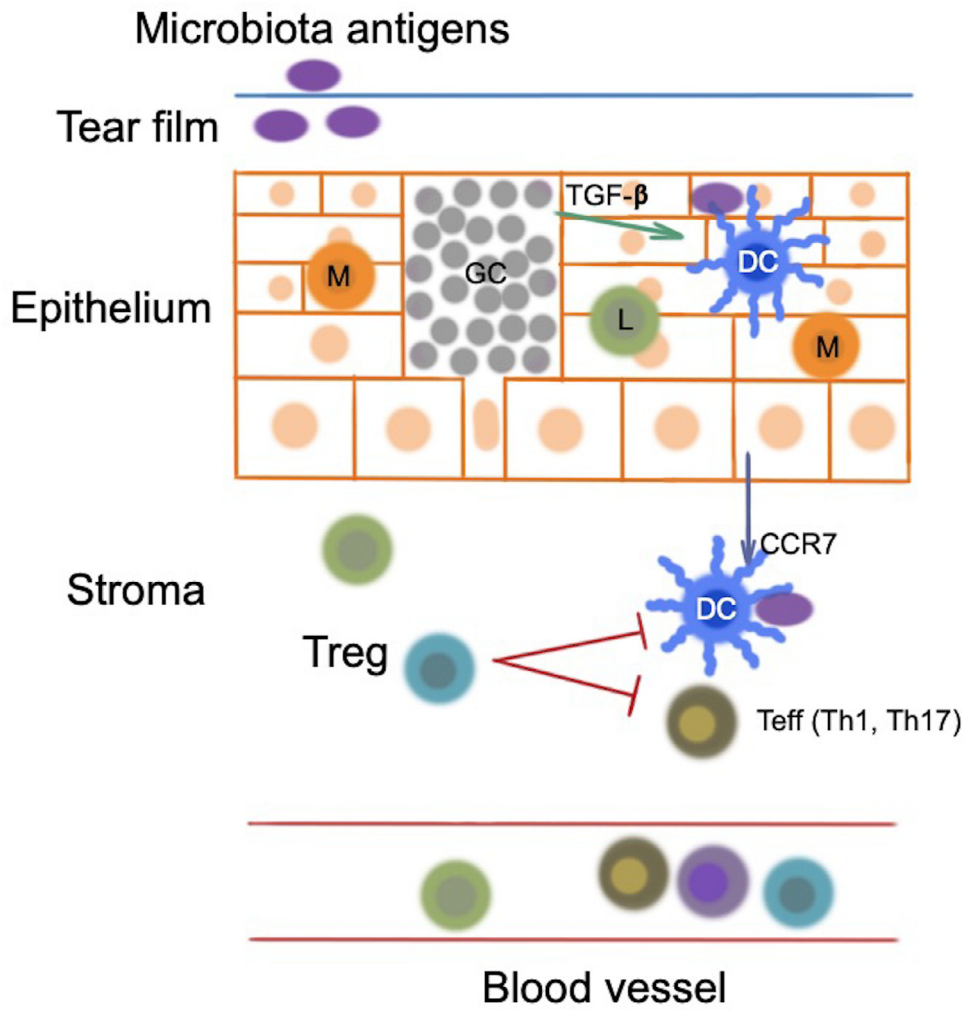

Fig. 2 – Mechanisms of epithelial immunologic tolerance toward microbiota. L = lymphocytes; M = monocytes; GC = goblet cell; DC = dendritic cell; Treg = regulatory T cell; Teff = effectors T cells; TGF-β = transforming growth factor-β; CCR7 = chemokine receptor 7.

proteins and sugars, and their functions depend on sugar composition, binding type, and sulfation pattern. The sugars can be glucosamine and/or galactosamine, the binding between molecules can occur in several positions, and the sulfation pattern also can vary, as is the case in heparan sulfate and chondroitin sulfate.[117] Bacteria can develop adhesion properties in two ways: inhibition of GAG synthesis and enzymatic degradation of GAGs. In fact, some GAGs act as receptors for bacteria in corneal epithelial cells.[87] In particular, heparan sulfate residues are responsible for bacterial adhesion through the expression of sulfate domains;[113] they are found in syndecans and glypicans (Fig. 3).[1,116] Syndecans are the main receptors on corneal epithelial cells used by bacteria to attach to the cell surface. Specific sulfate domains, such as N-sulfation and 6-sulfation of glucosamine, are more involved in this interaction.[84]

Polysaccharides are nutrients attracting *Demodex* infestation (classically of two types: *Demodex folliculorum* [*D. folliculorum*] and *Demodex brevis* [*D. brevis*]).[90]

3.2. *Bacterial organization: the biofilm*

A biofilm includes any syntrophic consortium of microorganisms in which cells stick to each other and, often, also to a surface.[16,73,119] These adherent cells become embedded within a slimy extracellular matrix that is composed of polymeric substances (polysaccharides, proteins, lipids, and DNA).[22,58,112] This results in a three-dimensional structure that represents a community organization for microorganisms: they live there, and they are protected from the environment and from antibiotics. Biofilms can attach to surfaces of a different nature and may include single species or a diverse group of microorganisms.[85] Biofilms are usually found on a solid substrate submerged in or exposed to an aqueous solution. In the biofilm, bacteria can share nutrients and are sheltered from harmful factors in the environment, such as desiccation, antibiotics, and the host's immune system.[70] During surface colonization, bacterial cells are able to communicate using quorum-sensing (QS) products like N-acyl homoserine lactone (AHL).[12,97,100] Substances like mucins, drugs, or enzymes able to disaggre-

