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THE OCULAR SURFACE, A TARGET FOR ONE HEALTH INDICATORS: THE MIRROR OF MICROBIAL INTERACTIONS BETWEEN PETS AND OWNERS AND AN INDICATOR OF AIR POLLUTION

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Abstract

One Health aims at tackling health issues with a comprehensive approach, encompassing human, animal and environmental health. Relationships between human health and the health of wildlife or livestock animals have been explored, however very few studies focus on the relationship between owner and companion animal. Sharing of allergic traits and of skin, oral, and gut microbiomes between dogs and owners has been demonstrated in multiple studies. Further evidence of pathogens sharing, including antimicrobial-resistant pathogens, between companion animal and owner highlights the existence and the importance of both pathogenic and non-pathogenic microbial exchanges and calls for a One Health approach to study the microbiomes. With the increasing number of households with pets and changing habits in the human-animal bond, a better understanding of these interactions is necessary, including at the microbial level. Microbial exchanges between pets and owners within the household have been explored in gut, oral, and skin microbiome, but not yet on the ocular surface.

Urbanization of built environment has led to modifications in the exposome surrounding humans and animals, including at the microbial scale, and could impact human and animal health. Exposure to air pollutants causes changes in gut and skin microbiome, particularly in cases of chronic exposure. Outdoor air pollutants are known to have a negative impact on the ocular surface, however, their impact on the ocular surface microbiome, hosted by the conjunctiva, is not yet known.

The conjunctiva has the particularity of being the only mucosa of the body directly exposed to the external environment. This direct exposure to the exposome also makes it easily accessible for non-invasive examinations and sampling. For this reason, if it is affected by, or if it can reflect, interactions between, humans, animals, and the environment, it could act as a sentinel of the body identifying these interactions.

The goal of this research was to apply a One Health approach to the study of the ocular surface microbiome, by combining several disciplines to investigate microbiome similarities between pet and owner, and the impact of pollutant exposure on the ocular surface.

The research began with the implementation of a pipeline for the sampling, processing, and sequencing of the ocular surface microbiome in both dogs and owners. Conjunctival swabs were collected from 15 dogs and their owners for subsequent DNA extraction and 16S rRNA sequencing. Ocular surface microbiome composition and alpha and beta diversity were

determined for dogs and owners. Dog-owner distances, i.e. beta diversity in each dog-owner pair, were calculated to estimate the level of similarity between dog and owner microbiomes. The impact of several lifestyle factors, and of pollution exposure on ocular surface microbiome alpha diversity and on similarities between dog and owner was investigated.

A protocol for the processing and sequencing of ocular surface microbiome samples, i.e. conjunctival swabs, was optimized. After thorough verification, it was determined that dog and owner samples could be processed following the same protocol.

Dog and owner microbiomes were found to be similar in overall composition, harboring the same main phyla and families, albeit forming two distinct clusters and dogs having a significantly more diverse microbiome. Small dogs tended to have a more similar ocular surface microbiome to their owner than large dogs. Pairs cohabiting with other pets had an ocular surface microbiome composition significantly more similar than the ones who did not.

This is the first research evaluating ocular surface microbiome interactions between pet and owner. It underlines the importance of key collaborations between physicians, veterinarians, biologists, engineers, and environmental agencies to effectively prevent the spread of pathogens, including antimicrobial-resistant pathogens, and detect the negative impacts of pollutants on human, animal, and environmental health. The ocular surface is proposed as a valuable indicator of pathogenic and non-pathogenic microbial exchanges, and pollution exposure.

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Abbreviations

AMR: Antimicrobial Resistance

ARPAE: Agenzia Regionale per la Prevenzione, l'Ambiente e l'Energia dell'Emilia-Romagna

ASVs: Amplicon Sequencing Variants

CO: Carbon Monoxide

DNA: Deoxyribonucleic Acid

ECST: Eye Community State Type

EPA: Environmental Protection Agency

HIV: Human Immunodeficiency Virus

MGD: Meibomian Gland Dysfunction

MRSA: Methicillin-Resistant *Staphylococcus aureus*

MRSP: Methicillin-Resistant *Staphylococcus pseudintermedius*

NGS: Next Generation Sequencing

NOx: Nitrogen Oxides

NIBUT: Non-Invasive tear Break-Up Time,

O_3: Ozone

oGVHD: ocular Graft-Versus-Host Disease

OPI: Ocular Protection Index

OSM: Ocular Surface Microbiome

PM: Particular Matter

SO_2: Sulfur Dioxide

TFOS: Tear Film and Ocular Surface society

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1. Introduction and background

1.1. One Health - tackling health issues of various scales

1.1.1. The One Health approach

One Health is a field at the crossroads between human, animal, and environmental health. It is an approach that considers the health of humans, domestic and wild animals, plants and the wider environment, including ecosystems, to be highly correlated and inter-dependent (One Health High-Level Expert Panel et al., 2022). Through an integrated and multi-disciplinary approach, One Health seeks to encompass the many factors that can impact health. It is not a new approach, but regained notoriety in recent years due to the rapid and significant changes in interactions between humans, animals, and the environment.

By emphasizing and analyzing these interactions, One Health aims at tackling not only the detection of global health threats, but also prevention, preparedness, response and management, i.e. the complete spectrum of disease control (WHO, 2024). The approach is mainly known for the tackling of antibiotic resistance, food safety, and zoonoses but is not limited to these issues. Climate change affects many, if not all, of the topics addressed by One Health. Its implications, such as temperatures, extreme weather events, and air quality, among many, have both direct and indirect impacts on health. Straightforward impacts of climate change, such as heat-related illnesses (Luber and McGeehin, 2008) or the negative effect of air pollution on respiratory airways (Xue et al., 2022) have been demonstrated plethora of times. However, climate change impacts many other health issues indirectly, that One Health aims at tackling. For example, rising temperatures undoubtedly have an effect on cardiovascular illnesses, but also have an effect on the regions inhabited by wild animals, including hosts to pathogenic microorganisms,

that can enter in contact with humans in new ways and more often. Keeping in mind that, although it is not the only explanation, the recent increase in detection of zoonoses is, most likely, in part due to the increase in the number and precision of detection tools (Beugnet and Chalvet-Monfray, 2013).

Interactions between ecosystems, humans, and animals are complex adaptive systems with often unpredictable behaviors due to a high number of inputs amounting to synergistic effects. These "effects" are in return fed back to modify the causes, nourishing a phenomenon of circular causality. With the recognition of these complex mechanisms, comes the question of the operationalization of One Health. A prominent method for this operationalization is multidisciplinary systems thinking combined with modelling approaches allowing for exploration and extrapolation of existing data (Arnold et al., 2024). Systems thinking encourages a holistic and comprehensive method involving the identification of key connections between parts of a system, identification and understanding of cause-effect feedback loops, non-linear relationships and dynamic behaviors within a system (Arnold and Wade, 2015). In the context of a disease outbreak, it can take the form of the following questions:

- **Who?** Who has the disease? Who are the stakeholders involved? Who is impacted? Who are the responders? etc.
- **Where?** Where is the disease spreading? Where was the first case? Where are the responders? Where could it spread? etc.
- **When?** When was the first case? How quickly is it spreading? When can responders react? etc.
- **How?** How is the disease transmitted? How serious is it? How can it be managed, controlled, prevented, cured? etc.
- **What?** What are the implications for human health, animal health, ecological health? etc.
- **Why?** Why did the outbreak occur? Why was it not detected sooner? etc.

Answering these questions requires the involvement of specialists from different fields (doctors, veterinarians, biologists, statisticians, epidemiologists, social scientists, urbanists etc.), policy makers, and community members. It shifts the health paradigm from an individual-centered approach to a community-based approach.

The relationship between humans, animals, and environment is a co-adaptive dynamic. To try and predict the unpredictability of such systems, it is necessary to understand the dynamics at stake before the occurrence of an outbreak. Microorganisms, including pathogens and parasites, are an invisible but integral part of this system that is highly impacted by the current anthropogenic changes. Thus, and to prepare for future potential outbreaks, studying how microorganisms, both pathogenic and non-pathogenic, move between human hosts, animal hosts, and the environment, is key to understanding the dynamics of pathogenic microorganisms' flow.

1.1.2. Microbial exchanges between humans and companion animals

Interactions between animals, humans, and the environment take place at multiple scales, from the population to the microscopic scale. The latter is the scale of microorganisms, mainly bacteria, viruses, and fungi, often organized in communities. The bacterial community of a given ecosystem is called microbiota, or microbiome if referring to the bacteria, their genes and theater of activity, and can be found in various human, animal, and environmental habitats such as the gut, skin, soil, or water, among many (Berg et al., 2020). Along with the growing accessibility of microbiome studies, more and more scientists have been able to characterize microbiomes. Investigating microbiomes with a One Health lens calls for the exploration of both pathogenic and non-pathogenic interactions at stake (Trinh et al., 2018). To explore these dynamics, relationships with a close human-animal contact, such as the ones between companion animals and their owners, or farmers and their livestock, are a precious resource. Since, in 2023, 46% of European households (FEDIAF, 2023) and 63% of United States households (APPA, 2024) owned a pet, understanding the possible microbial exchanges between pet and owner is a matter of public health.

Owners and pets live in close contact, sharing the same habits and lifestyles. This exposure over years of cohabitation most likely enables bacterial exchanges between humans and animals (Lehtimäki et al., 2020; Song et al., 2022b; Torres et al., 2017). Indeed, presence of household pets was associated with changes in the gut microbiome, with some bacterial species being significantly more abundant in pet owners and other significantly more abundant in non-pet owners (Kates et al., 2020). Exposure to household pets also increases the abundance of *Ruminococcus* and *Oscillospira* in the gut microbiome of infants (Tun et al., 2017) and has been associated with greater skin microbiome diversity (Ross et al., 2017; Song et al., 2013).

On top of the observed changes in pet owner microbiomes, several researches have demonstrated exchanges between pet and owner microbiomes. Two studies investigating cohabiting dogs and owners found that the skin microbiome of dog-owner pairs resembled more each other than that of a dog and a random owner or an owner and a random dog (Lehtimäki et al., 2020; Song et al., 2013). Lehtimäki et al. also found that dogs and owners with allergic traits tended to have allergic reactions or be healthy concurrently. Similarly, cohabiting cats, dogs, and owners share more oral and nares/nasal microbiomes with each other than with other humans and pets outside their household (Misic et al., 2015).

Exchanges of pathogenic bacterial strains between pets and owners within a household have also been described: cases of sharing of *Proteus mirabilis* strains, associated with urinary tract infections (Marques et al., 2021), as well as *Clostridium difficile* (Loo et al., 2016), *Klebsiella pneumoniae* (Marques et al., 2019) or *Escherichia coli* (Johnson et al., 2008; Naziri et al., 2022). Sharing of non-invasive zoonotic *Staphylococcus intermedius* from dog to owner has also been reported (Tanner et al., 2000). These results highlight the possible role of pets acting reservoirs of pathogenic bacteria for humans in the household, and vice-versa. Companion animal pathogens can impact human health in different ways, depending on the type of main reservoir. The pathogen reservoir for human infections can be the companion animal population, the environment contaminated by companion animals, wildlife, or, in the case of a lack of clear reservoir, pathogens thriving in both human and animal hosts (Giannelli et al., 2024).

Microbiome sharing between animals and humans also take place outside the household, as was demonstrated by a recent remarkable study analyzing the nasal and fecal microbiomes of 66 dairy farmers, 166 dairy cows from 37 American dairy farms and 60 non-farmers to act as controls. The study showed that the nasal microbiome of farmers was more compositionally similar to the one of cows than the one of non-farmers compared to cows. Shared microbial lineages were also identified between the gut microbiomes of farmers and cows (Mahmud et al., 2024).

These results highlight the existence of microbiome sharing between humans and animals, including that of pathogenic bacterial strains, as visible in **Figure 1**.

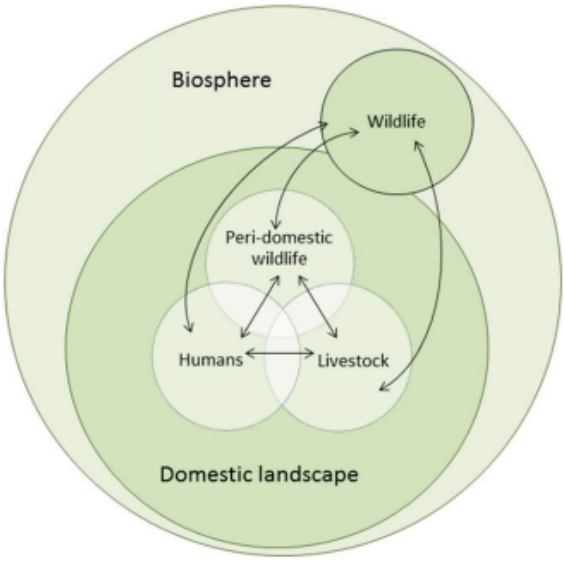

Figure 1. Bacterial flow at the domestic-wildlife-livestock-human interface. Arrows indicate direct, indirect, or vector-borne candidate pathogenic or non-pathogenic flow. The rate and direction of bacterial flow will depend on the nature and intensity of interactions between wildlife, livestock, and human compartments and the characteristics of the compartments. *(Adapted from "Zoonosis emergence linked to agricultural intensification and environmental change", Jones et al., 2013. Proc Natl Acad Sci U S A 110, 8399–8404.)*

1.1.3. The threat of Antibiotic Resistant-Bacteria sharing

Antimicrobial resistance (AMR) is an adaptive phenomenon by which bacteria become resistant to one or more (multidrug-resistance) families of antibiotic. It can occur naturally in bacteria or be the result of horizontal transfer, by way of bacterial transformation, phage-mediated transduction, or conjugation (Munita and Arias, 2016). AMR is one of the top ten global health threats of the 21st century (WHO, 2023). It is estimated to be directly responsible for 1.27 million deaths and to have contributed to 4.59 million deaths worldwide in 2019 (Murray et al., 2022). The rise of AMR is caused by the considerable and widespread use of antibiotics creating a selection pressure on bacteria. Among other drivers, use of the same antibiotics in humans and animals increases selection pressure, including the one exercised on microbiomes inhabiting human and animal bodies. Particularly in small animals, prescribed antibiotics are very similar to the ones prescribed to humans (Naziri et al., 2022). Close contact between

companion animals and owners can thus create opportunities for cross-species spreading of AMR.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), two of the most common community- and hospital-acquired infections, have been reportedly transmitted between companion animals and humans (Harrison et al., 2014; Nienhoff et al., 2009; Weese et al., 2006). A recurrently MRSA-infected couple that was healed only after MRSA was identified from their dog's nares and successfully eradicated, indicates the possibility of recurrent antimicrobial-resistant pathogens within the household (Manian, 2003), later confirmed in similar cases of household transmitted-MRSA (Sing et al., 2008; van Duijkeren et al., 2005). Carbapenem-resistant *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumanii,* other common pathogenic bacteria, have also been identified in companion animals (Naziri et al., 2022; Silva et al., 2022). Carbapenems are broad-spectrum antibiotics often used in humans as a last resort, therefore the rise in resistant bacteria is a serious threat for the treatment of multidrug-resistant patients.

In livestock as well the issue emerged, as shared microbial lineages between cows and farmers have been associated with antibiotic resistant genes (Mahmud et al., 2024).

Direct transfer of antibiotic resistant-bacteria between humans and animals and particularly direction of the transfer is often difficult to prove. However, there is clear evidence of the phenomenon and of its utmost importance for public human and veterinary health in the context on the global threat of AMR.

1.1.4. Microbiome exchanges between human/animals and the environment

Presence of humans or animals in an environment can affect the bacterial communities that are present, as more diverse bacterial communities have been found in the house dust of homes with pets (Dunn et al., 2013; Fujimura et al., 2010; Shan et al., 2019). Structure and composition of the human skin microbiome have also been proven to persist on surfaces in the household (Fierer et al., 2010). Moreover, sharing of skin, salivary, and fecal microbiomes between captive Komodo dragons and their environment shows that these exchanges likely occur in both directions (Hyde et al., 2016).

This can also be supported by studies demonstrating the impact of urbanization in human microbiomes, including gut, skin, vaginal, and milk microbiomes (Anumula et al., 2024; Das et al., 2018) as well as gut mycobiota (Kabwe et al., 2020).

Exchanges of microorganisms between humans, animals, and the environment have been evidenced in various bodily microbiomes, such as those of the skin, oral, and gut. However, exchanges at a particular mucosa of the body directly exposed to the external environment, the conjunctiva of the ocular surface, has not yet been explored.

1.2. The Ocular Surface

1.2.1. Anatomy and function of the ocular surface

The eye is composed of an internal and an external compartment. The internal compartment consists of the anterior and posterior chambers, iris, lens, vitreous cavity, retina, ciliary body, choroid, and intrinsic ocular muscles, whereas the external eye is made up of the eyelids, conjunctiva, sclera, cornea, limbus, and tear film (Kels et al., 2015).

The conjunctiva, home to the ocular surface microbiome, is a mucous membrane of nonkeratinized stratified squamous epithelium (see **Fig. 2**). It can be divided into three geographic zones: the palpebral conjunctiva, the bulbar conjunctiva, and the forniceal conjunctiva. The healthy, not-inflamed conjunctiva contains lymphocytes, neutrophils, macrophages, plasma cells, and mast cells. In the conjunctival epithelium, the Langerhans cells, a subpopulation of dendritic antigen-presenting cells, act as a sentinel of the ocular surface. The presence of immune cells and the blood and lymphatic vessels supplying the conjunctiva facilitates the trafficking of immune cells when an adaptive immune response is necessary (American Academy of Ophthalmology, 2023, 2019).

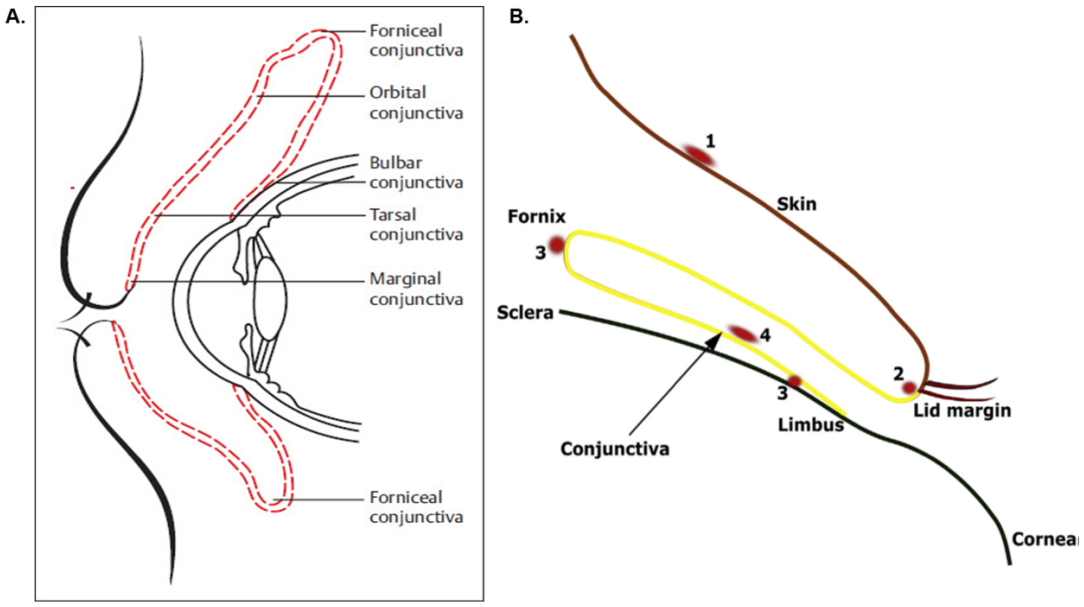

Figure 2. Anatomy of the conjunctiva. A. Description of the areas of the conjunctiva (Themes, 2022). B. Possible sampling sites of the conjunctiva. Site n°4 (Conjunctiva) is the one most used in studies characterrizing the ocular surface microbiome (Ozkan et al., 2019).

Along with the lacrimal glands and eyelids, the ocular surface is part of the lacrimal functional unit, responsible for the integrity, regulation, production and health of the tear film, the health of the ocular surface, and the quality of the image projected onto the retina. The tear film is a hydrophilic gel containing mucins, lipids, proteins, and salts, topped by a lipid layer (see **Fig. 2**). Among other functions, it removes pathogens, toxins, and others irritants from the ocular surface, maintains homeostasis of the resident ocular microbiome, and contributes to the antimicrobial defense of the ocular surface. Health of the tear film is essential for a healthy eye (American Academy of Ophthalmology, 2023).

To protect against infections, the external eye has anatomical and complex innate and adaptive immunoregulation defense mechanisms. The singularity of the ocular surface lies within the fact that it is the only mucosa of the body that is in direct contact with the external environment through the corneal and conjunctival epitheliums. When the lids are opened, it is directly exposed to the ambient air and solely protected by the tear film.

1.2.2. The ocular surface microbiome

The conjunctiva, part of the ocular surface, is the host of the ocular surface microbiome (OSM). Compared to other mucosal microbiomes, the OSM is a low-biomass and paucibacterial microbiome (Ozkan and Willcox, 2019). Possibly due to the antimicrobial compounds present in tears (McDermott, 2013) and action of the lids, this particularity makes it difficult to characterize the OSM with culture techniques and generally hard to define (Ozkan et al., 2017). Traditional culture techniques of ocular surface identified coagulase-negative Staphylococci as the predominant bacteria inhabiting the conjunctiva, as well as the *Corynebacterium, Propionibacterium, Staphylococcus, Streptococcus,* and *Micrococcus* genera (Dong et al., 2011; Willcox, 2013). These techniques, however, can often identify only a small number of organisms capable of growing in laboratory conditions. With Next-Generation Sequencing techniques, a more extensive description of OSM composition and diversity was made possible (An et al., 2022; Huang et al., 2016) and will be detailed in *1.4 The healthy human OSM*.

1.3. Characterization of microbiomes

1.3.1. What is the microbiome?

The “Human Microbiome” project, funded by the United States’ National Institute of Health between 2007 and 2016, brought many research groups together to uncover the human nasal, oral, skin, gastro-intestinal, and urogenital microbiomes. We now know that the human body hosts 10 to 100 trillion symbiotic microorganisms, most of which reside at the level of the intestinal tract (Turnbaugh et al., 2007). Each body site has a specific microbiome and each individual has their own unique microbiomes.

Microbial communities of specific ecosystems or body sites used to be named “flora”, however, the preferred term is today “microbiota”, referring to the collection of bacteria associated with a particular habitat. Although they have different meanings, “microbiota” is often used interchangeably with the term “microbiome”, that encompasses the collection of bacteria and their theater of activity, that is genetic material, microbial structural elements and metabolites, forming a specific ecological niche (Berg et al., 2020; Marchesi and Ravel, 2015). Inter-kingdom interactions between eukaryotes, prokaryotes, and viruses are necessary to maintain

diverse and balanced microbial communities and shape the development and function of the immune system (Clarke et al., 2014; Srinivasan et al., 2009).

Eubiosis is the state of equilibrium of a microbiome, i.e. a "healthy" microbiome. The eubiotic state of a specific microbiome is sensitive and precarious, site- and sometimes individual-specific (Iebba et al., 2016). Some symbiotic species that are part of a healthy microbiome can become pathogenic when overgrowing or colonizing a site where they are not usually present (Cho and Blaser, 2012; Flores-Mireles et al., 2015; Ma et al., 2012). Imbalance of a microbiome, or dysbiosis, due to a change in relative abundances of the present species or the introduction of new pathogenic species, can be caused by several factors, including, but not limited to, diet, antibiotic therapies, stress, hormones, and pathologies (Keeney et al., 2014; Ravel et al., 2011). To this date, the most studied dysbioses are those of the gastrointestinal tract (Fung et al., 2017; Levy et al., 2017) and urogenital (Bagga and Arora, 2020; Ceccarani et al., 2019; Łaniewski et al., 2020).

1.3.2. Culture-independent techniques to study microbiomes

Traditional culture methods were, for a long time, the only possible way to identify bacterial communities. However, not all bacteria can grow under laboratory conditions, which lead to an identification of bacteria that was only partial and resulting in a loss of up to 97-99% of information (Gordon, 2012). Since the invention of the first DNA sequencing method in 1977, several techniques have been developed. First-generation sequencing, or Sanger sequencing, is based on Sanger's chain-termination technique or Maxam & Gilbert chemical cleavage technique and enabled the sequencing of clonal DNA populations. Next-Generation sequencing (NGS), or second-generation sequencing, is based on pyrosequencing and can run many reactions in parallel, thus significantly increasing sequencing throughput. Third-generation sequencing is capable of sequencing single-DNA molecules, without requiring any amplification, such as Oxford Nanopore Technology (Heather and Chain, 2016). NGS revolutionized bacterial identification, enabling the identification of complex microbial populations by generating up to millions of DNA sequences per sample. The significant reduction in time and cost of sequencing technologies over the last decade have made the technology accessible to a broad range of laboratories.

Microbiome studies use two types of sequencing: metagenomic shotgun sequencing, that sequences the whole genome present in a sample, or 16S rRNA sequencing. The latter

sequences only a small and specific part of the genome, the 16S rRNA gene, that codes for the small subunit of prokaryotic ribosome. This particular gene is targeted because it is present in all prokaryotic cells and contains highly conserved regions and hypervariable regions, that enable to differentiate bacterial species. It is composed of 9 hypervariable regions, V1 to V9, only some of which are usually targeted in 16S rRNA sequencing, often V1-V3 or V3-V4 regions. Microbiome studies using metagenomic shotgun sequencing or 16S rRNA sequencing use several sequencing technologies, mainly Sanger sequencing, pyrosequencing (Illumina), or Oxford Nanopore sequencing (Kuczynski et al., 2011).

Since it is based on the amplification of a bacterial gene, 16S rRNA does not identify viruses, fungi, or prokaryotes (Doan et al., 2016; Ozkan et al., 2017) and can be subject to sequencing errors, particularly when working with paucibacterial microbiomes (Zhou et al., 2014). Finally, sequencing methods reveal microorganisms present in an environment, indifferent of whether they were alive or not when sampled.

The powerful current sequencing technologies have made possible the assessment and comparison of microbial communities between sites and individuals, and many studies have since demonstrated higher levels of bacterial detection and identification with NGS compared to traditional culture methods (An et al., 2022; Ham et al., 2018; Zhou et al., 2014). In the OSM also, 16S rRNA sequencing has been proven to be a more efficient tool (S. Li et al., 2019) and enables the identification of a higher microbial diversity compared to culture methods (Ozkan et al., 2017).

1.3.3. Laboratory practices in OSM studies: the need to standardize

16S rRNA library preparation and sequencing can be executed following different protocols. In a low abundance microbiome such as the OSM, even small differences in protocols can have a strong impact on the output and limit the comparisons that can be made between studies. Several general guidelines for microbiome studies have been published guidelines (Knight et al., 2018; Mirzayi et al., 2021). However, specific guidelines for OSM sampling and sequencing have yet to be proposed. Furthermore, no two studies by different research groups use the same protocol for OSM characterization.

Independently of the type of microbiome, sample collection faces numerous challenges. As a rule of thumb, limiting sampling to one operator to ensure reproducibility, limiting potential

contaminations and being able to identify them with the inclusion of negative controls, are necessary to ensure accurate sequencing (Eisenhofer et al., 2019). A graphical explanation of the steps of the OSM sequencing protocols that differ between studies is presented in **Figure 3**.

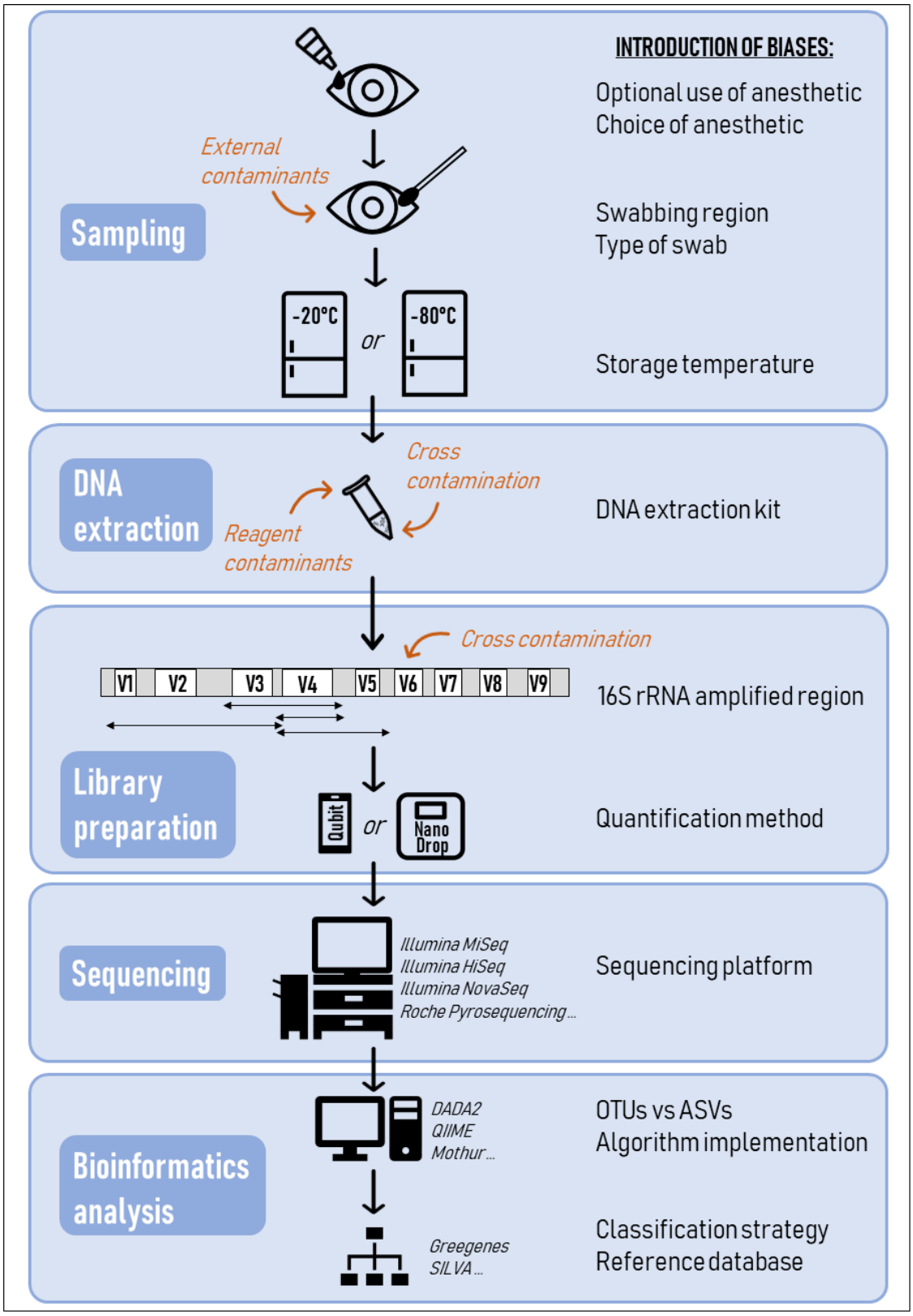

Figure 3. Steps that can introduce bias in ocular surface microbiome characterization. On the left, in blue boxes, are the name of each step from swabbing to data analysis. On the right, in black, are the choices within a step that can introduce a bias and that vary between studies.

Use of topical anesthetic. Although several articles do not provide any information regarding the use or not of anesthetic, many do apply a topical anesthetic before swabbing. Either the swab is soaked in anesthetic before sampling, or drops of anesthetic are applied directly to the ocular surface. The most common anesthetics used on the ocular surface are alcaine (proparacaine hydrochloride), oxybuprocaine hydrochloride, tetracaine hydrochloride and proparacaine. Use of anesthetic before sampling could have effects on the dilution of bacteria present in the area, and on how vigorous the operator can be when sampling, both of which could affect identification of the OSM. The first study comparing the use or not of anesthetic for OSM analysis purposes found it to decrease the detected intra-sample diversity (alpha diversity) and to alter bacterial community composition and structure (Shin et al., 2016). However, this result was contradicted by later findings of no significant difference between the OSM of participants that had previously received anesthetic drops compared to participants having received drops artificial tears (Delbeke et al., 2022). The latter study argued that the previous results of Shin et al. were based on comparison in different cohorts and could therefore be attributed to factors other than the anesthetic.

Bearing participants' comfort in mind, use of anesthetic can also impact how deep an operator can sample, which has an effect on the retrieved and sequenced OSM, and subsequent identified relative abundances (Dong et al., 2011).

Swabbing. Sampling the OSM is, most of the time, executed with a swab of various areas, that vary from study to study (see **Fig. 3**). The most commonly sampled areas are the superior and inferior fornices, and the conjunctiva. Some protocols also report the sampling of eyelid margins and meibomian glands. Similar to the differences observed between microbial communities in the fornix and on the conjunctival surface, different swabbing techniques could retrieve different aspects of the OSM (Ozkan et al., 2019, 2018). A precise definition of the swabbed region is therefore necessary for an accurate definition of the identified microbiome. Researchers should also take this into account when comparing their results to other studies, and assure themselves that comparisons are indeed possible.

The type of swab that is used also impacts the retrieved OSM, as significant differences in microbiome composition have been found when comparing sampling by conjunctival swabs and sampling with tear paper test strips (Chen et al., 2023), and when comparing calcium alginate swab, cotton-tipped applicator and Weck-Cell cellulose sponge (Katzka et al., 2021)

Swab storage temperature. After sampling, if not proceeding directly with DNA extraction, swabs need to be stored. In most cases, the chosen storage temperature is -80°C, however some prefer storing samples at -20°C, -70°C or +4°C. Although no study on the impact of storage temperature on ocular swabs has been conducted, it has been shown that, compared to keeping them at room temperature or at +4°C, freezing fecal samples for gut microbiome analysis immediately after sampling causes the least changes in sample composition and diversity (Song et al., 2016). Furthermore, a +4°C storage temperature encourages fungal growth and should therefore be avoided, when possible.

DNA extraction kits. DNA extraction is mostly performed with the use of a kit, of which there are different typologies developed for different biomass concentration and origin of samples (soil, blood, tissues etc.). More than 20 different kits have been reportedly used for DNA extraction from ocular swabs. The most used kits are the MasterPure Complete DNA and RNA Purification Kit (Epicentre), the DNeasy PowerSoil kit (Qiagen) and the MicroElute Genomic DNA kit (Omega Bio-Tek). Delbeke et al. compared processing of ocular swabs with 5 different DNA extraction kits and found distinct differences in yield (quantity of obtained DNA), observed alpha diversity after sequencing, and repeatability (Delbeke et al., 2023). The influence of DNA extraction method on microbial characterization has also been demonstrated in the gut (Costea et al., 2017; Panek et al., 2018) and oral (Teng et al., 2018) microbiomes.

DNA quantification. DNA retrieved from ocular samples is most commonly quantified by fluorometry, with a Qubit, QubitFlex, Quanti-it, or QuantiFluor fluorometer. Fluorometry is also the recommended quantification method in Illumina preparation protocols, Illumina being one of the top producers of sequencing reagents and instruments used in research.

Sequencing platform. Although most studies reporting on OSM sequencing use an Illumina sequencing platform, not all use the same type of sequencer. However, once sequencing data is generated, the choice of sequencing technique should not impact the throughput.

