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# The spectacle microbiota and its hygienic relevance

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## List of abbreviations

| | |
|---|---|
| ASV | Amplicon sequence variants |
| CFU | Colony forming units |
| CMV | Cytomegalovirus |
| ct | Cycle threshold |
| DNA | Deoxyribonucleic acid |
| dsDNA | double-stranded DNA |
| EDC | European Centre of Disease Prevention and Control |
| HAdV | Human adenovirus |
| HAI | Hospital-acquired infections |
| HSV | Herpes simplex virus |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| MALDI-TOF MS | Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry |
| MDRO | Multidrug-resistant organisms |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MRSE | Methicillin-resistant *Staphylococcus epidermidis* |
| NGS | Next-generation sequencing |
| NTC | No template control |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative PCR |
| rRNA | Ribosomal ribonucleic acid |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| Tm | Melting temperature |
| TRBA | Technical Rules for Biological Agents |
| VZV | Varicella zoster virus |
| WHO | World Health Organisation |

# 1 Introduction

## 1.1 The human microbiome

Microorganisms are ubiquitous present in high diversity in, on and around humans. Approximately $10^{13}$ microorganisms are estimated to inhabit an average human being of 70 kg, thereby equaling the number of eukaryotic human cells (Sender et al. 2016). The most densely colonized parts of the human body are the large intestine (colon) and dental plaque with about $10^{11}$ bacteria per ml content. The estimates for saliva range from $10^{9}$ to $10^{11}$ bacteria per ml (Sender et al. 2016) and for human skin from $10^{3}$ up to $10^{6}$ organisms per $cm^2$ (Cundell 2018). These organisms form a complex community, while interacting with each other within their community and their mammalian host. However, the majority of microorganisms colonize the human body as benign mutualists or commensals, meant i.e. without being harmful nor clearly beneficial/symbiotic. Nevertheless, many of the human associated microorganisms are known to be potentially pathogenic and may cause severe infections.

It turned out that a healthy microbiome is a complex assembly, often highly diverse and specially balanced. A shift in the microbial community's composition and/or functionality may stimulate pathogenic behaviour within microorganisms. Further, perturbations to the complex microbial communities, referred to as "dysbiosis", can affect the immune system, which in turn can correlate with symptoms of illnesses (Petersen and Round 2014; Berg et al. 2020).

The term "microbiota" represents all microorganisms present in a defined environment, including bacteria, archaea, fungi and other unicellular eukaryotes (protists), while the role of viruses is controversially discussed.

"Microbiome" includes all microorganisms, their genomes, metabolites and the specific physio-chemical properties of the respective habitat, forming a distinct ecological niche and a partly fragile ecosystem (Marchesi and Ravel 2015).

Great research effort has been expended on understanding the microbiome-human relationship, as well as analyzing the hygienic relevance of the microbiota in different habitats and their impact on health and disease. According to the WHO World Health

Organization (WHO 2021) the term “hygiene” “*refers to conditions and practices that help to maintain health and prevent the spread of diseases*”.

Methodological developments during the last 20 - 30 years, such as massive 16S rRNA gene amplicon sequencing or shotgun metagenomic sequencing, have greatly facilitated microbiome research. This allows for the analysis of yet uncultivable bacteria, their virulence factors and resistance genes. Such progress allows for a deeper and more comprehensive understanding of a microbial community’s composition and functionality, and their interactions with the human host in health and disease.

These understandings open up various opportunities, for instance for therapeutic purposes (Sorbara and Pamer 2022), such as manipulating the gut or skin microbiota in order to influence human health and wellbeing. In the case of surfaces in contact with the human body, the development of new antimicrobial cleaning and coating processes is also more conceivable if the respective microbial community is well understood.

The following work addresses the microbiota on various ophthalmologically relevant surfaces, such as spectacles, microscope oculars and slit lamps. For a comprehensive picture, the microbiota of human body parts in close vicinity to these objects is also described initially.

## 1.2 The human skin as a microbial habitat

The human skin functions as a protective barrier between the outside environment and the inner human body and is colonized by about 10 billion of microorganisms (Sender et al. 2016). In recent years, research has increasingly been devoted to the analysis of this complex community. Sequencing-based analyses have shown that the outer layer, the stratum corneum, is inhabited by a diverse microbiota, which comprises all three domains of life: bacteria, archaea, eukaryotes (fungi, metazoic parasites) and viruses (Egert et al. 2017).

Grice and Segre (2011) summarize in their work, that the outer layer of the skin may represent a harsh habitat for microorganisms, since it is relatively dry and cold. The excretion of sweat results in a high osmolarity and low water activity. Acids produced by the skin cells lead to a low pH value. These conditions limit the growth of many microorganisms. Even though the adverse conditions inhibit the growth of most pathogenic (skin) microorganisms, the outer layer is densely colonized. The resident

commensal or even symbiotic members of the skin microbiota form a complex and diverse community, and are dominated by gram-positive bacteria such as propionibacteria (cutibacteria), corynebacteria and staphylococci. But gram-negative genera have also been detected in rather high shares, depending on skin topography.

The maximum microbial load of a healthy, adult human's skin is estimated to be as high as $10^{11}$ microorganisms (Sender et al. 2016), many of which contribute to human health and wellbeing. For example, *Cutibacterium acnes*, a common colonizer of the human skin, which frequently resides in pilosebaceous units, has the ability to hydrolyze sebum triglycerides, thereby releasing free fatty acids onto the skin. This also lowers the pH value, which may prevent some potentially pathogenic bacteria from colonizing these areas, although promoting the growth of commensal coagulase-negative staphylococci or corynebacteria (Grice and Segre 2011).

Cultivation-based analyses revealed an uneven distribution of bacteria among the distinct body habitats ranging from approx. $10^2$ $cm^{-2}$ (fingertips, back) to $10^6$ $cm^{-2}$ (forehead, around the ears) (Egert et al. 2017; Skowron et al. 2021), but also a varying phylogenetic diversity.

The most diverse skin sites are the dry areas, such as the volar forearm, dominated by *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteriodetes*, with a significant share of gram-negative species. Sebaceous (oily) sites, like the face and scalp, carry a relatively low-diverse community, dominated by lipophilic propionibacteria (cutibacteria) (Platsidaki and Dessinioti 2018). Staphylococci and corynebacteria are present at moist sites in rather high percentages along with propionibacteria (Grice 2014; Byrd et al. 2018). Figure 1 displays the relatively most abundant bacterial taxa on typical skin areas, where contact with the surfaces of spectacles or other optical devices is likely to occur.

It has been shown that variability and diversity within different body sites on one person are greater than intrapersonal variability (Grice and Segre 2011). Nevertheless, the microbiota also differ greatly between individuals, mainly driven by factors such as age, sex, genetics, nutrition and personal hygiene (Grice 2014; Ying et al. 2015; Shibagaki et al. 2017). Otherwise, some bacteria, which in a healthy state usually represent commensal or transient members of the skin's microbiota, may become opportunistic pathogens in certain situations, e.g. if the microbial community becomes imbalanced

(Byrd et al. 2018). This is described for instance, in relation to *Staphylococcus aureus* and its role in atopic dermatitis (Ederveen et al. 2020).

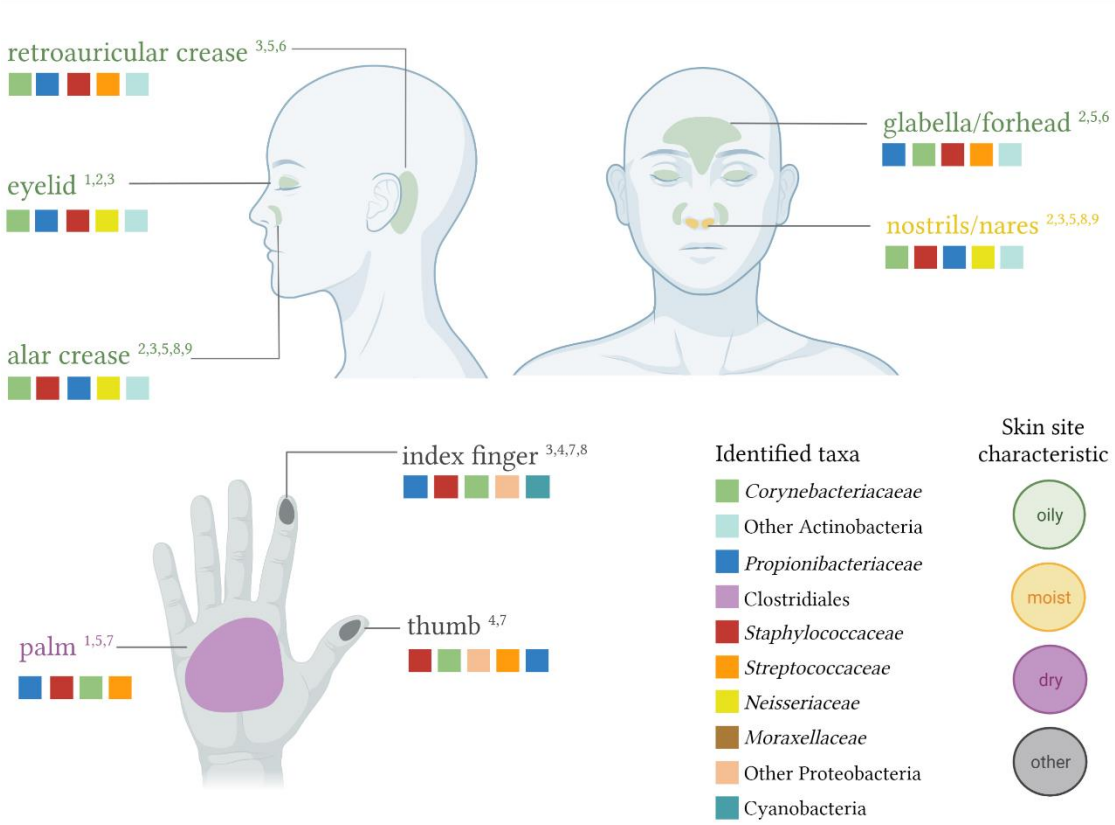


***Figure 1**: Prevalent bacterial taxa on typical skin areas, where contact with spectacles and other ophthalmic devices is likely to occur. Own illustration based on: 1) Grice et al. (2009); 2) Kong (2011); 3) Meadow et al. (2014); 4) Edmonds-Wilson et al. (2015); 5) Ross et al. (2017); 6) Byrd et al. (2018); 7) Cundell (2018) 8) Escapa et al. (2018). Illustration was created using biorender.com.*