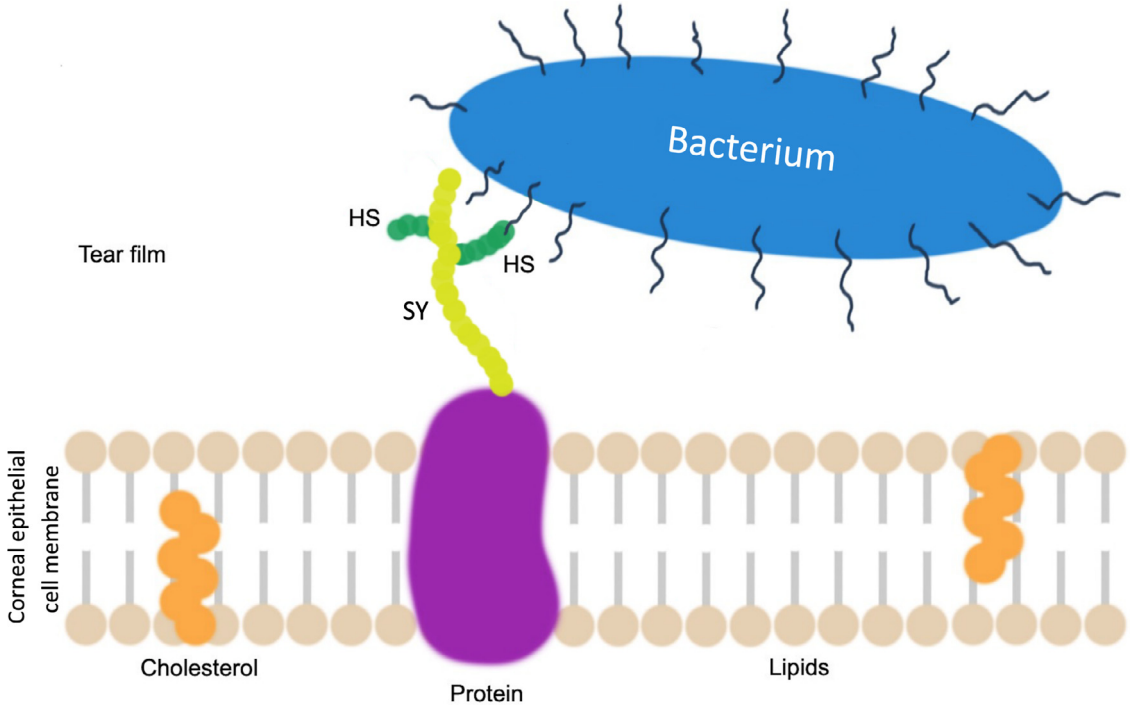

Fig. 3 – Schematic representation of the interaction between a bacterium and the corneal epithelial cell membrane. The receptor is a heparan sulfate residue (HS) present on the syndecan molecule (SY) linked to a membrane protein.

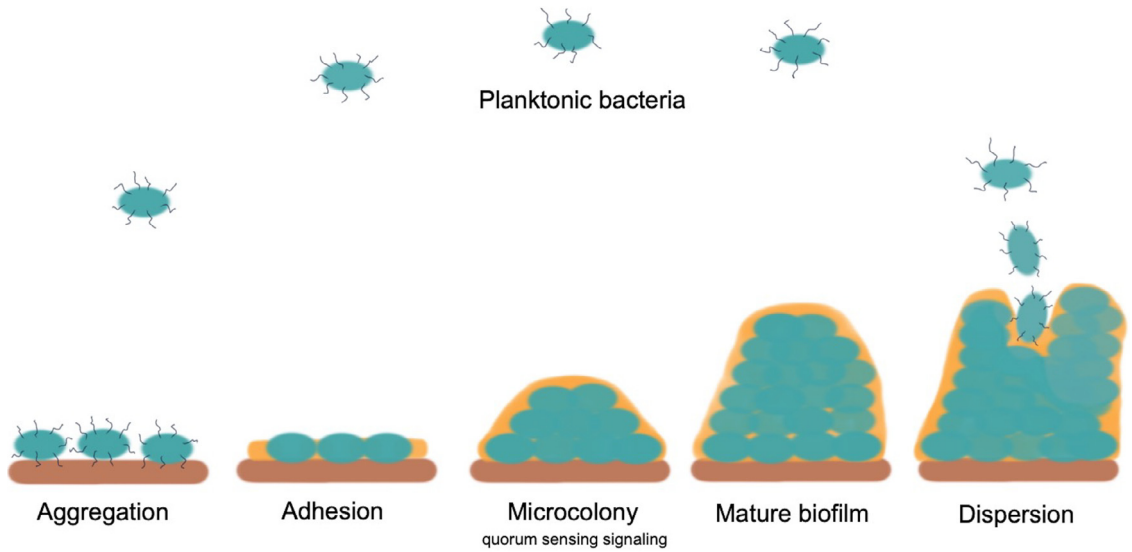

Fig. 4 – Biofilm formation and evolution.

gate bacterial products can be used to prevent or destroy biofilm formation.[32]

The dispersion of cells from the biofilm colony is an essential stage of the biofilm life cycle. It enables biofilms to spread and colonize new surfaces.[16] Enzymes that degrade the biofilm extracellular matrix, such as dispersin B, deoxyribonuclease, or cis-2-decenoic acid, together with nitric oxide, may contribute to biofilm dispersal. Biofilms grow through contiguous spreading or shedding of planktonic bacteria.[12,100] A clump of biofilm may detach from the original cluster and then seed onto surrounding surfaces, resulting in dissemination of infection (Fig. 4). Infections associated with biofilm growth are usually challenging to eradicate, since biofilms form a sort of shield, protecting bacteria from the external environment and host defenses.[21] Bacterial biofilms influence host response, inflammation, or susceptibility to infection. They produce not only enzymes, but also exotoxins, cytolytic toxins, and superantigens (Fig. 5).[55]

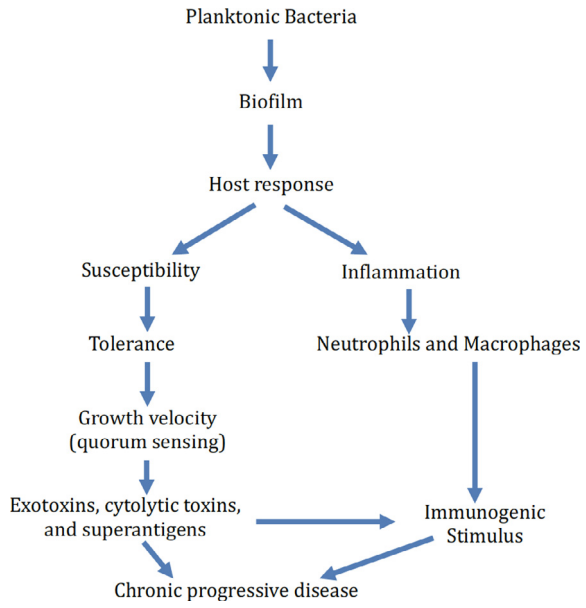

Fig. 5 – Role of bacteria biofilm in the development of the ocular surface chronic progressive disease.