Sequenced region. The most commonly sequenced 16S rRNA hypervariable regions are V3-V4. Some protocols amplify V4, V4-V5, or V1-V3 regions. A few OSM studies also performed metagenomic sequencing, allowing for a more precise characterization of the OSM, as it can produce bacterial identification at a higher taxonomic resolution, albeit at a higher economic cost. Although no research has yet compared OSM characterization with different 16S rRNA target regions, significant differences in the number of recognized OTUs, alpha diversity, and

relative abundance of certain phyla has been found in the gut microbiome when comparing samples sequenced targeting the V3-V4 and V4-V5 16S rRNA regions (Rintala et al., 2017). Differences in the retrieved microbial profiles when targeting V1-V3 or V3-V4 regions have also been evidenced in the human milk microbiota (Ruiz et al., 2021). Therefore, it is possible that targeted region also has an impact on the sequenced OSM.

Reference database. Several databases are used and continuously updated for bacterial taxonomy assignment. SILVA (Quast et al., 2013) and Greengenes (McDonald et al., 2012) are the most commonly used for 16S rRNA taxonomy. However, databases are built and developed with what has already been sequenced and identified, and therefore represents mostly organisms from ecosystems that have already been extensively characterized. For this reason, although many bacteria composing human microbiomes have been identified, thanks to projects such as the Human Microbiome Project, bacteria residing on other ecosystems or animals might not be assigned to a taxon yet. Analyzing the gut and nasal microbiomes of cows and farmers, Mahmud et al. recently provided evidence of a human-bias in these databases, suggesting that some of the resident bacteria in animal microbiomes might be hidden by the current data availability (Mahmud et al., 2024).

As evidenced, there is a great variability in the protocols followed for OSM characterization, from swabbing method to sequencing platform, and each of these steps can have an impact on the generated data. Clear discrepancies in microbiome composition and diversity have been evidenced when comparing methods, and many differences in OSM composition and diversity persist between studies. Although it would be complicated, and perhaps presumptuous, to want to define a single “good” protocol for OSM research, there is a need for the definition of best practices, frameworks and/or guidelines in the field of OSM research to increase the possibility of comparison between study and, in doing so, of our overall understanding of the OSM.

1.3.4. Tools and metrics for microbiome characterization

Increase in sequencing power required the development of equally powerful computational tools to handle the amount of data to process (Kuczynski et al., 2011; Ursell et al., 2012). Several tools have been developed for the analysis of microbiomes, such as DADA2 (Callahan

et al., 2016), QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010), or Mothur (Schloss et al., 2009). These pipelines are free, open-source, and designed for the handling and analysis of high-throughput sequencing data. They allow users to import raw sequencing data, in the form of *fastq* files, trim the necessary part of reads, filter them based on their quality, identify Operational Taxonomic Units (OTUs) or Amplicon Sequencing Variants (ASVs) and assign them a taxonomy using a reference database. Following this, the pipelines can provide information on composition, diversity and richness of samples.

Composition of microbiomes can be defined at different taxonomic resolutions, the lowest being the phylum level, and the highest depending on the method used. The species level can be identified with whole genome sequencing, however with 16S rRNA sequencing, the highest obtained taxonomic level is the genus, just one level above species.

Microbial diversity in a given habitat is high, possibly higher than what is currently known, and various indices have been developed to measure it, be it intra- or inter-sample diversity. Alpha diversity pertains to the bacterial diversity within a sample and is characterized by evenness, i.e. the number or abundance of taxonomic groups, and/or richness, i.e. the distribution of abundances of the groups. Richness can be simply measured by the number of observed species, OTUs, or ASVs, or with indices like Faith's Phylogenetic diversity (Faith, 1992) or Chao1 index (Chao, 1984). Other commonly used alpha diversity such as the Shannon index (Lemos et al., 2011), or the Simpson index (Simpson, 1949) take both richness and evenness into consideration. However, some argue that these "historical" and commonly used alpha diversity indices are not always used in the correct way and that an emphasis should be put on using estimates that account for unobserved species and model errors (Willis, 2019).

Diversity between samples is measured by beta diversity, referring to the degree of similarity in community membership or structure between them (Kuczynski et al., 2010). Common metrics used for microbiomes include Bray-Curtis dissimilarity (Bray and Curtis, 1957), unweighted UniFrac distance (Lozupone and Knight, 2005), weighted UniFrac distance (Lozupone et al., 2007) and, to a lesser extent, Jaccard index (Jaccard, 1912). Jaccard index is a binary dissimilarity metric, that considers only the absence or presence of taxa. It is easy to calculate and interpret but does not consider abundance nor does it account for phylogeny. Bray-Curtis dissimilarity is based on taxa counts in each sample, and considers both the presence/absence of taxa and their relative abundances. UniFrac, for "unique fraction metric", measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction

of the branch length of the tree that leads to descendants from either one environment or the other, but not both (Lozupone and Knight, 2005). Unweighted UniFrac considers the presence or absence of taxa and the branch length fraction that is unique to a community, whereas weighted UniFrac incorporates relative abundance of taxa and weights the branch lengths accordingly (Chen et al., 2012). All four distance-metrics vary from 0, if two samples share all the same taxa, to 1, if two samples do not share any taxa and, if accounted for, share no evolutionary history. Beta diversity is commonly visualized with ordination plots, mainly Principal Coordinates Analysis (PCoA) or Nonmetric Multi-Dimensional Scaling (NMDS), allowing for the clustering of samples with similar bacterial communities.

Finally, gamma diversity pertains to the diversity within an entire ecosystem. However, it is not yet of habit to measure it in OSM studies.

1.4. The healthy human OSM

1.4.1. Composition of the OSM

The three dominant phyla found in the OSM of healthy participants are Proteobacteria, Firmicutes, and Actinobacteria, usually followed by Bacteroidetes (Delbeke et al., 2021; Peter et al., 2023; Willcox, 2013). As illustrated by **Figure 4**, differences in mean relative abundances of these phyla vary between studies.

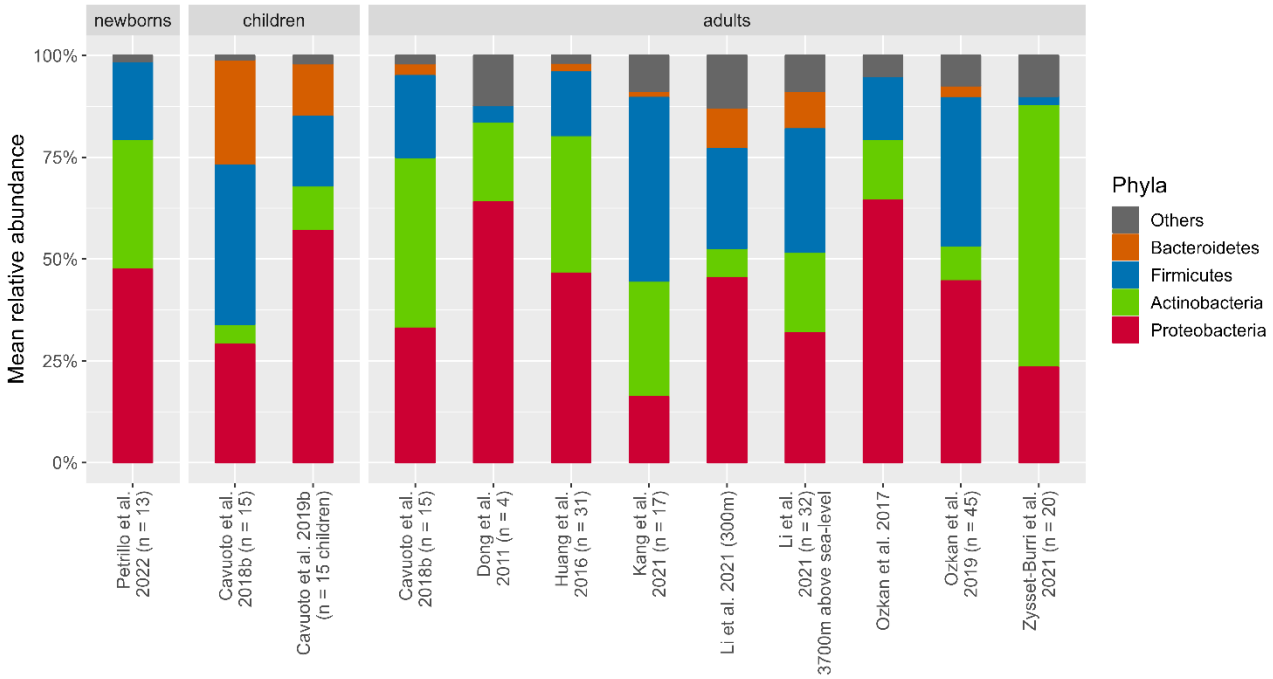

Figure 4. The healthy human ocular surface microbiome at the phylum level. (Cavuoto et al., 2018b, 2018a, 2019b; Dong et al., 2011; Huang et al., 2016; Kang et al., 2021; Li et al., 2021; Ozkan et al., 2017, 2019; Petrillo et al., 2022).

At the genus level, the OSM is characterized by the presence of *Propionibacterium*, *Corynebacterium*, *Staphylococcus,* and *Streptococcus*, and, to a lesser extent, *Acinetobacter*, *Brevundimonas*, *Pseudomonas*, *Aquabacterium* and *Sphingomonas* (Ozkan et al., 2017). Some of the bacteria identified on the conjunctiva has also been found in adjacent areas such as the face skin and oropharynx, and could, at least partially, derive from them. Indeed, with a light-pressure tamponing of the ocular surface, transient opportunistic environmental bacteria *Rothia*, *Herbaspirillum*, *Leptothrichia*, and *Rhizobium* can be uncovered. A "deeper" sampling, applied with more pressure can however isolate Staphylococci, Corynebacteriaceae, and Proteobacteria, therefore different levels of swabbing are necessary for a comprehensive characterization of the OSM (Dong et al., 2011). Recently, Borroni et al. also proposed the definition of 9 "Eye Community State Type" (ECST) of the healthy human OSM, based on the analysis of samples from 137 participants. Each ECST is characterized by a few predominant taxa and their mean relative abundances. For example, ECST 1 was enriched in *Bacteroides,* ECST 2 in *Staphylococcus* and *Corynebacterium,* and ECST 3 had a high abundance of *Staphylococcus* and bacteria of the Bacillales order (Borroni et al., 2022). The proposal of these

ECST mirrors the definition of enterotypes defined in the gut (Arumugam et al., 2011). However, this classification has, for the moment, only been used in this study and, since participants were recruited in Spain and Italy, the classification would need to be studied in other populations before being applied to other populations.

Metataxonomics showed that the bacterial population of the healthy OSM is originally identified in the environment (34%), human body (24%), plants (9%), animals (5%), and food (3%) and might surprisingly harbor more bacterial taxa than the intestines (Dong et al., 2022).

Characteristics of studies investigating the healthy human OSM are detailed in **Appendix 1**.

1.4.2. Characteristics impacting the OSM

To get a better grasp on what influences the composition of the OSM, and because such mechanisms are known in other microbiomes, several studies have compared the OSM of participants of different ages, genders, at different seasons, and living in different areas. Possible differences in OSM between the two eyes of a participant have also been explored.

Eye(s). The question of differences in OSM composition or diversity between right and left eye is important, as it impacts the sampling needed to study it properly. Wen et al. found that the bacteria identified in the right and left eye of healthy adults was statistically indistinguishable (Wen et al., 2017a). Choice of both or only one eye(s) for OSM sampling should therefore not impact the identified microbiome, unless one eye is affected by a certain pathology and the other is not.

Age. Investigation of the OSM at birth showed that it is mainly composed of Streptococci, coagulase-negative Staphylococci, and *Propionibacterium* (Eder et al., 2005), as well as *Massilia, Acinetobacter* and *Delftia* genera (Petrillo et al., 2022), similar to bacterial composition of the uterine cervix. Two days after birth, the OSM continues to evolve up to pediatric age, when its composition resembles more that of the adult OSM and is predominated by Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes (Cavuoto et al., 2019b). Although a greater diversity in the OSM of children over 6 months compared to younger infants has been described (Cavuoto et al., 2018a), as well as in children < 10 years compared to adults (Zhou et al., 2014), the question of higher or lower richness and diversity compared to adults is still debated. Differences in hygienic behaviors, state of immunity, and interpersonal contacts could explain these differences (Cavuoto et al., 2018b; Wen et al., 2017a). Nevertheless, the

healthy OSM remains stable and with a low intra-individual variability throughout adulthood (Cavuoto et al., 2019a; Kugadas and Gadjeva, 2016; Ozkan et al., 2017; Willcox, 2013).

Gender. The impact of gender on the OSM is still debated, as studies have found contradicting results. Wen et al. did not find any influence of gender on phyla-level composition, but identified a higher relative abundance of *Propionibacterium acnes* and *Staphylococcus epidermidis* and lower relative abundance of *Escherichia coli* in men compared to women (Wen et al., 2017b). Two studies have also reported alpha-diversity differences, however one study found it to be higher in women compared to men (Liang et al., 2021b), whereas the other one found it to be lower in women (Ozkan et al., 2023), highlighting necessary further investigation.

Seasons. In healthy Gambian participants aged > 10 years old, richness and alpha diversity of bacteria composing the OSM were found to be higher in dry season compared to wet season (Zhou et al., 2014). Similarly, a reduction in richness and diversity in wet season compared to dry season was found in Australian microbial keratitis patients (Stapleton et al., 2007). Among participants recruited in The Gambia, a higher diversity in healthy patients compared to trachomatous patients in the dry season but not in the wet season was also identified (Zhou et al., 2014). However, a study found no significant difference in OSM samples collected in different seasons from Spanish allergic conjunctivitis patients and healthy controls (Zarzuela et al., 2022).

Geography. Comparison of the OSM of healthy adults living in three Chinese cities with different temperatures, humidity, and air quality, yielded significant differences in composition and metabolic function (Deng et al., 2020). Differences were also found when comparing the OSM of inhabitants of cities at 300m and 3700m above sea-level. Although predominant phyla were similar, at the genus level, *Corynebacterium, Staphylococcus* and *Anaerococcus* were significantly more abundant in the people living at the highest altitude compared to the ones living at the lower altitude (Li et al., 2021).

Despite the evidenced differences in relative abundances, most, if not all, studies identified the same predominant phyla, (Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes) and the *Corynebacterium, Staphylococcus, Propionibacterium, Streptococcus, Acinetobacter, Pseudomonas* and *Bacillus* genera.

1.5. Human OSM alterations in ocular and systemic diseases

A growing body of evidence suggests that commensal bacterial communities colonizing the ocular surface play an important role in maintaining the homeostasis of healthy eyes. The resident microbiome plays a protective immunoregulatory role, protecting the ocular surface against over-representation of pathogenic species and dysbiosis that may lead to diseases (Cavuoto et al., 2019a). Changes in OSM composition and diversity have been observed in patients with various conditions, either systemic or specific to the ocular surface (Cavuoto et al., 2019a; Kugadas and Gadjeva, 2016). In most cases, it is however unclear if these changes precede or follow disease onset. A microbial imbalance on the ocular surface can modify immunoregulatory mechanisms and increase the inflammatory response triggering pathogenic mechanisms. Similarly, a pathological condition can disturb the immunological pathways, available metabolites and anatomical structure of the ocular surface, thereby leading to changes in OSM composition. Changes in OSM observed in patients with local (i.e. contact lens wearing, dry eye disease, conjunctivitis etc.) or systemic conditions (i.e. diabetes, HIV etc.) are detailed here, and reported in **Appendix 2**.

1.5.1. OSM alterations in ocular diseases

Dry eye disease and associated pathologies. Dry eye disease is a highly prevalent multifactorial pathology of the ocular surface. It is characterized by a loss of tear film homeostasis and inflammation, often presented as dryness, redness, foreign body sensation, and/or burning sensation (DEWS, 2007). Association of dry eye diseases with inflammatory disorders, such as Meibomian Gland Dysfunction (MGD), blepharitis, or ocular graft-versus-host disease (oGVHD), and autoimmune disorders such as Sjögren's Syndrome, have been established (Hernández-Zulueta et al., 2023) and will therefore be reported together in this section.

The severity of MGD has been positively associated with higher bacterial isolation rate, number of identified bacterial species (Jiang et al., 2018), and bacterial diversity (Z. Li et al., 2019).

Demodex blepharitis cases have been associated with a significantly higher relative abundance of Cyanobacteria (Wang et al., 2021; Yan et al., 2020). As shown in **Figure 5**, in some populations, higher relative abundances of Firmicutes (Yan et al., 2020) or Proteobacteria and Actinobacteria (Wang et al., 2021) were identified. In other populations, however, *Demodex*

blepharitis was associated with lower relative abundances of Firmicutes (Fu et al., 2022) or Actinobacteria (Wang et al., 2021), highlighting the need for further research on the topic.

At a higher taxonomic resolution, higher relative abundances of *Staphylococcus* were found in Stevens-Johnson syndrome and (Kittipibul et al., 2020; Ueta et al., 2021) and MGD patients (Dong et al., 2019). The *Staphylococcus hominis* species has also been associated with aqueous tear-deficient dry eye (Liang et al., 2021b). Compared to healthy controls, dry eye and associated pathologies have been characterized with higher abundances of genera such as *Sphingomonas* (Dong et al., 2019), *Bacillus*, *Brevundimonas* (Ji et al., 2022) and *Streptococcus* (Kittipibul et al., 2020; Ueta et al., 2021), and lower relative abundances of *Pseudomonas*, *Corynebacterium* (Gupta et al., 2023; Z. Li et al., 2019), *Bifidobacterium* and *Acinetobacter* (Ji et al., 2022).

Although some studies report no significant differences in alpha diversity in dry eye patients compared to healthy controls (Clougher et al., 2023; Dong et al., 2019), others report a lower diversity in dry eye patients (Z. Li et al., 2019), in Sjögren's syndrome patients (Kim et al., 2022; Song et al., 2022a), and in Stevens-Johnson Syndrome patients with severe ocular complications (Ueta et al., 2021). Some also report a higher diversity in post-HSCT patients with severe ocular GVHD compared to patients with mild to moderate oGVHD (Li et al., 2022), and in Stevens-Johnson Syndrome patients (Kittipibul et al., 2020). These contradicting results make it difficult to determine a clear impact of dry eye and associated diseases on OSM alpha diversity.

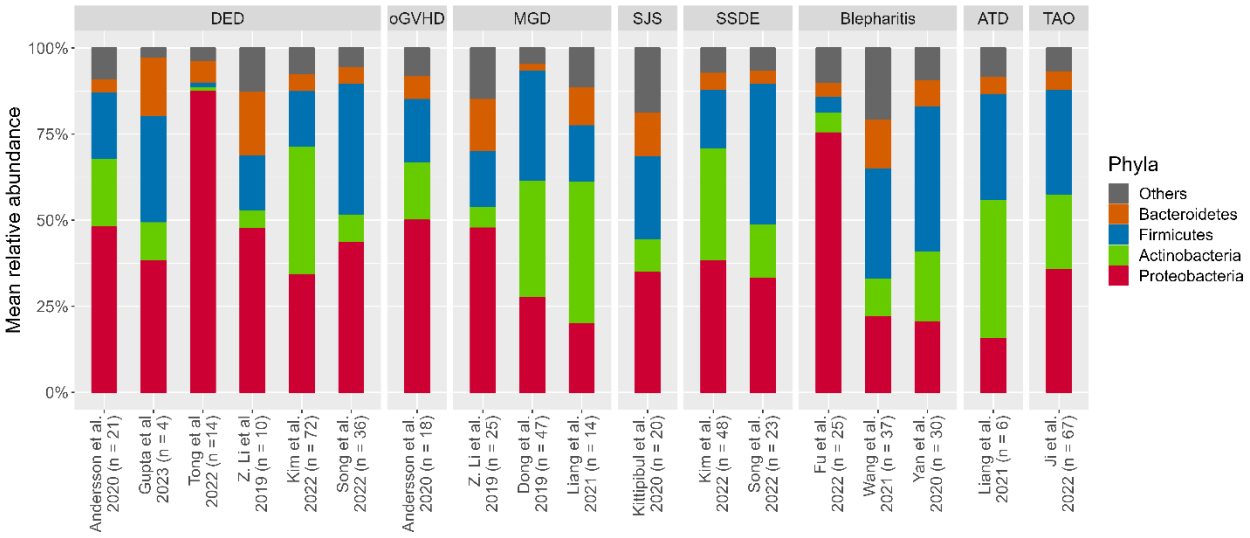

Figure 5. Phylum-level ocular surface microbiome composition of patients with dry eye disease and associated pathologies. ATD: Aqueous Tear Deficiency, DED: Dry Eye Disease, GVHD: Graft-Versus-Host Disease, MGD: Meibomian Gland Dysfunction, NSSDE: Non Sjögren's Syndrome-associated Dry Eye, SSDE: Sjögren's Syndrome-associated Dry Eye, SJS: Stevens Johnson Syndrome (Andersson et al., 2020; Dong et al., 2019; Fu et al., 2022; Gupta et al., 2023; Kim et al., 2022; Kittipibul et al., 2020; Z. Li et al., 2019; Liang et al., 2021b; Song et al., 2022a; Tong et al., 2022; Wang et al., 2021; Yan et al., 2020).

Allergic conjunctivitis, rhinoconjunctivitis, and atopic keratoconjunctivitis. Unfortunately, and much like in other pathologies, there are contradicting results regarding changes in OSM diversity in cases of conjunctivitis. Both higher (Liang et al., 2021a; Song et al., 2022b; Zarzuela et al., 2022) and lower (Inada et al., 2022; Wang et al., 2023) alpha diversities compared to healthy controls have been reported.

Composition-wise, allergic conjunctivitis, rhinoconjunctivitis, and atopic keratoconjunctivitis are associated with higher relative abundances of *Streptococcus* and *Haemophilus* (Hur et al., 2021; Liang et al., 2021a; Yau et al., 2019). Furthermore, *Pseudomonas* has been identified as a marker for allergic conjunctivitis by Wang et al. (2023). Major differences between studies persist nonetheless, with reports of mean relative abundances of Proteobacteria ranging from 78% (Hur et al., 2021) to close to 38% (Song et al., 2022b; Yau et al., 2019), as shown in **Figure 6**.

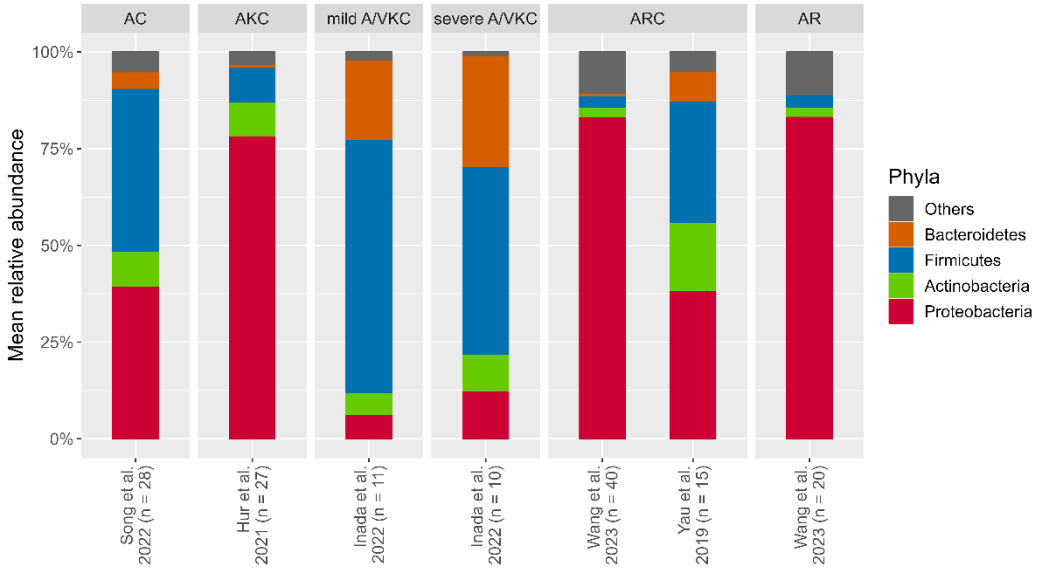

Figure 6. Phylum-level ocular surface microbiome composition of patients with conjunctivitis or rhinitis. AC: Allergic Conjunctivitis, AKC: Allergic Keratoconjunctivitis, ARC: Allergic Rhinoconjunctivitis, AR: Allergic Rhinitis, A/VKC: Allergic Keratoconjunctivis or Vernal Keratoconjunctivis (Hur et al., 2021; Inada et al., 2022; Song et al., 2022b; Wang et al., 2023; Yau et al., 2019).

Microbial keratitis. Microbial keratitis is a bacterial or fungal infection of the cornea (Ung et al., 2019) characterized by an increase in pathogenic bacteria and decrease in commensal organisms (Cavuoto et al., 2021), as well as a lower alpha diversity and a lower relative abundance of Actinobacteria (see **Fig. 7**) (An et al., 2022; Ren et al., 2021; Shivaji et al., 2021). Genus-level composition characterizing bacterial keratitis is harder to determine, since differences in reported cases persist: a higher abundance of *Pseudomonas* (Cavuoto et al., 2021) and both higher (Ren et al., 2021) and lower (Shivaji et al., 2021) relative abundances of *Escherichia-Shigella* have been reported. Fungal keratitis seems to be characterized by lower abundances of *Corynebacterium* and *Staphylococcus* and higher abundances of *Pseudomonas, Achromobacter, Caulobacter* and *Psychrobacter* (Ge et al., 2019).

Trachoma. Conjunctival scarring caused by trachoma, a severe infection due to *Chlamydia trachomatis* serovars A-C, is associated with a lower diversity and higher relative abundances of *Corynebacterium* and *Streptococcus* (Butcher et al., 2017; Pickering et al., 2019; Zhou et al., 2014). As shown in **Figure 7**, low relative abundances of Proteobacteria and high relative abundances of Firmicutes and Actinobacteria have been identified in adults with trachoma. In

children, however, there seems to be no significant change in OSM between children with and without trachoma (Butcher et al., 2017; Pickering et al., 2019).

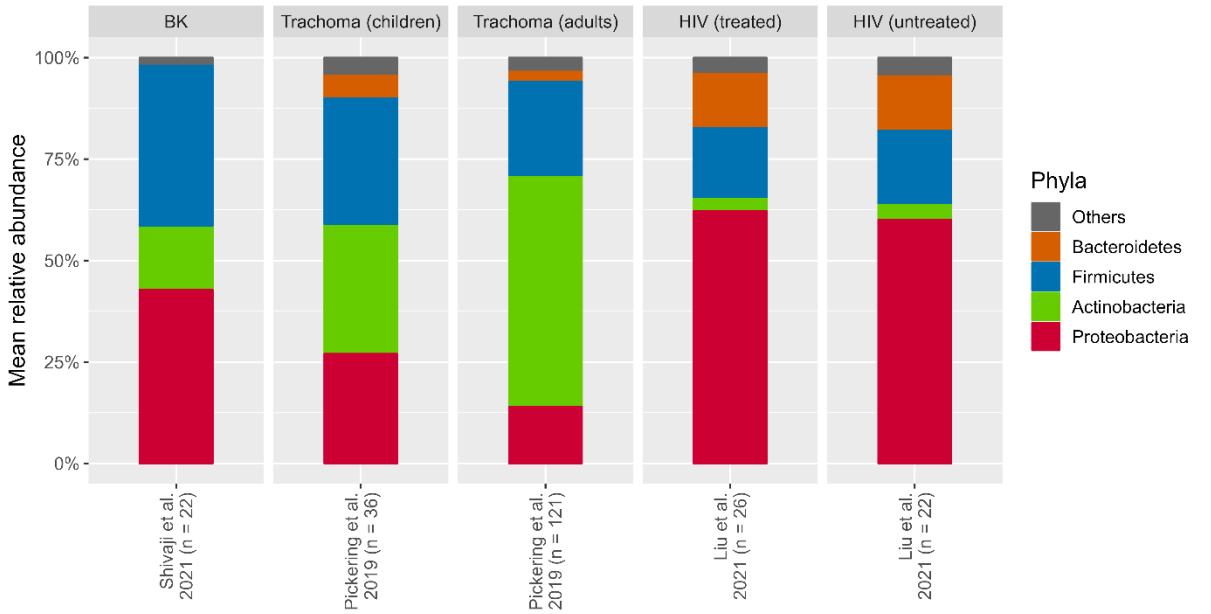

Figure 7. Phylum-level ocular surface microbiome composition of patients with infections - HIV, bacterial keratitis, or trachoma. BK: Bacterial Keratitis (Pickering et al. 2019; Liu et al. 2021; Shivaji et al. 2021).

Contact lens wear. With higher relative abundances *Methylobacterium*, *Lactobacillus*, *Acinetobacter*, and *Pseudomonas,* and lower abundances of *Haemophilus*, *Streptococcus*, *Staphylococcus*, and *Corynebacterium,* contact lens wearers tend to have an OSM composition more similar to the one of the skin microbiota (Shin et al., 2016). This could possibly be the result of a more frequent contact between users' hands and the ocular surface. However, later studies did not find any significant differences in relative abundance of these genera between contact lens-wearers and non-wearers (Xiao et al., 2023; Zhang et al., 2017). In certain cases, the OSM may also harbor opportunistic pathogens causing contact-lens-associated bacterial keratitis (Andersson et al., 2021).

Traumatic corneal ulcer. The OSM of traumatic corneal ulcer patients, a defect of the corneal epithelium, harbors an overrepresentation of Proteobacteria with, at the species level, higher

relative abundances of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Kang et al., 2020).

Mucosa-associated lymphoid tissue lymphoma. In patients affected with mucosa-associated lymphoid tissue lymphoma, the OSM presents a higher relative abundance of the *Delftia* genus, as well as lower relative abundances of the Bacteroidetes family and *Clostridium* genus (Asao et al., 2019).

Keratoconus. Keratoconus is an ocular disorder of the cornea characterized by its deformation, thus affecting vision. In keratoconus patients, relative abundances higher in the Bacteroidetes phylum and lower in *Escherichia, Enterobacter,* and *Bacillus* genera have been identified. The condition is also associated with lower bacterial diversity and richness (Tunç et al., 2023).

1.5.2. OSM alterations in systemic diseases

Diabetes. Various complications are associated with diabetes mellitus, the two major causes of visual impairments being diabetic retinopathy and cataract (Ham et al., 2018). Additionally, diabetic patients are more susceptible to sight-threatening infections and their risk of developing dry eye disease tends to be higher than in non-diabetic patients (Zhang et al., 2021).

At the phylum level, as shown in **Figure 8**, higher abundances of Proteobacteria and Bacteroidetes have been reported in diabetic patients compared to healthy patients (Ham et al., 2018; S. Li et al., 2019; Zhang et al., 2021; Zhu et al., 2021). At the genus level, a higher abundance of the *Acinetobacter* genus was identified by Li et al. and Ham et al. The former also identified an increase in *Pseudomonas,* in accordance with Suwajanakorn et al., whereas the latter found an increase in *Burkholderia, Rheinheimera*, and *Micrococcus.* Contradictory results regarding the relative abundance of *Pseudomonas* were found by a recent Zhang et al. study, who found it predominant in non-diabetic patients affected with dry eyes. Neisseriaceae and *Escherichia-Shigella*, potentially pathogenic taxa, were also predominant in diabetic patients, especially those exhibiting diabetic retinopathy (Suwajanakorn et al., 2022). So far, there is no evidence of a difference between Type I and Type II diabetic patients' OSM.

Variations in OSM diversity in diabetic patients are still under discussion since contradictory results have been found, with some studies reporting a higher alpha diversity compared to healthy controls (Chen et al., 2022; Zhang et al., 2021; Zhu et al., 2021), whereas other report

a lower alpha diversity (Ali et al., 2023; S. Li et al., 2019). Interestingly, Li et al. also reported more variation in OSM diversity between diabetic patients than between healthy individuals.

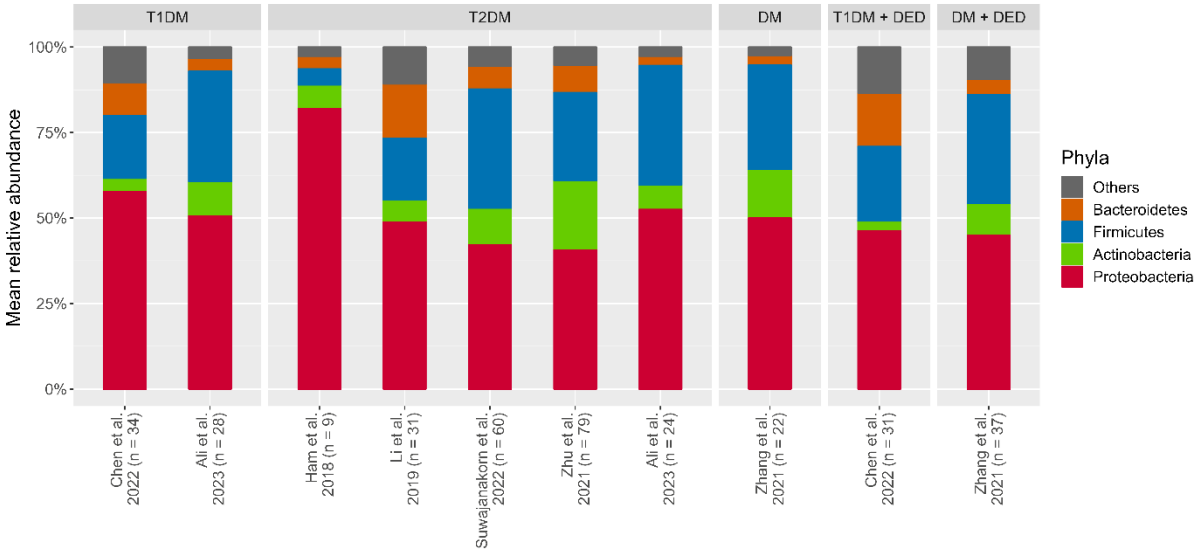

Figure 8. Phylum-level ocular surface microbiome composition of diabetes mellitus patients with or without dry eye disease. DM: Diabetes Mellitus; DED: Dry Eye Disease; T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus (Ali et al., 2023; Chen et al., 2022; Ham et al., 2018; S. Li et al., 2019; Suwajanakorn et al., 2022; Zhang et al., 2021; Zhu et al., 2021).

HIV. HIV (Human Immunodeficiency Virus) infection is associated with several lesions of the ocular surface and compositional and structural differences in OSM. It is characterized by higher relative abundances of the Proteobacteria and Bacteroidetes phyla (see **Fig. 7**) (Liu et al., 2021).

COVID-19. Only one study investigated the OSM in cases of SARS-CoV-2 infections, by comparing the OSM of healthy controls, with that of COVID-19 positive patients, and that of patients who had recovered from COVID-19. A significantly different structure and composition of OSM in positive and recovered patients compared to controls was found, highlighting persistent effects of COVID-19 on the OSM (Lin et al., 2024).

Ocular symptoms such as sensation of burning eyes, foreign body and tearing, as well as signs of conjunctival hyperemia and/or chemosis, blepharitis and meibomian orifices alterations are common in patients infected with COVID-19 (Nasiri et al., 2021). Detection of the SARS-CoV-2 virus in ocular swabs is low, ranging from 0 to 11.11%, and not always positive in the case of ocular symptoms (Cheong, 2020). Ocular swabs however have the peculiarity of being able

to detect SARS-CoV-2 positivity for over two weeks after the negativity of nasopharyngeal swabs (Bernabei et al., 2020). SARS-CoV-2 has been identified in tears, making the cornea and conjunctiva possible infections sites and reservoirs for diffusion (Barnett et al., 2020; Güemes-Villahoz et al., 2020; Ho et al., 2020). Involvement of the conjunctiva is also the most common ocular manifestation of COVID-19 (Ling et al., 2020).