Nevertheless, more important in the context of fomites and infections, are (cutaneous) infections as a result or cause of (chronic) sores. Many skin and soft tissue infections are associated with streptococci and staphylococci with a high prevalence of antibiotic resistant taxa (Sukumaran and Senanayake 2016). Through wounds or insufficient hygiene when using items (e.g. catheters, contact lenses, etc.), these opportunistic pathogens may be transferred to other body regions, and cause different infectious diseases there.

Although the skin also harbors different fungi (such as *Malassezia*), protozoa and viruses (Byrd et al. 2018), these organisms are not described here in detail. This thesis aims to focus on bacteriota as they dominate the human microbiome by mass and number.

## 1.3 The human eye as a microbial habitat

The microbiota of the eye's surface and the surrounding skin areas can be divided into the microorganisms of the ocular surface, the conjunctiva and cornea, as well as the adjacent skin areas, such as the meibomian glands, lid margins and eyelash bases.

The ocular microbial community is mostly composed of resident microorganisms which consist mainly of a few genera: most abundant are *Staphylococcus* species, while bacteria affiliated with *Corynebacterium*, *Pseudomonas*, *Acinetobacter*, *Propionbacterium (Cutibacterium)* and *Streptococcus*, as well as fungi have also been detected (Willcox 2013; Delbeke et al. 2021). Viruses have been found in the eyes of asymptomatic subjects as well, suggesting that they may persist in the eye, too (Doan et al. 2016). Similar to skin microbiota, it has been reported that the community composition varies between males and females, as well as with age and personal habits (Wen et al. 2017; Li et al. 2020).

For the intestine and the skin, symbiotic microbiota play a vital role in the regulation of host physiology, activation of the immune system and response to pathogens. The same is theorized for the eye microbiota (Li et al. 2020). However, the exposed position of the eyes to the environment increases the likelihood of pathogens entering and causing ocular infections and inflammation. Common eye infections include conjunctivitis (conjunctival inflammation), keratitis (corneal inflammation) or blepharitis (eyelid inflammation), mostly caused by bacteria or viruses. Fungal or parasitic diseases of the eye are also reported, though they are not discussed further here, considering that the focus of this thesis in context of the eye is on bacteria and, to some minor extent on viruses. Common microorganisms known to be affiliated with eye diseases are displayed in table 1.

Due to limited regenerative ability, immunological mechanisms causing tissue damage are not feasible for the eye. Therefore, immunological reactions have to be supressed or rather controlled, for instance by separating the eye from the immune system via the blood-retina barrier, but also with the ability to actively regulate and control the immune reaction, known as the ocular immune privilege (Caspi 2013).

***Table 1:*** *Frequent and common microorganisms, known to be affiliated with eye diseases.*

| **Potential pathogen** | **Origin** | **Eye disease** |
|---|---|---|
| *Cutibacterium acnes* | skin | endophthalmitis[1,2] |
| *Corynebacterium sp.* | environment/skin/ mucosa | different species known to cause endophthalmitis, conjunctivitis[3,4] |
| *Haemophilus influenzae* | mucosa (nasopharynx)[5] | conjunctivitis[8] |
| *Streptococcus pneumoniae* | respiratory tract | keratitis[6], conjunctivitis[8] |
| *Staphylococcus epidermidis* | skin | conjunctivitis, endophthalmitis[7] |
| *Staphylococcus hominis* | skin/axillae/pubic | endophthalmitis[8] |
| *Staphylococcus aureus* | environment/skin | blepharitis, endophthalmitis, keratitis, conjunctivitis[6,9] |
| *Neisseria gonorrhoeae* | urogenital tract | conjunctivitis[6] |
| *Pseudomonas aeruginosa* | ubiquitous | keratitis[10] |
| *Micrococcus luteus / sp.* | environment, skin | endophthalmitis[11], keratitis[12] |
| Human adenovirus (HAdV) | | conjunctivitis[13] |
| Herpes simplex virus | | keratitis[8] |
| Varicella zoster virus | | keratitis, conjuncivitis[13] |
| *Acanthamoeba* | environment/water/ poor hygiene using contact lenses | keratitis[14] |

*Table based on: 1) Cogen et al. (2008); 2) Ovodenko et al. (2009); 3) Kuriyan et al. (2017); 4) Hinić et al. (2012a); 5) Slack (2015); 6) Watson et al. (2018); 7) Flores-Páez et al. (2015); 8) Wong and Rhodes (2015); 9) O'Callaghan (2018), 10) Stern (1990); 11) Cartwright et al. (1990); 12) Taneja et al. (2019); 13) Ritterband and Friedberg (1998); 14) Marciano-Cabral et al. (2000)*

However, as a mechanism of mucosal defence against microbial infections, tears are an important component of the eye's innate antimicrobial response. They contain compounds such as lysozyme and lactoferrin, immunoglobulin A and lipocalin (Lu and Liu 2016; Hanstock et al. 2019), all with negative effects on pathogen growth and survival.

The combination of a homeostatic eye microbiome with antimicrobial agents plays an important role in preventing ophthalmic diseases. In addition, microbiomes from other parts of the body may also be involved in certain ocular diseases, e.g. the gut microbiome may be associated with uveitis and the oral microbiome with glaucoma (Lu and Liu 2016).

## 1.4 The role of frequently touched surfaces as fomites and for the spread of infectious diseases.

Dynamic and constant transfers are reported between the microorganisms of our body and devices used on a daily basis, and vice versa. Various studies have shown that there is a link between frequently touched objects and human bacteria, mostly originating from skin and epithelia. Consequently, these microbes have been found on items such as smartphones (Egert et al. 2015; Di Lodovico et al. 2018), tablets (Manning et al. 2013), public keypads (Bik et al. 2016), keyboards (Anderson and Palombo 2009) and others in rather high percentages. Many of these bacteria may also carry antibiotic resistant potential (Brady et al. 2007; Anderson and Palombo 2009; Di Lodovico et al. 2018; Cave et al. 2019; Gohli et al. 2019). A systematic review by Pace-Asciak and colleagues (2018) revealed that healthcare professionals' neckties are contaminated with bacteria originating from mostly skin and the environment. In addition, pathogens associated with hospital-acquired infections have been found on contaminated healthcare textiles (Owen and Laird 2020). An overview of bacterial counts on typical medical items used on a daily basis is displayed in table 2.

Although a few epidemiological investigations have confirmed fomite transmission causing, for instance, a norovirus outbreak (Stephens et al. 2019), studies about a proven bacterial infection directly related to surface contact are scarce. It is rather difficult to associate a major disease outbreak with exactly one particular surface and the related bacterial transfer, as many of the (bacterial) pathogens do not cause rapid and severe illnesses (Di Battista et al. 2021). Nevertheless, transmission of pathogens is likely to occur because they are present in high numbers on different surfaces and are persistent for longer periods of time. In particular, sites regularly touched by human hands must consequently be regarded as fomites, i.e. "*inanimate objects that become colonized with microbes and serve as potential intermediaries for transmission to/from humans*" (Stephens, 2019).

Fomites are of particular concern in clinical environments (Weber et al. 2010; Christoff et al. 2019) or in long-term care facilities, such as nursing homes, promoting the spread of infectious diseases, which is especially problematic for ill, elderly or otherwise immunocompromised persons.

Clearly, the most important vectors for microbial transmission are human hands. There is a known infection risk when the hand touches facial areas and mucous membranes, such as the eye, mouth and nose (Nicas and Best 2008; Zhang et al. 2020).