It is possible to control bacterial infections, since they are immunogenic, through an inflammatory reaction.[32] If the infection is not overcome, it can produce a chronic disease related to the biofilm presence. Various diseases are related to such a structure: chronic sinusitis, middle-ear infections, dental plaque, gingivitis, atopic dermatitis, onychomycosis, prostheses infection, endocarditis, chronic osteomyelitis, cystic fibrosis, bacterial vaginosis, urinary tract infections, and prostatitis.[12]

In the eye lid margin, biofilms produce blepharitis, meibomian gland dysfunction (MGD), and dry eye disease (DED) (Fig. 6). Moreover, biofilms can be responsible for keratitis and conjunctivitis in CL wearers. *S. aureus* produces exotoxins, which can participate in the development of punctate keratopathy, and if the immune system is highly stimulated, phlyctenular keratitis and marginal infiltrates may develop (Fig. 7). It is possible to treat and destroy biofilms through mechanical debridement, detergents (chlorhexidine 0.2–0.1%, povidone iodine, hypochlorous acid, etc.), mucolytic enzymes (N-acetylcysteine), iron chelating proteins (lactoferrin), and ultimately by chemical antibiotics (requiring high concentrations, preferably in gel/ointment formulations).[3,34,47,71,90,93]

4. Ocular surface microbial flora in contact lens wear

Several studies have demonstrated that CL wear can influence the epithelial characteristics and the microbial flora of the ocular surfaces.

Using conjunctival impression cytology, Aragona and coworkers demonstrated that the type of lens worn can affect conjunctival changes.[4] The paper describes epithelial cell alterations associated with the different types of lenses (rigid, gas-permeable, and soft) in asymptomatic CL wearers and in CL wearers who exhibited CL intolerance. In the asymptomatic group, major conjunctival alterations were detected in patients wearing rigid and gas-permeable CLs, while soft lenses seemed to cause minor changes on the conjunctival epithelium. In patients with CL intolerance, an opposite pattern of alterations was observed: major alterations were found in conjunctival impression cytology specimens from soft lens wearers. Regardless of CL tolerance, CL wear was associated with metaplastic cellular changes that resulted in reduced adhesion of the conjunctival epithelium to the filter used to obtain the conjunctival imprints, so that isolated cells were observed, rather than the epithelial patches typical of the normal conjunctiva. This indicates that CL wear induces a modification of the conjunctival epithelium morphology. The soft lenses included in this study were of low water content, nonionic. This characteristic reduces the diffusion of oxygen through the lens, but also limits the tendency to accumulate deposits that could be responsible for an allergic sensitization of the conjunctiva and for less deposition of microbial contaminants.

Table 3 – Conjunctival-lid margin changes of microbiota in contact lens wearers.

(a) Sankaridurg et al, 2009	(b) Shin et al, 2016
Coagulase-negative staphylococci	*Methylobacterium*
Propionibacterium sp.	*Lactobacillus*
Bacillus sp.	*Acinetobacter*
Streptococcus sp.	*Pseudomonas* sp.
Micrococcus sp.	*Corynebacterium*
S. aureus	*Staphylococcus*
Corynebacterium sp.	*Streptococcus*
	Haemophilus sp.

Many studies have been conducted to characterize how CL wear affects the ocular microbiota. Sankaridurg and coworkers employed longitudinal monitoring of microbial presence at the conjunctiva-lid margin area and reported that in CL-wearing children aged 8 to–14 years, microbial overgrowth was detected in 36% of the conjunctival swabs and in 54% of the lid swabs.[91] The microbial taxa detected were (in order of frequency) CoNS, *Propionibacterium* sp., *Bacillus sp.*, *Streptococcus* sp., *Micrococcus* sp., *S. aureus*, and *Corynebacterium* sp. (Table 3a). There was no difference in the microbial taxa recovered from non-CL wearers and wearers of HEMA-based soft lenses over a period of 2 years.[91] In contrast, in adults daily wear of soft CLs over a period of 1 year produced alterations in the conjunctival microbiota by increasing the number of isolated commensal organisms. Consistently, an increase in the viable *in vitro* bacteria, including *Corynebacterium* sp. and *P. acnes*, was observed in the eyes of former CL lens wearers who stopped wearing lenses for an average of 10 months compared to the control group.[17] Extended wear of HEMA-based hydrogel CLs were shown to expand the conjunctival and lid margin microbiota. Individuals carrying Gram-positive bacteria on lenses, such as CoNS and *Corynebacterium* sp., were more likely to develop CL-induced peripheral ulcers, whereas carriers of Gram-negative bacteria on lenses were more likely to develop CL-induced acute red eye.[35]

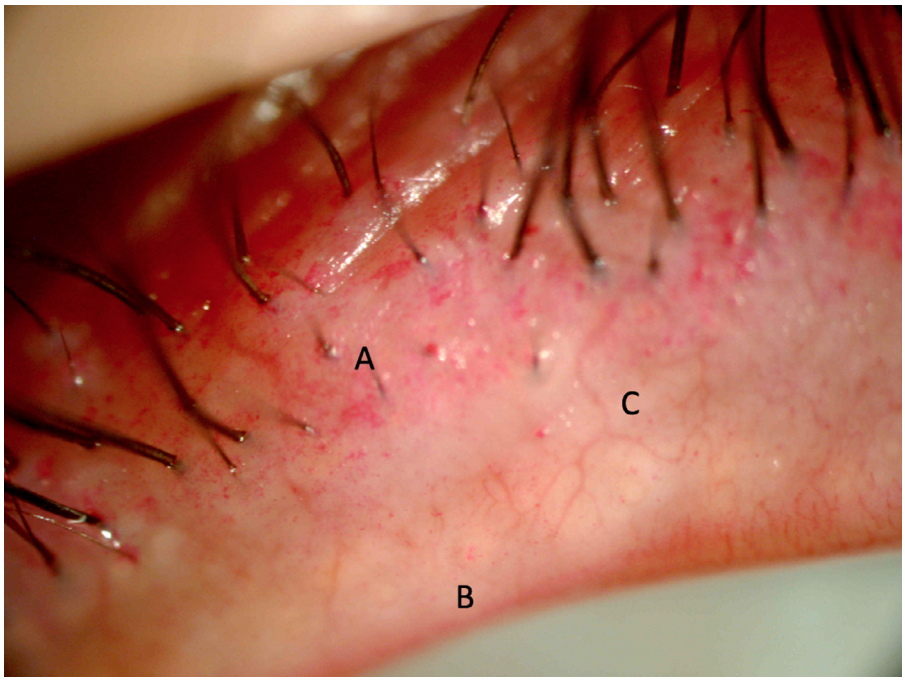

Fig. 6 – Effect of bacterial biofilm on the lid margin in chronic blepharitis. (A) Epithelial damage shown by rose bengal stain; (B) severe MGD with orifice obstruction with gland congestion demonstrated by the presence of whitish secretion seen in the background; (C) evident telangiectatic vessels.