Frequent use of masks during the height of the COVID-19 pandemic as one of the preventive measures worldwide has been associated with ocular irritation and discomfort, possibly caused by the upward air flowing increasing temperature of the exhaled air, humidity, tear film evaporation and perhaps even OSM dysbiosis (Burgos-Blasco et al., 2023), leading to the coining of the acronym "MADE", for "Mask-Associated Dry Eye" (Boccardo, 2022). Despite investigation on other medical devices and veils showing an increase in bacterial growth and infection, there is very little information on the effects of prolonged face mask used by nonhealthcare individuals. Further research is needed to determine if the conditions inside and outside masks can develop microbiomes and/or cause dysbiosis in close microbiomes, such as the ocular, oral, and cutaneous microbiomes (Brooks et al., 2022).

1.6. The OSM of companion animals

1.6.1. The healthy canine OSM

Although the ocular bacterial flora of dogs has been described with traditional culture methods, only three studies report its identification with NGS. These studies were conducted on canine populations composed of different breeds and living in different environments. It is still unknown whether these characteristics have an impact on the canine OSM, however some dog breeds, notably brachycephalic dogs, are particularly prone to ocular diseases (Sebbag and Sanchez, 2023). Banks et al. and Rogers et al. investigated populations of privately-owned dogs of several breeds (Banks et al., 2020; Rogers et al., 2020), while Leis & Costa only included Coonhound cross living together in a single colony of the Western College of Veterinary Medicine (Leis and Costa, 2019). Moreover, the canine populations of the three studies were of different ages, and different male-to-female ratios. Although no evidence of the effect of sex or age on the canine OSM has been demonstrated so far, it has been evidenced in humans and could be the case in animals as well.

Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria were identified as the main phyla of the canine OSM, albeit in different proportions (see **Fig. 9**). At the family level, Leis and Costa identified the Bifidobacteriaceae, Lachnospiraceae, Moraxellaceae, Corynebacterium families in 4.8% to 9.1% of all reads. As these were identified in more than 80% of samples and accounted for over 4.5% of all reads, they were considered components of the core canine OSM. In their client-owned dog population, Rogers et al. found that most prevalent families were Pseudomonadaceae, Micrococcaceae, Pasteurellaceae, Microbacteriaceae, Enterobacteriaceae, Neisseriaceae, and Corynebacteriaceae. Banks et al. did not report on higher taxonomic resolutions. Traditional culture techniques have also identified the presence of Staphylococcaceae and Streptococcaceae (Mironovich et al., 2022).

At the genus level, *Staphylococcus, Streptococcus, Acinetobacter,* and *Corynebacterium* have been identified in several dog populations, both using NGS (Leis and Costa, 2019) and culture techniques (Mironovich et al., 2022; Nadăș et al., 2021; Prado et al., 2005; Wang et al., 2008). Other frequently identified genera in dog OSM, albeit in lower prevalence, are *Pseudomonas, Neisseria, Micrococcus,* and *Bacillus* (Leis and Costa, 2019; Mironovich et al., 2022; Nadăș et al., 2021; Wang et al., 2008).

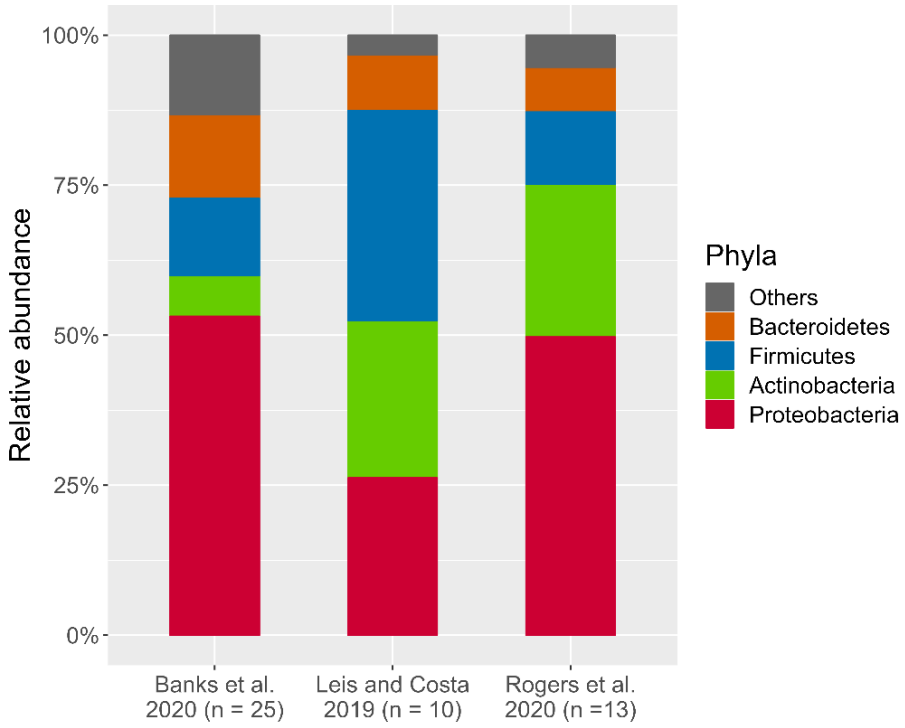

Figure 9. The healthy canine ocular surface microbiome at the phylum level. (Banks et al., 2020; Leis and Costa, 2019; Rogers et al., 2020).

1.6.2. The healthy feline OSM

Similarly, the feline OSM has been mostly identified by traditional culture techniques and only three studies have reported on it using NGS. A study by Darden et al. included 12 privately owned short-haired female cats (Darden et al., 2019). The two other studies, by Lucyshyn et al. and Weese et al. did not provide any information regarding the animals' breeds, however they indicated that the cats respectively lived in an animal shelter and in a sanctuary or privately-owned house (Lucyshyn et al., 2021; Weese et al., 2015). Age ranges of the feline populations also differed significantly, as Darden et al.'s study included cats from 1 to 1.5 years old, whereas as Weese et al. included 5 to 12-year-old cats. As for the canine OSM, impact of breed, living situation, sex, or age on the feline OSM has not been investigated yet.

The main two phyla of the feline OSM are Proteobacteria and Firmicutes (see **Fig.10**). Even at this low taxonomic resolution, different results were obtained between studies, as two also found Actinobacteria and Bacteroidetes in the main phyla, but not the third one. As shown in

Figure 10, relative abundances were different in each study, and do not allow the clear identification of a core feline OSM at this stage.

Commonly identified genera in healthy cats include *Mycoplasma, Streptococcus* and *Pseudomonas*, identified in all samples analyzed by Lucyshyn et al., and *Staphylococcus*, found in the samples reported by Weese et al. Other commonly identified genera with traditional culture techniques include *Staphylococcus, Streptococcus, Micrococcus,* and *Corynebacterium* (Aftab et al., 2019; Büttner et al., 2019; Hariharan et al., 2011).

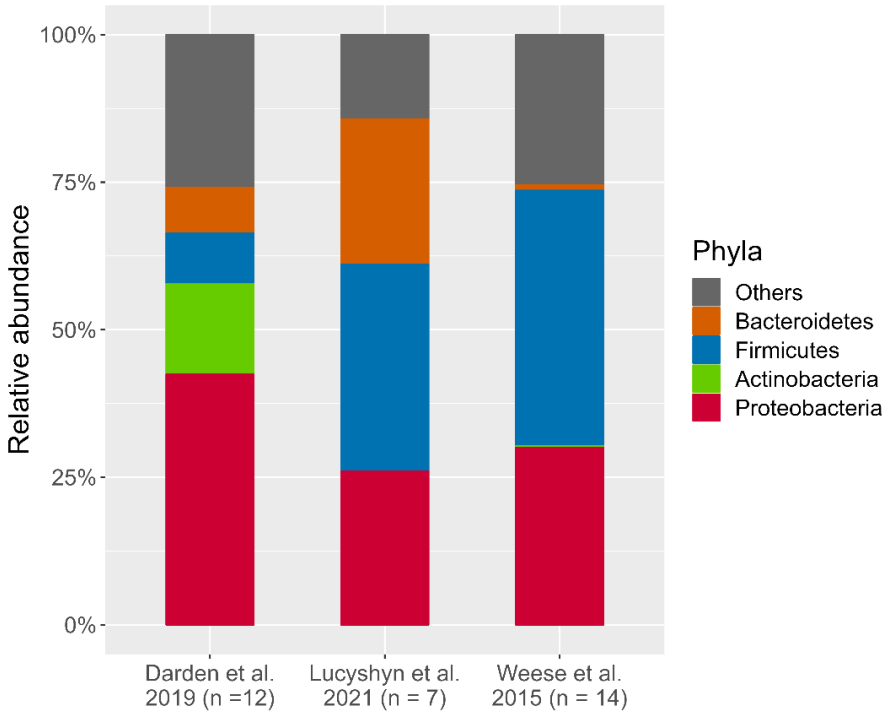

Figure 10. The healthy feline ocular surface microbiome at the phylum level. (Darden et al., 2019; Lucyshyn et al., 2021; Weese et al., 2015)**.**

Although the little amount of data on cat and dog sequenced OSM does not permit the definition of a clear core OSM, especially at high taxonomic levels, it provides a base for future studies. Interestingly, cat and dog OSM are composed of the same main phyla as the human OSM. Similarities can also be observed at the family and species level.

1.7. The impact of outdoor air pollution on the ocular surface

1.7.1. Outdoor air pollution

Ambient air pollution is one of the main environmental risk factors humans and animals are exposed to worldwide. The American Environmental Protection Agency (EPA) identified Carbon Monoxide (CO), lead, Nitrogen Oxides (NOx), Ozone (O_3), Particulate Matter (PM), mainly PM smaller than 10µm (PM_{10}) and PM smaller than 2.5µm ($PM_{2.5}$), and Sulfur Dioxide (SO_2) as the major outdoor air pollutants affecting health. Environmental pollution derives from both natural, such as volcanic eruptions or fires, and anthropogenic activities, such as industrialization, traffic, land use, agriculture, energy production, forestry, transportation, and waste generation (Owusu and Sarkodie, 2020). Air quality guidelines based on the observed effects of these pollutants on health have been devised by the World Health Organization (World Health Organization, 2021). It is estimated that between 1990 and 2017, none of the 195 countries and territories worldwide met the WHO guideline of a maximum 10µg/m^3 of $PM_{2.5}$ (Owusu and Sarkodie, 2020), and were therefore exposed to harmful concentrations of pollutants. It is now well-known that air pollution affects immune, inflammatory, and metabolic pathways (Arias-Pérez et al., 2020; Glencross et al., 2020; Rider and Carlsten, 2019), increasing the risks of cardiovascular diseases (Bhatnagar, 2022; Jia et al., 2023; Meo and Suraya, 2015), cancer within the respiratory tract (Xue et al., 2022), but also skin pathologies (Puri et al., 2017) and ovarian cancer (Dehghani et al., 2023), among many others.

As the most external part of the eye, the ocular surface is in direct contact with the environment through corneal and conjunctival epithelium, and therefore is completely exposed to air pollutants when the eyelids are opened. Consequently, and unsurprisingly, air pollution affects the ocular surface.

1.7.2. Correlation between outdoor air pollution and ocular surface diseases

At the macroscopic level, hospital and health insurance records allow for a retrospective analysis of ocular surface pathologies' cases. The number of patients can be correlated with measured air pollutants concentrations. With such methods, researchers have been able to demonstrate significant positive correlations between dry eye disease patients and levels of O_3, (Hwang et al., 2016; Lu et al., 2023), NO_2 (Mo et al., 2019; Song et al., 2019; Zhong et al.,

2018), and PM_{10} (Vehof et al., 2021; Yu et al., 2019). A study among 3.41 million United States veterans identified an overall higher risk of being diagnosed with dry eye disease for people living in urban areas with a relatively high concentration of aerosol optical depth, like Chicago or New York, compared to those living in rural or less polluted urban areas (Galor et al., 2014). Similar correlations with pollutants levels have been identified for conjunctivitis patients. Emergency department visits for conjunctivitis are significantly associated with average O_3 concentrations (range: 1.2 – 50.9 ppb) in females, especially with a delayed effect of 5 to 8 days (Szyszkowicz et al., 2019, 2012), highlighting the acute effects that O_3 can have on the ocular surface. Likewise, NO_2, O_3, SO_2 and $PM_{2.5}$ were found to be positively associated with emergency department or outpatient visits for conjunctivitis (Chang et al., 2012; Chiang et al., 2012; Szyszkowicz et al., 2016; Van Roosbroeck et al., 2008; Zhong et al., 2019). Among studies investigating the correlation between PM concentrations and conjunctivitis cases, many identify at least a trend, if not a significant positive association in adults (Aik et al., 2020; Chen et al., 2021; Fu et al., 2017; Larrieu et al., 2009; Lee et al., 2018; Lu et al., 2019; Wang et al., 2022) and children, for which, although conjunctivitis is more frequent than in adults, diagnoses are often difficult to carry out (Anderson et al., 2010; Chien et al., 2014; Nucci et al., 2017; Zhou et al., 2022). A Taiwanese cohort study also found that an increase in quartile concentrations of CO, NO, NOx, CH_4, and total hydrocarbon was associated with an increased risk of uveitis (Bai et al., 2021). All these results show a clear negative impact of both short-term and long-term outdoor air pollution on the ocular surface.

1.7.3. Clinical and sub-clinical manifestations of ocular surface pathologies

The ocular surface requires a complete tear film to maintain its health and function; adequate production, retention, and balanced elimination of tears are necessary for this process. Any imbalance of these components can lead to the condition of dry eye. Normal homeostasis of the ocular surface requires regulated tear flow, the primary driver of which is osmolarity, that is the end product of variations in tear dynamic. An increase in tear osmolarity is considered the best marker of dry eye (DEWS, 2007).

Subclinical changes of the ocular surface associated with dry eye diagnosis are presented in patients complaining of ocular discomfort (Versura et al., 1999) or travelling through highly polluted areas (Sarita et al., 2012; Saxena et al., 2003). In most cases, however, patients deem

the discomfort not strong enough to consult an ophthalmologist, bearing the question of whether the burden of air pollution on ocular surface pathologies is underestimated. Eye dryness can be considered a consequence of chronic subclinical inflammation due to increasing pollution level. The observed subclinical changes associated with high levels of air pollutants range from increase in inferior tarsal conjunctival cell count and MUC5AC gene expression, a gene coding for the mucin 5AC glycoprotein that is responsible for tear film hydration (see **Fig. 11**) (Novaes et al., 2007; Torricelli et al., 2014). With long-term exposure to air pollutants, there seems to be an adaptive response promoting mucin 5AC expression through an unknown pathway, allowing the ocular surface to maintain homeostasis and patients to remain temporarily symptom-free (Torricelli et al., 2013). The low abundance of ocular symptoms observed in patients exposed to high levels of air pollutants is an indication of the perceptual adaptation to chronic air pollution.

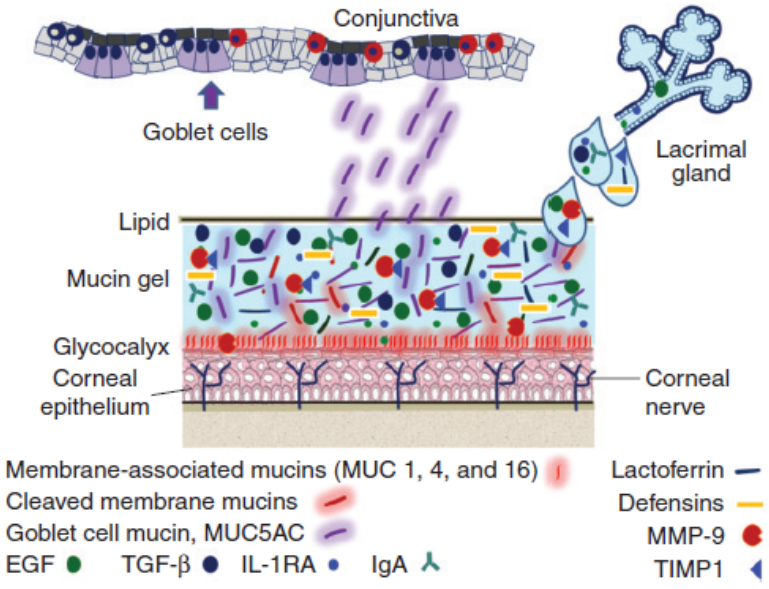

Figure 11. Structure of the tear film. The tear film consists of a mixed mucin/aqueous layer produced by the lacrimal glands, conjunctival goblet cells, and surface epithelium. It is topped by a lipid layer produced by the meibomian glands. *Adapted from "External Disease and Cornea. Basic and Clinical Science Course 2023-2024", American Academy of Ophthalmology. European Board of Ophthalmology subcommittee. Copyright © 2023 American Academy of Ophthalmology.*

Tear film osmolarity is also affected by ambient air pollution. Indeed, a significant negative correlation between tear film osmolarity level and $PM_{2.5}$ and NO_2 levels (Torricelli et al., 2013). Moreover, a more reduced tear break-up time has been observed in people living in urban areas compared to people living in rural areas (Gupta et al., 2002; Saxena et al., 2003). Changes in composition of the lipid layer, that stabilizes the tear film and prevents evaporation by sealing its aqueous layer, are also affected by air pollutants, as high levels PM_{10} destabilize the proportions of saturated and unsaturated fatty acids, causing inflammation of the ocular surface (Gutierrez et al., 2019)

These studies exhibit the fact that although many people, that could be considered healthy – or "apparently healthy" – have ocular discomfort or some attenuated symptoms, they do not, most of the time, consider these symptoms serious enough to consult a physician, even though they present subclinical signs of inflammation, tear film reduction, and other dry eye indicators.

1.7.4. Unmet needs

In the large majority of retrospective studies analyzing large datasets of hospital or insurance records to correlate them with pollutants concentrations, a correlation with at least one of the 6 main pollutants identified by the EPA has been found, confirming the negative effect of outdoor pollutants on the ocular surface. The last Tear Film and Ocular Surface society (TFOS) "TFOS Lifestyle Report: Impact of environmental conditions on the ocular surface" reviews all the possible correlations between environmental conditions and ocular surface, highlighting the negative effect of outdoor air pollutants. The authors outline some limitations of their review such as the heterogeneity in how exposures were assessed and/or categorized, how outcomes (e.g., dry eye disease) were defined, the lack of clear definitions and classification systems for environmental hazards lack of robust data and consistent studies focusing on the potential associations between environmental exposure and ocular surface diseases (Alves et al., 2023). Studies involving patient, i.e. studies not based on hospital or health insurance records, suggest the presence of subclinical symptoms identifiable by physicians but not considered critical enough for a patient to consult. Many of the patients included in these studies were labelled as "apparently healthy" but were found not be. On the other hand, the retrospective analyses and models using data from hospital or insurance records only included patients that did consult a physician regarding their ocular health. This leads us to believe that the impact of air pollution on the ocular surface estimated by the latter articles, severely underestimate the population

affected by ocular surface pathologies caused by air pollution. Subclinical parameters seem to appear long before patients seek treatment for eye conditions caused by outdoor air pollution. We can assume that the impact of air pollution on the eye could be detected before patients have significant symptoms and damage to the ocular surface is too important.

1.8. Aims

The One Health concept underlines the complex relationships between human, animal and environmental health. Applying the One Health approach to the microbiome allows for consideration of both pathogenic and non-pathogenic microbial transfer between humans, animals, and the environment. The ocular surface of humans and animals is exposed to the environment, and contains microorganisms (including bacteria, fungi, viruses, archaea, and protozoa). Albeit exposed to external contamination and unlike other exposed biological sites (i.e. the oral mucosa), the ocular surface surprisingly contains commensal microorganisms limited in number and degree of diversity. As is the case in the gut microbiome, microorganisms within the ocular surface could play a key role at the level of local adaptive and innate immunity. Despite the interest, reports on the human OSM are still few, small-scaled and inconsistent in technical standards and subject stratification. Furthermore, no consensus has been reached to establish a core OSM (remaining constant).

Although there are fewer investigations in animals, in veterinary medicine it is suggested that the ocular microbial population helps maintaining ocular health and immunity. There is evidence that the environmental microbiome, as well as the microbiome of animals in close contact, can affect the human microbiome and human health outcomes. People living in a household with pets have greater similarities in their nasal, oral, and skin microbiomes compared to people who do not have pets, suggesting the influence of pets on promoting microbial exchange. With the increasing number of households with pets and changing habits in the human-animal bond, a better understanding of these interactions is necessary, including at the microbial level.

Urbanization of built environments leads to changes in the environmental microbiome which could impact human and animal health. Exposure to airborne pollutants in the environment can induce dysbiotic changes to gut and skin microbiome composition. In humans, chronic exposure to environmental pollutants can also alter functional capacities of the skin microbiome, possibly

impacting skin homeostasis. At present, little is known about environmental pollutants' effects on the OSM of both animals and humans. The distribution of pollutant concentration is highly variable depending on the specific urban morphological context and vicinity to sources. The correct evaluation of the exposure of both animals and humans to environmental pollution is made by addressing local atmospheric circulation and sources. Pollutants such as respirable PM, NO_2, CO, and hydrocarbons are directly emitted by vehicles and the most affected group is urban inhabitants and their domestic animals, especially the population residing in close vicinity of the urban roadways and streets, and pedestrians. The study of pollutant dispersion in the built environment involves a multi-disciplinary approach. Pollutants emitted in the atmosphere are dispersed over a wide range of horizontal length scales, from macro-scale to meso-scale and to micro-scale. Pollutants either transported to the city or locally emitted are dispersed at different horizontal local scale. Their final spatial distribution is determined by several factors, such as the meteorology and the morphological characteristics of the city, as well as the population density and the type, nature and spatial location of sources. Important parameters for dispersion around buildings are their geometry and morphology, wind speed, wind direction, turbulence, atmospheric stability, temperature, humidity and solar radiation, together with the presence of obstacles such as trees, low barriers and parked cars. Consequently, local-induced wind fields consist of complex flow features such as recirculation zones and stagnation points which in turn govern the dispersion of pollutants. There is much that can be gathered from larger scale studies of the eye in health and disease within human and animals living in the same environment.

The conjunctiva has the particularity of being the only mucosa of the body directly exposed to the external environment. This direct exposure to the exposome also makes it easily accessible for non-invasive examinations and sampling.

Therefore, **the aim of this research** was to apply a One Health approach to the study of owner and pet’s OSM interactions with potential transmission of harmful microorganisms. The research combines several disciplines such as human medicine, veterinary medicine, microbiology, bio-informatics, and physics (Urban meteorology and air quality dynamics). A transdisciplinary approach has also been applied through the involvement of non-academic partners such as ARPAE (Agenzia Regionale per la Prevenzione l’Ambiente e l’Energia dell’Emilia-Romagna) with whom the research groups involved in this project were connected in data sharing. Results on transient OSM changes could serve for applications that could

enhance human and animal patients' care and optimize treatment plans with particular attention to AMR. Such enhanced understanding could lead to innovative interventions to prevent and manage a variety of human health and disease states.

To reach this goal, the first step was to set up and implement a protocol for the sampling, treatment, and analysis of OSM samples. This included determining whether canine and human OSM samples can be collected, prepared, and analyzed with the same protocol. The second step, once sequencing data was obtained, was to characterize the similarities and differences between dog and owner OSM and which parameters impact them. The final step was to explore the effect of air pollutant concentration and levels of exposure on OSM composition and/or diversity in dogs and owners.

2. Materials and methods

2.1. Implementation and troubleshooting of an OSM sequencing pipeline

Preparation of libraries for sequencing had never been conducted in the Laboratorio Analisi Cornea Superificie Oculare e Ricerca Traslazionale. A new organization of the laboratory and troubleshooting of all steps of OSM sequencing, from DNA extraction to bioinformatics were therefore necessary and required 1.5 years of work. The work conducted during this troubleshooting is presented in this section.

2.1.1. DNA extraction

The OSM being a paucibacterial microbiome, DNA extraction from ocular swabs is a step that requires particular attention and troubleshooting, in order to retrieve enough material (DNA) for further steps, while avoiding introduction of contaminants. Two DNA extraction kits were compared: the Qiagen DNeasy Blood & Tissue kit (Qiagen; Hilden, Germany) and the Zymo DNA Microprep kit (Zymo Research; Irvine CA, USA). DNA from 4 samples was extracted with both kits. Yields obtained with the 2 kits were similar for all samples, however the Zymo DNA Microprep kit is more time-consuming and requires a specific instrument for bead-beating. The Qiagen DNeasy Blood & Tissue kit had also been used for the DNA extraction of OSM samples extracted for a previous project and sequenced at by an external facility (Clougher et al., 2023), proving its efficiency for this type of sample. Therefore, the Qiagen DNeasy Blood & Tissue kit was chosen for all extractions.

Initial troubleshooting for DNA extractions with the Qiagen DNeasy kit was conducted following the pre-treatment for Gram-positive bacteria, which adds a lysozyme-based lysis, and

spin-column protocol of the DNeasy Blood & Tissue Handbook. The protocol recommends a final elution of the DNA in 200 µl or 100 µl of Buffer AE (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0). Quantification of extracted samples with a Nanodrop spectrophotometer or Qubit fluorometer were impossible due to concentrations lower than what can be detected with the instruments. The recommended initial DNA concentration for library preparation is 5 ng/µl. As the initial concentrations obtained after DNA extraction were lower than this recommendation, the following extractions were eluted in 30µl Buffer AE instead of 100 µl. Although DNA concentrations obtained with the lower elution volume were still lower (from 0.06 to 1.06 ng/µl) than the recommended 5 ng/µl, eluting in a lower volume would substantially reduce the obtained volume and risk not having enough sample if libraries need to be prepared multiple times. OSM of the right and left eyes are not significantly different (Wen et al., 2017b), therefore, to further increase DNA extraction, samples from both eyes of a participant were pooled together, when available. Samples from both eyes are pooled in the first centrifugation step of DNA extraction, resulting in a single sample per participant per elution (2 elutions in total).

2.1.2. 16S rRNA library preparation

16S rRNA library preparation was executed following the Illumina 16S Metagenomic Sequencing Library Preparation protocol. The first libraries prepared with DNA extracted from conjunctival swabs yielded concentrations ranging between 0.24 nM and 0.62 nM, which is too low for the required minimum concentration of 2 nM for a v2 chemistry MiSeq run. Therefore, to increase library preparation yield, and reach the required concentration, changes were made in 3 steps of the manufacturer's protocol:

- 2 cycles were added to the Amplification PCR, reaching 27 cycles instead of 25, to increase the number of amplicon copies while limiting contaminant over-representation. This resulted in an amplification PCR with the following program: initial denaturation at 95°C for 3 minutes, 27 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 5 minutes.
- cleaned-up products of the Amplification PCR were eluted in 32.5 µl of 10 mM Tris pH 8.5 instead of 52.5 µl.
- Index PCR was carried out with the maximum amount of DNA possible, i.e. 15 µl DNA and no water, instead of 5 µl DNA and 10 µl water.

The large majority of libraries prepared with this modified protocol had concentrations superior to 4 nM (i.e. the optimal concentration for MiSeq v2 loading). Thus, these modifications to the protocol were kept for all OSM samples.

2.1.3. Identification and removal of contaminants

After identification of contaminant families in samples prepared with a specific enzymatic lysis buffer and lysozyme during DNA extraction, a sequencing run containing negative controls to identify the contaminated product was conducted. A specific lysozyme tube was identified as the source of this contamination and eliminated. The lysozyme in question had previously been used in vaginal swab DNA extraction for microbiome characterization but, as the vaginal microbiome is a lot richer than the OSM, the relative abundance of these families, was very low and did not impact vaginal microbiome characterization. This once again underlines the need to include negative controls when working with the OSM, as it is highly sensitive to contamination. DNA extraction blanks and library preparation blanks were added to each sample batch.

2.1.4. Bioinformatics pipeline troubleshooting

In order to assess the quality of the bioinformatic processing of sequenced data, the first sequencing run contained ocular swabs that had never been sequenced before and ocular swabs that had been sequenced by an external company, allowing for the comparison between data analyzed by the company and our own results. The samples that had previously been sequenced were ocular swabs from pre- and post-hemopoietic stem cell transplant patients reported in a published article (Clougher et al., 2023), as well as ocular swabs from two pilot dog-owner pairs. These pilot pairs were used to verify that dog and owner ocular swabs could be processed following the same protocol.

Initial reports from the Illumina pipeline embedded on the MiSeq platform indicated a high number of unclassified reads and a great variation between samples, ranging from 3.7% to 92.98%. To understand why such a high number of unclassified reads was obtained, and because parameters of the Illumina pipeline cannot be modified, bioinformatic analysis of the obtained sequences was performed in collaboration with the Department of Medical and Surgical Sciences (DIMEC) team led by Prof. Gastone Castellani. The chosen pipeline for the analysis was DADA2, an open-source software package “for modeling and correcting Illumina-

sequenced amplicon errors" (Callahan et al., 2016; Rosen et al., 2012). Different values of the parameters regulating the stringency of read quality control during the trimming step, denoising, chimera removal, and the bootstrap confidence during taxonomy assignment were tested until the reach of an optimal balance between maintaining good read quality and assigning as mean reads as possible to known bacterial taxa. A bootstrap confidence of 0.8 ensures a 95-98% of sequences correctly classified to genus of the V3-V4 regions (Claesson et al., 2009) and has been repeatedly used in the analysis of ocular swab data for OSM characterization (Ren et al., 2022, 2021; Zhu et al., 2021). Thus, a 0.8 bootstrap confidence was selected for our analyses. **Figure 12** illustrates the significant improvement in data retrieval when using the DADA2 pipeline compared to the pipeline embedded in the Illumina MiSeq platform.

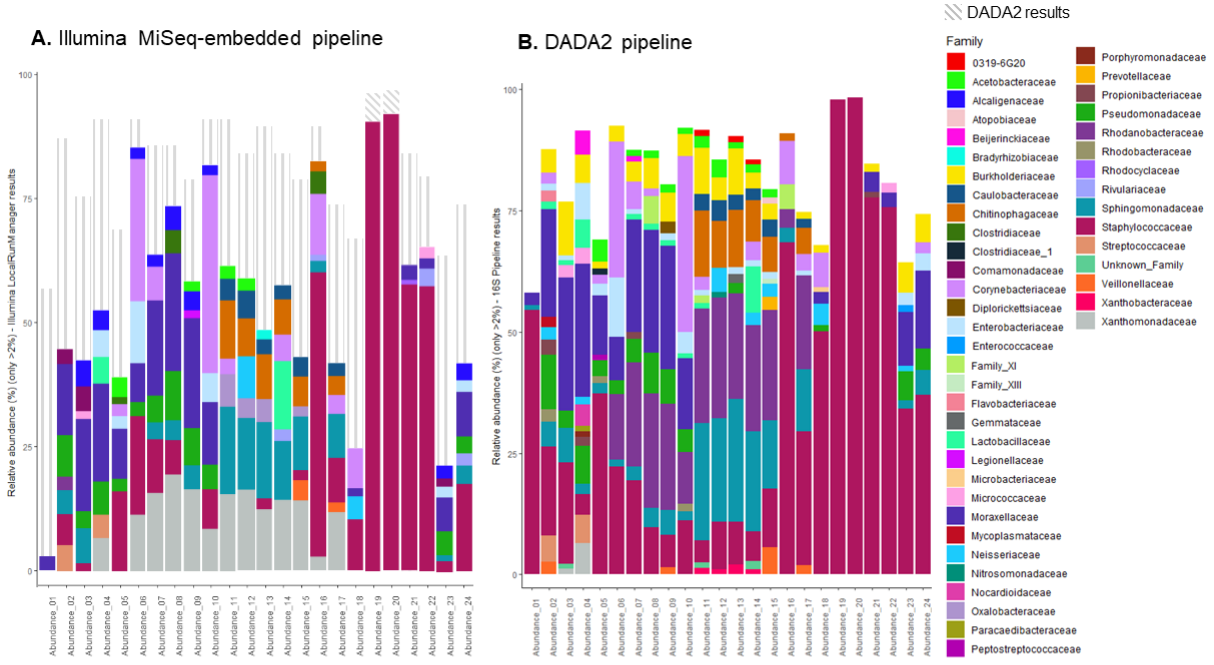

Figure 12. Improvement of bioinformatic data analysis with the DADA2 pipeline. A. Taxonomy assignment of ASVs at the family level obtained with the Illumina MiSeq embedded-pipeline. B. Taxonomy assignment of bioinformatics analysis with the DADA2 pipeline. Both pipelines were used with the same *fastq* input files.

2.2. Participant recruitment

Sixteen volunteer participants and their dogs were enrolled in the study. Exclusion criteria were the followings: dogs under 12 months old, owners under 18 years old, daily wear of contact lenses, use of topical antibiotics in the last 6 months (by dog and/or owner), eye surgery in the last 12 months (by dog and/or owner), dog-owner pair cohabiting for less than 1 year, owner in frequent contact with other animal species (i.e. veterinarians, veterinary nurses, people working in animal refuges etc.). To have the same number of dogs and owners, participants owning more than one dog had to choose one to enroll in the study. Sampling occurred between March and July 2023. Participants provided written informed consent. The study was approved by the Bioethics Committee of the University of Bologna Prot. N.0299234 on 09/11/2022, in accordance with the principles of the Declaration of Helsinki. The study was conducted on privately-owned animals with the informed consent of their owners, falling within the cases excluded from the regulations on the use of animals for scientific purposes (Legislative Decree No. 26/2014) pursuant to Art. 2. obtaining a favorable ethical and scientific opinion from the Committee for the animal welfare of the University of Bologna (Prot. No. 164586).

2.3. Data collection

Owners were asked to answer questions pertaining to the following parameters:

- Regarding the owner: age, gender, systemic diseases, ocular diseases, use of topical antibiotics in the last 6 months, glasses wear, smoking status, average daily time spent in front of a computer, occupation, address (for outdoor pollution exposure assessment).
- Regarding the dog: age, sex, breed, weight, sterilization status, systemic diseases, ocular diseases, use of topical anesthetics in the last 6 months, frequency of time spent outdoors.
- Regarding the dog-owner relationship: amount of time they have been cohabiting, presence of other pets in the household (and if so, which pets), frequency of exposure to other animals, whether the dog was allowed on the owner's bed and/or sofa, whether the dogs slept in their owner's bed.

Additionally, diameter of dogs' eyes was measured. The number of daily hours spent outside was estimated according to owners' occupation occupations and frequency of time spent outside for the dogs.in the following way:

- 2 hours/day for owners working in an indoor office.
- 5 hours/day for retired owners.
- 2 hours/day for dogs staying indoors during the day.
- 7 hours/day for dogs living in a house with a garden they frequently used.

Outdoor air pollution data was available thanks to the contribution of Maryam Safraz, Dr. Francesco Barbano, Dr. Erika Brattich and Prof. Silvana Di Sabatino from the Department of Physics and Astronomy Augusto Righi (DIFA). The methodology they developed is based on the hypothesis that road traffic is the major source of air pollution. They estimated daily average traffic counts for each owner's address and combined it with daily concentrations of 3 air pollutants, PM_{10}, $PM_{2.5}$ and NO_2, collected from ARPAE stations publicly available data. The mean daily exposure to PM_{10} and $PM_{2.5}$ was provided by Dr. Roberto Battistini from the Department of Civil, Chemical, environmental, and Materials Engineering (DICAM). It is based on the integral underlying the curve of PM concentration and considers an average number of daily hours spent outside, i.e. directly exposed to outdoor air pollution. Positive associations between hospital visits for conjunctivitis in women and O_3 concentrations 8 to 9 days before the visits (Szyszkowicz et al., 2019, 2012), as well as NO_2 and $PM_{2.5}$ concentrations 7 to 8 days before the visits (Szyszkowicz et al., 2016). Additionally, there is a cumulative effect of NO_2 concentrations on conjunctivitis cases for up to 11 days (Bao et al., 2021). Therefore, the 10 days prior to sampling were considered to assess outdoor air pollution exposure of dogs and owners.