***Table 2:*** *Overview of total bacterial counts (CFU = colony forming units) on different daily used items in clinical- and non-clinical environments. [*]CFU/internal surface of the shirt pocket; [+]CFU/Key; [#]CFU/front of necktie.*

| Item | Average bacterial counts (CFU $cm^{-2}$) |
|---|---|
| **Shopping trolley[1]** | 753 |
| **Mobile phone[2,3]** | 1 - 10 |
| **Paper-money[4]** | 10 - 25000 |
| **Computer touchscreens[5]** | 3 |
| **Computer keybord[6]** | 6 - 430[+] |
| **Hospital bedrails[7]** | 30 |
| **Doctor's shirts[8]** | 51[*] |
| **Doctor's neck ties[8]** | 95[#] |
| **Slit lamp lenses[9]** | 71 |
| **Slit lamp ophthalmology helmet[9]** | 3 |

*Table based on: 1) Carrascosa et al. (2019); 2) Egert et al. (2015); 3) Di Lodovico et al. (2018); 4) Vriesekoop et al. (2010); 5) Gerba et al. (2016); 6) Anderson and Palombo (2009); Messina et al. (2011); 7) Schmidt et al. (2019); 8) Lopez et al. (2009); 9) Sobolewska et al. (2018)*

Hospital-related studies have revealed that most cross-contamination occurs through the hands of healthcare workers when they touch other patients or contaminated surfaces (Pittet et al. 2006; Weber et al. 2010). In 2017, 8.3% of all European patients in an intensive care unit caught hospital-acquired infections (HAI) (EDC – European Centre for Disease Prevention and Control 2019). Over 2.5 million patients suffer from HAI each year in the European Union/European Economic Area (Cassini et al. 2016). For surgical site infections, the percentage varied between 0.5% and 10% (EDC – European Centre for Disease Prevention and Control 2019). It is estimated that about 20% to 40% of HAI in intensive care units are caused by hand-to-hand transmission (Weinstein 1991).

Multidrug-resistant organisms (MDRO) are a particular problem in clinics. Over the last two decades, the density of MDRO among pathogens has increased substantially

(Remschmidt et al. 2017), while 10% of acute care patients acquire multi-drug resistant microorganisms during their stay (Cao et al. 2016).

Juthani-Mehta and Quagliarello (2010) posit that similar assumptions may apply for nursing homes for elderly people or long-term care facilities. Nursing home residents often suffer from multiple comorbidities alongside a generally weaker immune system, sometimes cognitive and functional decline and a poorer hygiene. They may also require invasive medical devices, often reported to carry (antibiotic-resistant) pathogens. Given that inhabitants often share rooms and regularly used devices and come into contact with different healthcare workers and visitors, this may result in a generally high risk of acquiring infectious diseases.

## 1.5 The potential role of spectacles and other ophthalmologically relevant surfaces as fomites

In Germany, 67% of all adolescents and adults need spectacles to aid vision. About 30% of them also or exclusively use spectacles for monitor-based work. Additionally, 80% of all persons wear sunglasses and about 10% use special glasses for sportive activities (Zentralverband der Augenoptiker und Optometristen 2019). Therefore the global eyewear market is a constantly growing field, with the spectacle lenses sector accounting for almost the half of the market (Lüdemann 2021).

In addition to vision aids, work-related protective eyewear and face shields are often worn to protect against eye injuries and (eye) infections, while 3D glasses or video eyewear are used during leisure time activities.

Besides eyewear, other devices come into the vicinity of the eye, such as slit lamps, phoropters or tonometers, used for medical ocular examinations. Microscopes are especially interesting, as extremely widespread analytical instruments, present in almost all clinical and/or biological laboratories. Many of these devices are used by more than one person. A study (Reigoto et al. 2021) on the application of microscopy (mostly bright field and fluorescence) in scientific publications revealed, that almost 50% of pharmaceutical publications and almost all the articles (97%) in cell biology journals reported use of this technique. This indicates the importance of these instruments, at least in several fields of research.

In summary, ophthalmologically relevant objects are widely used in various applications, which is why it is necessary to consider their hygienic relevance. Due to their close contact with the human body, these items are thought to be contaminated with a diverse microbiota. The microbial contamination of eyewear and other optical instruments is potentially influenced by several factors (figure 2), such as sharing or individual use, the general setting (e.g. personal space, hospital environment, personal microbiota and hygiene of users), cleaning and cleaning intervals. Common sources of microbial contamination are typically human skin (touching or direct skin contact), mucosa (coughing, breathing) or eye-related body parts, such as eyelids or eyelashes (direct contact). But an environmental origin is also possible (wind, dust). Transmission of pathogens from inanimate objects to the human body and vice versa is likely, too.

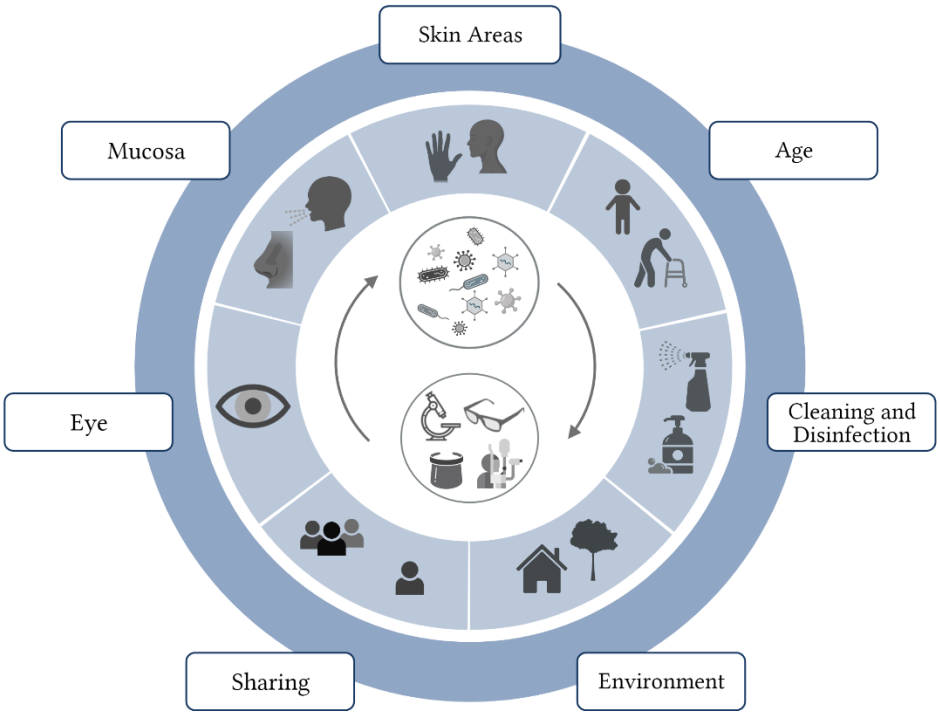


***Figure 2:*** *Factors influencing the microbial composition of ophthalmologically relevant items. Own illustration, created using biorender.com.*

However, until now, comprehensive studies on microbial contamination of spectacles and other ophthalmologically relevant objects have been scarce, and therefore little is known about their role as fomites.

One of the few cultivation-based studies by Giusti and collegues (2015) addressed the microbiological load of glasses distributed at 3D movie theatres, as a case of eye-irritation was reported after use. These spectacles were examined prior to and after use, and were found to be 8 times more contaminated with bacteria and fungi afterwards.

Protective or magnifying eyewear in clinical environments might be of special concern. Previous cultivation-based studies revealed a significant and diverse bacterial load on surgeons' loups and eyeglasses (Butt et al. 2012; Graham et al. 2019) and slit lamps' reusable tonometer tips (Hillier and Kumar 2008; Sobolewska et al. 2018). Bacteria were also detected at moderate (20 - 100 CFUs per item) to high numbers (>100 CFUs per item) at dental loupes (Zwicker et al. 2019) and generally in areas where splashes or aerosols occur, such as in dental treatments or in operating rooms. Lange (2014) concluded that eyewear may prevent bacterial transmission into the eye, but also may act as a potential reservoir for microorganisms causing nosocomial infections.

All of the studies revealed that optical items are contaminated with significant amounts of bacteria. Consequently, it was recommended that these devices be disinfected properly on a regular basis.

Hygienic and health-related challenges posed by spectacles in non-clinical settings and for healthy people might be moderate. Spectacles are usually used by single persons and probably predominantly contain a person's own microbial community. However, if considered as fomites, they might be problematic in clinical environments and for infection-susceptible groups of persons, such as immunocompromised or elderly people. In addition, spectacles serving as a reservoir for antibiotic-resistant strains are conceivable. Nevertheless, sharing optical devices can be considered more precarious, given the risk of recurrent eye infections. A previous cultivation-based study (Olcerst 1987) suggested that direct contact with microscope eyepieces significantly increases the risk of reoccurring eye infections, such as conjunctivitis.

## 1.6 Aim and objectives of the thesis

Generally, there are no or only a few scientifically-based findings about the microbial load on ophthalmologically relevant surfaces. Therefore, the aim of the thesis was to identify and quantify the microbial load and the composition of personal-use spectacles

and ophthalmologically relevant devices, with an extended focus on different groups of spectacle wearers, as well as on shared and clinically relevant objects.

The outcome of this thesis is relevant for all spectacle wearers and consumers, but also for groups of persons that have frequent contact with such surfaces, such as opticians, ophthalmologists, hygienists or healthcare professionals.

This thesis provides a solid basis to better evaluate the hygienic relevance of optical devices. It reveals the most important bacterial taxa on optical devices, which allows for more precisely designed antibacterial efficacy evaluations, cleaning tests or antimicrobial coatings and it recommends suitable cleaning measures.

The specific objectives of this thesis were

i) to establish a robust library preparation protocol for high throughput 16S rRNA gene amplicon sequencing from little DNA input material. Harvesting sufficient template DNA for such analyses is challenging, especially since most ophthalmic surfaces are smooth and relatively small.

ii) to comprehensively analyse the spectacle bacteriota using cultivation-dependent techniques, as well as cultivation-independent high throughput 16S rRNA gene amplicon sequencing.

iii) to analyze the effect of environmental parameters on the spectacle microbiota (age of spectacle wearers, sampling site).

iv) to investigate the antimicrobial efficacy of common spectacle cleaning methods.

v) to analyze similar ophthalmologically relevant reference surfaces (microscope oculars, slit lamps) with the established protocol.

vi) to evaluate, whether ophthalmologically relevant surfaces might serve as reservoirs for antibiotic-resistant bacteria.