Shin and coworkers, using 16S rRNA metagenomic sequencing to analyze samples from CL wearers and non-CL wearers, observed in the conjunctival microbiota of the CL wearers a relatively higher abundance of *Methylobacterium, Lactobacillus, Acinetobacter*, and *Pseudomonas* sp., and lower relative abundance of *Corynebacterium, Staphylococcus, Streptococcus*, and *Haemophilus* sp. compared to controls, suggesting that CL wear alters the microbial structure of the conjunctiva, making it more similar to that of the skin microbiota (Table 3b).[95]

Iskeleli and coworkers studied 29 eyes of 15 asymptomatic users of continuous-wear silicone-hydrogel lenses and found that, after 30 days of continuous wear, the number of subjects with culture negative swabs was significantly lower. Traditional culture methods showed that the most frequently encountered bacteria in the conjunctival sac were CoNS and diphtheroid rods, suggesting that extended wear of these lenses may modify the conjunctival microbial flora.[53]

In summary, the influence of CL wear on microbial commensal communities of the eye depends on the CL type, the duration of wear (e.g., daily wear versus extended wear), and the age group.[114]

5. Ocular microbiome/microbiota and eye diseases

Several factors can influence the type of bacteria present on the ocular surface, among these, both topical and systemic diseases.

5.1. *Dry eye*

5.1.1. *Clinical dry eye*

Colonizing bacteria produce enzymes, like lipases, and toxins that can provoke cellular damage at the ocular surface, inducing an alteration of the lipid layer of the tear film with instability, ocular surface inflammation, and irritative symptoms. These symptoms are similar to those occurring in dry eye (DE), without an evident infective status.

Zhang and coworkers, studying the ethnic differences in ocular surface microbiota in two different Chinese ethnic groups living in the same area of China, namely Han and Qiang, showed, using cultures performed on conjunctival swabs, different compositions of ocular flora: the Han population had a higher abundance of *Corynebacterium* sp., *Proteus* sp. and *Micrococcus* sp., while the Qiang population had a predominance of *S. epidermidis, Sphingomonas, and Staphylococcus xylose*.[123]

Graham and colleagues investigated the ocular surface flora in normal and DE patients by impression cytology, conventional culture techniques, and 16S rDNA sequencing.[41] Swabs were positive in 75% of normal subjects and 97% of DE patients; the mean number of bacteria per single culture was higher in DE patients. In both groups, the predominant bacteria species was CoNS that was present in 100% of positive samples and among which *S. epidermidis* was the most prevalent. DNA sequencing showed that the microbiome of normal subjects included CoNS, *S. epidermidis, Rhodococcus erythropolis*, uncultured bacteria, *Corynebacterium* sp., *Klebsiella* sp., *Propionibacterium, Bacillus*, and *Erwinia*. The microbiome of DE patients showed CoNS, *S. epidermidis, Rhodocossus* sp., uncultured

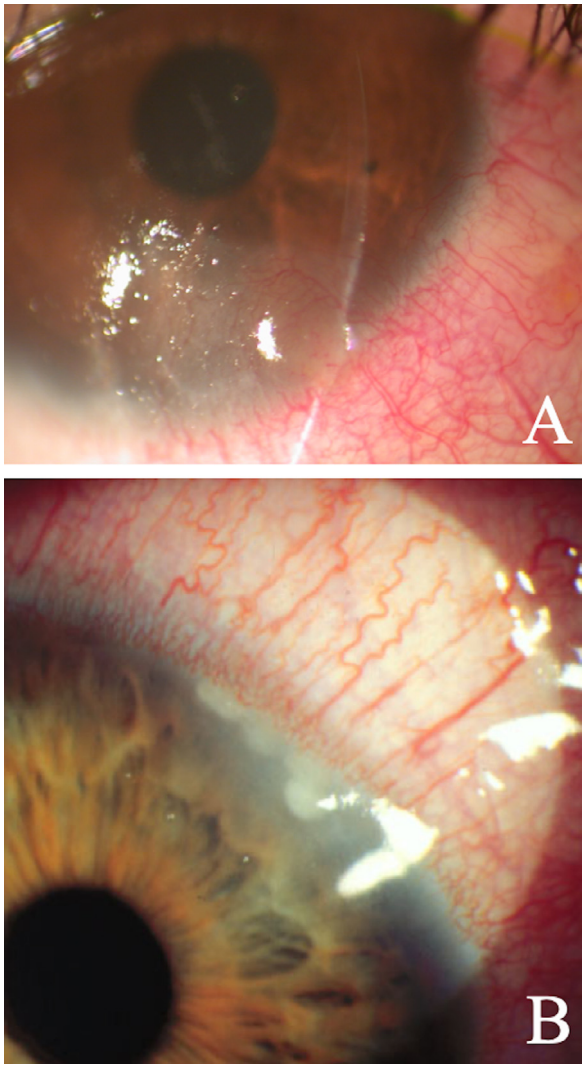

Fig. 7 – *Staphylococcus aureus*-induced phlyctenular keratitis (A) and marginal infiltrates (B).

bacteria, *Corynebacterium* sp., *Klebsiella* sp., *P. acnes*, and *Bacillus* sp. By impression cytology, it was found that in DE patients there was an inverse correlation between goblet cell density and bacterial population. The authors concluded that molecular biology-based results evidenced a higher bacterial population, although the exact clinical relevance of this finding needs to be better elucidated.