The 5 outdoor air pollution measures that were therefore considered were:

- Mean daily concentration of PM_{10} in the 10 days before sampling ($\mu g/m^3$)
- Mean daily concentration of $PM_{2.5}$ in the 10 days before sampling ($\mu g/m^3$)
- Mean daily concentration of NO_2 in the 10 days before sampling ($\mu g/m^3$)
- PM_{10} exposure in the last 10 days ($h*\mu g/m^3$)
- $PM_{2.5}$ exposure in the last 10 days ($h*\mu g/m^3$)

2.4. Sampling

Sampling of dog owners was performed at Ophthalmology Unit, DIMEC, University of Bologna, Italy. A brief assessment of the ocular surface of both eyes, measuring Ocular Protection Index (OPI) and Non-Invasive Tear Break-Up Time (NIBUT) with the CA-800

Corneal Analyzer (Topcon Healthcare; Tokyo, Japan) was performed. NIBUT is the number of seconds between the last blink and the appearance of dry spots in the tear film. A NIBUT > 5 indicates a normal tear film stability index, qualified hereafter as a “normal NIBUT”; a NIBUT ≤ 5 indicates a low tear film stability index, qualified hereafter as an “impaired NIBUT”. OPI is the ratio between NIBUT and inter-blink interval. An OPI ≥ 1 indicates a protected tear film, qualified hereafter as a “normal OPI”; an OPI < 1 indicates an exposed ocular surface, qualified hereafter as an “impaired OPI” (Ousler et al., 2008).

To ensure reproducibility, sample collection was ensured by a single operator. Two drops of oxybuprocaine 0.4% sterile eyedrops were applied to each eye before sample collection with a sterile flocked swab (eSwab®, Copan Diagnostics; Murrieta, CA, USA) in each eye. Swabs were immediately placed at -80°C.

Sampling of dogs was performed by a veterinarian at the owner’s house. To ensure reproducibility, sample collection was ensured by a single operator. No ocular assessment visit was performed before sampling, and, to avoid further disturbing and containing of the animals, no anesthetic was applied. Sampling of the inferior fornix of the conjunctiva was performed by pressing the swab against the mucosa using one continuous circular movement, to expose the entire sampling surface of the swab to the conjunctival surface. Samples were kept on ice for a maximum of 2 hours, then at +4°C for a maximum of 24 hours and finally placed at -80°C.

2.5. DNA extraction

DNA extraction was done in batches of samples, each batch contained both dog and owner samples of different pairs to identify possible batch effects and not mistake them for signatures of dog OSM or owner OSM.

Samples were defrosted and vortexed to ensure transfer of bacteria collected on the swab into the liquid. Sample from one eye was transferred to 1.5 ml tube to centrifuge at 5,000 x g for 10 minutes. Supernatant was discarded and sample from the second eye was added to the tube for a second centrifugation at 5,000 x g for 10 minutes. Supernatant was discarded and bacterial pellet was resuspended in 180 µl enzymatic lysis buffer (20 mM Tris HCl, pH = 8,0; 2 mM EDTA; 1,2 % Triton X-100; 20 mg/ml lysozyme) and incubated at 37°C for 30 minutes. After incubation, 25 µl proteinase K and 200 µl Buffer AL (guanidine hydrochloride 30-50 %; maleic acid 0.1-1 %) were added to the sample and further incubated at 56°C for 30 minutes. 200 µl

ethanol 100% was added and mixture was pipetted into a DNeasy Mini spin column and centrifugated at 6,000 x g for 1 minute. To purify the DNA, columns were first washed with Buffer AW1 (guanidine hydrochloride 50-70 %) and centrifugated at 6,000 x g for 1 minute, then washed with Buffer AW2 and centrifugated at 16,000 x g for 3 minutes to remove any residual ethanol. Purified DNA was eluted a first time in 30 µl Buffer AE, and a second time in 50 µl buffer AE to retrieve all extracted material. DNA extracts were quantified by fluorometry and stored at -20°C.

2.6. 16S rRNA library preparation

Library preparation of samples was done in batches that differed from the DNA extraction ones, in order to identify possible batch effects and cross contaminations.

V3-V4 hypervariable regions of bacterial 16S rRNA gene were amplified using the following primers: 16S Fw: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNG-GCWGCAG; 16S Rev: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTAC-HVGGGTATCTAATCC. A modified version of the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina; San Diego, CA, USA) was followed. Specifically, initial Amplicon PCR was prepared with 2.5 µl DNA, 5 µl 16S PCR Forward primer, 5 µl 16S PCR Reverse primer, 12.5 µl 2X KAPA HiFi HotStart Ready Mix (Roche; Basel, Switzerland). Amplicon PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, 27 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 5 minutes. Amplicon PCR products were purified with AMPure XP Beads (Beckman Coulter Life Sciences; Brea, California, USA) and eluted in 32.5 µl of 10 mM Tris pH 8.5. Index PCR was set up with 15 µl DNA, 5 µl Nextera XT Index Primer 1, 5 µl Nextera XT Index Primer 2 (Illumina; San Diego, CA, USA), 25 µl 2X KAPA HiFi HotStart Ready Mix. Index PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

Libraries were quantified *via* fluorometry with the Qubit dsDNA High sensitivity Kit (Life Technologies; Carlsbad, California, USA), using 5 µl per library, and quality of the libraries was assessed on a 2100 Bioanalyzer using the Agilent DNA 1000 Kit (Agilent Technologies; Santa Clara, California, USA).

Samples were diluted to 2 nM, pooled, denatured and diluted to 6pM following Illumina MiSeq System Denature and Dilute Libraries Guide. Specifically, 5 µl of 2 nM library pool was combined to 5 µl freshly diluted 0.2 N NaOH, vortexed briefly, centrifugated at 280 x g for 1 minute, and incubated at room temperature for 5 minutes to denature DNA into single strands. 990 µl of pre-chilled HT1 was added, resulting in a 10 pM denatured library. To dilute it to 6 pM, 360 µl of 10 pM denatured library was combined to 240 µl pre-chilled HT1, mixed and pulse centrifugated. PhiX control V3 (Illumina; San Diego, CA, USA) was denatured following the same steps as the library, and diluted to the same concentration of 6 pM, as recommended by the manufacturer for low diversity libraries. 480 µl of 6 pM denatured libraries were combined with 120 µl of 6 pM denatured PhiX control, resulting in a 20 % PhiX spike-in, and kept on ice until heat denaturation. Just before loading onto the reagent cartridge, the combined library and PhiX control tube was incubated at 96°C for 2 minutes for heat denaturation, then inverted twice to mix, and immediately placed in an ice-water bath for 5 minutes. The 600 µl of heat denatured combined library and PhiX control was loaded onto the reagent cartridge and onto the MiSeq sequencer. A 2 x 251 paired-end run was performed on an Illumina MiSeq platform using MiSeq Reagent Nano kit v2 (Illumina; San Diego, CA, USA).

2.7. Bioinformatics and statistics

Paired-end fastQ files were analyzed using the DADA2 pipeline (Callahan et al., 2016). Obtained reads were trimmed, filtered, and clustered into ASVs. Taxonomy assignment of ASVs was performed against the SILVA v.132 database (Quast et al., 2013). Contaminant ASVs were identified and removed with the R package *decontam* version 1.22.0 (Davis et al., 2018) using the frequency method and a 0.5 threshold. The phylogenetic tree obtained with DADA2 was rooted with the R package *ape* version 5.7-1.

Alpha diversity, describing the diversity within a sample or group, was assessed by the number of observed ASVs, Shannon index, and Simpson index *via* the R package *phyloseq* version 1.46.0. The alpha diversity of dog samples was compared to the one of owner samples.

Beta diversity to compare dogs and/or owners was estimated by 3 metrics, in line with ones most used in OSM literature: weighted UniFrac distance (Lozupone et al., 2007), unweighted UniFrac distance (Lozupone and Knight, 2005), and Bray-Curtis dissimilarity (Bray and Curtis, 1957). The 3 were calculated with the *phyloseq* package. Significance of clustering in the

ordination plots was estimated by analysis of variance with the *adonis* function of the R package *vegan* version 2.6-4. When the 3 metrics displayed the same trend and Wilcoxon rank-sum test significance level, only weighted UniFrac was represented in the figures, as it is the metric that considers relative abundances while also taking phylogeny into account. When different trends and/or significance level differed, plots obtained with three metrics are displayed

Beta diversity between an owner and their dog will be referred to as "**dog-owner distance**" and beta diversity between an owner one of the other 14 dogs in the study will be referred to as "**dog-random-owner distance**".

Additionally, to produce a comprehensive metric showing the impact of metadata on dog-owner distance, dog-owner pairs were ranked from most (1) to least similar (15) for each of the 3 beta diversity measures. The mean rank of each pair was calculated and considered an indicator of the level of similarity between an owner's microbiome and their dog's. Differences in beta diversity were estimated with Wilcoxon rank-sum test, considering a p.value < 0.05 as significant.

Indicator species analysis was executed with the R package *indicspecies* version 1.7.14 (De Cáceres and Legendre, 2009).

3. Results

3.1. Study population

Sixteen dog-owner pairs were recruited. However, one dog was not sampled due to schedule incompatibility, and the dog-owner pair was excluded, resulting in a total of fifteen included pairs. During sampling, owners underwent an ocular examination that confirmed all owners were free of ocular pathologies at time of sampling and therefore considered healthy.

The dog-owner pairs had been living together for a mean time of 4.6 (± 2,9) years. All dogs were privately-owned and lived, at least partially, indoors. 11 out of 15 dogs were mixed breeds, other breeds were 1 Chihuahua, 1 Pinscher, 1 Shih Tzu, and 1 Siberian Husky. Main characteristics of dogs and owners are reported in **Table 1**.

Table 1. Characteristics of the dog and owner population

		Mean (± SD) or (%)
Owners	Gender	13: women (87 %) 2: men (13 %)
	Age	47 (± 18) years
	Smoking status	6: yes (40 %) 9: no (60 %)
	Use of contact lenses	1: frequent wear (7%) 2: occasionally (13%) 1: 2 never (80%)
	Glasses wear	11: yes (73%) 4: no (27%)
	Use of computer	8: more than 6 hours/day (53%) 4: 2 to 6 hours/day (27%) 3: less than 2 hours/day (20%)

	NIBUT	6: normal in both eyes (40%) 5: impaired in 1 eye (33%) 4: impaired in both eyes (27%)
	OPI	7: normal in both eyes (47%) 3: impaired in 1 eye (20%) 5: impaired in both eyes (33%)
	Mean daily PM_{10} concentration in the 10 days before sampling	16.7 (± 2.3) µg/m^3
	Mean daily $PM_{2.5}$ concentration in the 10 days before sampling	10.0 (± 1.6) µg/m^3
	Mean daily NO_2 concentration in the 10 days before sampling	26.2 ± (16.4) µg/m^3
	PM_{10} exposure in the last 10 days (h*µg/m^3)	344.9 ± (158.7) µg/m^3
	$PM_{2.5}$ exposure in the last 10 days (h*µg/m^3)	208.4 ± (103.6) µg/m^3
Dogs	Sex	11: females (73 %) 4: males (27 %)
	Age	6,5 (± 3,6) years
	Breed	11: Mixed breeds 1: Chihuahua 1: Pinscher 1: Shih Tzu 1: Siberian Husky
	Weight	16 (± 11) kg
	Size	7: small dogs (< 10 kg) 4: medium dogs (10 to 25 kg) 4: large dogs (> 25 kg)
	Sterilization	3: not sterilized (20 %) 12: sterilized (80 %)
	Eye diameter	17 cm ± 5
	Mean daily level of PM10 in the 10 days before sampling	12.3 (± 2.2) µg/m^3
	Mean daily level of PM2.5 in the 10 days before sampling	6.7 (± 1.3) µg/m^3
	Mean daily level of NO2 in the 10 days before sampling	28.6 (± 19.3) µg/m^3
	PM_{10} exposure in the last 10 days (h*µg/m^3)	301.1 (± 235.7) µg/m^3
	$PM_{2.5}$ exposure in the last 10 days (h*µg/m^3)	168.9 (± 145.3) µg/m^3
Dog-owner pair	Time dog and owner have lived together	4,6 (± 2,9) years
	Presence of other pets in the house	7: no other pets (47%) 2: 1 dog (13%) 3: 1 cat (20%) 1: 2 cats (6%) 1: 3 cats (6%) 1: 1 cat, birds (6%)
	Frequent contact with other pets	5: yes (33%) 10: no (67%)
	Outside of walks, is the dog mostly indoors or outdoors?	12: indoors (80%) 3: indoors and outdoors (20%)
	Is the dog allowed on the bed?	11: yes (73%) 4: no (27%)
	Is the dog allowed on the sofa?	13: yes (87%) 2: no (13%)

NIBUT: Non-Invasive tear Break-Up Time, OPI: Ocular Protection Index

3.2. Sequencing results

A total of 1,495,056 reads was obtained after sequencing. Reads were trimmed, denoised, merged, and filtered for chimera removal before taxonomy assignment. In the taxonomy assignment step, most reads (83%) were classified up to the family level, however only 67% were assigned a genus and less than 0.5% were assigned a species (see **Fig. 13**). Thus, the results will focus on analysis up to the family level and only composition will be detailed for the genus level.

After contaminant removal, a total of 423,101 ASVs remained and was used in the data analysis. Samples had between 2,862 and 40,683 ASVs, with a mean of 14,103 ASVs. There was no significant difference in the number of reads obtained in dogs compared to owners, nor was there a correlation between dog-owner pair and number of reads.

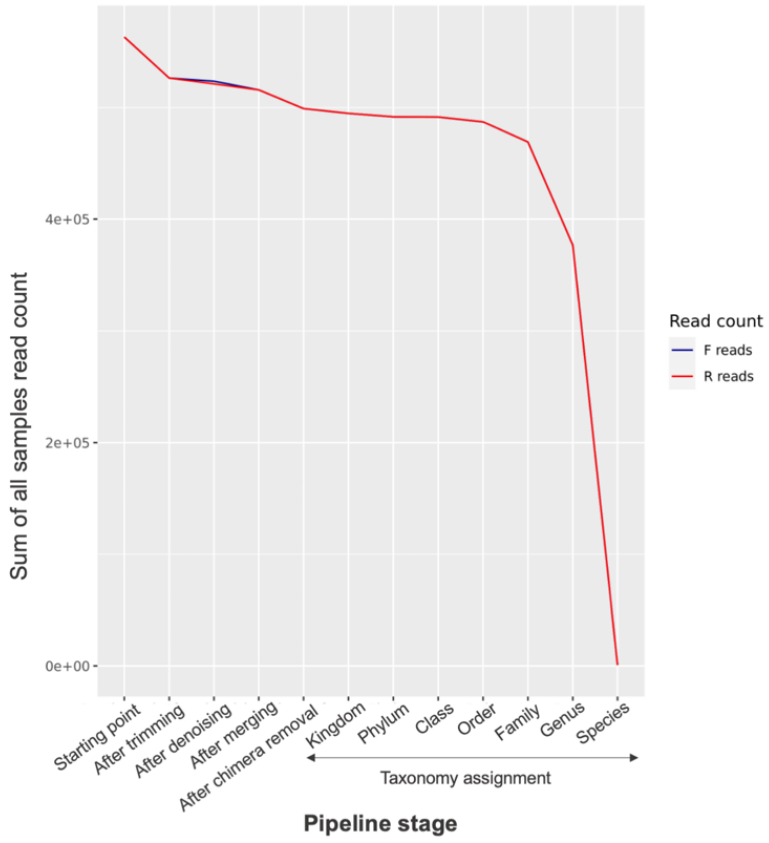

Figure 13. Read loss plot of sequencing data according to pipeline stage.

3.3. Composition of dog and owner OSM

Phylum. The main phyla identified in the OSM of dogs and owners were the same, with Proteobacteria, Actinobacteria, and Firmicutes accounting for 60 to 80% of the relative abundance in each sample (see **Fig. 14A**). 22 phyla were found in the dog population and the owner population. The phylum Dependentiae was only identified in 1 owner, whereas 10 phyla were only identified in dogs (see **Fig. 14E**).

Class. Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacilli and Bacteroidia accounted for more than 75% of classes identified in dogs and owners (see **Fig. 14B**). 37 classes were found in both owner and dog samples, 38 were found only in dog samples only and 3 only in owner samples (see **Fig. 14F**).

Order. The 5 most abundant orders in the dog population were, from highest to lowest, Burkholderiales, Pseudomonadales, Corynebacteriales, Micrococcales, and Staphylococcales. In the owner population, they were Corynebacteriales, Staphylococcales, Xanthomonadales, Rhizobiales, and Burkholderiales in owners (see **Fig. 14C**). Main orders in dogs and owners were the same but in different relative abundances.

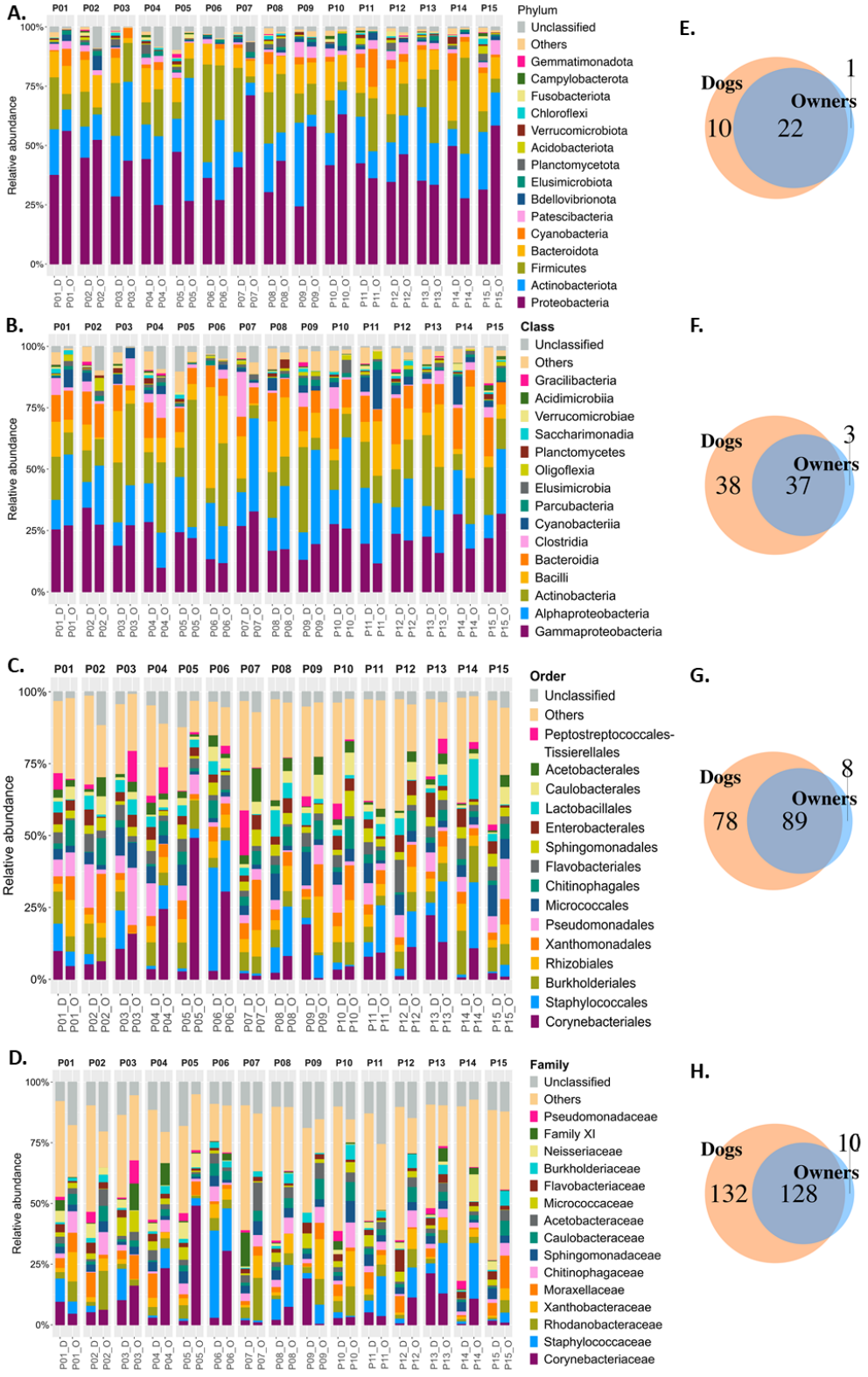

Figure 14. Composition of the ocular surface microbiome of dog-owner pairs. Relative abundances of the 15 most abundant phyla (A.), classes (B.), orders (C.), and families (D.). Number of taxa found in the dog population alone, the owner population alone, and the both populations at the phylum (E.), class (F.), order (G.), and family (H.) level.

Family. The main families in dogs were *Staphylococcaceae, Corynebacteriaceae, Moraxellaceae*, *Sphingomonadaceae* and, to a lesser extent, *Pseudomonadaceae, Micrococcaceae, Pasteurellaceae*, and *Neisseriaceae*. The main families in owners were *Corynebacteriaceae, Staphylococcaceae, Rhodanobacteraceae, Xanthobacteraceae*, and *Chitinophagaceae* (see **Fig. 14D**). The vast majority of families identified in owners were also identified in dogs (127 out of 137). 10 families were only identified in owners, whereas 133 families were only identified in dogs (see **Fig. 14H**).

Core OSM at the family level. A systematic review on the OSM defined the core microbiome as the genera present in at least 5 of the 11 included studies with a relative abundance of at least 1%. Adapting this definition, the core microbiome will be here defined, separately for dogs and owners, as the families present in at least 8 out of 15 samples with a relative abundance of at least 1%. In owners, the sequenced core OSM is (from least to most abundant) *Corynebacteriaceae, Staphylococcaceae, Rhodanobacteraceae, Xanthobacteraceae, Chitinophagaceae, Caulobacteraceae, Acetobacteraceae, Moraxellaceae, Sphingomonadaceae, Burkholderiaceae, Enterobacteriaceae, Propionibacteriaceae,* and *Flavobacteriaceae* (see **Fig. 15A**). In dogs, the core OSM is composed of *Staphylococcaceae, Corynebacteriaceae, Moraxellaceae, Sphingomonadaceae, Micrococcaceae, Flavobacteriaceae, Rhodanobacteraceae, Neisseriaceae, Streptococcaceae, Chitinophagaceae, Porphyromonadaceae, Pasteurellaceae, Xanthobacteraceae, Pseudomonadaceae, Acetobacteraceae, Rhodobacteraceae, Caulobacteraceae, Comamonadaceae, Weeksellaceae,* and *Microbacteriaceae* (see **Fig. 15B**). Interestingly, in owners, more than 60% of the OSM is composed of the 13 core families, with *Corynebacteriaceae* and *Staphylococcaceae* accounting for more than 23% (see **Fig. 15C** & **Table 2**). In dogs, however, the 20 core families account for less than 50% of the OSM (see **Fig. 15C** & **Table 2**), that could indicate a higher diversity, richness, or number of rare families in dogs, or a higher variability between dogs compared to between owners.

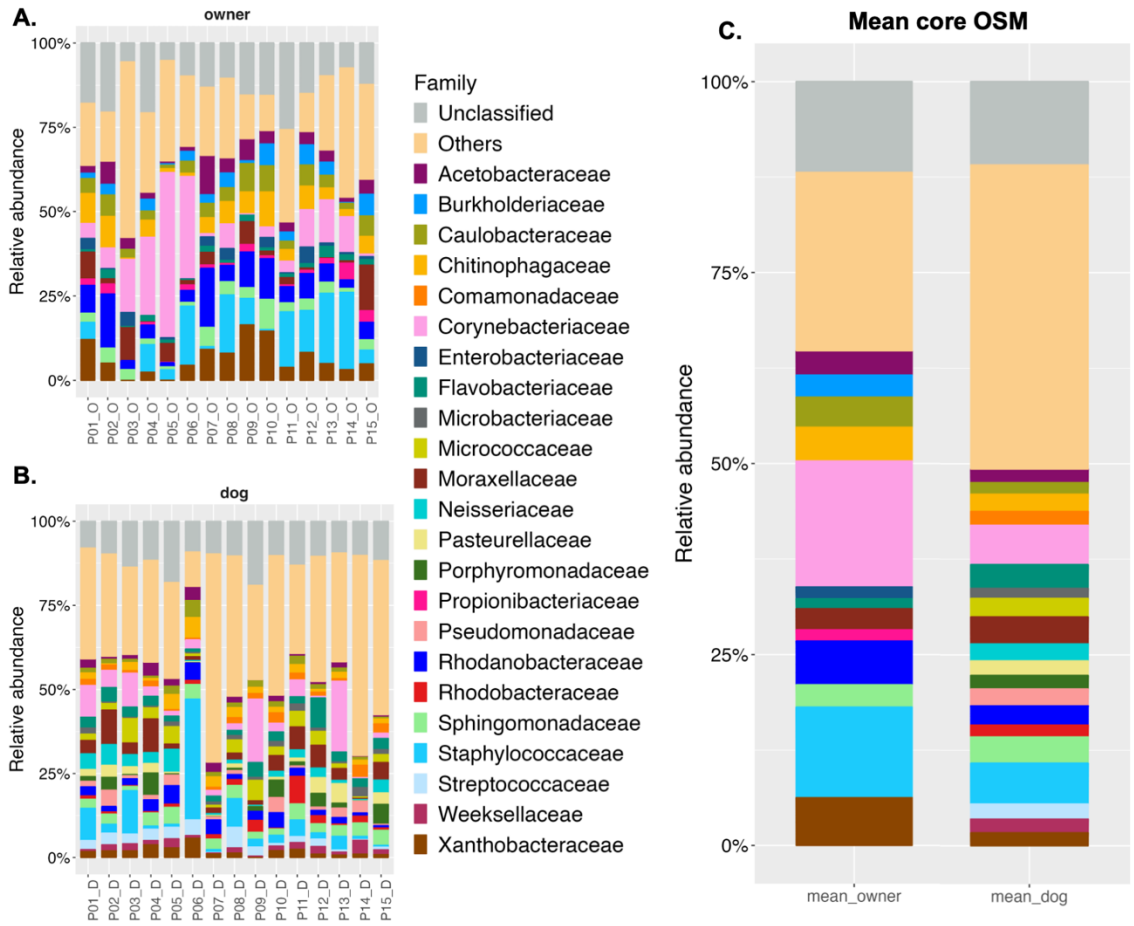

Figure 15. Core families of the dog and owner ocular surface microbiome. A. Relative abundances of core ocular surface microbiome families of owners. B. Relative abundances of core ocular surface microbiome families of dogs. C. Mean relative abundances of core families of the canine and human healthy ocular surface microbiome.

Table 2. Mean relative abundances of core ocular surface microbiome families in dogs and owners. Mean relative abundance (± standard deviation).

Family	**Dogs**	**Owners**
Acetobacteraceae	2,1 (± 1,4)	4,7 (± 3,2)
Burkholderiaceae		3,5 (± 2,5)
Caulobacteraceae	1,9 (± 1,2)	5,2 (± 2,6)
Chitinophagaceae	2,6 (± 1,7)	5,9 (± 3,9)
Comamonadaceae	1,9 (± 1,1)	
Corynebacteriaceae	6,5 (± 7,6)	13,5 (± 14,4)
Enterobacteriaceae		2 (± 2)
Flavobacteriaceae	3,6 (± 2,1)	1,6 (± 1,1)

Microbacteriaceae	1,2 (± 1)	
Micrococcaceae	3,7 (± 2,5)	
Moraxellaceae	4,7 (± 3,5)	4,3 (± 4,7)
Neisseriaceae	2,9 (± 2,5)	
Pasteurellaceae	2,2 (± 2)	
Porphyromonadaceae	2,4 (± 2,6)	
Propionibacteriaceae		1,8 (± 1,5)
Pseudomonadaceae	2,1 (± 1,8)	
Rhodanobacteraceae	3,1 (± 1,8)	8,4 (± 6)
Rhodobacteraceae	1,9 (± 2,3)	
Sphingomonadaceae	3,8 (± 1,3)	3,8 (± 2,4)
Staphylococcaceae	6,7 (± 10)	10,6 (± 9,2)
Streptococcaceae	2,7 (± 1,9)	
Weeksellaceae	1,8 (± 1,1)	
Xanthobacteraceae	2,1 (± 1,6)	7,7 (± 5,8)

Genus. The 3 most frequently identified genera in all dog and owner samples were *Rhodanobacter, Corynebacterium*, and *Staphylococcus.* Other frequent genera found in the dog population were *Streptococcus, Porphyromonas, Conchiformibius,* and *Sphingomonas*.

Core OSM at the genus level. Following the same definition of core microbiome that was used for the family level, a genus-level core microbiome of the dog and owner populations was determined. In owners, the identified genus-level core OSM is (from least to most abundant *Staphylococcus, Corynebacterium, Rhodanobacter, Vibrionimonas, Acidocella, Bradyrhizobium, Acinetobacter, Ralstonia, Sphingomonas, Cutibacterium*, and *Flavobacterium* (see **Fig. 16A**). In dogs, the genus-level core OSM is composed of *Staphylococcus, Corynebacterium, Rhodanobacter, Pseudomonas, Streptococcus, Sphingomonas, Conchiformibius, Porphyromonas, Acidocella, Flavobacterium, Moraxella, Acinetobacter, Kocuria, Vibrionimonas, Ralstonia,* and *Bradyrhizobium* (see **Fig. 16B**).

Similarly to what was observed at the family level, the genus-level core OSM in owners is made up of less families (11 genera compared to 16 for dogs) that account for a bigger mean relative abundance (close to 50% in owners compared to less than 30% in dogs) (see **Fig 16C** & **Table 3**).

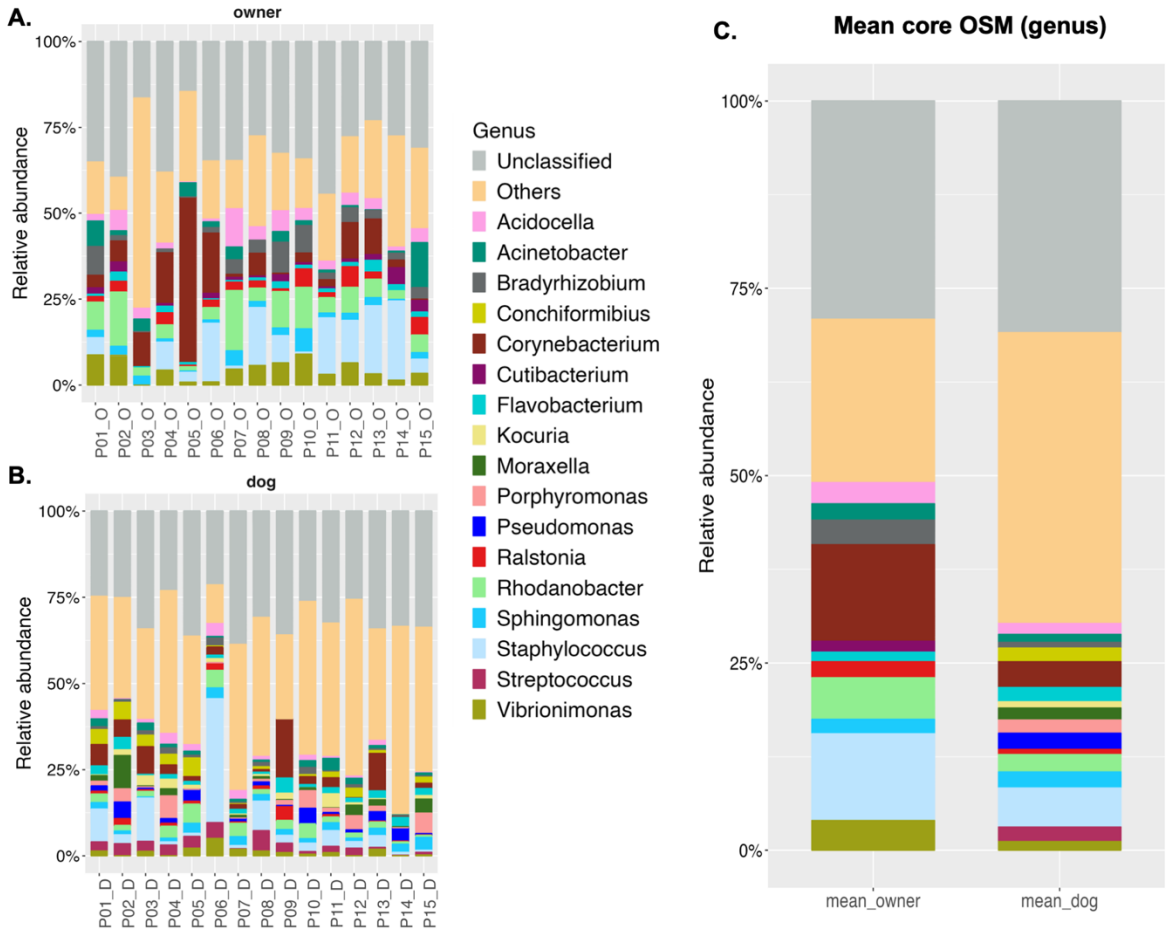

Figure 16. Core genera of the dog and owner ocular surface microbiome. A. Relative abundances of core ocular surface microbiome genera of owners. B. Relative abundances of core ocular surface microbiome genera of dogs. C. Mean relative abundances of core genera of the canine and human healthy ocular surface microbiome.

Table 3. Mean relative abundances of core ocular surface microbiome genera in dogs and owners. Mean relative abundance (± standard deviation).

Genus	Dogs	Owners
Acidocella	2,5 (± 1,7)	5,9 (± 4,3)
Acinetobacter	1,9 (± 1,6)	4 (± 5,3)
Bradyrhizobium	1,1 (± 1)	5,5 (± 4,3)
Conchiformibius	2,8 (± 2,7)	
Corynebacterium	6,6 (± 7,2)	12,4 (± 15,1)
Cutibacterium		2,5 (± 2)
Flavobacterium	2,5 (± 1,8)	2 (± 1,3)
Kocuria	1,7 (± 1,8)	
Moraxella	2,2 (± 3,3)	

Porphyromonas	2,8 (± 3,2)	
Pseudomonas	3,2 (± 3,2)	
Ralstonia	1,2 (± 1,7)	3,9 (± 2,7)
Rhodanobacter	3,7 (± 2,2)	11 (± 8)
Sphingomonas	2,9 (± 1,4)	3,3 (± 2,6)
Staphylococcus	7,6 (± 11,5)	14,1 (± 11,4)
Streptococcus	3,1 (± 2,4)	
Vibrionimonas	1,6 (± 1,7)	7,2 (± 4,4)

Overall, main taxa composing the OSM of dogs and owners are very similar from phylum to family. Interestingly, there are more taxa present in dogs and not owners than vice versa.

To analyze the diversity within each sample, i.e. alpha diversity, the focus will first be put on comparing the OSM of dogs and owners as 2 separate populations. Afterwards, the entire population of dogs and owners will be analyzed together to investigate the impact of metadata, i.e. dog and owner characteristics and habits, on OSM diversity and similarities within a dog-owner pair.

3.4. Comparison of dogs and owners

3.4.1. Alpha diversity

Alpha diversity, pertaining to diversity within a sample, was estimated in each single sample with 3 metrics: the number of observed ASVs, the Shannon diversity index, and the Simpson index. Mean alpha diversity in dog samples was compared to mean alpha diversity in owner samples. Simpson diversity index was significantly higher in dogs compared to owners (p.value = 0.0453). Observed ASVs and Shannon index were also higher in dogs, albeit without significance (see **Fig. 17**).