The following summary highlights the main findings of publications I - IV, which are subsequently placed in a larger context. For detailed descriptions of the methods and discussion of the results, please see the individual papers. Each of the above mentioned objectives were addressed in the individual papers.

In **publication I** the spectacle bacteriota a was analysed with MALDI-TOF MS-based identification (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) of isolated bacteria, with the aim of quantifying the living bacteria. From this, statements could be made about the pathogenic potential of the spectacle surface. It was assumed that spectacle hygiene might be more important for elderly people due their weaker immune systems. Furthermore, the potentially impaired vision of elderly persons was assumed to result in less frequent spectacle cleaning and a higher bacterial load. Therefore, the spectacles of two different groups of persons (younger vs. older spectacle wearers) were analysed. Additionally, the antimicrobial efficacy of four widespread spectacle cleaning methods was investigated by using test bacteria that had previously been identified as dominant on these items.

**Publication II** focused on the comprehensive analysis of the spectacle bacteriota at different spectacle sites using high throughput 16S rRNA gene amplicon sequencing, to better account for aerotolerant anaerobes, slow growing and yet-uncultivated bacteria.

As bacterial transmission between human beings is more likely if surfaces are touched by different persons, **publications III and IV** analyzed microscope oculars and slit lamps as reference surfaces to spectacles. These items are used widely in many laboratories or clinical areas. Slit lamps are of certain concern for the diagnosis of eye diseases and are shared by different persons (doctors and patients), which increases the probability of transmitting potentially pathogenic microbes. In **publication III** polyphasic approaches using cultivation and molecular methods were applied. **Publication IV** also addressed antibiotic-resistant bacteria on slit lamps.

Additional, yet unpublished, research (**chapter 2.5**) focused on establishing an assay to determine the load of human adenovirus on spectacles, a known pathogen for highly contagious and severe keratoconjunctivitis.

# 2 Results

The following section contains four original publications, published in international peer-reviewed journals, referred to with roman letters within the thesis. The articles are reprinted with permission from the respective journals. These publications summarize the research conducted to answer the research questions introduced in the first section.

## 2.1 Publication I: A view to a kill? – Ambient bacterial load of frames and lenses of spectacles and evaluation of different cleaning methods

Fritz, B.*, Jenner, A.*, Wahl, S., Lappe, C., Zehender, A., Horn, C., Blessing, F., Kohl, M., Ziemssen, F. and Egert, M., *PLoS ONE.* 13 (11), e0207238, 2018.
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PLOS ONE

RESEARCH ARTICLE

# A view to a kill? – Ambient bacterial load of frames and lenses of spectacles and evaluation of different cleaning methods

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## Abstract

Surfaces with regular contact with the human body are typically contaminated with microorganisms and might be considered as fomites. Despite spectacles being widespread across populations, little is known about their microbial contamination. Therefore, we swab-sampled 11 worn spectacles within a university setting as well as 10 worn spectacles in a nursing home setting. The microbial load was determined by aerobic cultivation. All spectacles were found to be contaminated with bacteria, with nose pads and ear clips having the highest density, i.e. at sites with direct skin contact. Summed over all sites, the median microbial load of the university spectacles ($1.4 \pm 10.7 \times 10^3$ CFU $cm^{-2}$) did not differ significantly from the spectacles tested in the nursing home ($20.8 \pm 39.9 \times 10^3$ CFU $cm^{-2}$). 215 dominant bacterial morphotypes were analyzed by MALDI biotyping. 182 isolates could be assigned to 10 genera, with *Staphylococcus* being the most common. On genus-level, bacterial diversity was greater on nursing home spectacles (10 genera) compared to the university environment (2 genera). Four cleaning methods were investigated using lenses artificially contaminated with *Escherichia coli*, *Micrococcus luteus*, a 1:2 mixture of *E. coli* and *M. luteus*, and *Staphylococcus epidermidis* (the dominant isolate in our study), respectively. Best cleaning results (99% -100% median germ reduction) were obtained using impregnated wipes; dry cleaning was less effective (85% -90% median germ reduction). Finally, 10 additional worn university spectacles were cleaned with wipes impregnated with an alcohol-free cleaning solution before sampling. The average bacterial load was significantly lower ($0.09 \pm 0.49 \times 10^3$ CFU $cm^{-2}$) compared to the uncleaned university spectacles previously investigated. Spectacles are significantly contaminated with bacteria of mostly human skin origin—including significant amounts of potentially pathogenic ones and may contribute to eye infections as well as fomites in clinical environments.

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## Introduction

The human body is colonized by approximately $10^{13}$ microorganisms. Cell densities on the human skin can vary from $10^2$ $cm^{-2}$ up to $10^6$ $cm^{-2}$ [1, 2]. Therefore, surfaces regularly touched by humans or those in close contact with the human body can consequently become contaminated with microorganisms and these surfaces can be considered fomites. For instance, mobile communication devices and the touchscreens of computers, tablets, and smartphones are notorious for contributing to fomites in clinical environments [3, 4]. Recently, we reported the ambient bacterial load of smartphone touchscreens from a non-clinical university environment [5]. Uncleaned touchscreens were just moderately (1.37 CFU (colony forming unit) $cm^{-2}$) contaminated with bacteria of mostly human origin, including significant amounts of potentially pathogenic ones. Cleaning with alcohol-impregnated lens wipes effectively reduced bacterial contamination by 96%, thereby lowering any potential risk of infection.

Spectacles are globally widespread optical devices that aid human vision. Due to their environmentally exposed position in the center of the human face, their close contact to the human skin, nose and mouth and regular contact with human hands, it is safe to assume that spectacles are contaminated with microorganisms. Thus far, only a few studies have analyzed the microflora of spectacles. In clinical environments, surgeons' spectacles were identified as fomites [6]. Their spectacles were highly contaminated with *Staphylococcus epidermidis* and it has been suggested that this represents a risk to patients during operations. Consequently, it was recommended that surgeons disinfect their spectacles on a regular basis. Another recent study [7] addressed the microbiological safety of glasses distributed at 3D movie theatres and the study compared manual vs. automated sanitation systems. The spectacles under investigation were discovered to be contaminated with bacteria and fungi, however, the study did not clearly recommend an effective sanitation system.

In this study, we quantified the microbial load of 31 worn spectacles at 7 different sampling sites, each, and subsequently identified the dominant bacteria. 11 spectacles were obtained from a university environment, 10 spectacles from a nursing home and another 10 spectacles were obtained from the university environment, but were cleaned prior to investigation. In effect, we analyzed spectacles from two different populations–from students and employees at a non-clinical university environment and from inhabitants of a local nursing home. Moreover, we investigated the antimicrobial efficacy of 4 widespread spectacle cleaning methods by using test bacteria that had been previously identified as being dominant on spectacles and smartphone touchscreens. To the best of our knowledge, this is the most comprehensive study on the microflora of spectacles thus far. This study intends to create a solid basis that will invite a deeper understanding of the hygienic relevance of these widespread objects and of the evaluation of suitable cleaning and disinfection measures.

## Material and methods

### Microbial load of worn spectacles

The sampling of worn spectacles was performed in Villingen-Schwenningen, Baden-Württemberg, Germany during the summer of 2015. The spectacles for swab-sampling were kindly provided by 11 students and employees (mean age ± standard deviation: 43.8 ± 19.8 a, 8 females and 3 males) of Furtwangen University, Campus Villingen-Schwenningen, and 10 inhabitants of a local nursing home (mean age: 89.6 ± 6.3 a, 10 females). Spectacles and usage data were provided voluntarily. Informed consent to use the obtained data for scientific purposes was obtained orally. The sampling done in the nursing home was communicated and supported by the directorate of the institution. Personal data of the participants was not recorded, rendering

it impossible to assign spectacle microbiota to a specific wearer. Moreover, the spectacle wearers provided neither directly health-related data, nor were the analyses aimed at detecting directly health-related bacteria, such as obligate pathogens or MRSA. Therefore, we believe that the study was performed in an ethically appropriate manner.

Sampling was performed in the field, i.e. within the university and nursing home. Each pair of spectacles was sampled at 7 sites: lenses (left and right, front side and back side, respectively), ear clips (left and right side, respectively) and nose pads. The area of each sampled site was calculated by measuring the geometry of the spectacles. Microbial loads were determined according to DIN 10113–1:1997–07 –Part 1 [8]. Surfaces were meander sampled using sterile cotton swabs (Deltalab, Barcelona, Spain). Each area was sampled twice, first with a wet swab and then with a dry swab. A sterilized medium (wetting medium) containing 1.5 g of casein peptone (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and 12.75 g of sodium chloride (Carl Roth) per 1500 ml of water was used for wetting the swabs and for subsequent dilution series. After sampling, wet and dry swab heads were combined and microorganisms were extracted by rigorous shaking in a defined volume of wetting medium. The volume of wetting medium used for extraction depended on the sampled area: 2 ml of medium were used per 1 $cm^{-2}$. Germ numbers were determined from that suspension by serial decadal dilution and subsequent plating of 50 μl of each dilution on Tryptic Soy Agar with neutralizers (TSA; Carl Roth). Germ numbers were determined after aerobic incubation at 37˚C for 3 d, referred to the sampled area, and then expressed as colony-forming units (CFU) $cm^{-2}$.