Using 16S rRNA sequencing, Li and coworkers compared the microbiome composition of the ocular surface in Chinese subjects with or without DE. They found a statistically significant difference between DE and non-DE (NDE) patients at both phylum and genus levels.[67] Ten bacterial phyla were the most represented in both groups: *Proteobacteria* (47.6%; 51.7%), *Firmicutes* (17.2%; 16.9%), *Bacteroidetes* (16.5%; 13.6%), *Actinobacteria* (6.2%; 6.1%), *Cyanobacteria* (2%; 1.7%), *Acidobacteria* (1.7%; 1.7%), *Chloroflexi* (1.6%; 1.5%), *Planctomycetes* (1.4%; 1.4%), *Epsilonbacteraeota* (1%; 1.2%), and *Verrucomicrobia* (0.9%; 1.1%) accounted for 97% of all sequencing reads, although the first three (*Proteobacteria, Firmicutes*, and *Bacteroidetes*) accounted for 92%. At the genus level, the following were common in both

Table 4 – Genus-level representation of ocular surface microbiome of dry eye patients with or without Meibomian gland dysfunction in Chinese patients. In brackets are reported the percentages of positive sample presenting the specific taxa.

(a) Li et al[67]	(b) Dong et al[30]	
DED	MGD	MGD
Pseudomonas (11.5)	*Pseudomonas (12.6)*	*Staphylococcus (20.7)*
Acinetobacter (7.8)	*Bacillus (7.7)*	*Corynebacterium (20.2)*
Bacillus (7.1)	*Acinetobacter (7.1)*	*Propionibacterium (9.3)*
Corynebacterium (2.6)	*Corynebacterium (2.9)*	*Sphingomonas (5.7)*
Chryseobacterium (2.2)	*Chryseobacterium (2.8)*	*Snodgrassella (4.2)*
	Pedobacter (2.7)	*Streptococcus (2.8)*

DED = dry eye disease; MGD = meibomian gland dysfunction.

DE and NDE: *Pseudomonas* (11.5%; 17.7%), *Acinetobacter* (7.8%; 8.8%), *Bacillus* (7.1%; 7.6%), *Chryseobacterium* (2.2%; 2.8%), and *Corynebacterium* (2.6%; 2.7%) (Table 4a). A greater diversity was demonstrated in the NDE subjects (Shannon index $P < 0.05$) with a more dominant microbiome than the DE ($P < 0.05$). Furthermore, the DE group had increased levels of *Bacteroidia* and *Bacteroidetes*, suggesting that these bacteria could be typical of the DE condition, while *Pseudomonas* and *Proteobacteria* were reduced.

5.1.2. *MGD and blepharitis*

In the same paper, Li and coworkers, dividing the DE group into MGD and non-MGD patients, found that no statistically significant differences between the two groups.[67] *Pseudomonas, Bacillus, Acinetobacter,* and *Corynebacterium* (Table 4a) were the most represented, although *Bacilli* showed a higher relative abundance in the MGD group and could be considered typical pathogens of MGD.

Watters and colleagues studied patients with several degrees of MGD, among which CL wearers were included.[110] Meibum expression did not modify the bacterial profile. CoNS were the most prevalent organisms isolated, present in two thirds of the population studied, although their presence was not correlated to the severity of MGD. CL wearers showed an increased presence of *P. acnes* that was correlated to MGD severity. No differences in microbiota were observed related to the severity of anterior blepharitis or CL wear.

Dong and coworkers, using 16S rDNA sequencing, investigated the microbiome of 47 Chinese MGD patients compared with a control group of 42 sex- and age-matched subjects without MGD.[30] They found significant differences at the phyla level, with a significantly higher abundance of *Firmicutes* (31.7% vs. 19.7%) and *Proteobacteria* (27.5% vs. 14.7%), and a lower abundance of *Actinobacteria* (34.2% vs. 57%) in MGD vs control patients. At the genus level, the numbers of *Staphylococcus* (20.7% vs. 7.9%) and *Sphingomonas* (5.7% vs. 0.8%) were significantly higher in patients with MGD vs controls, while the numbers of *Corynebacterium* (20.2% vs. 46.4%) were significantly lower. The meiboscores showed a significant direct correlation with the abundance of *Staphylococcus* in MGD patients. They concluded that patients with MGD can have bacterial microbiota alterations in the conjunctival sac, with a potential

role of *Staphylococcus, Corynebacterium*, and *Sphingomonas* sp. in MGD pathophysiology (Table 4b).

In a previous study carried out on a small group of patients with blepharitis, Lee and coworkrs demonstrated that blepharitis might be associated with a change in microbial composition, namely, greater quantities of *Streptophyta, Corynebacterium,* and *Enhydrobacter* species (Table 4c).[65] It was suggested that cytotoxicity and inflammatory components, triggered by bacterial growth, may contribute to the pathological process of MGD. Zheng et al confirmed the changes of ocular surface flora in MGD patients showing a wide bacterial profile and positive aerobic and anaerobic culture, significantly higher in both meibomian secretion and conjunctiva of MGD patients than in controls.[121]

The ocular surface inflammatory condition occurring in MGD and blepharitis could be mediated by the induced expression of acidic mammalian chitinase (AMCase) that is considered an immune response activator and is over expressed in MGD.[39,77] It can be hypothesized that changes in ocular surface microbial flora can mediate AMCase upregulation.

5.1.3. *Microbiota changes in experimental dry eye*

A potential role of commensals as triggering factors that promote inflammation in DE was suggested by a study of ocular microbiota changes in thrombospondin-1-deficient mice [TSP-1(-/-)], a strain that develops Sjögren's syndrome-like disease.[104] Conjunctival swabs were collected from TSP-1(-/-) and wild-type C57BL/6 mice and analyzed for microbial composition. In the conjunctiva of TSP-1(-/-) mice, colonization with *S. aureus* and CoNS species significantly increased with age, and this colonization developed earlier than in wild-type C57BL/6 control mice. This study suggests that, in mice, alterations in the microbiota composition occur in the early stages of Sjögren's-like disease, advancing the hypothesis that TSP-1 can play a significant role in regulating immunity to commensals.[104]

Contributions of the ocular microbiota to ocular surface homeostasis include the priming of immune activity on ocular surface epithelial cells and the regulation of mucin composition and production.[14] Other findings explore the emergent role of ocular microbiota cross-talk with pattern recognition receptors (PRR) to protect and strengthen local and adaptive mucosal immunity while preserving vision. Thus, the alteration of microbiota composition significantly affects ocular homeostasis, and thus, at least in theory, its normalization could improve the prognosis of ocular surface involvement in related diseases.[122] Although data in this specific area are still scarce, deciphering the functional role of microbial communities at the ocular surface could bring new insights and clarify the epidemiology and pathology of ocular surface dynamics in health and disease.[74]