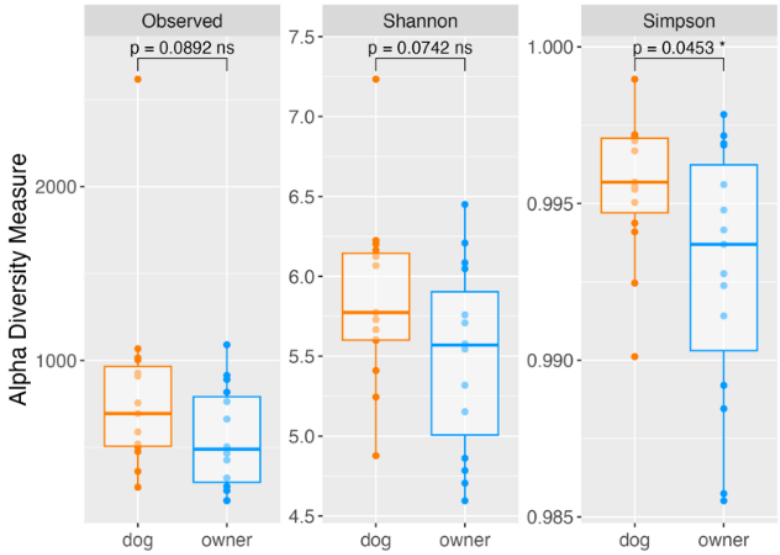

Figure 17. Comparison of ocular surface microbiome alpha diversity in dogs and owners. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

As can be observed in **Figure 17**, one dog had a much higher alpha diversity than the others. Interestingly, when removing this sample and performing Wilcoxon rank-sum tests to compare alpha diversity indices in dogs and owners, none of the p.values were significant.

3.4.2. Beta diversity

Beta diversity, pertaining to diversity between samples, was first compared between dogs and owners. Principal Coordinate Analysis (PCoA) plots showed separate clusters of dogs and owners, with a statistically significant difference when performing analysis of variance for the three tested distance-metrics: Bray-Curtis dissimilarity, weighted UniFrac distance, and unweighted UniFrac distance ($p = 0.001$) (see **Fig. 18**). The plots obtained with Bray-Curtis dissimilarity and with weighted UniFrac distance, the two metrics taking relative abundances into account, show a wider dispersion of owners along the two axes compared to dogs. This could indicate that beta diversity among owners is higher than beta diversity among dogs.

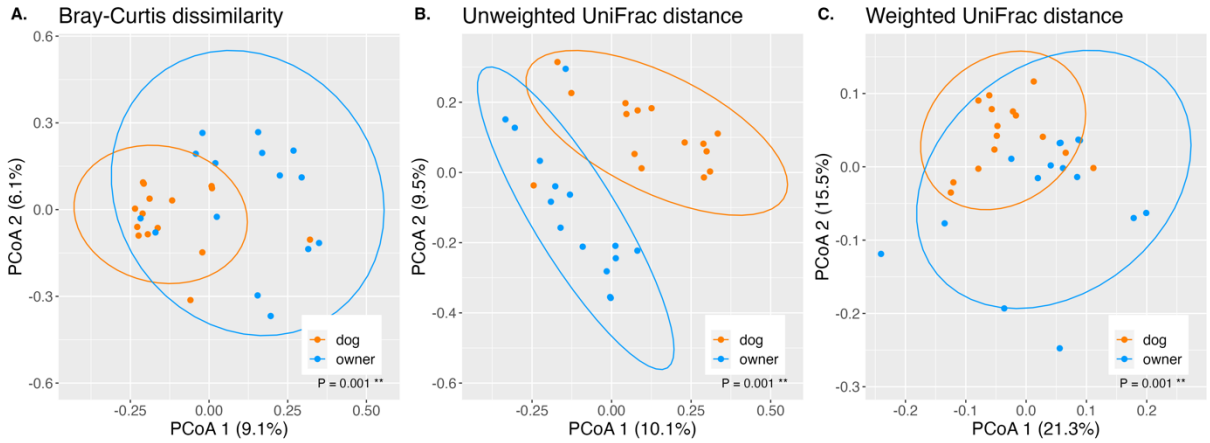

Figure 18. Principal Coordinate Analysis plots exhibiting separate clusters of dogs and owners. P: p-value estimated with adonis. ** $p < 0.001$.

Figure 19 shows that the beta diversity between any 2 dog samples (dog-dog distance) is significantly higher than between any 2 owner samples (owner-owner distance) when estimating it with Bray-Curtis dissimilarity (p.value = 1.31e-27). Interestingly, when estimating beta diversity with weighted UniFrac, that also accounts for phylogeny, the opposite result was observed: beta diversity between any 2 dog samples was significantly lower than between any 2 owner samples (p.value = 4.1e-9). The latter confirms what was observed in weighted UniFrac PCoA plot (see **Fig. 18**) and evidences a more similar OSM among dogs compared to owners. No significant difference was observed when comparing dog-dog and owner-owner unweighted UniFrac distances.

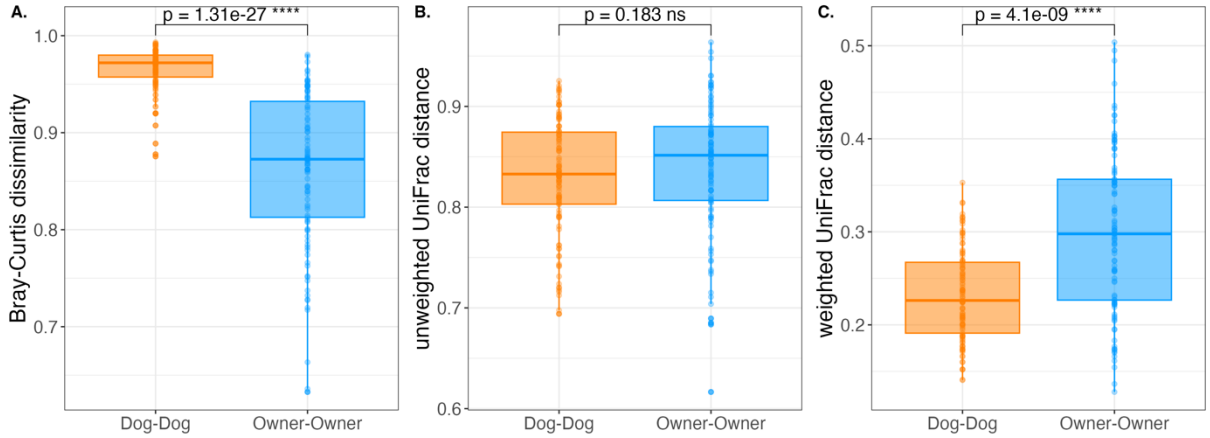

Figure 19. Comparison of beta diversity between dog samples and owner samples. Wilcoxon rank-sum test p.value; ns not significant, *$p < 0.05$.

3.4.3. Indicator species analysis

Indicator species are determined by an analysis of the relationship between taxa occurrence or abundance values in a set of sample groups and the classification of samples in these groups. Only indicators with a significant p.value were considered. To characterize indicators, 2 parameters are estimated, the specificity and the fidelity. Specificity, of positive predictive value, is an estimate of the probability that a sample belongs to the target group given the fact that the taxa has been found. The fidelity is an estimate of the probability of finding the taxa in samples belonging to the group.

When comparing dog and owner samples, indicators were found for dogs from the phylum to family level. At the phylum level, among the 34 phyla found in the whole population (dogs and owners), 8 phyla were identified as indicators of dog samples: Verrucomicrobiota, Acidobacteriota, Fusobacteriota, Chloroflexi, Gemmatimonadota, Deinococcota, Spirochaetota, and Campylobacterota. Interestingly, none of these phyla are among the 10 that were only found in dogs and not owners.

At the class level, 19 classes were identified as indicators of dogs. The best indicator classes are Thermoleophilia, with a high specificity, indicating that all samples where it was found were dog samples, Acidimicrobiia, with a fidelity of 1, meaning that it was found in all dog samples.

At the order level, 35 indicators of dogs have been found. The best indicators with a high specificity and fidelity, were Cytophagales, Solirubrobacterales, Bacteroidales, and Microtrichales.

At the family level, 52 indicators of dogs were identified among the 276 families found in the whole population. The 5 best indicators, with high specificity and fidelity, were *Cytophagales, Solirubrobacterales, Bacteroidales, Microtrichales*, and *Clostridiales.* None of these families are part of the previously defined core OSM of dogs.

3.5. Impact of dog and owner characteristics and habits on OSM

3.5.1. Alpha diversity

All comparisons of alpha diversity between groups was assessed for Oberserved ASVs, Shannon diverity index, and Simpson index. For simplification purposes, when trends and significance were the same for the 3 indices, only one (Shannon diversity index) was represented in figures.

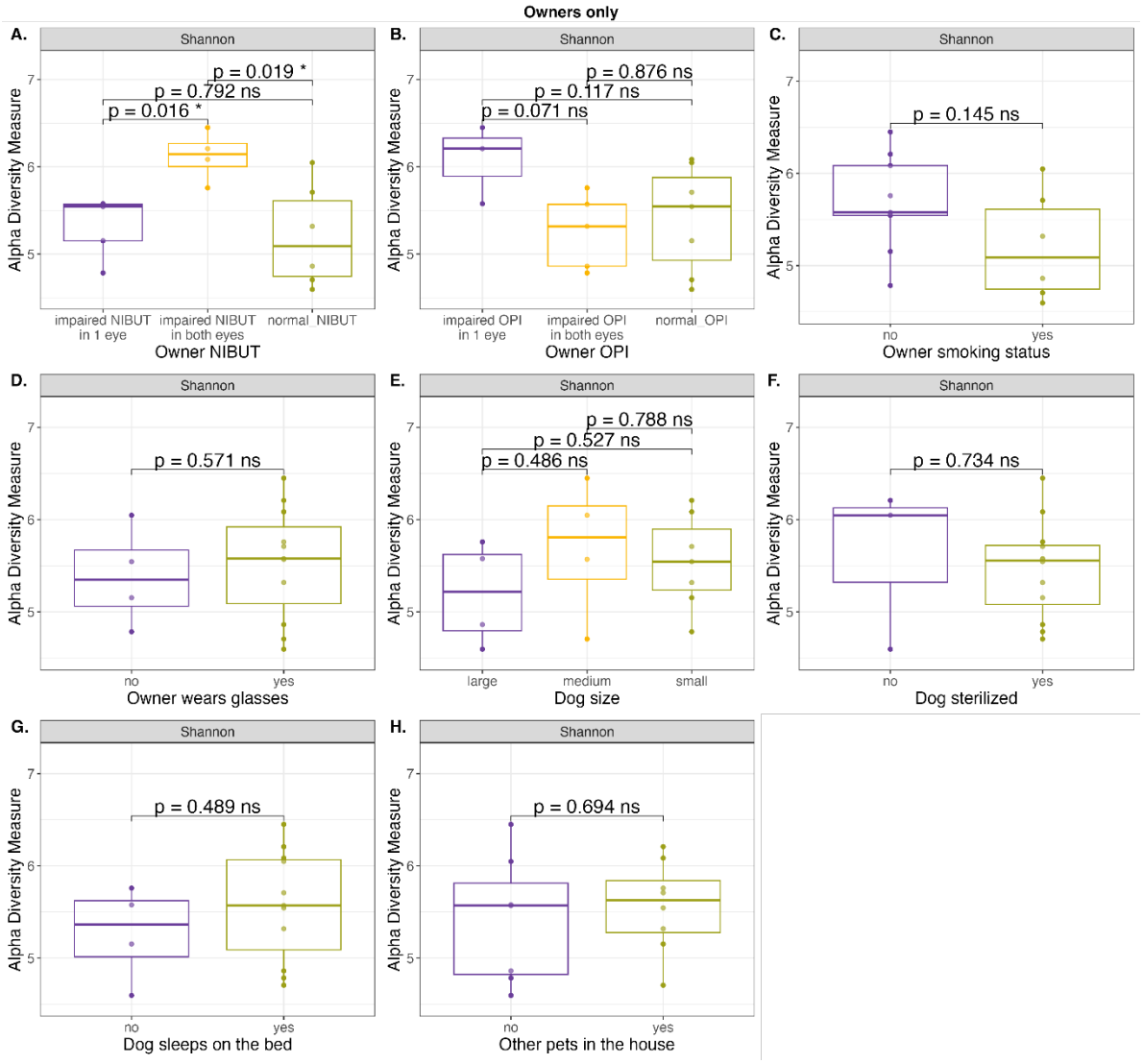

Figure 20. Comparison of owner ocular surface microbiome alpha diversity according to various metadata parameters. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

Stratification according to metadata parameters in owners. As shown in **Figure 20A**, owners with an impaired NIBUT in 1 eye had a significantly lower alpha diversity than owners with an impaired NIBUT in both eyes (p.value < 0.0009). Oddly, no significant difference in alpha diversity between owners with a normal NIBUT in both eyes and owners with an impaired NIBUT in one or both eyes was observed. No significant difference in alpha diversity was found when comparing owners with a normal OPI to owners with an impaired OPI in one or both eyes (see **Fig. 20B**). Regarding smoking status, no difference was found (see **Fig. 20C**), neither according to whether the owner wore glasses (see **Fig. 20D**). No difference was found either in owner alpha diversity when stratifiying according to dog size (see **Fig. 20E**), dog sterilization status (see **Fig. 20F**), whether dog slept on the owner's bed (see **Fig. 20G**), or presence of other pets in the household (see **Fig. 20H**). Nonetheless, owners that allowed their dog to sleep with them tended to have a higher Shannon diversity index, indicating a more diverse OSM compared to those never sleeping with their dog or only sometimes. Shannon diversity index also tended to be higher in owners allowing their dog on their bed.

As shown in **Figure 21A**, in the dog population, dog size does seems to have an impact on alpha diversity. Dogs that were not sterilized tended to have a higher alpha diversity, albeit not significantly (see **Fig. 21B**). Additionnaly, the dog population is made up of 12 sterilized dogs and 3 not sterilized, which unables proper comparison according to sterlization status. Sleeping in their owner's bed resulted in a higher Shannon diversity index in dogs, albeit not significantly (see **Fig. 21C**). Dogs that lived in households with other pets present also tended to have a higher shannon diversity index compared to the ones living in households where they were the only pets, but not significantly (see **Fig. 21D**).

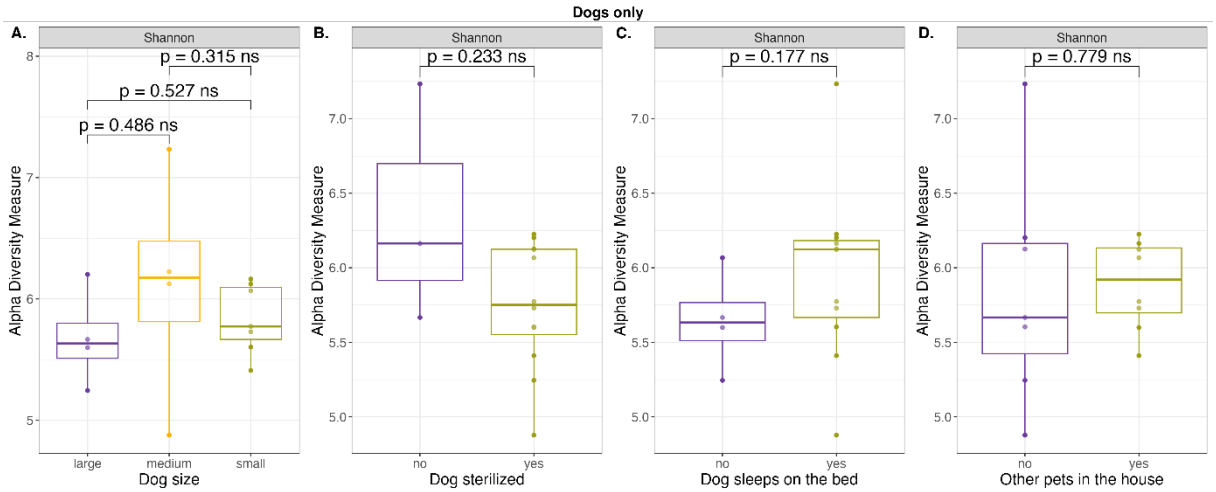

Figure 21. Comparison of dog ocular surface microbiome alpha diversity according to various metadata parameters. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

As shown in **Figure 22A**, a moderate positive correlation (0.3 > R > 0.5) was observed between owner's age and Shannon diversity index. In dogs, however, the observed linear correlation between age and Shannon diversity index was a moderate negative correlation (-0.3 > R > -0.5) (see **Fig. 22B**). None of these 2 linear correlations were significant.

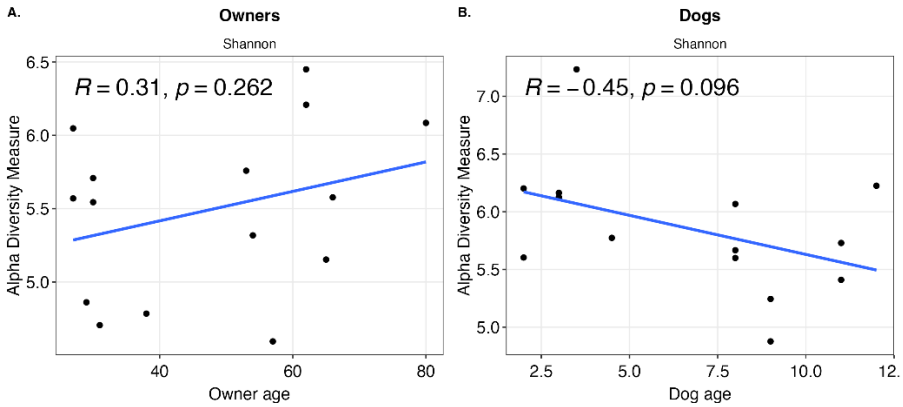

Figure 22. Linear regression between Shannon diversity index and age in owners (A) and dogs (B). R: Pearson correlation coefficient; p: Pearson correlation test p.value.

A strong negative correlation between the amount of time dog and owner have been living together and dog samples alpha diversity was observed (0 < R < -0.3) (see **Fig. 23B**). In dogs, the Pearson linear correlation is significant (p.value = 0.021), but not in owners, for which the

correlation is only weak (see **Fig. 23C**), or when considering the entire dog and owner population (see **Fig. 23A**). Possible correlations between age and alpha diversity in both dog and owner population, separately, were explored but there was no statistically significant correlation.

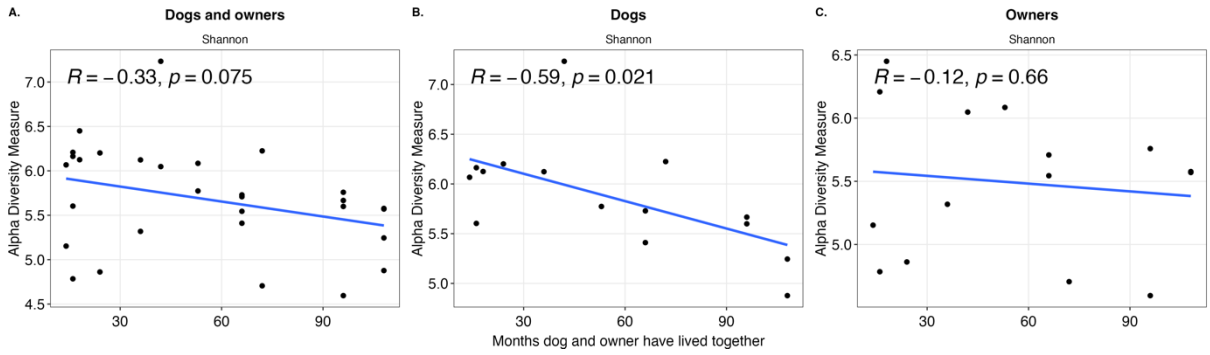

Figure 23. Linear regression between Shannon diversity index and time dog and owner have lived together. A. In the dog and owner populations. B. In the dog population only. C. In the owner population only. R: Pearson correlation coefficient; p: Pearson correlation test p.value.

3.5.2. Beta diversity

3.5.2.1. Characteristics of dog-owner pairs

"Dog-owner distance" refers to the distance measuring the similarity between the sample of a dog and that of their owner, i.e. the beta diversity between the 2 samples. The smaller the distance, the most similar dog and owner OSM are. This distance is here calculated with Bray-Curtis dissimilarity, weighted UniFrac distance, and unweighted UniFrac distance.

Table 4 shows that according to the distance metric used, pairs with the most and least similarities highly differ. Pairs P05, P08, P06, P07, and P14 ranked particularly differently in each metric, with a 10 to 14 rank difference. Contrarily, pairs P10, P13, P02, and P11 had similar ranks in each metric, only varying from 3 to 5 ranks.

Table 4. Comparison of dog-owner Bray-Curtis dissimilarities, unweighted UniFrac distances, and weighted UniFrac distances. For each distance-metric, the pairs are ranked from smallest (rank = 1) to biggest (rank = 15) distance, i.e. from the pair with the most similarities to the pair with the least. 'Max rank difference' indicates the maximum rank difference between the 3 distance-metrics.

Bray-Curtis dissimilarity			Unweighted UniFrac distance			Weighted UniFrac distance			Pair	Max rank difference
distance	pair	pair rank	distance	pair	pair rank	distance	pair	pair rank		
0,7192	P06	1	0,7621	P05	1	0,2254	P08	1	**P01**	6
0,8631	P07	2	0,8170	P10	2	0,2294	P10	2	**P02**	4
0,9071	P13	3	0,8202	P09	3	0,2311	P01	3	**P03**	7
0,9161	P01	4	0,8353	P06	4	0,2432	P12	4	**P04**	7
0,9220	P10	5	0,8380	P14	5	0,2451	P11	5	**P05**	14
0,9295	P08	6	0,8520	P13	6	0,2511	P13	6	**P06**	10
0,9375	P04	7	0,8538	P12	7	0,2547	P15	7	**P07**	10
0,9395	P11	8	0,8604	P07	8	0,2801	P03	8	**P08**	11
0,9419	P09	9	0,8663	P01	9	0,2811	P14	9	**P09**	7
0,9503	P02	10	0,8715	P11	10	0,2850	P09	10	**P10**	3
0,9604	P05	11	0,8718	P15	11	0,2891	P06	11	**P11**	5
0,9668	P12	12	0,8835	P08	12	0,3369	P07	12	**P12**	8
0,9710	P03	13	0,8869	P04	13	0,3430	P02	13	**P13**	3
0,9762	P15	14	0,9009	P02	14	0,3878	P04	14	**P14**	10
0,9763	P14	15	0,9023	P03	15	0,4403	P05	15	**P15**	7

As all 3 metrics reflect a different aspect of the beta diversity between samples and have their strengths and weaknesses, a "mean rank" measure was introduced, for a more comprehensive approach to the comparison of dog-owner distances. "Mean rank" is assessed by calculating the mean rank of a dog-owner pair across three beta diversity metrics (Bray-Curtis distance, weighted UniFrac distance, unweighted UniFrac distance), and reflects how similar dog and owner microbiomes are. Pairs sharing the most similarities in OSM have the lowest mean rank.

Possibly due to the small size of population, no evident marker of dog-owner pairs with the highest or lowest rank was identified. However, some interesting features can be observed. The 4 dog-owner pairs with the lowest mean rank were all pairs with a small dog (< 10 kg), allowed on their owner's bed and that lived with other pets in the household (see **Table 5**). These 4 pairs also have some of the highest dog eye size/weight ratios of the population.

Among the 4 dog-owner pairs with the highest ranks, 3 were pairs with a large dog, and 1 with a small dog (Chihuahua). For 2 of these pairs, the owner allowed their dog on the bed. Interestingly, none of them had other pets living in the house (see **Table 5**).

Table 5. Dog-owner pairs characteristics ranked from most to least similar.

Dog-owner pair	Mean rank	Dog size	Dog eye size (mm)	Dog eye size/weight ratio (mm/kg)	Years lived together	Presence of other pets in the house	Dog allowed on bed	Dog daily environment
P10	3	small	10	2	5,50	yes (1 cat)	yes	indoors
P13	5	small	15	1,87	1,33	yes (1 dog)	yes	in & outdoors
P01	5,33	small	13	2,17	3	yes (1 cat)	yes	indoors
P06	5,33	small	23	6,57	4,42	yes (1 cat)	yes	indoors
P08	6,33	medium	15	0,68	1,50	no	yes	in & outdoors
P07	7,33	medium	20	1,18	6	yes (1 cat)	yes	indoors
P09	7,33	medium	15	0,75	9	no	yes	indoors
P11	7,67	large	27	1	8	yes (3 cats)	no	indoors
P12	7,67	small	NA	NA	1,17	yes (1 cat and birds)	no	indoors
P05	9	small	15	1,87	5,50	yes (1 dog)	yes	indoors
P14	9,67	medium	13	0,62	3,50	no	yes	indoors
P15	10,67	large	21	0,78	2	no	yes	indoors
P04	11,33	large	17	0,57	9	no	no	indoors
P03	12	small	11	2,89	1,33	no	yes	in & outdoors
P02	12,33	large	NA	NA	8	no	no	indoors

3.5.2.2. Stratification of dog-owner pair distances by metadata

When the 3 metrics showed the same trend and Wilcoxon rank-sum test significance level, only weighted UniFrac is represented in the figures. When different trends and/or significance level differ, plots obtained with the 3 metrics are displayed.

With Bray-Curtis dissimilarity and unweighted UniFrac, dog-random-owner distance tended to be slightly higher than dog-owner distance, implying that the OSM of an owner and their dog is more similar than the OSM of an owner and another random dog in the population (see **Fig. 24A** & **Fig. 24B**). With weighted UniFrac, the opposite trend can be observed (see **Fig. 24C**). However, none of these differences were significant.

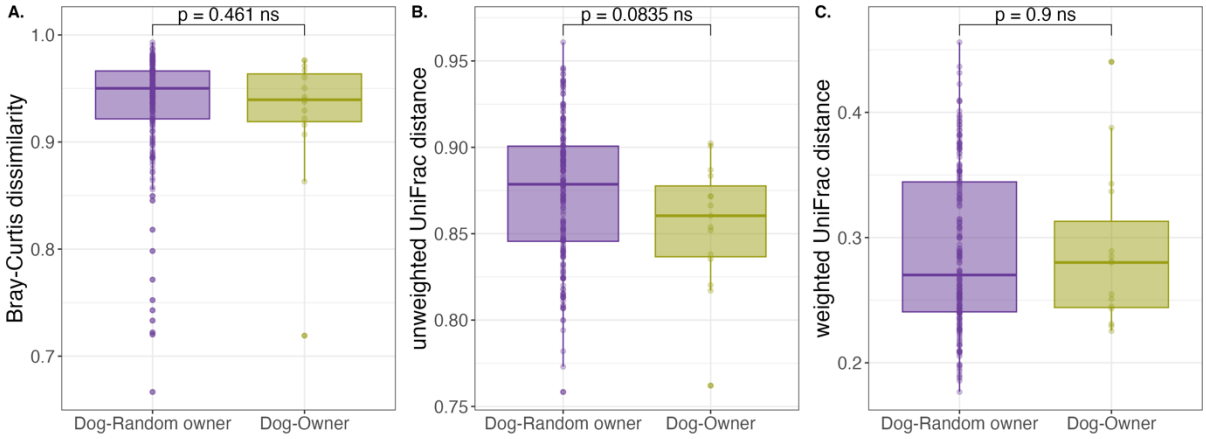

Figure 24. Comparison of dog-owner distance between dog-owner pairs and dog-random-owner pairs. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

Dog size does not have a significant impact on dog-owner distance, although dog-owner pairs with large dogs (> 25 kg) tended to have a higher dog-owner distance, and therefore shared less similarities compared to pairs with medium-sized dogs (10 to 25 kg) and small-sized dogs (< 10 kg) (see **Fig. 25A**). Additionally, no significant difference in dog-owner distance was observed between pairs in which the owner allowed their dog on their bed, and pairs with owner who did not (see **Fig. 25B**).

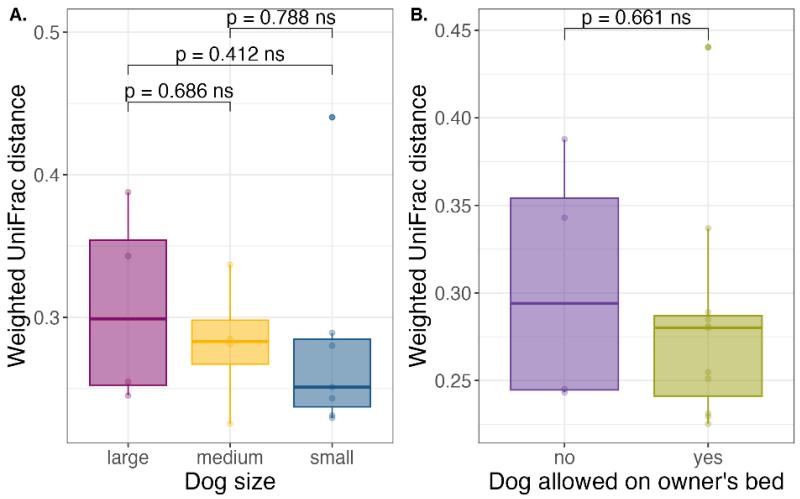

Figure 25. Comparison of dog-owner weighted UniFrac distance according to dog size and dog allowed on owner's bed. A. Stratified by dog size: small: < 10kg; medium: 10 to 25kg, large: > 25kg. B. Stratified by whether dog is allowed on the owner's bed. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

Dog-owner distance in pairs living with other pets at home is significantly lower than in pairs that do not have other pets when calculating it with Bray Curtis distance (see **Fig. 26A**). The same trend was observed when calculating with unweighted and weighted UniFrac, although not significantly (see **Fig. 26B** & **Fig. 26C**).

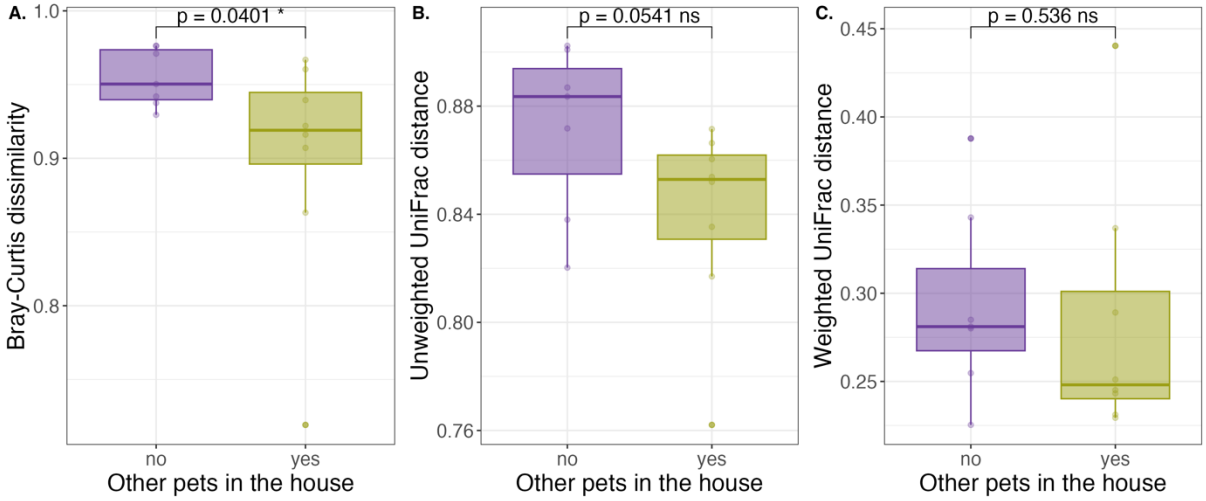

Figure 26. Dog-owner Bray-Curtis dissimilarity (A.), unweighted UniFrac distance (B.), or weighted UniFrac distance (C.) according to the presence of other pets in the household. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

A weak negative linear correlation between (0 > R > -0.3) dog-owner distance and the amount of time dog and owner have lived together was observed when using Bray-Curtis dissimilarity (see **Fig. 27A**) and unweighted UniFrac (see **Fig. 27B**). Interestingly, a strong positive correlation (0.3 > R > 0.5) was observed when using weighted UniFrac distance (see **Fig. 27C**). However, no statistically significant linear trend was found for neither of the 3 distance-metrics.

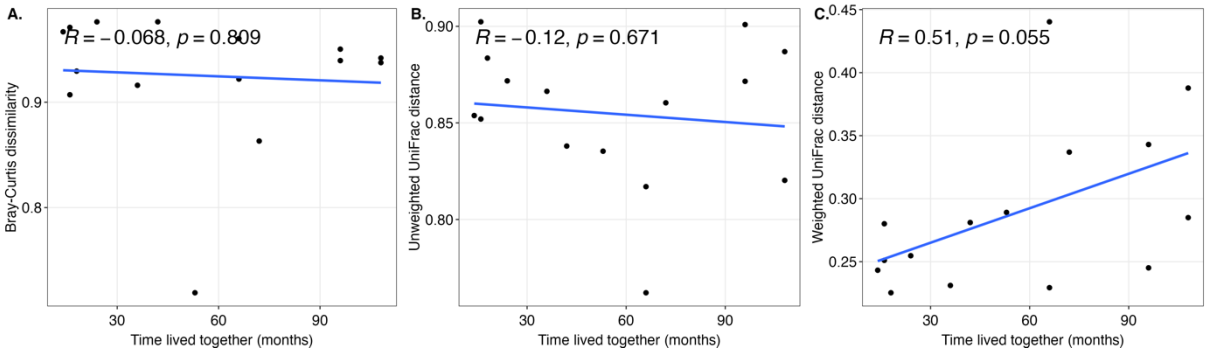

Figure 27. Linear regression between dog-owner distance and time dog and owner have lived together. R: Pearson correlation coefficient; p: Pearson correlation test p.value.

When using Bray-Curtis dissimilarity, dog-owner distance was significantly higher in owners that do not wear glasses compare to owners that wear them (see **Fig. 28A**). A similar trend, albeit not significant, was observed when using unweighted (see **Fig. 28B**) and weighted UniFrac (see **Fig. 28C**). No difference in alpha diversity between owners wearing glasses or not was found (see **Fig. 20D** on page 70), nor was there a difference in OSM community structure (*adonis* p. value > 0.05).

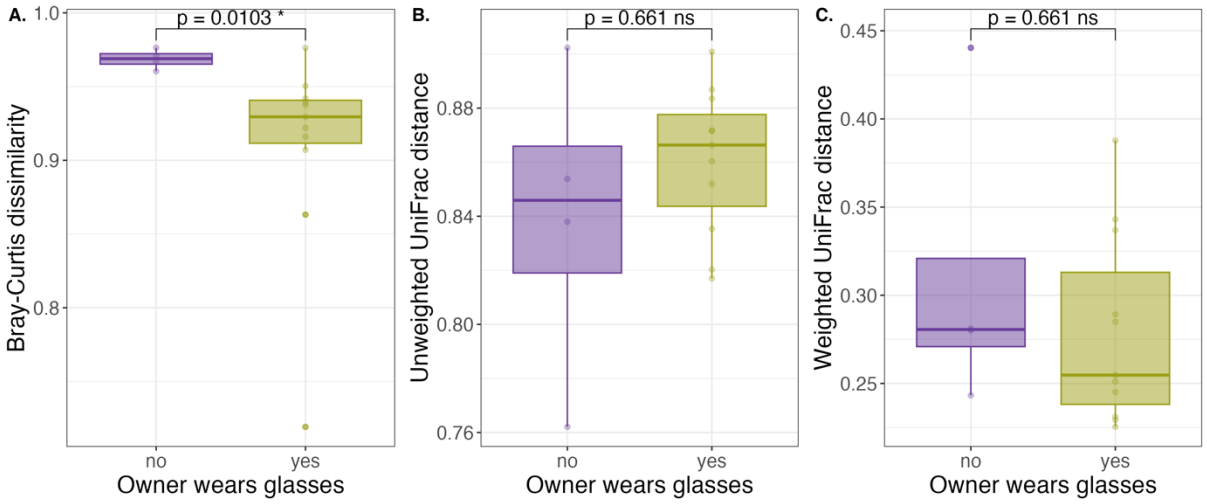

Figure 28. Dog-owner Bray-Curtis dissimilarity (A.), unweighted UniFrac distance (B.), or weighted UniFrac distance (C.) according to whether the owner wears glasses or not. Wilcoxon rank-sum test p.value; ns not significant, *p < 0.05.