## Identification of microbial isolates by MALDI biotyping

In order to get an overview of the microbial diversity on worn spectacles, a representative of each microbial morphotype was isolated per sampling site and spectacle, respectively, i.e. from the agar plates used for quantification of the microbial load. Morphotypes were visually differentiated based on colony size, color and morphology. Selected colonies were repeatedly T-streaked on TSA-Agar, cultivated at 37˚C, and then controlled for morphotype purity. A colony of each overnight culture was then suspended in 300 μl of sterile water and stored at -80˚C until further analysis.

The identification of isolated microorganisms was performed by means of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis using the MALDI Biotyper system (MALDI Biotyper Microflex, Bruker Daltonics GmbH, Bremen, Germany) according to the manufacturer's instructions. One day prior to the analysis, proteins were extracted from the frozen colony samples following the recommended protocol for ethanol-formic acid extraction [9]. The volumes of formic acid (Carl Roth) and acetonitrile (Carl Roth) were adapted as specified in the protocol for single, small colonies. Protein extracts were stored at 4˚C until further analysis. Subsequently, 0.7 μl of each protein extract was spotted onto the Biotyper steel target. After air drying, the samples were covered with 1 μl of alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonics), air dried again, and then measured. The fingerprint profiles that were obtained were matched against the internal MALDI Biotyper reference library (software version 3.3.1.0, 4613 entries). Similarities were expressed as score values ranging from 0.0–3.0. According to the manufacturer, scores $\geq$ 1.7 indicate secure genus identification, scores $\geq$ 2.0 indicate secure genus and probable species identification.

## Standardized cleaning tests

Cleaning tests were performed with *Escherichia coli* K12 (DSMZ 498), *Micrococcus luteus* (DSMZ 1605) and *Staphylococcus epidermidis* (DSMZ 20044) as test bacteria, obtained from the Leibniz Institute DSMZ (DSMZ—German Collection of Microorganisms and Cell

Cultures, Braunschweig, Germany). Bacteria were grown aerobically in liquid Lysogeny Broth (LB, Carl Roth). To determine the viable count, bacteria were decadal diluted in wetting medium. 50 μl of each suspension were plated on TSA-Agar with neutralizers and then were incubated aerobically at 37˚C. For further analyses, cells were adjusted to densities of $4 \times 10^8$ CFU $ml^{-1}$ (*E. coli*, *S. epidermidis*) and $4 \times 10^{10}$ CFU $ml^{-1}$ (*M. luteus*), respectively. *E. coli* and *M. luteus* were also used in a 1:2 mixture of these cell suspensions.

Test lenses typically used for spectacles (CR39 Index 1.5 with LotuTec Coating) were provided by Carl Zeiss Vision International GmbH (Aalen, Germany). Before and after microbiological testing, lenses were sterilized with 70% ethanol and a 20 min UV-C-treatment (253.7 nm).

All cleaning tests were performed under a laminar flow workbench. Lenses were fully submerged in the bacterial test suspensions for 1 minute and then dried for 30 minutes at room temperature. Subsequently, one half of each glass was cleaned while the other half of the glass remaining uncleaned. Standardized cleaning was performed by wiping the contaminated side of the glass five times up and five times down. 4 different cleaning products, representing widely used spectacle cleaning methods were investigated: 1. Cellulose-based, alcoholic lens cleaning wipes impregnated with ethanol and isopropanol (AN); 2. Cellulose-based, alcohol-free lens cleaning wipes impregnated with an amine-containing cleaning solution (A); 3. Dry cellulose-tissues without a cleaning solution (C); 4. Dry, fine-grained microfabric clothes (M). All cleaning products were supplied by Carl Zeiss Vision International GmbH. 1 minute after cleaning, swab sampling and quantification of bacteria from the cleaned and uncleaned glass sides was performed as described above. Cleaning tests were repeated 10 times for each test bacterium and each cleaning product, respectively.

In order to investigate the effect of cleaning naturally contaminated spectacles, 10 worn spectacles of students and employees (mean age: 40.6 ± 16.1 a) from a university environment were cleaned intensively by wiping the frames, nose pads and lenses with alcohol-free lens cleaning wipes (A). Subsequently, the microbial load was determined using the method described above.

### Statistical analyses

All microbial load data were expressed in the median ± interquartile range (IQR, between the 25% and 75% quartile). Statistical analyses (plots and statistical tests) were performed using R 3.4.1 [10]. Cleaning tests were examined for statistical significance between the microbial load of the cleaned and the uncleaned glass sides using the Wilcoxon signed rank test for differences between paired samples. The antimicrobial efficiency of different cleaning products and the microbial load of worn (and cleaned) spectacles of university members and nursing home inhabitants were compared using Wilcoxon-Mann-Whitney-U tests for independent samples. All tests were two-sided and p-values $< 0.05$ were considered to represent significant results. No adjustment of p-values for multiple testing was performed as the study was considered an exploratory pilot study.

## Results and discussion

The aim of this study was to investigate site-dependent microbial loads on worn spectacles using cultivation-based techniques. In addition, we investigated four different cleaning techniques for their efficacy in reducing microbial loads on spectacle lenses.

### Microbial load of worn spectacles

While many related studies from (non-) clinical and healthcare environments report diverse contamination rates of, for instance, mobile communication devices [3, 4, 6], quantitative data

on the microbial load of spectacles are scarce. For the first time, our study provides a comprehensive examination of spectacle microbiota by aerobic cultivation. We swab-sampled twenty-one spectacles of adult university students or staff (n = 11) and nursing home residents (n = 10).

Bacteriological analyses indicated that all spectacles were contaminated by bacteria. Summed across all sample sites, we determined a median microbial load of $1.4 \pm 10.7 \times 10^3$ CFU $cm^{-2}$ found on the university spectacles and a median of $20.8 \pm 39.9 \times 10^3$ CFU $cm^{-2}$ on the nursing home spectacles.

We did not find a statistically significant difference between the microbial loads of the two investigated environments ($p = 0.0821$; Fig 1), thereby confirming findings of Leyden and colleagues [11], whom did not find differences between the quantity of aerobic and anaerobic bacteria found on the foreheads and cheeks of adults and elderly people.

In both environments, the highest cell counts were found on the nosepads (median of $0.16 \pm 2.0 \times 10^3$ CFU $cm^{-2}$ on the university spectacles and a median of $2.6 \pm 14.1 \times 10^3$ CFU $cm^{-2}$ on the nursing home spectacles) while the lowest cell counts were measured on the lenses (median of $0.04 \pm 0.08 \times 10^3$ CFU $cm^{-2}$ on the university spectacles and a median of $0.23 \pm 0.28 \times 10^3$ CFU $cm^{-2}$ on the nursing home spectacles, with a significant statistical difference: $p = 0{,}015$). This finding matches our assumption that lenses may be more contaminated in the nursing home environment than in a university setting. Due to potentially impaired vision, elderly people may clean the lenses of their spectacle less often or less effectively than younger people with potentially better vision.

Recent studies [12–14] showed that the area behind the ears (retroauricular crease), the sides of the nostrils (alar crease) and the forehead represent the highest density of microbial colonization on the human face. The detected colony counts on nosepads and earclips were indeed similar to colony counts typically found on the human forehead ($10^3$ CFU $cm^{-2}$) and the scalp and cheek (about $10^4$ CFU $cm^{-2}$) [15]. Cell densities on spectacle lenses were low in comparison to cell densities on hands, skin, earclips and nosepads. This low count could be due to the smoothness of the lens surfaces, regular cleaning measures, as well as distance to the facial skin.

## Identification of microbial isolates by MALDI biotyping

MALDI-TOF fingerprints of 215 dominant bacterial isolates from all spectacles were generated by MALDI biotyping and used for species and genus-level differentiation. 182 out of 215 isolates could be securely assigned to 10 genera and 12 species. We found a higher number of genera on the spectacles of nursing home inhabitants (10 genera) than on those of the university members (2 genera). Previous studies [16] showed an effect of age on microbial community structure and richness on forehead and scalp, with higher diversity found on elderly individuals. This finding could be based on changes in hormone balances, pH value, and the sebum production [2, 16, 17]. Fig 2 shows the identified genera and the relative abundance of affiliated isolates.

The observed taxa mainly represent well-known colonizers of the human skin, such as staphylococci, micrococci, corynebacteria, brevibacteria or *Acinetobacter* sp. [18]. Staphylococci dominated the aerobic, cultivable spectacles microbiota with *Staphylococcus epidermidis* being the most common representative. Studies [19] have shown that skin areas with direct contact to spectacles, such as the retroauricular crease or the alar crease, are mainly colonized by corynebacteria and propionibacteria and are only sparsely colonized by staphylococci. Given that spectacles represent an aerobic environment, our study was performed under aerobic cultivation conditions. Aerotolerant anaerobes will likely not thrive well under such conditions [20]. Consequently, we did not detect any propionibacteria and we only detected a single isolate of *Corynebacterium* sp. Corynebacteria are aerobic, yet slow growing bacteria, and may have been outcompeted by staphylococci under the used cultivation conditions.