5.2. *Ocular allergies*

A pilot study addressing the modifications of microbial flora in patients with allergic conjunctivitis compared with an age- and sex-matched control group found that the microbial flora of allergic patients was characterized by a higher diversity of species. Conjunctival swabs of allergic patients demonstrated CoNS, *S. aureus, S. viridans, S. pneumoniae, Streptococcus alpha-emoliticus, Haemophilus,* and Gram-positive *Bacillus,* while the control group showed only CoNS, *S. aureus, S. viridans,* and Gram-positive *Bacillus*.[68]

5.3. *Stevens-Johnson syndrome*

In Stevens-Johnson syndrome (SJS), the normal flora can become pathogenic because of changes occurring in the ocular surface. Venugopal amd coworkers obtained conjunctival samples, with gentle swabs, from 176 eyes of 88 SJS patients and 124 eyes of 124 normal controls, and evaluated the swabs with traditional culturing methods. CoNS were present in both groups, while *S. aureus* and *Corynebacteria* were present only in the SJS group. Among the *S. aureus,* 21% were of the methicillin-resistant strain. Although the great majority of the swabs were positive for single isolates, 7.6% showed double positivity, among which *S. viridans, Enterobacter* sp., *Micrococci, S. aureus*, diphtheroids, and anaerobic spore-bearing bacteria were variously coupled.[106]

5.4. *Degenerative lesions of ocular surface system: pterygium and lid laxity*

Ozkan and coworkers studied the bacterial presence in different areas of the ocular surface of patients undergoing surgery for pterygium or lid laxity.[81] Tissues were obtained from the normal conjunctiva used for the transplantation in pterygium and from one excretory duct of each patient undergoing surgery for lid laxity; swabs were also taken from the conjunctiva and skin. In agreement with previous studies, the authors found no difference in richness and Shannon diversity for sex, but they did find differences related to ocular surface regions and age. Skin showed the highest alpha diversity, while lid margin and ocular surface showed moderate diversity. Conjunctival tissue had the lowest diversity.

According to the authors, the identified bacterial distribution could be divided into the following three groups:

Group 1: OTUs including *Corynebacterium* and *Staphylococcus* genera, mainly resident on the skin and lid margin;

Group 2: OTUs found on conjunctival swabs characterized by *Acinetobacter* and *Aeribacillus,* indicating that these bacteria are resilient to the tear antimicrobials;

Group 3: OTUs present in the conjunctival tissue and the lid margin, being represented by the *Pseudomonas* genus residing both in protected conjunctival niches and on lid margin; this indicates the ability of such bacteria to survive in harsh conditions, where they may form biofilms. A limitation of this study is that the sample collection was inconsistent among the groups, as the pterygium group did not have lid margin specimens and the lid laxity group did not have conjunctival tissue specimens.

5.5. *Trachoma*

Chlamydia trachomatis infection is characterized by an initial severe inflammatory response of the ocular surface that may lead to conjunctival scarring, trichiasis, corneal opacity, and blindness. This progression depends on several factors, including environmental and genetic background, characteris-

tics of conjunctival immune response, concurrent infection with other pathogens, and changes in ocular surface microbiota.

Some bacteria are associated with an increased inflammatory condition in subjects with trachoma.[50] In a study carried out on Gambian children with trachoma who were nonpositive for chlamydia, the most frequently encountered bacteria were *S. pneumoniae* and *H. influenzae*. These bacteria were suspected of maintaining high levels of inflammation on the ocular surface, thus contributing to the development of conjunctival scars.[125]

Another study in Gambia included a group of children with active trachoma (AT), adults with scarring trachoma (ST), and two age-matched control groups.[86] In the child subpopulation, microbiota composition was similar to that of the corresponding control groups and was characterized by a prevalence of *H. influenzae* and *S. pneumoniae*; only 7/49 patients of the AT group showed the presence of *C. trachomatis*. The adult group with ST did not show *C. trachomatis,* but had a reduced diversity of the microbiome with a prevalence of *Corynebacterium* that was detected in 332/364 (91.2%) samples. The 16S rRNA analysis identified four species of *Corynebacterium* (*C. accolens, C. mastitidis, C. tuberculostericum*, and *C. simulans*) that seem to be involved in the development of conjunctival scars.

5.6. *Conjunctival lymphoma*

A study carried out in Japan on patients with mucosa-associated lymphoid tissue (MALT) lymphoma showed a diverse composition of microbial flora; the genus *Delftia* was significantly more abundant and *Bacteroides* and *Clostridium* were less abundant than in healthy controls. The authors thought that *Delftia* might play a pathophysiological role in the development of MALT lymphoma, whereas *Bacteroides* and *Clostridium* might play a defensive role.[7] They concluded that further studies are needed to clarify the relationship between MALT lymphoma and microbial flora on the ocular surface.

5.7. *Diabetes*

Systemic disorders can affect the ocular surface flora. Martins and coworkers, using culture methods, compared a population of diabetic patients with normal subjects and found that diabetic patients had a higher culture positivity than nondiabetic patients. The most common genera found was CoNS and, among diabetic patients, those with diabetic retinopathy had a higher frequency of positive cultures.[72]

Using traditional culture methods to study the conjunctival flora in patients with type 1 or type 2 diabetes, Bilen and coworkers found that the most prevalent isolates were *S. epidermidis, S. aureus,* and *Corynebacterium* sp. in both groups.[15]

Together with other factors, like corneal nerve dysfunction and correlated ocular surface anomalies, increased levels of tear glucose in diabetes mellitus may induce overgrowth and colonization by potential pathogenic bacteria.[5] Li et al, based on 16S rRNA sequencing in Chinese patients with type 2 diabetes, suggested that ocular surface microbiota might change in accordance with diabetes control.[66] Ocular surface microbiota of diabetic patients demonstrated a composition that was statistically significantly different from that of the control group: *Bacteroidetes* were increased, while *Proteobacteria* were reduced ($P = 0.001$ and $P = 0.006$, respectively). High amounts of unclassified species were also detected. In the diabetic patients, the abundances of *Acinetobacter* and *Pseudomonas* were significantly reduced ($P = 0.015$ and $P = 0.001$ vs controls, respectively). Notably, the lower abundance of *Acinetobacter* had a linear inverse correlation with older age ($r = -0.343$, $P = 0.011$). The alpha diversity of ocular surface microbiota was significantly higher in the diabetic group, with also a higher richness and evenness than controls ($P = 0.04$ for Shannon index).