As shown in **Figure 29**, no significant correlation was observed between dog eye size and dog-owner distances, with neither of the 3 distance-metrics. Interestingly, although not significant, the linear correlation with Bray-Curtis dissimilarity was negative (see **Fig. 29A**) but positive with unweighted UniFrac distance (see **Fig. 29B**), or weighted UniFrac distance (see **Fig. 29C**).

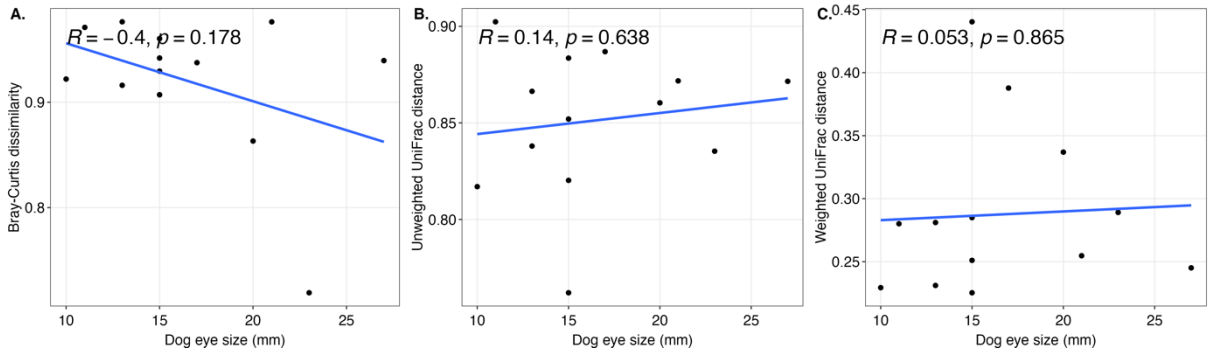

Figure 29. Linear regression between dog-owner distance and dog eye size (mm). A. Using Bray-Curtis dog-owner. B. Using unweighted UniFrac dog-owner distance. C. Using weighted UniFrac dog-owner distance. R: Pearson correlation coefficient; p: Pearson correlation test p.value.

Since dog eye size might have different effects on the OSM based on the dog's size, dog eye size/weight ratio was used to reflect the diameter of the eye compared to the size of dogs. The hypothesis is that large dogs with small eyes, i.e. with a low eye size/weight ratio, share less OSM similarities with their owners compared to small dogs with large eyes, i.e. with a high eye size/weight ratio. When using Bray-Curtis dissimilarity to assess dog-owner distances, there was a statistically significant strong negative correlation between dog-owner distance and dog eye/weight ($R < -0.5$; p.value < 0.001) (see **Fig. 30A**). However, when using weighted (see **Fig. 30B**) or unweighted UniFrac (see **Fig. 30C**) distances there was only non-significant weak correlation.

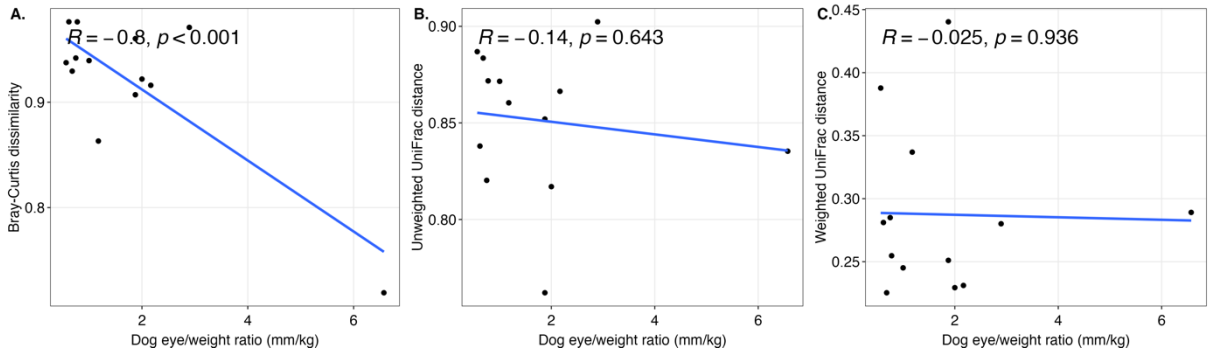

Figure 30. Linear regression between dog-owner distance and dog eye size/weight ratio (mm/kg). A. Using Bray-Curtis dog-owner dissimilarity. B. Using unweighted UniFrac dog-owner distance. C. Using weighted UniFrac dog-owner distance. R: Pearson correlation coefficient; p: Pearson correlation test p.value.

3.6. OSM and air pollution

The exposure to outdoor air pollution in the 10 days prior to sampling was estimated with 5 measures: mean daily PM_{10}, $PM_{2.5}$, and NO_2 concentrations ($\mu g/m^3$), and the mean daily levels of PM_{10} and $PM_{2.5}$ participants were exposed to ($h*\mu g/ m^3$).

In the 10 days prior to sampling, 27 out of 30 participants (15 dogs and 15 owners) were exposed to mean daily $PM_{2.5}$ concentrations above the 5 $\mu g/m^3$ WHO air quality guideline. 17 were exposed to PM_{10} above the 15 $\mu g/m^3$ guideline and 14 were exposed to NO_2 concentrations above the 20 $\mu g/m^3$.

No significant linear correlation was found between Shannon diversity and neither of the 5 air pollution measures in the whole population, the dog population only, or the owner population only. When investigating the whole dog and owner population, there was a weak negative association ($0 > R > -0.3$) between mean daily PM_{10} and $PM_{2.5}$ concentrations and Shannon index, albeit not significant (see **Fig. 31A** & **Fig. 31B**). A weak positive correlation was observed between PM_{10} and $PM_{2.5}$ exposure and Shannon index (see **Fig. 31D** & **Fig. 31E**). However, there is no correlation between mean daily NO_2 concentration and Shannon index (see **Fig. 31C**). The same non-significant trends were observed when investigating linear correlations between the 5 pollution measures and Simpson index.

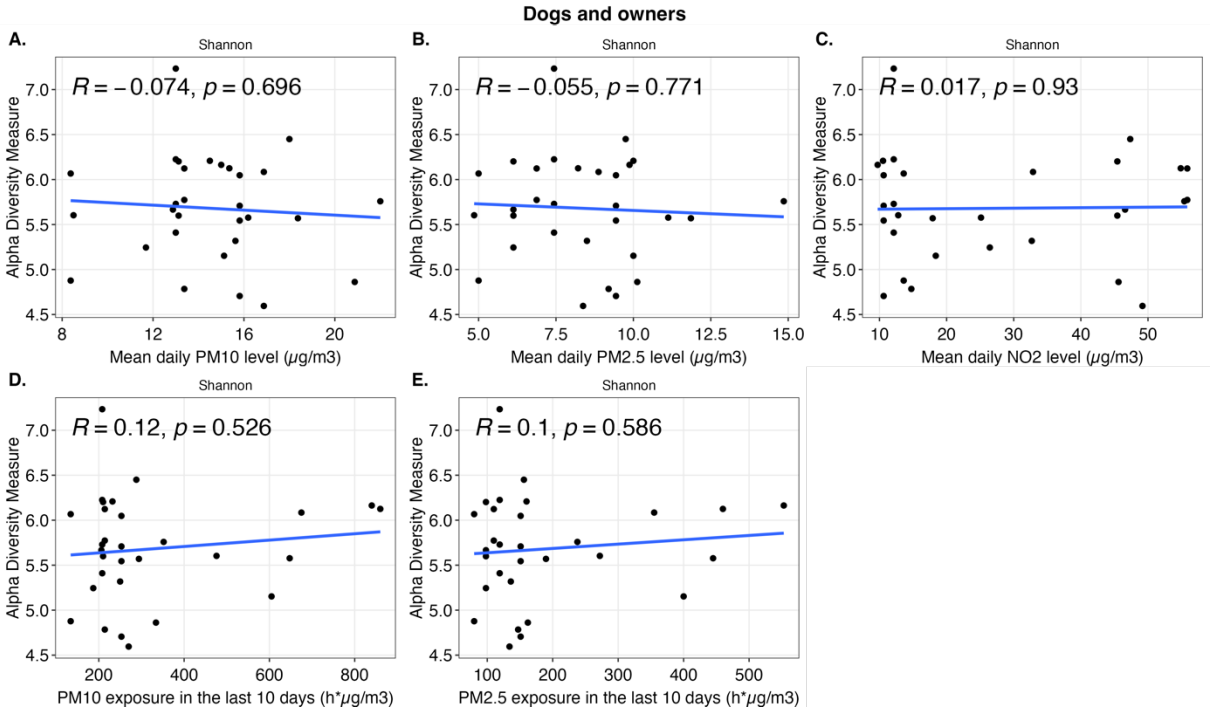

Figure 31. Linear regression between dogs and owners Shannon diversity index and outdoor air pollution measures. A. Correlation with mean daily PM_{10} concentration (µg/m^3) in the 10 days before sampling. B. Correlation with mean daily $PM_{2.5}$ concentration (µg/m^3) in the 10 days before sampling. C. Correlation with mean daily NO_2 concentration (µg/m^3) in the 10 days before sampling. D. Correlation with mean daily levels of PM_{10} participants were exposed to (h*µg/ m^3). E. Correlation with mean daily levels of $PM_{2.5}$ participants were exposed to (h*µg/ m^3). R: Pearson correlation coefficient; p: Pearson correlation test p.value.

In the owner population, there was a weak positive association (0 > R > -0.3) between mean daily PM_{10} and $PM_{2.5}$ concentrations and Shannon index (see **Fig. 32A** & **Fig. 32B**). A weak positive association was also observed between PM_{10} and $PM_{2.5}$ exposure and Shannon index (see **Fig. 32D** & **Fig. 32E**). However, there was no correlation between mean daily NO_2 concentration and Shannon index (see **Fig. 32C**). The same correlations, still non-significant, were observed when investigating linear correlations between the 5 pollution measures and Simpson index.

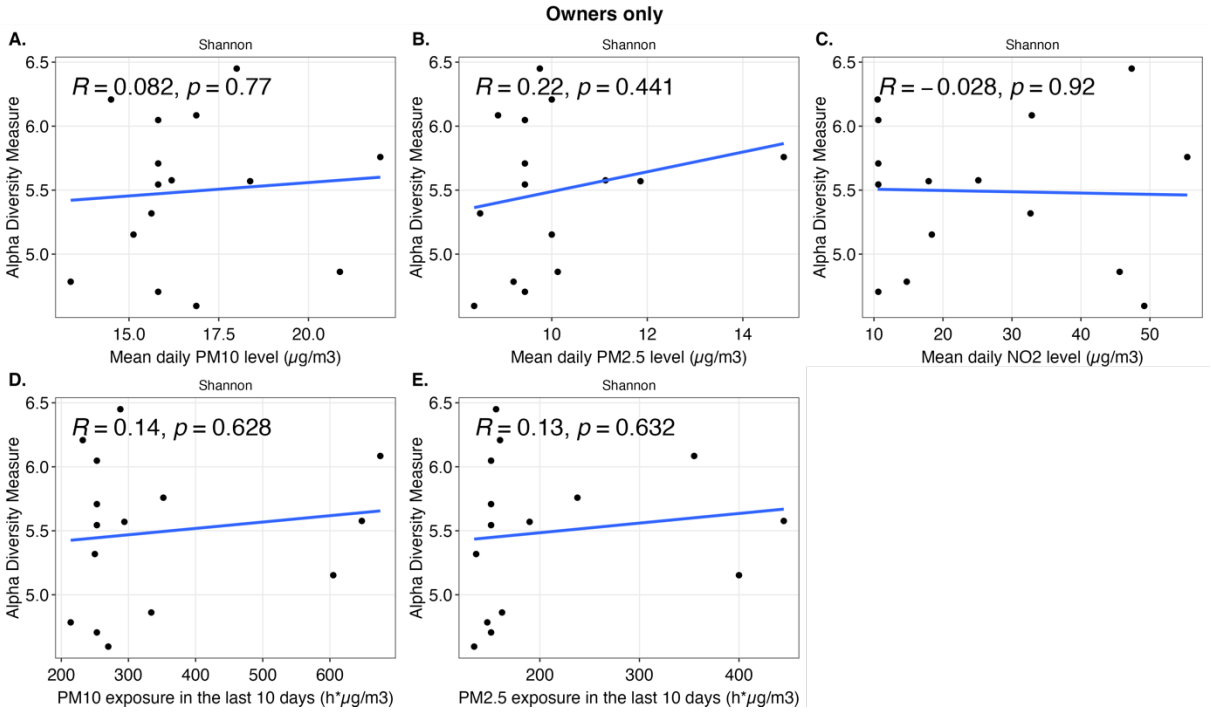

Figure 32. Linear regression between owners Shannon diversity index and outdoor air pollution measures. A. Correlation with mean daily PM_{10} concentration (µg/m^3) in the 10 days before sampling. B. Correlation with mean daily $PM_{2.5}$ concentration (µg/m^3) in the 10 days before sampling. C. Correlation with mean daily NO_2 concentration (µg/m^3) in the 10 days before sampling. D. Correlation with mean daily levels of PM_{10} participants were exposed to (h*µg/ m^3). E. Correlation with mean daily levels of $PM_{2.5}$ participants were exposed to (h*µg/ m^3). R: Pearson correlation coefficient; p: Pearson correlation test p.value.

In the dog population, there was a moderate positive association (- 0.3 > R > -0.5) between mean daily PM_{10} and $PM_{2.5}$ concentrations and Shannon index (see **Fig. 33A** & **Fig. 33B**). A weak positive association was also observed between PM_{10} and $PM_{2.5}$ exposure and Shannon index (see **Fig. 33D** & **Fig. 33E**). However, there was no correlation between mean daily NO_2 concentration and Shannon index (see **Fig. 33C**).

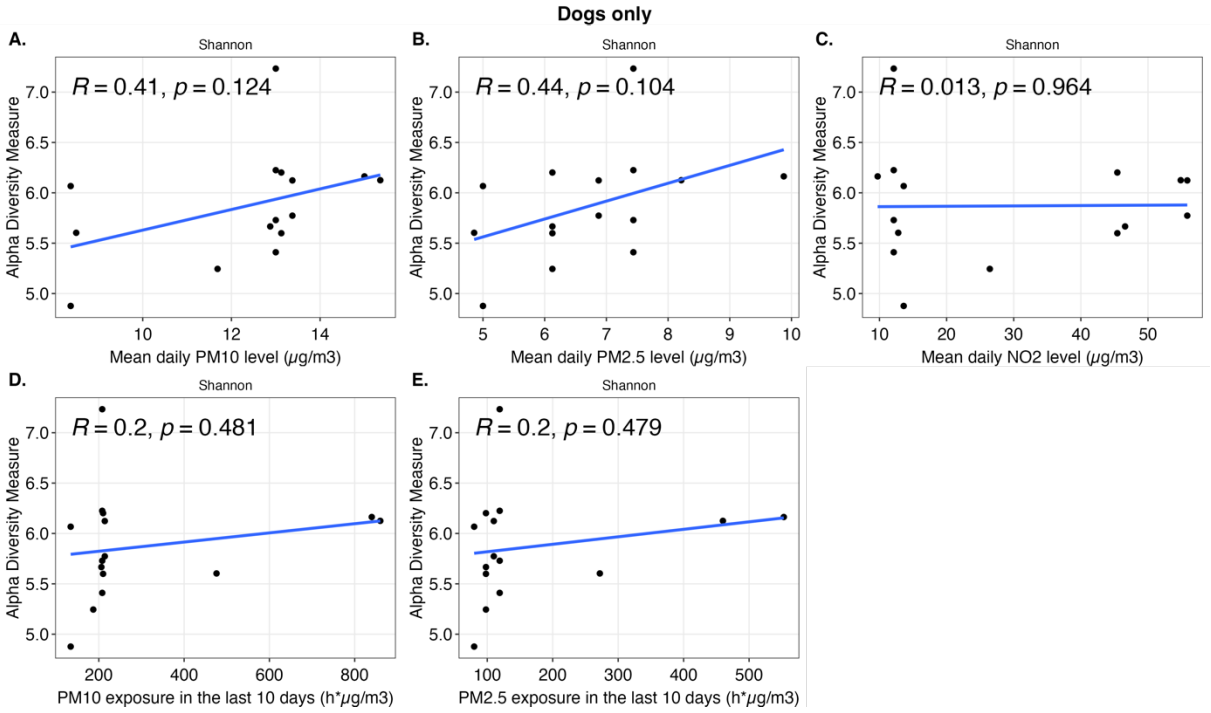

Figure 33. Linear regression between dogs Shannon diversity index and outdoor air pollution measures. A. Correlation with mean daily PM_{10} concentration (µg/m^3) in the 10 days before sampling. B. Correlation with mean daily $PM_{2.5}$ concentration (µg/m^3) in the 10 days before sampling. C. Correlation with mean daily NO_2 concentration (µg/m^3) in the 10 days before sampling. D. Correlation with mean daily levels of PM_{10} participants were exposed to (h*µg/ m^3). E. Correlation with mean daily levels of $PM_{2.5}$ participants were exposed to (h*µg/ m^3). R: Pearson correlation coefficient; p: Pearson correlation test p.value.

For the whole population, the dog population, and the owner population, similar results were found when investigating the correlation between the 5 air pollution measures and the number of observed ASVs or the Simpson index (figures not displayed).

Linear correlations parameters between the 5 outdoor air pollution measures and the 3 dog-owner distance-metrics are summarized in **Table 6**. Only 2 out of the 45 computed linear regressions had a significant p.value. They show that daily mean NO_2 concentration in the 10 days prior to sampling has a moderate positive correlation (0.3 > R > 0.5) with dog-owner unweighted UniFrac distance among the entire population (dogs and owners), and a strong positive correlation (R > 0.5) with dog-owner unweighted UniFrac distance in the owner population

Table 6. Summary of linear correlation coefficients and significance between dog-owner distance and air pollution measures. Pearson correlation coefficient (R) and p.value are reported for the 3 dog-owner metrics: Bray-Curtis dissimilarity, unweighted U UniFrac distance, and weighted UniFrac distance

	Pollution measure	Bray-Curtis dissimilarity		Unweighted UniFrac distance		Weighted UniFrac distance	
		R	p.value	R	p.value	R	p.value
Dogs and owners	Mean daily concentration of PM_{10} in the 10 days before sampling ($\mu g/m^3$)	-0.086	0.651	0.017	0.928	-0.09	0.637
	Mean daily concentration of $PM_{2.5}$ in the 10 days before sampling ($\mu g/m^3$)	-0.0098	0.959	-0.062	0.746	-0.059	0.755
	Mean daily concentration of NO_2 in the 10 days before sampling ($\mu g/m^3$)	-0.79	0.309	**0.46**	**0.011***	-0.25	0.186
	PM_{10} exposure in the last 10 days ($h*\mu g/m^3$)	-0.18	0.355	0.18	0.343	-0.14	0.469
	$PM_{2.5}$ exposure in the last 10 days ($h* \mu g/m^3$)	-0.12	0.524	0.15	0.435	-0.11	0.564
Dogs	Mean daily concentration of PM_{10} in the 10 days before sampling ($\mu g/m^3$)	-0.3	0.28	-0.03	0.916	-0.067	0.812
	Mean daily concentration of $PM_{2.5}$ in the 10 days before sampling ($\mu g/m^3$)	-0.26	0.347	-0.24	0.396	-0.06	0.831
	Mean daily concentration of NO_2 in the 10 days before sampling ($\mu g/m^3$)	-0.33	0.223	0.41	0.132	-0.26	0.355
	PM_{10} exposure in the last 10 days ($h*\mu g/m^3$)	-0.0098	0.972	0.26	0.348	-0.32	0.251
	$PM_{2.5}$ exposure in the last 10 days ($h*\mu g/m^3$)	-0.014	0.96	0.2	0.474	-0.3	0.283
Owners	Mean daily concentration of PM_{10} in the 10 days before sampling ($\mu g/m^3$)	0.047	0.867	0.077	0.785	-0.19	0.506
	Mean daily concentration of $PM_{2.5}$ in the 10 days before sampling ($\mu g/m^3$)	0.19	0.499	0.024	0.934	-0.12	0.68
	Mean daily concentration of NO_2 in the 10 days before sampling ($\mu g/m^3$)	-0.027	0.923	**0.52**	**0.046***	-0.24	0.385
	PM_{10} exposure in the last 10 days ($h*\mu g/m^3$)	-0.43	0.108	0.069	0.806	0.12	0.672
	$PM_{2.5}$ exposure in the last 10 days ($h*\mu g/m^3$)	-0.28	0.314	0.084	0.765	0.15	0.604

4. Discussion

4.1. The need to standardize OSM research

Although a relatively low number of studies on the OSM have been carried out compared to other microbiomes, such as the ones included in the Human Microbiome Project (Turnbaugh et al., 2007), many different protocols to characterize it have been and are currently being used. As highlighted in **Figure 3**, many steps from sampling to bioinformatics analysis differ between studies. Since these differences impact OSM characterization (Delbeke et al., 2023; Ozkan et al., 2018; Shin et al., 2016), it can be difficult to properly compare study results. Particularly, since variations in DNA extraction kits and protocols have the largest effect on observed fecal microbiome composition (Costea et al., 2017), it is reasonable to believe that similar effects of DNA extraction kits can be observed in the OSM. Additionally, the presence of contaminants is ubiquitous among DNA extraction kits and can severely impact sequence-based analysis of low-biomass microbiomes (Cheema et al., 2021; Delbeke et al., 2023; Salter et al., 2014). Protocols should therefore include the detection of contaminants present in reagents and kits, i.e. the kitome. Because the OSM is a low biomass microbiome, DNA present in extraction kits and laboratory reagents can be present in concentrations similar to that of the actual microbiome samples and can be mistaken for the studied microbiome (Eisenhofer et al., 2019; Glassing et al., 2016). Adding multiple negative controls such as sampling blank, DNA extraction blank, and no-template-amplification controls should be considered in each batch to identify contaminants (Eisenhofer et al., 2019). The need for a standardization of sequence-based OSM identification methods has been expressed in a few articles (Delbeke et al., 2021; Peter et al., 2023; Scott et al., 2021) and is the subject of our review, accepted and currently in press at New

Microbiologica. Nonetheless, no recommended protocol or golden standard for OSM sequencing has been determined to this day.

Regarding bioinformatic analysis of OSM sequencing data, most published studies do not provide information on the treatment of unclassified reads. Elimination of unclassified reads before statistical analysis impacts relative abundances and is also reflected in the calculated alpha and beta diversities. For this reason, a standardization of bioinformatic and statistical analysis of OSM sequencing data is warranted for a proper comparison between studies.

As pointed out in paragraphs *1.4 The healthy human OSM* and *1.5 Human OSM alterations in ocular and systemic diseases,* OSM composition reported in literature varies greatly from one study to the other. Opposite results have also been found when comparing differences in alpha diversity between healthy controls and patients with ocular diseases. These discrepancies could, at least partially, be explained by differences in protocols.

In their review combining results from studies on different populations, Delbeke et al. proposed mean phylum-level OSM compositions for the US, Asian, and Australian populations. US and Asian populations displayed similar compositions, pointing towards the OSM not being influenced by geographical and environmental background and/or ethnicity. The Australian population, however, showed a considerably higher abundance of Firmicutes, and lower abundance of Actinobacteria compared to US and Asian populations. However, results from the Asian population came from 8 studies, the US population from 3 studies, and the Australian population from only 1 study. The observed differences could therefore also be attributed to differences in methods and not geography (Delbeke et al., 2021). Further investigation is needed to elucidate the potential variability in OSM between populations and environmental expositions, which could play a role in the differences observed between studies. These results show how protocol differences between studies prevent a clear understanding of the causes behind the different obtained results.

4.2. Study design in One Health pet-owner studies: benefits and challenges

One Health studies on companion animals, humans, and the environment they share call for a specific study design that takes into consideration the peculiarities of the pet-owner relationship. Simultaneously to the carrying out of this research on dog and owner OSM, a

similar study on dog and owner gut microbiome sharing was conducted at the University College Dublin - Department of Veterinary Sciences by Dr. Dagmara Niedziela and Prof. Grace Mulcahy. Along with data analysis, my 3-months visiting period in their department allowed us to reflect together on lessons learned from our respective studies. Based on our experience and on results from other published studies focusing on pet-owner microbiome sharing, we proposed a review of the opportunities, challenges and potential benefits of such studies, as well as a general framework of key considerations and best practices (Clougher et al., 2025).

4.2.1. Data collection challenges

Investigating pet-owner relationships implies the need to characterize the subjects (age, gender, sex, breed, diseases etc.) as well as their interactions, more specifically the ones that might impact the studied microbiome. The nature, quantity and quality of these interactions may have a significant impact on pet and owner microbiome and should therefore be thoroughly characterized in the data collection process. In order to capture best the complexity of pet-owner relationships, the focus should not only be on direct pet-owner interactions, like petting habits or amount of time they have lived together, but also on more "indirect" interactions (Rodriguez et al., 2021). Our experience leads us to consider relevant the following questions:

- Does the pet move freely around the house?
- How many hours a day does the pet spend outside?
- Do pet and owner do any kind of training or playing together? If so, what type and how often?
- Are the pet and/or owner in frequent contact with other animals? Could that impact the studied microbiome?
- Are there other cohabiting pets?
- Is the pet allowed on sofas and beds?
- Did the pet have other owner(s) before?
- Do pet and owner live in an urban or rural setting?

Other examples of useful data to collect in these projects are included in **Table 7**. Specific data to be collected should be considered in accordance with the aims of each study. Moreover, to best identify metadata impacting microbiome sharing between pet and owner, we recommend the inclusion of people that do not cohabit with any pets, as controls.

Table 7. Overview of different types of data that can be collected in pet-owner microbiome studies, with examples.

Data/Question type	Owner	Pet
General data	Gender	Sex
	Age	
	Occupation (if relevant; pre-select from occupation types)	Breed (pre-select to make working consistent)
	Weight / height (voluntary)	Weight and/or size
	Country of birth	Previous owners
	Country of residence	
Inclusion / Exclusion data	Antibiotic intake in the last 6 months	
	Foreign travel in the last 2 months	
Health data	Allergies	
	Systemic diseases	
	Diseases relevant to the studied microbiome	
	Smoking status	
	Drinking alcohol	
	Reproductive status (if relevant)	Sterilization/reproductive status
Exercise data	Exercise – how many hours per day, how many days per week	
	Exercising together with dog/owner	
	Types of exercise (multiple choice)	
	Exercise indoors or outdoors	
Behavioral data	Amount of time spent with dog	Amount of time spent with owner
	Being the dog's primary caregiver	Licking owner's hands / face
	Hours of sleep	Sleeping in owner's bed
	Stress levels	Behavior with humans – friendly, afraid of strangers
	Days working on-site or from home	Behavior with other animals – stressed, aggressive, friendly
Household / environment data	Other pets in the household (if so, which pets)	
	Type of dwelling (rural / urban)	
	Number of household members	
	Living with family or roommates	
Nutritional data	Diet type (vegetarian, vegan, omnivore, ketogenic, prescription diet etc.)	Diet type (raw, commercial, prescription diet, human food, etc.)
		Wet or dry food
		Dog food brand
	Supplements	
	Probiotics / prebiotics	

	Type of drinking water (bottled, tap, filtered etc.)

Power calculations, although often considered difficult and not mentioned in many microbiome studies, are necessary for a thorough estimation of the necessary sample size. This is particularly relevant when working with a limited number of samples that cannot be replicated, for example when data protection applies in the case of human samples. An underpowered study that requires repeating could even be considered unethical. In microbiome studies, methods to calculate sample size do exist, and include the use of pairwise distances and PERMANOVA (micropower R package) (Kelly et al., 2015) or alpha and beta diversity metrics (Casals-Pascual et al., 2020) to estimate sample size based on statistical power and effect size. However, these methods rely on availability of data from similar projects and projects on pet-owner interactions are rare and would require estimating sample size of the pet and owner cohorts separately.

4.2.2. Sample collection challenges

Sample collection in microbiome studies faces numerous challenges, such as limiting sampling to one operator to ensure reproducibility, sample storage temperature (especially if participants collect the samples themselves at home), or limiting potential contaminations (Eisenhofer et al., 2019). Storage temperature has been shown to have an impact on downstream sequencing: compared to samples kept at room temperature or +4°C, better results are obtained in fecal samples stored at -20°C or stored with preservation methods such as EtOH or RNALater (Song et al., 2016).

Additionally, having both human and animal participants could require sampling pets and owners in separate locations and/or at different times, such as having to sample owners in a hospital and pets in their home or vice versa, as was the case for this project. In such cases, sampling sites could have different environmental conditions (humidity, pollutants etc.) and contaminants, that should be reduced to a minimum and accounted for with negative controls. Sampling location can also impact stress and anxiety of both owner and pet, highlighting the importance of choosing locations minimizing these effects (Tang et al., 2020). These precautions are particularly important when working with paucibacterial microbiomes, easily skewed by the presence of contaminants, such as the OSM. Different “appointments” for pet and owner sampling may also increase loss to follow-up that should be considered in the power calculations. Sample storage conditions should also be as similar as possible.

4.2.3. Laboratory challenges

Due to the potential differences in composition and diversity between human and animal microbiomes, avoiding cross-contamination is crucial. If samples are processed in batches, randomization of owner and pet samples should be considered so that batch effects can later be identified if present. Adding multiple negative controls such as sampling blank controls, DNA extraction blank controls, and no template amplification controls should also be considered in each batch (Eisenhofer et al., 2019). Due to the presence of a kitome in most DNA extraction kits, and the different sequencing results that they can produce (Cheema et al., 2021; Delbeke et al., 2023), choice of DNA extraction kit should be made accordingly to the studied microbiome and its average biomass. This is particularly important when working with low biomass microbiomes, in which the DNA present in extraction kits and laboratory reagents can come in concentrations similar to that of the samples and can therefore be mistaken for the studied microbiome (Eisenhofer et al., 2019; Glassing et al., 2016)

4.2.4. Bioinformatics pipeline challenges and recommendations

Analysis of sequencing data requires bioinformatic pipelines that can be divided into primary and secondary analysis. Primary, or "upstream", analysis processes raw output data to count matrices, using OTUs or ASVs. It is recommended to use reproducible pipelines, such as nf-core pipelines (https://nf-co.re/ampliseq/2.9.0/docs/usage), allowing for easy comparison between studies and an accurate description of the pipeline version and parameters used.

For metagenomic data, a skew towards human microbiomes has been found in several databases, highlighting the need for the development of databases tailored toward animal sequences (Mahmud et al., 2024; Smith et al., 2022).

4.2.5. Data analysis challenges

As detailed in section *1.3.4 Tools and metrics for microbiome characterization,* several alpha and beta diversity metrics are used in microbiome analysis. For beta diversity metrics, the main difference between the Bray-Curtis dissimilarity, weighted and unweighted UniFrac distances is that UniFrac incorporates phylogenetic information, that is not reflected in Bray-Curtis dissimilarity. However, this requires the construction of a phylogenetic tree, that can be time-consuming. As these metrics have strengths and limitations, their use can depend on the question asked and/or data availability, there is no agreement on which to use for microbiome

data analysis. Some studies choose one, whereas others report on beta-diversity with multiple metrics (Chang et al., 2022; Kang et al., 2022; Z. Li et al., 2019; Yau et al., 2019).

More specifically for pet-owner studies, beta-diversity can be used to estimate the distance, i.e. the "level" of similarity, between pet and owner. As measures all have their strengths and limitations (see **Table 8**), a comprehensive understanding of similarities in pet-owner pairs can be achieved by ranking pairs according to each measure and using an average rank to identify the least and most similar pairs. For this approach, we recommend using Bray-Curtis dissimilarity, unweighted UniFrac, and weighted UniFrac.

Table 8. Comparison of characteristics, strengths, and weaknesses of three distance metrics: Bray-Curtis dissimilarity, unweighted UniFrac distance, and weighted UniFrac distance.

	Accounts for relative abundance of taxa	**Accounts for phylogeny***	**Strengths**	**Weaknesses**
Bray-Curtis dissimilarity	Yes	No	Easy to calculate (no phylogenetic tree) Used in many studies Relative comparison between groups Sensitive to abundance differences	Does not account for phylogeny Abundant species are weighted more than rare ones
Unweighted UniFrac distance	No (only presence/ absence)	Yes	Accounts for phylogenetic relationships among taxa	Requires a rooted phylogenetic tree Binary test of presence/absence of taxa Sensitive to sequencing depth
Weighted UniFrac distance	Yes	Yes	Accounts for phylogenetic relationships among taxa Accounts for rare taxa	Requires a rooted phylogenetic tree

* Requires a phylogenetic tree.

Non-parametric tests, such as Wilcoxon tests, as well as multivariate methods, such as analysis of similarity (ANOSIM) or multivariate analysis of variance with permutation (PERMANOVA), have previously been used for comparisons of alpha and beta diversity between pets and owners (Song et al., 2013). Specifically, ANOSIM describes whether dissimilarity between selected groups is significantly different than the dissimilarity within each group (Xia and Sun, 2017). It is important to note that when looking at pet-owner microbiome interactions, ANOSIM can be used to compare beta diversity between groups within the

metadata (for instance, dogs versus owners, between breeds of dogs, or between family units); however, it cannot be used to compare group-to-group distances with one another. Comparisons of specific group-to-group distances (for instance pet-owner versus pet-random-owner) should be done using non-parametric Wilcoxon tests, and data manipulations may be required for such comparisons. PERMANOVA on the other hand is a distance-based method to test the association of microbial composition with covariates of interest (Tang et al., 2016).

4.2.6. Proposed framework

The proposed framework for pet-owner microbiome studies, based on the various considerations and recommendations described, can be found in **Table 9**.

Table 9. Proposed framework for experimental design of One Health relationships between companion animal, human, and environment.

	Key considerations/best practices
Study organization/ Recruitment	Which body sites/microbiome(s) will be sampled? How many times?
	How many pet-owner pairs are needed? (power calculations)
	Are non-pet owners (controls) included?
Data collection	What "basic" information is needed on pets and owners? How many are needed?
	Which pet-owner interactions might impact the microbiome(s)? Establish the necessary metadata.
Sample collection	Are pets and owners sampled at the same time and/or in the same place? Determine the biases that may arise from these differences.
	Try to limit batch effect and cross-contamination, insert multiple negative controls.
	When working with low-biomass microbiomes: particular attention must be put on the choice of DNA extraction kit.
Laboratory work	How many and which negative controls are included?
	Choice of DNA extraction method.
Bioinformatics	Use reproducible, well-established packages and pipeline, note package versions and document code. Version control on github is highly recommended.
	Determine if batch correction is needed.
	Consider ASV to be the current gold standard for 16S analysis.

	If possible, use metagenomic data, and complement with 16S.
Data analysis challenges	Rank pet-owner pairs based on multiple beta diversity metrics.

4.3. Rare taxa of the OSM

After identification of *Rhodanobacteraceae* as a contaminant family in the dog and owner samples, I proceeded with the removal of the responsible contaminated reagent, addition of negative controls, and removal of contaminant ASVs in the downstream analysis of sequencing reads. However, some *Rhodanobacteraceae* reads were still present in the decontaminated ASVs, calling for an investigation on its origin, as it is a family rarely found in the OSM.