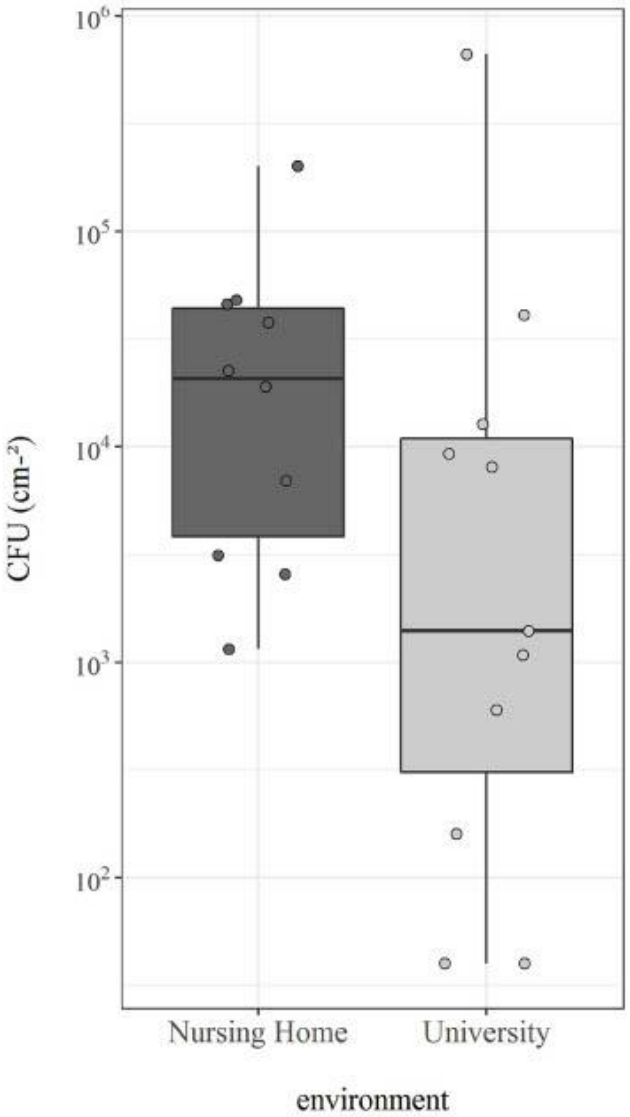


**Fig 1. Box-whisker plot showing the determined microbial counts (CFU cm$^{-2}$) of the two investigated environments (nursing home, n = 10, and university, n = 11).** Displayed are the median, 25% and 75% quartiles and outliers (open circles). Whiskers represent the lowest and highest microbial counts within the 1.5 fold of the interquartile range (IQR) (the 25% and 75% quartile). An observation is marked as an outlier if it was more than 1.5 times of the IQR away from the 25% or 75% quartile, respectively.

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*S. epidermidis* is a typical skin commensal, colonizing predominantly the axillae, palms, head and nares [15]. Typically, it does not have strong pathogenic potential, rather, it maintains a commensal or even beneficial relationship with its host. Nevertheless, *S. epidermidis* can also lead to severe infections. In addition, it might represent a reservoir for antibiotic resistant genes, which can be transformed into the closely related and more virulent *S. aureus* [21]. With

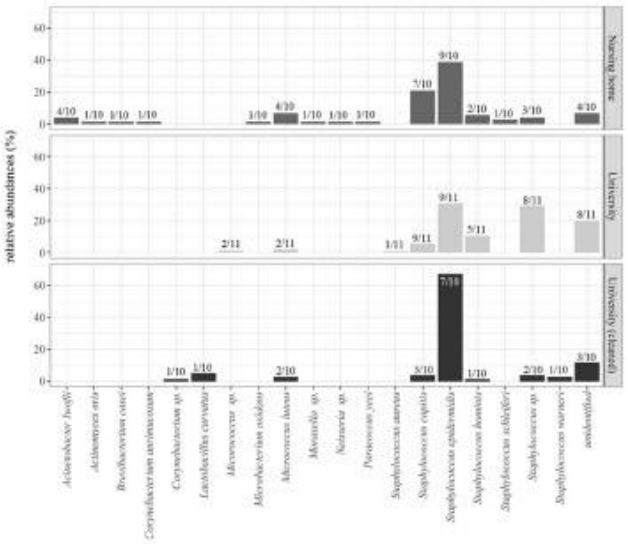


**Fig 2. Barplot of identified bacterial taxa isolated from spectacles of two environments.** Bars show the relative abundance of isolates from university spectacles (n = 11, 143 isolates), nursing home spectacles (n = 10, 72 isolates) and cleaned university spectacles (n = 10, 76 isolates). "Unidentified" indicates a MALDI identification score below 1.7, where a reliable identification of genus-level was not made possible. Numbers (x/n) on top of the bars indicate the number of spectacles that the respective taxon was detected on.

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*Staphylococcus aureus*, *Staphylococcus epidermidis* and others, we identified many species known to comprise antibiotic resistant strains [22], such as MRSA (*Methicillin-resistant Staphylococcus aureus*) or MRSE (*Methicillin-resistant Staphylococcus epidermidis).* Hence, further investigations should examine spectacles as carriers of antibiotic resistant bacteria in more detail, an area which could be of particular hygienic relevance in clinical environments.

In order to address the overall pathogenic potential of spectacle microbiota, the identified bacterial species were classified into biosafety risk groups (RG). 60% of the identified bacteria from university spectacles and 64% of the bacteria from nursing home spectacles were affiliated with risk group 2 (RG 2), such as *S. epidermidis*, *S. hominis*, *S. aureus* and *S. schleiferi*. The remaining species were classified as non-pathogenic (RG1). RG 2 organisms are harmless for those with intact immune systems but could cause severe diseases for newborns, immunocompromised patients, pregnant women, or elderly persons.

*S. epidermis*, *S. hominis* and *S. aureus* are also related to eye diseases. For instance, both endolphtalmitis and conjunctivitis are often caused by *S. epidermidis* [21]. *S. hominis*, and *S. aureus* had also been associated with intraocular and external ocular infections [23].

Overall, we have shown that spectacles are contaminated with bacteria of mostly human skin origin, including potentially pathogenic ones and should therefore be considered fomites.

## Standardized cleaning tests

Cleaning tests were performed with *Micrococcus luteus* (as a gram positive representative), *Escherichia coli* K12 (as a gram negative representative), a 1:2 mixture of *E. coli* and *M. luteus* and *Staphylococcus epidermidis*. The latter was chosen as it was the dominant spectacle isolate in our study. Test lenses were artificially contaminated with bacteria and then cleaned using 4 different cleaning procedures.

All tested procedures removed a significant amount of bacteria from lens surfaces ($p < 0,001$). Fig 3 shows the measured germ reductions.

Across all test bacteria, the impregnated cellulose wipes (A and AN) showed the most germ reduction (means of 99% - 100%). The efficacy of the alcoholic lens wipes (A) was slightly higher when compared to the alcohol-free formulation (AN), however, differences were not significant ($p = 0,228–0,746$).

Dry cleaning with cellulose tissues (C) and microfabric clothes (M) showed reduced cleaning success (mean germ reduction of 85%– 90%) when compared to cleaning with the impregnated wipes. The observed differences between these products (dry and impregnated) were mostly statistically significant ($p < 0.05$, $p < 0.01$, respectively). Evidently, wet cleaning was more effective than pure mechanical cleaning with dry wipes.

The cleaning performance of the alcohol-impregnated wipes is presumably largely based on their ethanol or isopropyl alcohol content, which are well known antimicrobials. Ethanol and isopropyl alcohol destroy the bacterial cell wall and plasma membrane by denaturing proteins and dissolving lipids, leading to subsequent interference with metabolism and cell lysis. Alcoholic formulations represent effective antimicrobial agents against vegetative bacteria, mycoplasms, fungi and viruses [24].

The investigated non-alcoholic lens wipes contain detergents such as alkyl-dimethylamine oxides, which have also been shown to have antimicrobial effects. These substances act as amphoteric surfactants by disrupting and perturbing the bacterial cell membrane [25]. Consequently, these wipes showed similar cleaning efficacy compared to the alcohol impregnated wipes. However, using non-alcoholic products to clean spectacles may be favorable so as to protect sensitive parts of the spectacles, such as the frame material, from damage.

In order to verify the measured germ reduction for naturally contaminated spectacles, 10 worn spectacles from university staff and students were, prior to swab-sampling, thoroughly cleaned using wipes impregnated with the alcohol-free cleaning solution. Following cleaning, 3 spectacles showed no bacterial contamination at all, 4 spectacles showed slight contamination on the nosepads and earclips and 3 spectacles showed germs on the lenses. Compared to the total germ count of uncleaned spectacles from university members (see above), we calculated 94% less bacteria on the cleaned spectacles (median $0,09 \pm 0.49 \times 10^3$ CFU $cm^{-2}$). In addition, we identified less but largely the same species, on university and nursing home spectacles. We also found two additional species, *Lactobacillus curvatus* and *Staphylococcus warneri*, which also represent typical human skin commensals (Fig 2).

## Conclusion

Our results provide the first insight into the aerobic and cultivable spectacle microbiota. All investigated worn spectacles were found to be contaminated by bacteria of mostly human skin origin, in particular at sites with direct skin contact. The bacterial community was highly dominated by staphylococci, in particular *S. epidermidis*. No propionibacteria were found, which is likely due to the aerobic cultivation conditions. Many of the identified bacteria represented potential pathogens and some of them are known to cause skin and eye diseases. Hence, spectacles should certainly be seen as fomites, particularly in clinical environments where transmission of pathogens could occur through spectacle contamination. In addition, spectacles could represent a reservoir for pathogens causing recurring eye infections. However, we also demonstrated that superficial cleaning with impregnated lens wipes can reduce microbial load by ~ 2 log scales and thus help prevent bacterial transfer.