A significant correlation of microbiota with Ocular Surface Disease Index (OSDI) score and glycemic control was evident in patients with diabetes. Linear regression analysis showed a direct correlation with OSDI score for *Acidobacteria* ($r = 0.457$, $P = 0.01$) and *Bacteroidetes* ($r = 0.645$, $P < 0.001$) and an inverse correlation for *Proteobacteria* ($r = -0.358$, $P = 0.048$). The correlation study between the microbiota composition and glycemic control demonstrated, at the phyla level, a direct correlation for *Bacteroidetes* ($r=0.461$, $P < 0.001$) and inverse correlation for *Proteobacteria* ($r = -0.429$, $P = 0.001$); at the genus level, an inverse correlation for *Acinetobacter* ($r = -0.518$, $P = 0.003$) and *Pseudomonas* ($r = -0.376$, $P = 0.037$) was demonstrated. These data suggest that changes of the microbiota might depend on metabolic alterations of the conjunctival microenvironment.

6. Effect of treatments on the ocular surface microbiome

6.1. *Prophylactic treatment in children*

Systemic treatments can affect the composition of the ocular surface microbiome. In a population of Nigerian children aged from 1 to 59 months, the biannual administration of oral azithromycin (1 dose of 20 mg/kg every 6 months for 2 years) for prophylaxis against trachoma induced an increased diversity of the ocular surface microbiome with a Shannon index of 183 versus 107 in the control group.[28] The study pooled metagenomic RNA sequencing results from material obtained with conjunctival swabs taken at baseline and 6 months after last drug administration. Before azithromycin treatment, the predominant bacterial genera were *Haemophilus, Moraxella, Lactobacillus*, and *Streptococcus*, while 24 months later (6 months after the fourth treatment), a significant change in the bacterial community was observed, with a reduced predominance of *Haemophilus* sp. ($P = 0.03$). The nature and distribution of viruses were not modified during the study period, suggesting that the modification of the microbiota was a direct consequence of the azithromycin treatment. The microbiome richness demonstrated by the Shannon index, which appears to be much higher than in other studies, may be due to environmental factors or to genetic material coming from the skin or other mucosae.

6.2. *Treatment of acute conjunctivitis in adults*

Acute conjunctival infections can induce changes in the ocular surface flora. A multicenter trial carried out in Italy on

209 patients with acute conjunctivitis compared the efficacy of two treatments, netilmicin and gentamicin. A total of 121 patients had positive baseline conjunctival swab cultures and were randomly assigned to receive one of the two treatments for 10 days.[82] The swab results demonstrated the presence of Gram-positive and Gram-negative bacteria. Gram-positive organisms comprised 89% of the isolates while Gram-negative organisms constituted 11%. The main Gram-positive bacteria were *S. epidermidis* (44.8% of cases) and *S. aureus* (36.9%), CoNS (15.8%), *Micrococcus* sp. (1.7%), and *Streptococcus* sp. (0.8%). Among the Gram-negative bacteria, *Acinetobacter* sp. (28.6%), *P. aeruginosa* (21.4%), *Serratia* sp. (14.3%), *Pasteurella* sp. (14.3%), *Haemophilus* sp. (14.3), and *Neisseria* sp. (7.1%) were represented, indicating that even germs considered part of the normal flora can become pathogenic.

6.3. *Treatment of microbiota in Stevens-Johnson syndrome*

In the course of SJS, the modified ocular surface environment can produce changes in the normal ocular flora as a consequence of the inflammatory insult. This can lead to an increase in opportunistic flora and an increased rate of antibiotic resistance. Among the opportunistic bacteria, *S. aureus*, especially of the methicillin-resistant strain, is particularly common.

A study carried out on an Indian population described the microbiota and its response to antibiotics. It demonstrated that all bacteria isolates were sensitive to gatifloxacin and moxifloxacin, while the 99% were sensitive to chloramphenicol and 98% were sensitive to gentamicin. When considering *S. aureus*, ciprofloxacin, gentamicin, cloxacillin, and cefazolin had the highest rates of resistance (25%, 10%, 10%, and 10%, respectively).[106]

6.4. *Changes in ocular flora after topical antimicrobial treatment*

Alterations of the normal conjunctival flora due to prolonged or repeated use of antibiotics can have important clinical consequences. Dave and coworkers found that, in patients undergoing intravitreal (IVT) injections, ocular surface flora changed according to the antibiotic treatment performed.[23] The antibiotic treatments studied compared azithromycin 1%, gatifloxacin 0.3%, moxifloxacin 0.5%, and ofloxacin 0.3%, administered q.i.d. for the 4 days after the IVT procedure and repeated after each procedure. In the azithromycin-treated group, *S. epidermidis* was significantly enhanced, while *S. aureus* was significantly reduced. In the fluoroquinolone-treated groups, both *S. epidermidis* and *S. aureus* were increased as percentage of isolates; however, in these groups, the percentage of Gram-negative bacteria significantly decreased.

Hsu and coworkrs studied the effect on the ocular flora of povidone-iodine (PVI) 5% in 13 patients undergoing IVT injections repeated for a minimum of 3 consecutive months. Cultures were performed before each treatment, PVI 5% was applied, and no antibiotics were used before or after injections. Among the 77% positive cultures carried out at the end of the study, no antibiotic resistance had developed.[49] The most frequently isolated bacterium was CoNS both at baseline and following serial IVT injections. PVI 5% treatment was able to prevent changes to the ocular surface microbial flora, also preventing the development of antibiotic resistance.

Yin and coworkers reported that antibiotic resistance is frequently encountered after IVT injection followed by the administration of antibiotics;[118] however, they found that PVI 5% administered before injection provided no additional benefits in terms of bacterial conjunctival colonization. Given the increasing amount of antibiotic resistance against second- and third-generation fluoroquinolones, the authors discouraged the widespread use of prophylactic antibiotic treatment after IVT injection.