Only 2 studies investigating the human OSM reported the presence of the *Rhodanobacter* genus, member of the *Rhodanobacteraceae* family, in their manuscript or figures (Lee et al., 2012; Shivaji et al., 2021). Lee et al. found *Rhodanobacter* to be the 16th most prevalence genus in a population of left and right eyes of 7 blepharitis patients and 4 healthy controls. Their study identified a higher prevalence of *Rhodanobacter* in eyelash samples compared to tear samples, however this difference was not discussed in the study. Shivaji et al. also identified *Rhodanobacter* in the OSM of healthy controls, with a median relative abundance of 0.23%. In patients with bacterial keratitis*, Rhodanobacter* was found in conjunctival swabs (OSM) with a median relative abundance of 0.01%, and in corneal scraping samples, with a median relative abundance of 0.65%. These relative abundances are much lower than the ones identified in our population, nonetheless both studies confirm the possible presence of *Rhodanobacter* in the OSM. Notably, Shivaji et al. report thorough verifications of the absence of contaminants in the reagents and kits used, with negative PCRs obtained with DNA extraction reads and failed sequencing with no template-DNA negative controls. Furthermore, the protocols and taxonomy assignment methods used in both studies differ between them and differ with the protocol here described, limiting the possibility of *Rhodanobacter* being identified as the result of the same contamination undetected by negative controls or erroneous taxonomy assignment. Thus, *Rhodanobacter* was considered a component of the OSM and not only a contaminant.

Rhodanobacter has also been identified in the gastrointestinal microbiota of humans (Rajilić-Stojanović and de Vos, 2014), pandas (Jin et al., 2020), and mice (Chen et al., 2020; Joseph et

al., 2021), and in the meconium (first stool of a newborn after their birth) of Italian newborns of the Pisa Birth Cohort (Guzzardi et al., 2022). Its low prevalence in the gastrointestinal tract makes it likely to be a transient member of this microbiota (Rajilić-Stojanović and de Vos, 2014). It has also been identified in humans in the carotid microbiome (Brun et al., 2021), oral microbiome (Pan et al., 2023), and microbiome of the saliva and plaque (Rafeek et al., 2019). These last microbiomes are located in areas of the face close to eyes, therefore transmission from these areas to the ocular surface is a possible explanation for its presence on the ocular surface. Finally, *Rhodanobacter* was found in the human urinary tract microbiome (De Seta et al., 2022; Hilt et al., 2014), semen microbiome (Farahani et al., 2021), and milk microbiome (Ruiz et al., 2021). Interestingly, the latter study, identifying *Rhodanobacter* in human milk microbiome, reports on the comparison between 2 different sample processing and analysis methods. They compared previous results obtained by targeting the V1-V3 regions and using the SILVA v. 132 reference database, with new processing targeting the V3-V4 regions and the updated SILVA v.138 database used for taxonomy assignment. They found considerable differences in the relative abundances of some genera, including *Rhodanobacter*, of which the relative abundance was much higher in the new analysis. Ruiz et al. argue that targeted 16S rRNA regions and choice of reference database play an important role in identified bacteria. Specifically regarding *Rhodanobacter*, they identify a possible mix up with the *Dyella* genus. As highlighted in the paragraph *1.3.3 Laboratory practices in OSM studies: the need to standardize,* reference database used for taxonomy assignment differs between OSM studies and could also be an explanation for the scarce identification of *Rhodanobacter*.

Presence of *Rhodanobacter* in dog and owner OSM samples could also be attributed to its presence in soil and plant microbiomes (Igwe et al., 2024; Li et al., 2023; Xiao et al., 2024). The bacteria could be picked up from dogs while playing outside and later transmitted to their cohabiting owners. The fact that *Rhodanobacter* has a lower relative abundance in dogs compared to owners could be explained by the higher taxa richness in dog samples, lowering the relative abundance but not the absolute count. Comparison with humans not cohabiting with pets would be necessary to verify this hypothesis.

Much like *Rhodanobacter*, other rare genera of the OSM, in the sense that they are present in low relative abundances, might have not yet been identified as part of the OSM and considered contaminants. Standardization of protocols, with the introduction of negative controls at each

stage, and the implementation of studies on large populations are necessary to identify these rare genera and gain a more complete understanding of this particular microbiome.

4.4. Study population and sample collection

As previously detailed, One Health studies exploring interactions between humans, companion animals, and the environment they share require a specific and thorough study design. In this research, no power calculations were executed before recruitment of dog-owner pairs. The minimum number of pairs was determined according to population size in studies characterizing the canine OSM. Banks et al. reported results on the OSM of 25 dogs, Leis & Costa on 10 dogs, with only 7 included in the analysis, and Rogers et al. on 13 dogs (Banks et al., 2020; Leis and Costa, 2019; Rogers et al., 2020). Previous studies on companion animal microbiota and mycobiota have also demonstrated that a sample size of 10 to 12 animals is sufficient (Meason-Smith et al., 2017, 2015; Older et al., 2017).

Having both dog and owner participants required 2 separate sampling times and locations for each dog-owner pair, since owners were sampled at the Ophthalmology Unit, where dogs are not allowed. Unfortunately, this increased the risk of loss to follow-up and resulted in missing a dog sample and the following exclusion of their dog-owner pair. But because sampling location can impact stress and anxiety of both dog and owner (Tang et al., 2020), the choice to sample dogs in a location where these effects were minimized, i.e. their home, was made.

Tests of DNA extraction, library preparation, and sequencing realized during the implementation and troubleshooting phase showed the possibility of analyzing dog and owner OSM samples following the same protocol. Regarding the use of anesthetic before sampling, although it has been shown to not alter 16S rRNA sequencing of the OSM (Delbeke et al., 2022), using anesthetic for sampling of owners and not dogs could have impacted our results.

4.5. Comparison of dog and owner OSM composition and alpha diversity

In the owner population, 23 phyla, 40 classes, 98 orders, 138 families, and 214 genera were identified. These numbers are in line with the number of taxa identified in other studies characterizing the human OSM (Chen et al., 2022; Kang et al., 2021; Tunç et al., 2023). In dogs

also, the identification of 32 phyla, 75 classes, 167 orders, 260 families, and 525 genera, is also in line with other canine OSM studies (Leis and Costa, 2019; Rogers et al., 2020).

The main phyla identified in owners were Proteobacteria, Actinobacteria, and Firmicutes. Classes with the highest relative abundances were Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacilli and Bacteroidia. The 5 orders with the highest relative abundances were Corynebacteriales, Staphylococcales, Xanthomonadales, Rhizobiales, and Burkholderiales. Families with the highest relative abundances in owners were *Corynebacteriaceae, Staphylococcaceae, Rhodanobacteraceae, Xanthobacteraceae*, and *Chitinophagaceae*. These findings are in line with what is currently known of the healthy human OSM (Delbeke et al., 2021; Hernández-Zulueta et al., 2023; Peter et al., 2023).

The defined genus-level core human OSM was composed of *Staphylococcus, Corynebacterium, Rhodanobacter, Vibrionimonas, Acidocella, Bradyrhizobium, Acinetobacter, Ralstonia, Sphingomonas, Cutibacterium*, and *Flavobacterium.* In a review combining results from 11 studies, Delbeke et al., from which is derived the aforementioned definition of the core OSM, defined a genus-level core OSM that contained similar mean abundances of *Corynebacterium* (10% in the review, here 12%), *Acinetobacter* (6% in the review, here 4%), and, albeit in a smaller relative abundance, *Staphylococcus* (6% in the review, here 14%) (Delbeke et al., 2021). Other genera identified in the review but that are not part of the here-defined human core OSM were *Pseudomonas, Propionibacterium,* and *Streptococcus*. However, these genera were not identified in all of the studies used for the definition of this core OSM. Similar proportions were found in other reviews on the human OSM (Aragona et al., 2021; Peter et al., 2023). Interestingly, *Staphylococcus* was identified in all owner samples, as was the case in a cohort of Italian and Spanish healthy volunteers, albeit in higher relative abundances (Borroni et al., 2022).

Regarding the canine OSM, the predominant phyla identified, Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes, are in line with results from other studies, albeit in different proportions (Banks et al., 2020; Leis and Costa, 2019; Rogers et al., 2020). Our results identified Proteobacteria as the most abundant phyla, much like the healthy human OSM, and alike previous studies on the canine OSM (Banks et al., 2020; Rogers et al., 2020). Leis & Costa, however, found Firmicutes to be the most abundant phyla (Leis and Costa, 2019). These discrepancies could be, at least partially, attributed to differences in the studied canine population, as Rogers et al. and Banks et al. included a heterogenous group of privately-owned

dogs, much like the ones included here, whereas Leis & Costa studied 10 colony-raised Coonhound crosses. They could also be the result of different sampling methods or different DNA extraction and/or library preparation protocols. At the family level *Corynebacteriaceae, Pseudomonadaceae, Micrococcaceae, Pasteurellaceae*, and *Neisseriaceae* are part or the defined core canine OSM and were identified in the populations studied by Rogers et al.

It is important to note that most available microbial databases are skewed towards human-hosted bacteria that could prevent from the identification of some dog-specific bacteria (Mahmud et al., 2024).

Our findings reveal a higher alpha diversity in the canine OSM compared to the human one. Similarly, two studies found that dogs have a greater alpha diversity than owners in skin microbiome (Lehtimäki et al., 2020; Song et al., 2013). These findings could reflect dogs' frequent exposure to more various sources of bacteria, or behavioral differences compared to their human companions. Access to the outdoor environment and different hygiene habits appear to have a significant impact dog skin microbiome (Rodriguez-Campos et al., 2020) and could likely do so for the OSM as well. There is also evidence that urban dogs, which have less contact with other animals than rural dogs, have a lower abundance of microbes, and their skin microbiome is dominated by human skin bacteria, whereas the skin microbiome of rural dogs tends to be richer in environmental microbes (Lehtimäki et al., 2018). Here, no difference was found in dog-owner pair similarities between pairs with dogs living indoors and pairs with dogs living outdoors. However, this result could be attributed to the limited number of participants.

Ordination plots revealed that although baring many similarities, dogs and owners have a distinct OSM. When using weighted UniFrac distance, dogs were found to have a more similar OSM to one another than humans, as is the case for the gut microbiome (Song et al., 2016). It has also been argued that dogs have more similar habits to one another than humans, thereby limiting differences in lifestyle factors between them and making them good subjects for the study of the impact of environmental factors on health (Lehtimäki et al., 2020). However, when using Bray-Curtis dissimilarity, the opposite result was found, i.e. dog samples were found to have a less similar OSM to one another than humans. The main difference between the 2 metrics is that, unlike Bray-Curtis dissimilarity, weighted UniFrac distance accounts for phylogeny. Therefore, an explanation to this difference can be that relative abundances of taxa differ more between dog samples than between human samples but the taxa present in dog samples are phylogenetically closer than taxa present in owner samples.

Indicator species analysis revealed 8 phyla, 19 classes, and 35 orders indicators of dog samples among the 30 dog and owner samples. Among the 52 indicator families identified, the 5 with highest specificity and fidelity were *Cytophagales, Solirubrobacterales, Bacteroidales, Microtrichales*, and *Clostridiales.* These families were not explicitly reported in the works of Leis & Costa, Rogers et al., and Bank et al., however they only mentioned the most frequent families, which the latter are not. Access to raw data from the 3 studies would be necessary to determine if the families were also present in their dog samples.

4.6. Impact of dog and owner characteristics on alpha diversity

Results indicate a strong negative correlation between Shannon index in dog samples and amount of time dog and owner have lived together. This would indicate that the longer dogs have lived with their owners, the lower their alpha diversity is. No causal link can however be established here. The tendency for alpha diversity to also decrease with the increase of dog's age, albeit not significantly, can explain this result. The effect of age on canine OSM has not yet been explored, however one study reporting on the skin microbiome of dogs found no effect on age in several body sites, including the skin close to the conjunctiva (Rodrigues Hoffmann et al., 2014).

No other statistically significant result linking alpha diversity to other dog characteristics, owner characteristics, or dog-owner interactions or habits was found.

4.7. Beta diversity metrics

Three distance metrics were chosen for the analysis of beta diversity. Both weighted and unweighted UniFrac distances incorporate phylogenetic information, that is not reflected in Bray-Curtis dissimilarity. There is no agreement on which to use for OSM data analysis, some studies choose one (Cavuoto et al., 2021; Zhang et al., 2021), whereas others report on beta-diversity with multiple metrics (Chang et al., 2022; Kang et al., 2022; Z. Li et al., 2019; Yau et al., 2019). Interestingly, when comparing dog-owner distances with the 3 metrics, most pairs ranked very differently in each metric. Only 4 pairs kept similar ranks (differences of 3 to 5 ranks) in the 3 metrics,

4.8. Impact of dog and owner characteristics and interactions on dog-owner distance

Dog-owner distance was found to be smaller in pairs with owners who wear glasses (Bray-Curtis dissimilarity p.value = 0.0103), indicating that, in pairs with owners who wear glasses, dog and owner OSM are more similar than in pairs in which the owner does not wear glasses. As the impact of wearing glasses on the OSM has not yet been investigated, it is possible that this difference can simply be attributed to differences in owner OSM due to glasses-wearing, regardless of having a dog or not. However, no difference in alpha or beta diversity was found here between owners with and without glasses. A possible interpretation could be that, in order to limit the risk of breaking the glasses, owners wearing them might allow less contact between their dog's face and their own.

Song et al. and Lehtimäki et al. found the skin microbiota of dog-owner pairs to be more similar than the one of dog-random-owner pairs (Lehtimäki et al., 2020; Song et al., 2013). For the OSM, only a non-significant trend was observed when comparing similarities between dog-owner and dog-random-owner pairs. This could indicate that the OSM of dogs and humans share many similarities regardless of their cohabitation, or that people who own a dog tend to share a similar microbiome with their dogs. Another explanation could be that the OSM is not influenced by cohabitation with a dog. However, due to results demonstrating otherwise on the skin, oral and nasal microbiotas (Misic et al., 2015), and the similarities and direct exposure of these microbiotas to the external environment, it seems unlikely. The studies of Song et al. and Lehtimäki et al. were conducted on much larger populations (respectively, 17 families for a total of 159 people and 36 dogs, and 168 dog-owner pairs), which could also explain why similar results might not be visible on this population of 15 dog-owner pairs.

Dog-owner pairs with small dogs (< 10 kg) tended to have more similar microbiomes than pairs with large dogs (> 25 kg), possibly attributable to a more frequent face-to-face contact between owners and small dogs, that can easily be carried in one's arms. Another explanation for the impact of dog size on dog-owner distance lies in the fact that, in the 15 dog-owner pairs, smaller dogs were also more frequently aloud on beds than large dogs, which could increase the number of surfaces that dog and owner share within the household. A study on the impact of human-pet co-sleeping practices on owner's sleep has also identified that the likelihood of co-sleeping is higher with small dogs (< 11 kg) (Hoffman et al., 2021). Small dogs are more

likely to lie on laps or furniture (Westgarth et al., 2008), and sharing a bed adds sheets and pillows to the surfaces touched by both dog and owner faces, possibly creating a more "direct" contact between their OSM. Song et al argued that since skin microbiome structure and composition persist on surfaces (Fierer et al., 2010), it could be easily exchanged between human and animal inhabitants of a household.

Furthermore, it has been shown that the risk of sharing *Enterobacteriaceae* between pet and owner, easily transmitted through contact and identified in cats and dogs' fur and footbed, increases with bed-sharing practices (Zanen et al., 2022). This is also supported by the multiple studies demonstrating the impact of pets on house dust composition and increase in diversity in their presence (Fujimura et al., 2010; Shan et al., 2019). Although the ocular surface does not usually come into contact with these surfaces, hands do, and are often in contact with the eyes. On average, spontaneous touches of the T-zone occur 68.7 times per hour (Rahman et al., 2020). These self-touches represent an infectious risk but could also help maintain microbial diversity and prevent dysbiosis of the skin, ocular surface, gastrointestinal, and respiratory microbiomes (Spencer et al., 2021). Spontaneous face-touches could therefore pick up bacteria present on the household surfaces, deriving from all human and animal occupants, but also pick-up bacteria directly on the dog when petted, and make their way to the eyes. Furthermore, the risk of transmission of zoonotic pathogens from bed sharing, face licking or kissing pets, although uncommon, is real and has been documented even in cases of life-threatening diseases (Chomel and Sun, 2011).

The only lifestyle parameter with a significant impact on dog-owner distance was the presence of other pets in the house (Bray-Curtis dissimilarity p.value = 0.0401). Dog-owner pairs that cohabited with other pets (mostly cats and dogs) shared significantly more OSM than the pairs without any other pets. This could be attributed to an increased number of human and animal vectors adding and moving bacteria present on surfaces within the house.

Interestingly, there was no significant correlation between dog-owner distance the amount of time dog and owner have lived together for neither of the 3 distance-metrics.

4.9. Impact of pollution on the OSM

The WHO 2021 Report on Global Air Quality Guidelines recommends maximum annual levels of 15 $\mu g/m^3$ for PM_{10}, 5 $\mu g/m^3$ for $PM_{2.5}$, and 20 $\mu g/m^3$ for NO_2 (World Health Organization,

2021). In the 10 days prior to sampling, 27 out of 30 participants (15 dogs and 15 owners) were exposed to mean daily $PM_{2.5}$ concentrations above the 5 µg/m^3 guideline. 17 were exposed to PM_{10} above the 15 µg/m^3 guideline and 14 were exposed to NO_2 concentrations above the 20 µg/m^3.

There was no significant linear correlation between mean daily PM_{10} concentration, $PM_{2.5}$ concentration, NO_2 concentrations, mean daily PM_{10} exposure, or $PM_{2.5}$ exposure and alpha diversity (Simpson and Shannon indices). There were, however, weak to moderate positive correlations between alpha diversity and PM_{10} daily mean concentration, $PM_{2.5}$ daily mean concentration, PM_{10} exposure, and $PM_{2.5}$ exposure, although all were non-significant.

Daily mean NO_2 concentration in the 10 days prior to sampling is correlated to unweighted UniFrac dog-owner distance, with a significant moderate correlation among the entire population (dogs and owners) (R = 0.46, P.value = 0.011), and in the owner population (R = 0.52, p.value = 0.046). NO_2 is also the only air pollutant confirmed to have an significant impact on the increase of dry eye disease in the latest TFOS Lifestyle Report (Alves et al., 2023). We can therefore assume that NO_2 impacts not only the ocular surface and dry eye disease incidence, but also the microbiome that resides on the ocular surface, i.e. the OSM.

Data on OSM and pollution exposure came from a single sampling time and can therefore only be descriptive. There are currently no existing studies on the impact of exposure to air pollutants on the OSM. However, the important number of studies showing the negative effect of pollutants on the ocular surface and ocular disease prevalence (Chang et al., 2012; Chen et al., 2019; Novaes et al., 2010), as well as the OSM dysbiosis observed in many ocular diseases (Aragona et al., 2021; Arjunan and Swaminathan, 2022), a negative impact of air pollution on the OSM is more than plausible. Additionally, the ocular surface and the OSM are sensitive to humidity and seasonal changes (Stapleton et al., 2007; Zarzuela et al., 2022; Zhou et al., 2014) and are therefore likely both affected by composition of the external air. Further longitudinal studies evaluating this phenomenon are warranted for a better understanding. The negative impact of particular matter on the ocular surface has also been demonstrated on dogs (Jones et al., 2024).

4.10. Limits

An important limitation of this research is the population size. Furthermore, since the OSM seems to be impacted by gender (Liang et al., 2021b; Ozkan et al., 2023), a bias may have been introduced by the high percentage of female owners (87%). A similar bias was present in the dog population, however, the impact of sex on the canine OSM has yet to be investigated. Another bias introduced in the study is the reference database for ASVs taxonomy assignment, skewed toward the human population, therefore possibly identifying less bacteria in dogs microbiome (Mahmud et al., 2024). To better analyze the impact of outdoor air pollution on the OSM, the study would have needed to include several (at least) sampling times, with different levels of air pollution. Finally, the study would have benefitted from the inclusion of people not living with pets to act as controls.

4.11. Risks and benefits of the dog-owner relationship

Although One Health initiatives mainly focus on zoonoses from wildlife or livestock, companion animals are also reservoirs of diseases that can be transmitted to humans and can play a role in disease transmission (Day, 2010). Pet owners tend to have a good understanding of physical harm-associated risks that come with sharing their daily lives with a pet, but less regarding disease-related risks, i.e. zoonoses (Alho et al., 2018; Alrukban et al., 2022). In many households, high-risk behaviors such as feeding pets raw food, feeding pets in the kitchen, or letting pets face-lick children are still common (Stull et al., 2013). Up to 50% of owners let their pet (cat or dog) lick their faces and 60% letting them visit the bedroom (Overgaauw et al., 2009). These behaviors carry risks because of pet-related zoonoses, such as toxoplasmosis, fungal infections and bacterial infections (Day, 2016), and the numerous vector-borne diseases that infect both pets (cats, dogs) and humans, such as Leishmaniosis, Borreliosis, Bartonellosis, or Rickettsiosis (Day, 2011). Sharing of specific bacterial strains, such as urinary-tract infection-promoter *Escherichia coli,* between human and animal household members is already common (Johnson et al., 2008; Stenske et al., 2009). Shedding of antimicrobial resistant bacteria in pets has also been evidenced and is strongly associated to a raw pet food diet, due to a recurrent contamination of raw pet food by extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in cats (Baede et al., 2017) and dogs (Baede et al., 2015). In dogs, an association between raw meet diets and multidrug-resistant and third-generation

cephalosporin-resistant *Escherichia coli* has also been reported (Morgan et al., 2024). Evidently, lifestyle habits can have a strong impact on microorganism exchanges between pet and owner, particularly alarming when sharing antimicrobial-resistant pathogens. Although there is evidence of sharing of multi-drug resistant organisms between humans and pets within a household (Genath et al., 2024), it appears to still play a minor role in AMR sharing and, to this day, pet-ownership is not considered a risk factor for carriage of multidrug-resistant organisms (Hackmann et al., 2024). However, many of the pathogens shared between pets and owners have flu-like symptoms, often not recognized by family doctors, leading to a probable underdiagnosis (Overgaauw et al., 2020). Thus, the reported frequency of these infections might be underestimated and more research on pet-owner exchanges of antimicrobial-resistant microorganisms is needed to fully identify the scope of the issue and limit risks of future transmission.

Nonetheless, pet ownership can have many positive effects on human health. The term "zooeyia", from the Greek "zoion", animal, and "Hygeia", the ancient Greek goddess of health, was even coined to express the positive inverse of "zoonosis", i.e. the positive effect of animals on human health (Hodgson and Darling, 2011). For instance, pet ownership has been associated with increased mental health (Jennings, 1997; Marcial-Modesto et al., 2023), increased owner's physical activity (Martins et al., 2023), and decreased cardiovascular disease risk (Takashima and Day, 2014). Early-life exposure to pets is also negatively associated to later atopy-related disease and allergies (Hesselmar et al., 1999; Nafstad et al., 2001; Svanes et al., 1999).

Benefits for pets and owners can come from the pet-owner relationship and possible negative effects for owners are often discussed, however, this relationship can also be detrimental to pets. These negative effects can be related to changes in feeding practices, behavioral problems, breeding and animal welfare problems, or anthropomorphism (Overgaauw et al., 2020). Cases of zoo-anthroponotic transmission, i.e. transmission of pathogens from humans to animal species or humans to environment to animal species, have also been reported (Fernandes et al., 2018).

4.12. Public health measures and pet-owner relationships

There is an association between pet-owner closeness and the prevalence of shared bacterial species form the nasal mucosa, armpit, and interdigital spaces of the foot, highlighting the

crucial role of pet-owner interactions in microbial exchanges (Wipler et al., 2017). The results here-presented indicate a bacterial flow between dog and owner on the ocular surface, particularly increased in cases of multiple pets present in the household. Along with pet owners not being necessarily aware of these risks, a Canadian study on 641 respondents found that 60% of pet owners never had medical doctors or staff ask if they owned pets (Stull et al., 2012), indicating a possible lack of caution or awareness from health professionals as well. A key collaboration between veterinarians and physicians is necessary for an effective prevention of companion animal zoonoses, especially for immunocompromised patients (Friedmann and Son, 2009; Grant and Olsen, 1999). The importance of this collaboration is further supported by case reports of recurrent infections of antimicrobial-resistant bacteria in owners, successfully eradicated only after being also identified and eradicated in their companion animal(s) (Manian, 2003; Sing et al., 2008; van Duijkeren et al., 2005).

To reduce transmission of diseases from pets to humans, personal hygiene measures should be followed, such as washing hands after handling an animal, their feces, or their environment, protecting the skin when handling feces, avoiding contact with animal-derived treats, not allowing pets to lick open-wounds or medical devices (Stull et al., 2015). There is already an emphasis put on the risks of pathogenic species spreading from pet-feces in the form of street signs, mostly present in residential neighborhoods and in the vicinity of dog parks, asking owners to pick up their dog's feces to limit the possible spread of pathogens, such as the one shown in **Figure 34**. These signs help raise awareness in the public space, however no such awareness is present in the household. Along with the increased knowledge on microbiome- and pathogen-sharing dynamics between pets and owners, it is necessary to keep owners informed and implement public health policies reflecting these new findings, bearing in mind the positive effects of the pet-owner relationship.

Figure 34. Street sign to encourage dog owners to clean up after their dogs. Photo taken in November 2023 in Bray, Ireland by Suzanne B. Clougher

4.13. The ocular surface, a target for One Health indicators

The World Small Animal Veterinary Association's One Health Committee considers 3 key areas for companion animal-One Health: 1) the human-companion animal bond, 2) comparative and translational medicine, 3) zoonotic infectious diseases (Overgaauw et al., 2020). The domestication of dogs by humans began between 14,000 and 12,000 years ago (Morey, 1994) and no other species has such a diverse role and value in different cultures (Macpherson, 2005). Because of their unique closeness and the fact that they share the same exposome with their owners, companion animals have a valuable role to play as sentinels, be it incidental or intentional, in people's lives (Schmidt, 2009). This research shows that, as with skin, gut, and oral microbiomes, similarities in dog and owner OSM can be increased by their habits. Since several studies have also demonstrated exchanges of antimicrobial-resistant bacteria following the same pathways as the ones in urinary tract and gut microbiome exchanges (Harrison et al., 2014; Weese et al., 2006), sharing of antimicrobial-resistant ocular surface-bacteria between pet and owner, in the same way that they seem to share OSM, is possible.

Because it can be identified on the ocular surface *via* non-invasive sampling, identification of bacterial sharing between pet and owner is particularly easy. For this reason, the ocular surface is a promising target for the identification of microbial flow between pet and owner.

Regarding outdoor air pollution, a few studies on the effect of various air pollutants on the ocular surface included "apparently healthy volunteers" which revealed, at different levels,

signs and symptoms caused by pollutants exposure (Torricelli et al., 2013; Versura et al., 1999). The most relevant aspect from this literature review was the identified so-called "subclinical inflammation" which can be mainly asymptomatic or paucisymtpomatic and is therefore easily misdiagnosed. Several studies involving actual patient visits suggest the presence of subclinical symptoms identifiable by physicians, but often not considered critical enough for patients to consult (Sarita et al., 2012; Saxena et al., 2003). On the other hand, articles reporting on the impact of air pollutants concentrations on patient visits for ocular diseases based on hospital or insurance records only include the patients that actually visited clinics, i.e. patients that had symptoms severe enough for them to consult. This could imply that the impact of air pollution on the ocular surface estimated by the latter articles severely underestimates the population affected by ocular surface pathologies caused by air pollution. The absence of severe symptoms even in cases of exposure to high levels of pollution could also be attributed to a perceptual adaptation of the conjunctiva to the pollutants. Perceptual adaptation is the attenuation of neural and perceptual responses to sustained redundant stimulation, commonly noted in the auditory and olfactory systems, in which adaptation occurs by shifting the receptor sensitivity function to higher stimulus concentrations, thereby increasing stimulus intensity saturation levels. The relative absence of symptoms after exposure to high concentrations of pollutants could therefore also be an indication of adaptation to chronic exposure to air pollution.

Disease prevention is categorized into 3 levels of prevention: primary, secondary and tertiary. Primary prevention consists in interventions before the disease occurs, like immunizations or traffic reduction. Secondary prevention aims at identifying diseases as early as possible, generally achieved through the implementation of screening programs. Finally, tertiary prevention occurs once diseases have overt symptoms, and aims at reducing disease progression and/or consequences. Screening as we know it, was shaped by Wilson & Junger's 1968 article defining the principles for disease screening (Wilson et al., 1968). Many screening programs have been implemented since then and, following the practices and principles that were established, they have demonstrated the relevance and need for secondary prevention (Gini et al., 2020; Zielonke et al., 2020). Screening programs involving eye health are to this day limited to diabetic patients. However, the literature detailed in *1.7 The impact of outdoor air pollution on the ocular surface* showed that subclinical parameters seem to appear long before patients seek treatment for eye conditions caused by outdoor air pollution. Therefore, it is possible to assume that the impact of air pollution on the ocular surface could be detected before patients

have significant symptoms and damage to the ocular surface is too important. Moreover, as many diseases are unquestionably negatively impacted by outdoor air pollution (Arias-Pérez et al., 2020; Glencross et al., 2020), and because the effect of air pollutants on the ocular surface is observable through non-invasive tests, the ocular surface could act as target or a sentinel to monitor exposure to air pollution. Further research on dose-response links between air pollution and subclinical signs on the ocular surface, as well as on links between ocular surface deterioration and pathologies caused by air pollution are needed to better determine how the ocular surface could become and index for pollution exposure detection.

An overview of impacts of the exposome on the ocular surface, and the ties to climate change, is presented in **Figure 35**.

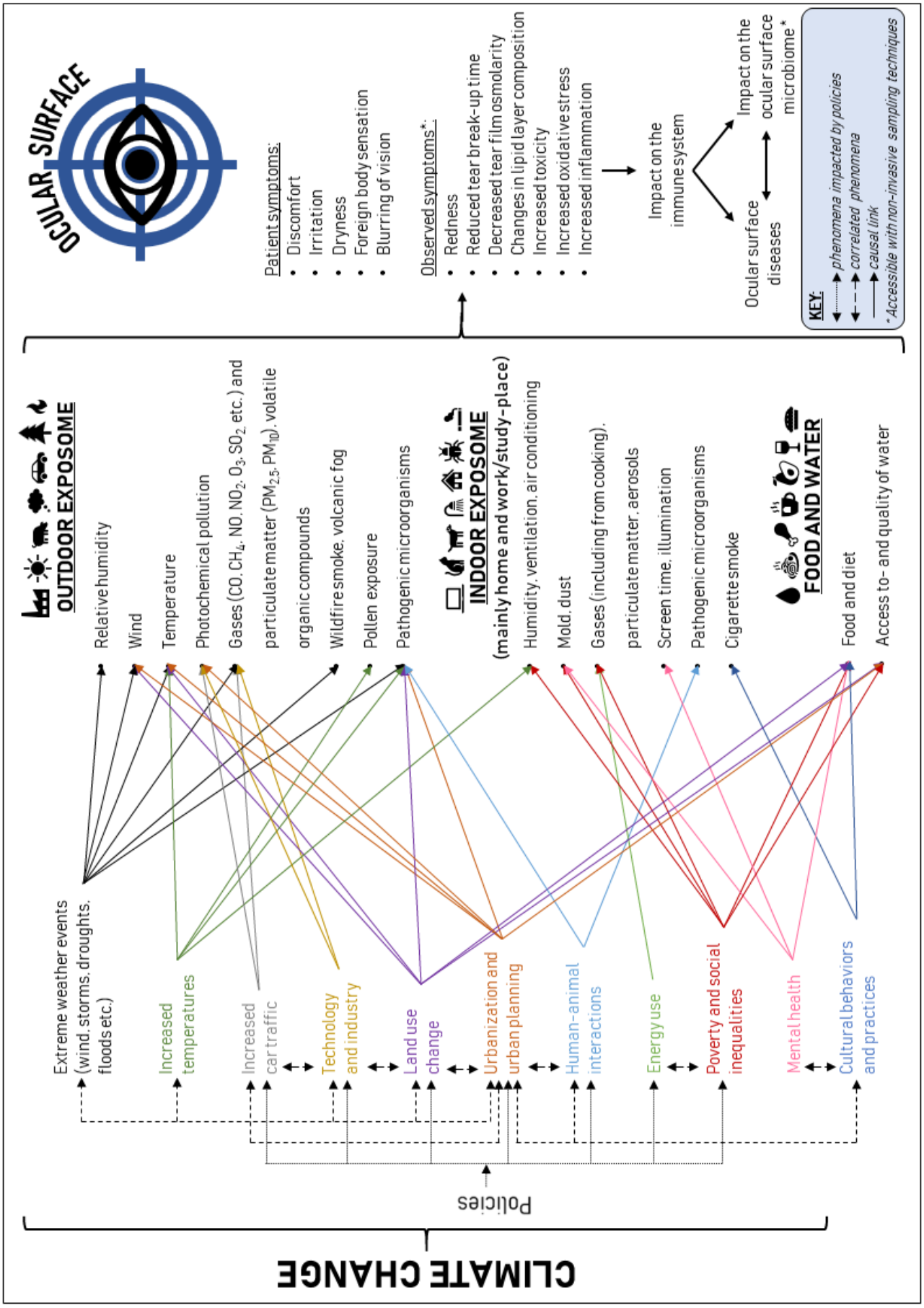

Figure 35. Impact of exposome on the ocular surface.

5. Conclusion

One Health studies on relationships between owner and companion animal are not very common. Intensification of the human-animal bond calls for an understanding of microbial flow, particularly in the case of pathogens and antimicrobial-resistant pathogen sharing.

To summarize results from the present study, the main outcomes can be outlined as follows:

- This is the first study evaluating microbiome interactions between pet and owner at the ocular level. Such interactions have been studied in the gut, mouth, and nose, however not yet on the ocular surface, often less considered, perhaps because there is no direct contact between pet and owner's ocular surfaces.
- This study is the first to investigate the correlation between OSM and air pollutant exposure, albeit in a descriptive manner, due to a single sampling time.
- Methods for OSM sampling and characterization through sequencing are very heterogenous, which hinders the possible comparisons between studies and the identification of a core microbiome. Through a collaboration between biologists, microbiologists and bioinformaticians, this study proposes an optimized protocol for OSM sampling and sequencing.
- The OSM of dogs and owners share a lot of similarities. These results add to previous evidence of microbial exchanges found between pets and owners in other body sites, such as the gut, mouth, and nose.
- Similarities in dog and owner OSM significantly increase when other pets are present in the household.
- Similarities between dog and owner OSM tend to be bigger in pairs with small dogs compared to large dogs. This is possibly due to difference in habits between dog and

owner when the dog is small, more often carried in the owner's arms, and allowed on sofas and beds.

- Within the household, this microbiome sharing occurs between humans, animals, and the surrounding environment, in all directions.
- NO_2, known to have a negative impact on the ocular surface, as it is positively correlated with dry eye disease incidence, is positively correlated with similarities in dog and owner ocular surface microbiome.