For future investigation we will conduct 16S rRNA gene next generation sequencing based analyses of spectacle microbiota in order to better account for aerotolerant anaerobic, slow-

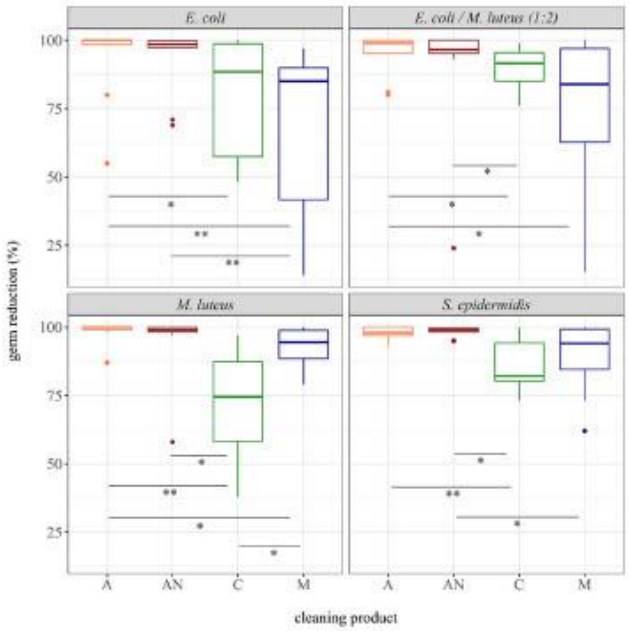


**Fig 3. Box-whisker plot showing the relative germ reduction of 4 different cleaning procedures, calculated from the microbial load before and after the cleaning.** A (orange) = cellulose-based, alcoholic lens cleaning wipes; AN (red) = cellulose-based, alcohol-free lens wipes, C (green) = dry cellulose-tissue; M (blue) = fine-grained microfabric cloth (n = 10 for all test bacteria and cleaning products, respectively). Displayed are the 25% and 75% quartiles, median and data outliers (open circles). Whiskers represent the lowest and highest microbial counts within the 1.5 fold of the interquartile range (IQR) (the 25% and 75% quartile). An observation is marked as an outlier if it was more than 1.5 times of the IQR away from the 25% or 75% quartile, respectively. Asterisks mark a statistically significant difference between the cleaning products: * $p < 0.05$; ** $p < 0.01$.

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growing and yet-uncultured microorganisms. In addition, particular emphasis will be placed on spectacles as carriers of multi-resistant bacteria in clinical and nursing environments.

## Supporting information

**S1 Table. Germ counts of the spectacles at the different sample sites and environments.**
(XLSX)

**S2 Table. Number of isolates from the spectacles and the identified bacterial taxa.**
(XLSX)

**S3 Table. Germ counts of the cleaning tests.**
(XLSX)

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## 2.2 Publication II: Site-specific molecular analysis of the bacteriota on worn spectacles

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# Site-specific molecular analysis of the bacteriota on worn spectacles

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**Regularly touched surfaces are usually contaminated with microorganisms and might be considered as fomites. The same applies for spectacles, but only little is known about their microbial colonization. Previous cultivation-based analyses from our group revealed a bacterial load strongly dominated by staphylococci. To better account for aerotolerant anaerobes, slow growing and yet-uncultivated bacteria, we performed an optimized 16S rRNA gene sequencing approach targeting the V1-V3 region. 30 spectacles were swab-sampled at three sites, each (nosepads, glasses and earclips). We detected 5232 OTUs affiliated with 19 bacterial phyla and 665 genera. *Actinobacteria* (64%), *Proteobacteria* (22%), *Firmicutes* (7%) and *Bacteroidetes* (5%) were relatively most abundant. At genus level, 13 genera accounted for 84% of the total sequences of all spectacles, having a prevalence of more than 1% relative abundance. *Propionibacterium* (57%), *Corynebacterium* (5%), *Staphylococcus* (4%), *Pseudomonas*, *Sphingomonas* and *Lawsonella* (3%, each) were the dominant genera. Interestingly, bacterial diversity on the glasses was significantly higher compared to nosepads and earclips. Our study represents the first cultivation-independent study of the bacteriota of worn spectacles. Dominated by bacteria of mostly human skin and epithelia origin and clearly including potential pathogens, spectacles may play a role as fomites, especially in clinical environments.**

About 48% of all individuals in Europe wear spectacles[1], i.e. spectacles are remarkably widespread in population. Due to their exposed position in the center of the human face, their close contact to the human skin, nose and mouth, and regular contact with human hands, spectacles are thought to be contaminated with a diverse microbiota. It is well known that surfaces with regular contact to the human body become easily contaminated with microorganisms and consequently can be considered as fomites. The same should apply for spectacles, but only little is known about their microbial load and the hygienic relevance resulting from it.

Previous studies showed that surgeons' eyeglasses represent fomites in clinical environments[2]. These spectacles were highly contaminated with *Staphylococcus epidermidis*, and it has been suggested that this contamination might represent a risk to patients during operations. Consequently, surgeons were advised to disinfect their spectacles on a regular basis.

To get a first glance into the composition of the spectacle microbiota, we recently performed a cultivation-based study using worn spectacles from university staff and students and from inhabitants of a nursing home for elderly people. We found significant amounts of bacteria on all investigated spectacles and could show that spectacles from elderly people had a more diverse taxonomic composition. Many of the identified bacteria represented potential pathogens that may cause skin and eye diseases[3]. This may be particularly problematic in clinical environments and for infection-susceptible groups of persons, such as immunocompromised or elderly people. We provided a first description of aerobic bacteria on spectacles, but many other groups remained elusive as only (aerobic) cultivation-based methods were used. Clearly, (aerotolerant) anaerobes, slow growing and yet-uncultivated bacteria were probably discriminated against with this approach.

In this study, we examined the bacteriota composition of 30 spectacles at 3 different sample sites (earclips, nosepads and lenses) using Illumina MiSeq-based 16S rRNA amplicon sequencing. All investigated spectacles were obtained from university staff or students. To the best of our knowledge, this is the first molecular study on the bacteriota of spectacles so far. We believe that it provides a solid, cultivation-independent basis for a deeper understanding of the hygienic relevance of these very widespread items that aid human vision.

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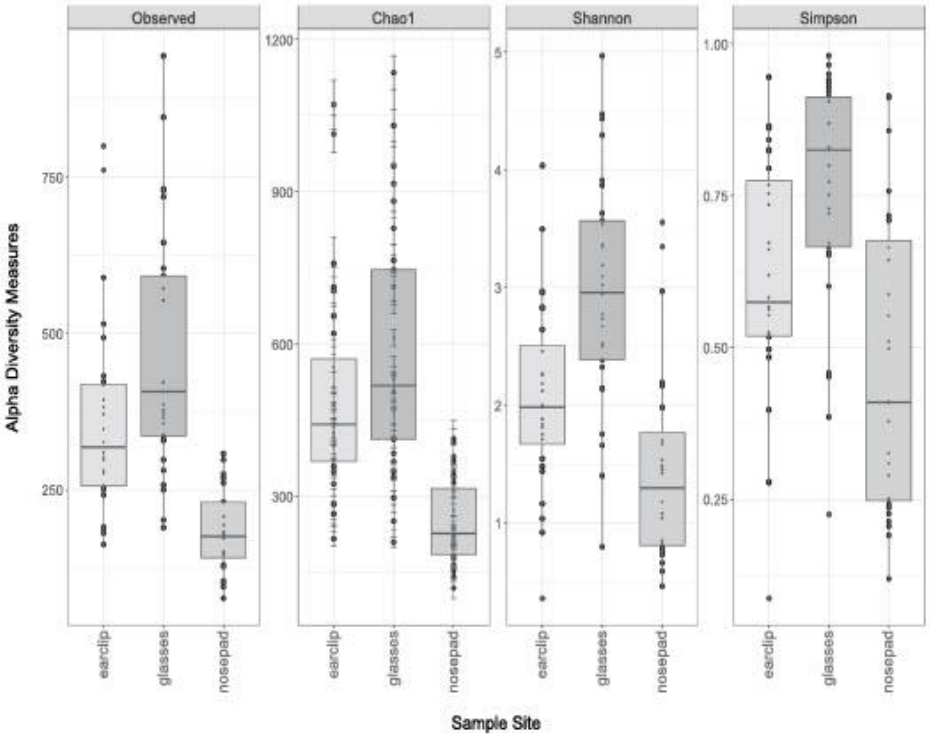


**Figure 1.** Comparison of alpha diversity measures between the three sample sites (earclips, glasses, nosepads). Differences are shown by four indices (observed taxonomic units, Chao1 estimated species richness, Shannon and Simpson diversity index). All differences were found statistically significant ($p < 0.05$).

## Results

**Sequencing results.** Out of 5707896 raw sequences, we obtained an average of 22193 operational taxonomic units (OTUs) per sample after quality filtering and deletion of chimeric sequences (11%). After rarefication to 21416 reads per sample and removal of singleton taxa, we identified 5232 OTUs from 85 samples across the three sample sites (28 earclips, 28 nosepads and 29 glasses). The taxonomic assignment of the OTUs revealed 19 bacterial phyla, 52 classes, 105 orders, 241 families and 665 genera. Metadata, original OTU table and taxonomic assignment for all OTUs can be found in the supplementary file 1.