7. Discussion

Many factors affect the identification of components of the microbiome/microbiota, including method of analysis. Two main techniques can be used. The first is more traditional, with different culture media that can select different bacteria (microbiota), promoting the growth of some and inhibiting others.[79] The advantage of such techniques is that they allow study of the sensitivity of bacteria to antibiotic treatment, which is not possible with more advanced molecular biology methods.

Molecular biology techniques have allowed a better identification of the genome present on the ocular surface (microbiome).[27,105] It is difficult to understand whether this material belongs to living microorganisms or is just residue of preexisting germs or the result of genetic material transferred from other close sites, such as the skin.[86] Therefore, when considering ocular surface microbiome/microbiota, it is almost impossible to discern clues about the exact composition of a core constituent present in all subjects. An important issue when assessing genetic material is contamination. The material must be processed very carefully to avoid presenting environmental contaminants.

Ocular surface homeostasis is imperative to avoid infections and preserve visual function, and homeostasis requires balance between the microbiota and the host immunological system. In fact, some components of the microbiota can have an intrinsic pathogenicity.[13] A subclinical inflammation can be considered a positive, para-physiological protective mechanism necessary to preserve the integrity of the ocular surface structures from environmental attacks of different origins, including inflammatory, infective, oxidative, and iatrogenic stresses.[10,11,32,102,108,115]

To maintain proper balance of the host/microbiota relationship, it is crucial to limit the contact between the microbiota and the epithelial cells. Several structures are involved in this task: tear film mucus, secretory-IgA, tear film peptides with antimicrobial activity, and ocular surface residing immune cells that form a defense structure similar to what in the gut has been termed "mucosal firewall."[69]

The ocular surface is a natural habitat for the commensal flora, but it can also be the site of infection when pathogens overcome its defense mechanisms. The microbiota resident on the ocular surface interacts with both pathogens and the immune system, regulating the active response of the latter. Microbiota is known to influence such mechanisms in various

mucosae, and some reports indicate a similar role for the ocular surface flora.[18,37,38] A recent study by St Leger and coworkers demonstrated that stable colonies of *C. mastitidis* inhabit the ocular surface and contribute to induction of neutrophil recruitment through the activation of $\gamma\delta$ T cells secreting interleukin-17, and the release of antimicrobial factors protecting against *Candida albicans* and *P. aeruginosa* infections.[101]

One of the most significant ways that microbiota and potentially pathogenic bacteria interact is in the competition for metabolites responsible for their nourishment, in what is considered a mechanism of resistance to infections. Microbial virulence can be also hindered by the metabolic activity of commensals; for example, *S. epidermidis* produces antimicrobial proteins and proteases that can interfere with biofilm production by the pathogen *S. aureus*.[13,54,92]

The microbiota residing on the healthy ocular surface helps to control infections with the ability to stimulate and regulate both innate and adaptive immunity.[76] It induces a sort of guidance for the immune system so that the barrier immunity is constantly reinforced, providing a self-limitation to its own growth and to pathogen invasion. Furthermore, the microbiota have the ability to produce a cellular reaction against infections. Changes in microbiota composition activate epithelial cell response either by interacting with Toll-like receptors or by a route independent from them[102]; in these ways an innate immune reaction can be activated and maintained. If the pathogenic stimulus is sufficiently strong or prolonged, the innate immune activation causes a recruitment of those cellular elements that will result in an adaptive immune response.

Resident microbiota is specific for each tissue type;[13] however, the composition of tissue-specific microbiota is unstable due to several external and internal influences.[26,120,123]

Sometimes pathogens can coopt microbial flora components to enhance their tissue penetration.[45] Various pathogens, including viruses, can be facilitated by the microbiota-induced production of IL-10, responsible for an immunological tolerance that can allow the transmission of viral infections.[57,62]

Since components of microbiota can be involved in acute infections, the immune system has developed specific ways to control it through the presence of neutrophils and monocytes in the conjunctival stroma. As a result of such infections, commensal-specific memory T cells develop, being indistinguishable from pathogen-induced memory cells.[43] This can be also considered the consequence of the enormous number of antigens expressed by microbiota components. Therefore, a significant part of the memory T cells can be microbiota-specific, but, like pathogen-specific CD4+ T cells, their concentration will decay with time.

The development of CD4+ T cells plays a significant role in the maintenance of an immunologic response in an ever-changing environment, allowing tolerance for the variable commensal population and, at the same time, a barrier function against pathogens.

8. Conclusions

In conclusion, the ocular flora appears to play a significant role in the regulation of ocular surface system function. It is, therefore, of the utmost importance to have a precise knowledge about its real composition. Hence, further studies addressing microbial commensal flora are necessary to: 1) assess whether its composition is stable or variable among individuals or populations, 2) determine if at least some components need to be present to guarantee ocular surface homeostasis, 3) elucidate how environment or other factors affect its composition, and 4) define the real connection between ocular flora and ocular surface dysfunction and diseases. To achieve these goals, are necessary studies including a great number of subjects, so overcoming a frequent limitation due to a rather low study population.

Finally, it is of the utmost importance to use the best techniques for the identification, isolation, growth of organisms, and study of therapeutic responses in order to better understand the effects of the ocular surface flora on the ocular surface health and disease.

9. Method of literature search

A literature search was performed using the PubMed search engine, covering the years 1980 to 2020, and using the following key words in various combinations: microbiome, microbiota, ocular surface, conjunctiva, ocular diseases, immunologic tolerance, glycosaminoglycans, biofilm, contact lenses, dry eye, MGD, blepharitis, ocular allergies, Steven-Johnson syndrome, pterygium, lid anomalies, trachoma, conjunctival lymphoma, diabetes, ocular treatment, antibiotics, antiseptics arranged in various ways. The search yielded 416 results; of those, articles contributing current knowledge related to healthy conditions, immunologic aspects (tolerance vs inflammatory reaction), bacterial/ocular surface interactions, biofilm organization, ocular surface disorders, and effect of treatments on microbial flora were included in this review, for a total of 126 references. Included papers focused on flora residing in the ocular surface structures and its regulatory activity on ocular surface system function. Several less pertinent articles, e.g., those focusing on gut microbiota and its possible effects on ocular diseases, etc., were excluded.

Conflict of interest

Prof. Maria Rescigno is founder of "Postbiotica" a microbiota company. The other Authors declare of not having any conflict of interest with the subject of the paper.

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