Results from the present study provide evidence for several impacts on various fields, outlined as follows:

- One Health studies on owner and companion animal can benefit from specific study design, considering the particularity of working with human and animal samples, and considering the bond between pet and owner.
- Closeness between pet and owner alters their microbiomes. Pet owners must be aware of the risk of sharing pathogens with their pet, not only through direct contact of the skin, but also through the ocular surface, mouth, nose, and gut. This alteration of microbiomes could also be beneficial, possibly triggering the immune system into a protective effect, or increasing a positive bacterial diversity. As some studies have shown, benefits of living with a pet might not only be psychological or physical, but also physiological.
- Public health policies aiming to keep owners informed of the risks and benefits of pet ownership, and of the behaviors and lifestyle choices that carry a higher risk of pathogen transmission must be implemented.
- Collaborations between physicians, veterinarians, and all medical staff are highly recommended to effectively detect and prevent the spread of pathogens and antimicrobial-resistant pathogens between human and animal inhabitants of a household. Knowledge on pet-owner microbiome exchanges must be shared and diffused to help physicians and veterinarians tackle these issues with a more comprehensive approach.
- Air pollutants have a negative impact on ocular surface homeostasis, and possibly also on the OSM. The negative impact of some air pollutants might be underestimated due

to symptoms often not disturbing enough for people to visit a physician and/or due to an adaptation of the ocular surface to the chronic exposure to air pollutants.

- The ocular surface of apparently healthy people is often revealed to be altered by air pollution. Characterization of this alteration before the appearance of severe symptoms or further complications through a brief and non-invasive examination of the ocular surface could help determine the extension of the negative impacts of air pollution. The ocular surface, especially the conjunctiva is a promising target for the definition of indicators that reveal the levels of air pollution one is exposed to.
- A collaboration between physicians/veterinarians and environment agencies is warranted to detect negative impacts of pollution on human, animal, and environmental health and protect them from it as best as possible. Due to limited differences between their individual "lifestyles" compared to humans, dogs could be valuable assets to study the impact of pollutants on microbiomes.

Future developments and perspectives:

- Studies on larger populations could provide a better understanding of microbiome-sharing dynamics between pet and owner and help pinpoint the factors that impact it most.
- The study of antimicrobial-resistant bacteria and AMR genes sharing between the OSM of pet and owner could provide more specific insights on the nature of the shared bacterial species.
- Further research is needed on the correlations between immune system and exposure to pets, and ocular surface immune system and air pollution.
- Longitudinal studies with OSM sampling in different seasons and with different levels of air pollutants could help elucidate the impact of pollution on the OSM.

The ocular surface is proposed as a valuable indicator of air pollution exposure, and of microbial exchanges, pathogenic and non-pathogenic, between humans, animals, and their surrounding environment. It interacts with the exposome and is accessible for an easy assessment and non-invasive sampling procedures, making it a suitable target for these One Health indicators.

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Appendix 1

Table App. A: Characteristics of studies characterizing the healthy OSM

Authors	Country	Population	Age (mean ± SD)	Exclusion criteria	Main results
Borroni et al. 2022	Italy, Spain	**137** 84 women 53 men	Range: 18-82 years	Ocular surgery, allergy or type of ocular inflammation, use of contact lens, ocular surface diseases, meibomian gland dysfunction, use of antibiotics in the past 6 months, use of tablet for systemic diseases, BMI index <18.5 or >24.9, hyperglycemia blood levels	Most abundant and prevalent genera: *Staphylococcus, Bacillus, Corynebacterium; Staphylococcus* present in all samples; Identified 9 eye community state types (ECST) characterized by different taxonomic compositions of the microbiota
Doan et al. 2016	USA	**107:** 62 under 30 years old (31 women 31 men) + 45 over 60 years old (24 women 21 men)	18 to 30 years or 60 and older	Obvious ocular surface disease/irritation, facial skin disease, history of recent contact lens wear, use of oral or topical antibiotics or prescription eye medication in the past 3 months, ocular surgery in the last 12 months, active ocular infection, dry eye condition, diabetes or immune-compromised state. Maximum Ocular Surface Disease Index Questionnaire (OSDI) score: 6	Predominant identified organisms: *Corynebacterium, Propionibacteria*, coagulase-negative *Staphylococci* and *Streptococcus;* the OSM is distinct from other close microbiota; torque teno virus (TTV) identified in 65% of all conjunctivas tested; 42 genera identified

Dong et al. 2011	USA	**4**	26 to 48 years	Contact lens wear, medical histories of ocular diseases, ocular traumas/transplantation, history of antibiotic treatment in the previous 6 months	5 phyla and 59 genera identified in total; 12 genera were ubiquitous: *Propionibacterium, Bradyrhizobium, Corynebacterium, Acinetobacter, Brevundimonas, Staphylococcus, Aquabacterium, Sphingomonas, Streptococcus, Streptophyta, Methylobacterium* (other 47 genera accounted for <4%)
Huang et al. 2016	China	**31** 15 women 16 men	67.5 ± 11.8 years (Range: 41-84 years)	Contact lens wear, medical histories of systemic disease, ocular surface disease, uveitis, glaucoma, retinal disease or ocular trauma/transplantation, use of eyedrops (antibiotics, corticosteroids, nonsteroidal anti-inflammatory drugs) in the previous 6 months	25 phyla and 526 genera identified; *Corynebacterium, Pseudomonas, Staphylococcus, Acinetobacter, Streptococcus, Millisia, Anaerococcus, Finegoldia, Simonsiella* and *Veillonella* accounted for 76% of the microbial community
Kang et al. 2021	China	**17** 8 women 9 men	women: 41.6 ± 13.7 years; men: 43 ± 13.3 years	History of systemic or ocular diseases, contact lens wearing, topical or systemic antibiotics, steroid, any eye drop (prescribed or over the counter) or probiotic treatment in the previous 6 months. Maximum OSDI score: 12	12 phyla, 70 genera and 140 species identified; species identification for each sample ranged from 6 to 47 indicating differences in microbial diversity among individuals; highly relative abundances and positivity rates of *Streptococcus pyogenes, Staphylococcus epidermis, Propionibacterium acnes, Corynebacterium accolens* and *Enhydrobacter aerosaccus*
Ozkan et al. 2017	Australia	**45** 22 women 23 men	38 ± 10 years	Under 18 years of age, contact lens wear 3 months prior to and during the study period, ocular or systemic disease, history of eye trauma or surgery (including refractive surgery), use of antibiotic, anti-inflammatory or immunosuppressive medication in the previous 6 months. Maximum OSDI score: 13	No microbial species found in all subjects at all times or in all subjects at any one time; *Corynebacterium* was the most commonly detected taxon; 26 taxa present in at least one or more subjects at all times including *Corynebacterium* and *Streptococcus;* ocular surface does not appear to support a substantial core microbiome; possibility of the existence of individual-specific core microbiomes; 16 genera and 4 phyla present in at least one or more subjects at all times
Ozkan et al. 2019	Australia	**45** 22 women 23 men	38 ± 10 years	Under 18 years of age, contact lens wear in the previous 3 months and during the study period, ocular or systemic disease, history of eye trauma or surgery (including refractive surgery), use of antibiotic, anti-inflammatory or immune-suppressive medication in the previous 6 months. Maximum ODI score: 13	Highest relative abundance at phylum level: Proteobacteria, Firmicutes, Actinobacteria; at genus level: Acinetobacter, Aeribacillus, Acetobacter, Neisseriaceae (F); the 4 sampled regions had a distinct bacterial biogeography
Pal et al. 2022	India	**15** 6 women 9 men	36.5 ± 7.6 (Range: 20-52 years)	Ocular allergy, inflammation and any other ocular surface diseases, used of antibiotics in the past 3 months, OSDI and Schirmer test results	No significant differences found in alpha diversities among the 3 age groups (20-30 years, 31-40, and 41-52); top ten predominant genera; *Lactobacillus, Bacillus, Corynebacterium_1, Staphylococcus, Mycobacterium, Cutibacterium, Streptococcus, Acinetobacter, Escherichia-Shigella, Clostridium_sensu_stricto_12*; Compared to

					conjunctival OSM found in Shivaji et al (2021): 144 genera identified in both, 22 only in conjunctiva, 1 only in tears; tear sampling seems like an alternative sampling method for OSM research
Zysset-Burri et al. 2021 *	Switzer-land	**20** 5 women 15 men	69.7 ± 8.3 years	History of recent (3 months) ocular surgery, use of systemic or topical antibiotics within the last 3 months, smoking, contact lens wear, use of systemic immunomodulators and corticosteroids	Dominant phyla: Actinobacteria and Proteobacteria; dominant genera: *Propionibacterium, Agrobacterium, Corynebacterium*; OSM was found to be associated with the tear proteome
Cavuoto et al. 2018a	USA	**50 children** 19 girls 31 boys	37 months ± 36 months (range 1-168 months)	Signs of obvious ocular surface disease/irritation, active infection, skin disease, concurrent contact lens use, or administration of oral or topical antibiotics or topical immunosuppressants within the prior 90 days	Greater diversity in children over 6 months; *Staphylococcus* sp. Predominant by culture and 16S sequencing; top 5 most abundant phyla: Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria and Bacteroidetes; top 7 most abundant families: *Staphylococcaceae, Streptococcaceae, Corynebacteriaceae, Moraxellaceae, Enterobacteraceae, Oceanospirillaceae,* and *Bacillaceae*
Cavuoto et al. 2019b	USA	**15 children**	3.7 years ± 31 months	Current ocular or intraocular infection, use of antibiotics (topical or oral) within the prior 30 days, ocular surgery within the prior 90 days, patient or guardian refused participation	Predominantly Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria; conjunctiva had a lower number and relative abundance of species than eyelid margin and periocular skin
Petrillo et al. 2022	Italy	**13 newborns** 7 girls 6 boys	Newborns	Ocular pathologies and/or altered general clinical picture, mothers presenting urogenital tract infections	Dominated by Proteobacteria, Actinobacteria and Firmicutes at birth; After treatment with gentamicin, observed a decrease in Proteobacteria and increase in Firmicutes, Bacteroidetes and Fusobacteria; Most representative species: *Cutibacterium acnes*, *Massilia timonae, Staphylococcus epidermis*
Cavuoto et al. 2018b	USA	**30** **Effect of age:** <u>15 children:</u> 4 girls 11 boys <u>15 adults:</u> 3 women 12 men	children: 44 ± 31 months (range 5-98 months), adults: 57 ± 17 years (range:29-83 years)	Active infection, skin disease, concurrent contact lens use, or administration of oral or topical antibiotics in the previous 90 days	Significant differences between adult and children OSM: diminished richness and diversity in adults; main phyla identified: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria; reduction in *Streptococcus, Staphylococcus,* and *Brachybacterium* in adult samples compared to children
Suzuki et al. 2020	Japan	**36** **Effect of age:** 9 young women (YW), 9 young men (YM), 9 elderly women (EW), 9 elderlies	YW: 25.9 ± 5.2 years; YM: 31.8 ± 3.8 years; EW: 64.0 ± 2.9 years; EM: 65.4 ± 2.6 years	Tobacco smoking, contact lens wear, ocular or systemic disease, medication at the time of the study	Conjunctival sac samples from young subjects exhibited microbiomes different from that of the skin; difference in young and elderly subjects' microbiomes; relative abundance of *P. acnes* in the conjunctival sac higher in younger subjects compared to elderly; relative abundance of *Corynebacterium* sp. in the conjunctival sac higher in elderly subjects compared to younger; main phyla present in the conjunctival sac: Actinobacteria, Proteobacteria, Firmicutes

		men (EM)			
Wen et al. 2017 *	China	**90** **Effect of age and sex:** 25 YW, 23 YM, 23 EW, 19 EM	young subjects: 27.9 years (Range 23-44 years), elderly subjects: 67.1 years (Range 47-84 years)	Smoking, signs of systemic disease or ocular disease, history of antibiotic treatment or contact lens wear in the previous 6 months	Significant beta diversity difference between male and females; *P. acnes* and *S. epidermis* decreased significantly from male to female while *E. coli* increased; core microbial species identified: *Propionibacterium acnes*, *Staphylococcus epidermis, Escherichia coli, Micrococcus luteus, Mycoplasma hyorhinis, Acidovorax ebreus, Acidovorax* sp., *Staphylococcus haemolyticus, Acinetobacter braumanii, Ochrobactrum anthropi, Xanthomonas campestris, Bacillus cereus*
Deng et al. 2020	China	**86** **Effect of geography:** 42 women 44 men (From 3 different Chinese cities: Guangzhou, Wenzhou, and Beijing)	26.9 ± 0.3 years	Smoking, medical history of systemic and ocular diseases, contact lens wear or antibiotic treatment in the previous 6 months	Predominantly *Staphylococcus epidermis* and *Propionibacterium*; identified core bacterial species (>1%) of the conjunctival surface: *Propionibacterium acnes, staphylococcus epidermis, Propionibacterium avidum, Escherichia coli, Micrococcus luteus, Acidovorax sp., Staphylococcus haemolyticus, Acidovorax ebreus, Pseudomonas aeruginosa, Ochrobactrum anthropic, Mycoplasma hyorhinis*; environment seems to shape people's OSM
Li et al. 2021	China	**62** **Effect of altitude:** (32 living at 3700m above sea-level, 30 living at 300m above sea-level; groups were sex- and age-matched)	N/A	Tobacco smoking, contact lens wear, glasses wear, eye and/or systemic disease, use of eye drops and/or oral antibiotics in the previous 6 months	Significantly decreased diversity and richness in samples from highlanders compared to lowlanders; 5 dominant ocular surface microbiota taxa in both groups: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Cyanobacteria; most common genus found *Corynebacterium, Staphylococcus, Pseudomonas, Anaerococcus, Acinetobacter, Streptococcus* and *Massilia* (different relative abundances between high and lowlanders)
Dong et al. 2022 **	China	**128** 77 women 51 men (divided into age groups)	Women: 42.92 ± 19.15 Men: 35.69 ± 18.61	Several systemic diseases, pregnant or breastfeeding women, systemic antibiotics or topical eye drops containing antibiotics, anti-inflammatory, and corticosteroid eye drop administration within 3 months, contact lenses, peri- and ocular infection in the previous 3 months, DED and other eye diseases	Most isolated genera: *Staphylococcus, Streptococcus,* and *Moraxella*; identified OSM mainly came from human body sites (34.55%), environment (33.33%), plants (9.05%), and animals (4.90%)

* Used whole metagenome shotgun sequencing; ** Used metataxonomics, culturomics and genome sequencing analysis, YW: Young Women, YM: Young Men, EW: Elderly Women, EM: Elderly Men, OSDI: Ocular Surface Disease Index Questionnaire

Appendix 2

Table App.2 Characteristics of case-control studies characterizing the OSM of healthy controls and patients with different pathologies

Authors	**Country**	**Disease/ Conditions**	**Case group** n (mean age ± SD in years)	**Control group** n (mean age ± SD in years)	**Main results**	**Shannon index** (diversity)	**Simpson index** (diversity)	**Chao1 index** (richness)
Ham et al. 2018	Korea	Type 2 diabetes	**9** 5 women, 4 men (60.7 ± 10.9)	**16** 4 women, 12 men (32.9 ± 4.78)	↑ Proteobacteria and ↓ Firmicutes; ↑*Actinobacteria, Bacteroidetes, Cyanobacteria* in diabetes	NA	NA	NA
Li et al. 2019	China	Type 2 diabetes	**31** 14 women, 17 men (56.68 ± 15.13)	**23** 10 women, 13 men (43.88 ± 17.43)	↑ *Acinetobacter* and *Pseudomonas* in diabetes	↓	ns	ns
Zhu et al. 2021	China	Type 2 diabetes mellitus (T2DM)	**79** 47 women, 32 men (67 ± 8)	**113** 61 women, 52 men (65 ± 10)	Significant ↑ number of observed species; ↑ phylum *Bacteroidetes* and *Fusobacteria,* ↑ genus *Haemophilus, Pseudomonas, Empedobacter*; ↓ *Streptococcus* in T2DM	ns	ns	ns
Suwajanakorn et al. 2022	Thailand	Type 2 diabetes mellitus (T2DM)	**60** 30 women, 30 men (55.55 ± 8.93)	**20** 10 women, 10 men (55.75 ± 9.54)	Potentially pathogenic bacteria Enterobacteriaceae, Neisseriaceae, *Escherichia-Shigella*, and *Pseudomonas* predominant in DM	ns	NA	ns
Ali et al. 2023	Egypt	Type 1 (T1DM) and Type 2 (T2DM) diabetes mellitus	**28** T1DM **24** T2DM (range: 18-60)	**18**	Firmicutes/Bacillota ratio higher in T1DM and T2DM than controls, Fusobacteria more abundant in T1DM than T2DM, *Streptococcus* and *Paracoccus* more abundant in T1DM and T2DM than in controls	↓	↓	↓
Zhang et al. 2021	China	DED ± diabetes mellitus (DED ± DM)	**37** DM with DED: 18 women (62 ± 5) 19 men (59 ± 3) **22** DM: 10 women (64 ± 6) 12 men (62 ± 3), **34** DED: 9 women (55 ± 8) 25 men (57 ± 4)	**22** 10 women (53.9 ± 4.5) 12 men (56.8 ± 3.4)	Most prevalent species in DM + DED group: *Ochrobactrum, Corynebacterium, Bacillus, Cupriavidus, Lactococcus*; unique core members of DM + DED group: unclassified *Ruminococcaceae, Bacteroides,* unclassified *Peptostreptococcaceae,* unclassified *Barnesiellaceae*	↑ for DM + DED; ns for DM and DED	↑ for DM + DED; ns for DM and DED	↑ for DM + DED and DM; ns for DED
Chen Z. et al. 2022	China	Diabetes Mellitus ± DED (DM ± DED)	**31** DM + DED: 20 girls, 11 boys (13.19 ± 2.67) **34** DM:	**33** 23 girls, 10 boys (13.28 ± 2.86)	Different composition and ↓ in Proteobacteria in DM and DM+DED groups; ↑ Bacteroidetes, Tenericutes, Firmicutes, and Acidobacteria in	↑ in DM and DM + DED	↑ in DM + DED; ns in patients with DM	↑ in DM and DM + DED

			22 girls, 12 boys (13.90 ± 2.76)		DM+DED; ↑ *Bacteroides* and *Clostridium* DM+DED			
Graham et al. 2007	Northern Ireland	Dry Eye Disease (DED)	**34** 21 women (46 ± 14) 13 men (52 ± 15)	**57** 33 women (38 ± 17) 24 men (50 ± 21)	No substantial differences between healthy and dry eye subjects	NA	NA	NA
Liang et al. 2021 *	China	DED (including ATD and MGD)	**47** 23 women, 25 men (40.0 ± 14.6)	**48** 25 women, 23 men (27.9 ± 4.0)	↑ inter-individual variation; *Staphylococcus aureus* and *S. capitis* associated with MGD, *S. hominis* associated with ATD	↓	NA	NA
Tong et al. 2022 *	Singapore	DED	**14** 10 women, 4 men (44.3 ± 16.2)	**10** 7 women, 3 men (44.1 ± 14.3)	Similar composition in mild DED and controls; abundance in *Staphylococcus* correlated with Schirmer readings; *Streptococcus spp.* increased with age	NA	NA	NA
Song et al. 2022	China	DED associated (SSDE) or not with Sjögren's Syndrome (NSSDE)	**23** SSDE: 23 women (48.09 ± 9.01) **36** NSSDE: 27 women, 9 men (39.89 ± 13.45)	**39** 27 women, 12 men (36.61 ± 11.03)	Top five abundant genera: *Acinetobacter, Staphylococcus, Bacillus, Corynebacterium, Clostridium_sensu_stricto_1*; Different Firmicutes/Bacteroidetes ratio significantly different in controls VS SSDE and SSDE VS NSSDE	↓	NA	↓
Kim et al. 2022	Korea	SSDE and NSSDE	**48** SSDE: 37 women, 11 men (51.71 ± 9.46)	**72** NSSDE: 68 women, 4 men (54.5 ± 13.6)	*Acinetobacter* significantly more abundant and *Xanthomonads* significantly less abundant in SSDE	↓	NA	ns
Andersson et al. 2020	Denmark	DED (± OGVHD)	**21** DED: 18 women, 3 men (Median age: 60) **18** DED +OGVHD: 11 women, 7 men (Median age: 61)	**28** 16 women, 16 men (Median age: 32)	*Bacilli* identified as a marker of aqueous tear-deficient dry eye; *Pseudomonas* as a marker for controls	↓	NA	NA
Li J. et al. 2022 *	China	DED post-HSCT (± OGVHD)	**50** oGVHD**:** 22 women, 28 men (36.1 ± 11.3) **26** non-oGVHD**:** 10 women, 16 men (31.9 ± 11.6)	**48** 25 women, 23 men (27.9 ± 4.0)	More viral species detected in allo-HSCT group; alpha diversity impacted by sex mismatch in male recipients; *Gordonia bronchialis* and *Pseudomonas parafulva* enriched in oGVHD patients	↓ in all post-allo-HSCT	NA	NA
Gupta et al. 2023	India	DED	**4** 1 woman, 3 men (51.5 ± 1.29)	**4** 4 men (42.25 ± 3.77)	↓Acinetobacteria, Actinobacteria, Proteobacteria, Thermi, Cyanobacteria in DED	NA	NA	NA
Jiang et al. 2018	China	Meibomian Gland Dysfunction (MGD)	**41** 24 women, 17 men (34.3 ± 10.8)	**29** 16 women, 13 men (31.8 ± 8.7)	↑ bacterial isolation rate, number of species and bacterial severity in MGD; *Corynebacterium macginleyi* was only detected in severe MGD	NA	NA	NA

Dong et al. 2019	China	MGD	**47** 38 women, 9 men (57.53 ± 15.10) mild, moderate, and severe MGD	**42** 28 women, 14 men (62.76 ± 9.73)	significant in females with MGD: ↑ *Staphylococcus* and *Sphingomonas*; ↓ *Corynebacterium*	ns	ns	ns
Li Z. et al. 2019	China	DED ± MGD	**35** 19 women, 16 men (57 ± 14)	**54** 24 women, 30 men (52 ± 16)	Phyla level: ↓ *Proteobacteria,* ↑ Bacteroidetes; genus level: ↑ Acinetobacter, *Pseudomonas* in DED	↓	↓	ns
Zhao et al. 2020 *	China	MGD	**61** 38 women, 23 men (44.2 ± 16.2)	**15** 11 women, 4 men (25.2 ± 2.7)	↑ *Campylobacter coli, Campylobacter jejuni*, *Enterococcus faecium* in MGD	↑ no data on significance	↑ no data on significance	↓ no data on significance
Ozkan et al. 2023	Australia	MGD ± lacrimal dysfunction (LD)	**15** MGD: 10 women, 5 men (42.9 ± 12.4) **17** MGD + LD: 12 women, 5 men (40.0 ± 14.2)	**15** 6 women, 9 men (35.5 ± 8.3)	Significant difference in bacterial community structure between groups, ↑ *Pseudomonas azotoformans, P. oleovorans,* and *Caballeronia zhejiangensis* in MGD+LD compared to MGD and controls, ↑ ASVs belonging to *Corynebacterium kroppenstedtii* and *C. macginleyi* in MGD compared to MGD+LD and controls	ns for all groups	NA	ns for all groups
Wang et al. 2021	China	Blepharitis (anterior, posterior, mixed)	**37** 28 women, 9 men (Anterior: 23.40 ± 15.53, Posterior: 51.77 ± 9.42, Mixed: 53.21 ± 18.90)	**20** 6 women, 4 men (59.16 ± 16.88)	phylum level: ↑ Actinobacteria, Cyanobacteria, Verrucomicrobia, Acidobacteria, Chloroflexi, Atribacteria and ↓ Firmicutes; genus level: ↑ *Lactobacillus, Ralstonia, Bacteroidetes, Akkermansia, Bifidobacterium, Escherichia-shigella, Faecalibacterium, Brevibacterium,* ↓ *Bacillus, Staphylococcus, Streptococcus, Acinetobacter* in Blepharitis	↑	ns	↑
Yan et al. 2020	China	*Demodex* Blepharitis	**30** 22 women, 8 men (41.07 ± 16.03)	**14** 11 women, 3 men (41.14 ± 15.81)	relative abundance of Staphylococcus epidermis positively correlated with *Demodex* amount; phylum level: ↑ Firmicutes, Cyanobacteria and: ↑ *Corynebacterium;* genus level: ↑ *Lactobacillus and Bifidobacterium* in *Demodex* blepharitis	ns	ns	ns
Fu et al. 2022 *	China	*Demodex* Blepharitis	**25** 13 women, 12 men (44.9 ± 12.5)	**11** 6 women, 5 men (28.0 ± 5.6)	*Acinetobacter guillouiae* and *Pseudomonas putida* related to more severe ocular surface parameters in Blepharitis; *Sphingobium* sp., *YG1* and *Acinetobacter guillouiae* identified as potentially pathogenic biomarkers for *Demodex* Blepharitis	ns	ns	↓
Kittipibul et al. 2020	Thailand	Stevens-Johnson Syndrome (SJS)	**20** 15 women, 5 men (44.5)	**20** 15 women, 5 men (44.2)	↑ positive cultures; ↑ pathogenic microorganisms in SJS	↑	NA	NA

Ueta et al. 2021	Japan	SJS/Toxic epidermal necrolysis (SJS/TEN) with severe ocular complications	**37** 22 women, 15 men (52.19 ± 16.90)	**9** 5 women, 4 men (43.33 ± 24.82)	↑ *Corynebacterium 1, Neisseriaceae uncultured,* or *Staphylococcus*, or simultaneous enrichment of *Propionibacterium, Streptococcus, Fusobacterium, Lawsonella,* and *Serratia* in SJS/TEN	↓	NA	↓
Ji X. et al. 2022	China	Thyroid-associated ophthalmo-pathy (TAO)	**67 samples** 39 women, 8 men (44.25 ± 13.44)	**22 samples** 15 women, 7 men (62.64 ± 7.11)	↑ *Bacillus* and *Brevundimonas;* ↓ *Corynebacterium*; *Paracoccus, Haemophilus, Lactobacillus,* and *Bifidobacterium* positively correlated with severity of TAO clinical manifestations	ns	NA	NA
Yau et al. 2019	China	Allergic rhino-conjunctivitis (ARC)	**15** 4 women, 11 men (9.0 ± 4.1)	**15** 8 women, 7 men (7.18 ± 0.6)	In both cases and controls: most abundant phyla: Proteobacteria, Firmicutes and Actinobacteria; most abundant genera: *Moraxella, Corynebacterium, Streptococcus, Staphylococcus, Dolosigranulum*	ns	NA	NA
Hur et al. 2021	Korea	Atopic kerato-conjunctivitis (AKC)	**27** (Age and sex not provided)	**37** (Age and sex not provided)	↑ taxonomic composition and↑ Beta diversity in AKC; ↑ *Ralstonia, Bifidobacterium, Proteus* and ↓ *Pseudomonas, Staphylococcus, Haemophilus* in AKC	ns	NA	NA
Liang et al. 2021 *	China	Allergic conjunctivitis (Seasonal/ Perennial allergic conjunctivitis SAC/PAC, Vernal kerato-conjunctivitis VKC)	**39** (21 with SAC/PAC, 18 with VKC) 14 women, 25 men (19.8 ± 16.7)	**48** 25 women, 23 men (27.9 ± 4.0)	↑ *Brevibacterium aurantiacum* in SAC/PAC; ↑ *Streptococcus, Haemophilus* in VKC	ns	NA	NA
Song et al. 2022	China	Allergic conjunctivitis	**28** 20 women, 8 men (31.69 ± 11.75)	**39** 27 women, 12 men (35.61 ± 11.03)	Top genera in AC: *Bacillus, Staphylococcus, Corynebacterium, Acinetobacter,* and *Ralstonia;* Firmicutes/Bacteroidetes ratio at the phylum level was similar between case and controls	↑	NA	ns
Inada et al. 2022	Japan	AKC or VKC	**11** mild A/VKC: 1 woman, 10 men (33.8 ± 12.4) **10** severe A/VKC: 1 woman, 9 men (20.1 ± 12.9)	**6** 2 women, 4 men (41.0 ± 13.0)	↑ Firmicutes and ↓ Proteobacteria; significant ↑ *Blautia* in severe compared to mild and of *Morganella* in severe compared to controls	↓ in AKC and VKC	NA	↓ in AKC and VKC
Zarzuela et al. 2022	Spain	SAC or PAC	**28** SAC: 10 women, 18 men	**35** 18 women, 17 men	*Kocuria* and *Propionibacterium acnes* colonization observed in the PAC group	ns for all groups	NA	NA

			(31.39 ± 18.15) **<u>32</u>** <u>PAC:</u> 22 women, 10 men (34.87 ± 14.79)	(45.71 ± 16.57)				
Wang et al. 2023	China	ARC or allergic rhinitis (AR)	**<u>40</u>** <u>ARC:</u> 25 women, 15 men (40.22 ±9.84) **<u>20</u>** <u>AR:</u> 6 women, 14 men (38.4 ± 8.86)	**34** 21 women, 13 men (38.47 ± 11.27)	No significant difference in taxonomic distribution between groups, identified biomarkers: *Pseudomonas* for ARC, *Ralstonia* for controls	↓ in AR	↓ in ARC and AR	↓ in ARC and AR
Andersson et al. 2021	Denmark	Bacterial Keratitis (BK) (contact lens associated)	**35** 21 women, 14 men (Median age: 33 in CL, 44 in CLBK)	**28** 14 women, 14 men (Median age: 32)	overall conjunctival microbial profile not altered by contact lens wear or BK. OSM could harbor commensals that may act as opportunistic pathogens	ns	NA	NA
Cavuoto et al. 2021	USA	BK (unilateral)	**17** 10 women, 7 men (49.3 ± 17.5)	**16** 4 women,12 men (56.6 ± 17.0)	↑ *Pseudomonas* and other Proteobacteria; ↑ potential pathogens and ↓ commensal organisms in unilateral BK. Alterations present in both eyes in unilateral BK patients.	↑	NA	↑
Ren et al. 2021	China	BK	**20** 10 women, 10 men (50)	**42** (Similar gender and age composition)	↓ *Actinobacteria* and *Corynebacteria;* ↑ *Gamma proteobacteria, Pseudomonas, Bacteroides, Escherichia-Shigella* in BK	↑ for healthy eyes of BK; ns for healthy eye	↑ for healthy eyes of BK; ns for healthy eye	↑
Shivaji et al. 2021	India	BK	**22** 4 women, 18 men (51.5, range 27-71)	**20** 7 women, 13 men (44.5)	↓ abundance of different phyla and genera, ↑ pathogenic bacteria in BK	↓	↑	↓
An et al. 2022	China	BK	**12** 2 women, 10 men (57 ± 19)	**18** 15 women, 3 men (62 ± 13)	↑ Proteobacteria, ↑ *Acinetobacter,* and ↑ *Enterobacteriaceae* and ↓ in *Staphylococcus* in BK	NA	↓	↑
Ge et al. 2019	China	Fungal keratitis (FK)	**8** (Total population: 58.3 ± 9.53)	**10** (Total population: 58.3 ± 9.53)	↑ *Pseudomonas, Achromobacter, Caulobacter, Psychrobacter* in FK	ns	NA	NA
Zhou et al. 2014	The Gambia	Trachoma (*Chlamydia trachomatis* infection)	**115** 75 women, 40 men (n=29 ≤ 10, n=86 > 10)	**105** 72 women, 33 men (n=21 ≤ 10, n=84 > 10)	↑ *Corynebacterium* and *Streptococcus* in trachoma	ns	NA	NA

Aoki et al. 2013	Solomon Islands		**257** 97 girls, 160 boys (5.6)	**257** 97 girls, 160 boys (5.5)	*Paracoccus* is one of the main genera in trachoma but not in controls, no significant differences between trachoma and controls	ns	ns	NA
Pickering et al. 2019	The Gambia	Trachoma (*Chlamydia trachomatis* infection)	**36** children: 14 girls, 22 boys (6, range: 1-14) **121** adults**:** 86 women, 35 men (53, range: 16-87)	**49** children: 19 women, 30 men (5, range: 1-13) **158** adults**:** 90 women, 22 men (55, range: 16-84)	active trachoma in children was not associated with significant changes in the ocular microbiome; reduced diversity and ↑ *Corynebacterium* in adults with trachoma	ns in children ↓ in adults (estimation of both richness and evenness using Hill number)		
Liu et al. 2021	China	HIV (26 treated with antiretroviral therapy and 22 untreated)	**48** 7 women, 41 men (Untreated group: 38.18 ± 14.24: treated group: 39.46 ± 11.71)	**27** 8 women, 19 men (32.22 ± 12.55)	compositional and structural difference between HIV positive and HIV negative patients; Proteobacteria and Bacteroidetes significantly more abundant in untreated HIV-positive patients	ns for untreated HIV; ↓ for treated HIV	NA	NA
Shin et al. 2016	USA	Contact lens wearing	**9** (Age and sex of conjunctival swabs participants not provided)	**11** (Age and sex of conjunctival swabs participants not provided)	↑ skin-like bacteria; ↑ *Methylobacterium, Lactobacillus, Acinetobacter, Pseudomonas* in contact lens wearers	NA	NA	NA
Zhang et al. 2017	China	Contact lens wearing	**42** 23 included in the analysis: 13 women, 10 men (16.5 ± 4.4)	**25** 12 included in the analysis: 6 women, 6 men (23.7 ± 7.3)	↓ *Delftia*, ↑ *Elizabethkingia* in soft contact lenses wearers	ns	NA	ns
Xiao et al. 2023	China	Contact lens discomfort: asymptomatic or symptomatic (CL) wearers (ACL)	**12** ACL: 11 women, 1 man (27.83 ± 4.71) **11** CL: 9 women, 2 men (28.82 ± 4.63)	**12** 9 women, 3 men (26.75 ± 7.42)	↓ Firmicutes in ACL compared to CL, ↓ *Bacillus* in ACL and CL compared to controls. Firmicutes positively correlated with OSDI score	ns for all groups	ns for all groups	ns for all groups
Asao et al. 2019	Japan	Mucosa-associated lymphoid tissue lymphoma (MALT)	**25** 18 women,7 men (61.7 ± 15.6)	**25** matched 18 women, 7 men (58.3 ± 13.0)	↑ *Delftia* genus and ↓ *Bacteroides* and *Clostridium* in MALT lymphoma patients	NA	NA	NA
Kang et al. 2020	China	Traumatic corneal ulcer (TCU)	**22** 8 women, 14 men (56.7 ± 7.8)	**20** 7 women, 13 men (56.4 ± 8.2)	↑ *Pseudomonas* in TCU	↓	NA	↓
Tunc et al. 2023	Turkey	Keratoconus	**10** 7 women, 3 men	**10** 5 women, 5 men	↑ Bacteroidetes in keratoconus, ↓ *Escherichia, Oceanobacillus, Citrobacter* and 17 other genera	ns	ns	ns

			(23.5 ± 2.3)	(27.0 ± 3.6)				

↓: diversity of the cases significantly lower than the one of the controls; ↑: diversity of the cases significantly higher than the one of the controls; ns: no significant difference in diversity between cases and controls; NA: no reports on the diversity index provided in the article. * Used metagenomic shotgun sequencing

AKC = Allergic Kerato-Conjunctivitis; BK = Bacterial Keratitis; DED = Dry Eye Disease; DM = Diabetes Mellitus; FK = Fungal Keratitis; GVHD = Graft-Versus-Host Disease; HIV = Human Immunodeficiency; Virus; MALT = Mucosa-Associated Lymphoid Tissue lymphoma; MGD = Meibomian Gland Dysfunction; OGVHD = Ocular Graft-Versus-Host Disease ; PAC = Perennial Allergic Conjunctivitis; SAC = Seasonal Allergic Conjunctivitis; SJS = Stevens-Johnson Syndrome; SS = Sjögren Syndrome; TAO = Thyroid-Associated Ophthalmopathy; TCU = Traumatic Corneal Ulcer; TEN = Toxic Epidermal Necrolysis; T2DM = Type 2 Diabetes Mellitus; VKC = Vernal Kerato-Conjunctivitis