**Diversity analyses.** To determine which surfaces hosted the most diverse communities, diversity metrics were calculated. Alpha diversity results revealed more species and a higher diversity on the glasses, compared to nosepads and earclips (Fig. 1). The differences within the factor "sample site" were statistically significant (ANOVA, Analysis of Variance, $p < 0.001$) for all diversity indices, using Holm-corrected p-values. Therefore, we performed a multiple pairwise comparison between the means of groups applying Tukey HSD (Honestly Significant Difference) as post-hoc test. We found statistically significant differences between the nosepads and glasses for all indices ($p < 0.05$), as well as significant differences between the nosepads and earclips ($p < 0.05$) and between glasses and earclips ($p < 0.05$, except for Chao1 with $p > 0.05$). Exact (adjusted) p-values can be found in the supplementary file 2 (Statistical analyses performed in R).

To assess beta-diversity, we calculated structural similarity and variation between the microbiota from the sample sites using weighted and unweighted UniFrac-Principal Coordinates Analysis (UniFrac-PCoA) and the UniFrac distance analysis. The PCoA plot of the unweighted UniFrac data suggests that the samples cluster according to glasses and nosepads/earclips. The PCoA plot of weighted UniFrac data rather indicates a clustering of nosepads and glasses/earclips (Fig. 2).

ANOSIM (Analysis of Similarities) on UniFrac distances revealed significant differences in beta diversity between the sample sites (unweighted UniFrac: $R = 0.316$, $p < 0.05$; weighted UniFrac: $R = 0.161$, $p < 0.05$). By comparing the different sites with each other, ANOSIM on UniFrac distances using Holm-corrected p-values revealed a statistical difference between all the tested sites (adjusted $p < 0.05$).

As shown in Figs. 1 and 2, spectacle lenses carried the most diverse bacterial community. Exact (adjusted) p-values can be found in the supplementary file 2 (Statistical analyses performed in R).

**Taxonomic composition.** The dominant bacterial phyla across all sample sites were *Actinobacteria* (64%), *Proteobacteria* (22%), *Firmicutes* (7%) and *Bacteroidetes* (5%) (Fig. 3a). At genus level, just 14 genera accounted

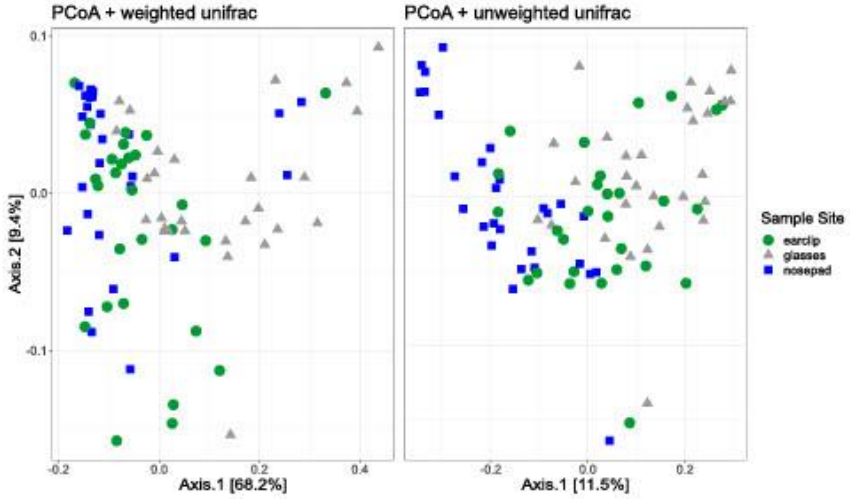


**Figure 2.** PCoA plots of weighted and unweighted UniFrac distances. Displayed are all samples, assigned on OTU level to the different sample sites (earclips, glasses, nosepads).

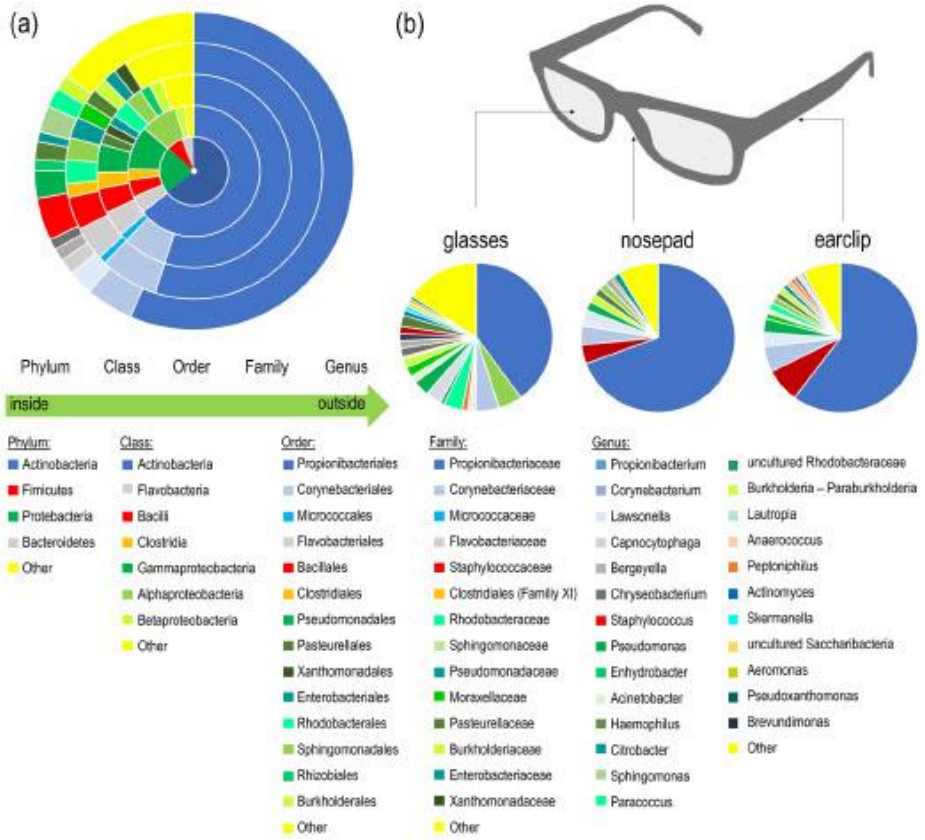


**Figure 3.** Taxonomic composition of the spectacle bacteriota. (**a**) Multi-level ring chart showing the taxonomic composition of the spectacle bacteriota, as delivered by Illumina-based 16S rRNA amplicon sequencing of 85 samples from 30 spectacles. (**b**) Pie charts showing the taxonomic composition of the different sample sites (earclips, n = 28, nosepads, n = 28, glasses, n = 29). For simplicity, only the taxa with a relative abundance of more than 1% are shown (**a**,**b**). All taxa with an individual relative abundance of < 1% are grouped as "others". Spectacle graphic art was obtained from https://freesvg.org with a CC0 licence.

| OTU | SILVA genus | Relative abundance (%) | BLAST result and accession no. | RG* | Sequence similarity (%) | eHOMD result and accession no. | RG* | Sequence similarity (%) | Origin |
|---|---|---|---|---|---|---|---|---|---|
| *denovo11578* | *Propionibacterium* | 55.96 | *Cutibacterium acnes* CP033842.1 | 2 | 99.8 | *Cutibacterium acnes* 530_5256 | 2 | 99.0 | skin |
| *denovo26270* | *Propionibacterium* | 0.82 | *Cutibacterium granulosum* LT906441.1 | 2 | 99.8 | *Cutibacterium granulosum* 114N078A | 2 | 99.8 | skin |
| *denovo21272* | *Staphylococcus* | 3.63 | *Staphylococcus epidermidis* MK542833.1 | 2 | 99.8 | *Staphylococcus epidermidis* 601_3363 | 2 | 99.8 | skin |
| *denovo11292* | *Staphylococcus* | 0.60 | *Staphylococcus hominis* MG254773.1 | 2 | 99.8 | *Staphylococcus hominis* 127N087A | 2 | 99.6 | skin/axillae/pubic |
| *denovo14106* | *Corynebacterium 1* | 2.84 | *Corynebacterium tuberculostearicum* KJ081533.1 | 2 | 99.8 | *Corynebacterium tuberculostearicum* 077N045A | 2 | 99.8 | skin/mucosa |
| *denovo120* | *Corynebacterium 1* | 0.50 | *Corynebacterium sp. strain CAU 1475* MG460588.1 | ND | 99.6 | *Corynebacterium coyleae 3* 41N142B | 2 | 99.8 | environment/mucosa/skin |
| *denovo16140* | *Lawsonella* | 2.76 | *Lawsonella clevelandensis* CP009312.1 | 2 | 98.5 | *Lawsonella clevelandensis* 173N105C | 2 | 99.8 | environment/skin /not fully defined |
| *denovo21616* | *Pseudomonas* | 1.58 | *Pseudomonas guaguanensis* KY471631.1 | ND | 100 | *Pseudomonas pseudoalcaligenes* 740_6666 | 1 | 98.8 | environment/water/not fully defined |
| *denovo16838* | *Enhydrobacter* | 0.84 | *Moraxella osloensis* CP024443.2 | 2 | 99.8 | *Moraxella osloensis* 711_5304 | 2 | 99.6 | skin/ mucosa /respiratory tract |
| *denovo4497* | *Haemophilus* | 1.12 | *Haemophilus parainfluenzae* LT695215.1 | 2 | 99.8 | *Haemophilus parainfluenzae* 718N000A | 2 | 99.8 | nasal/oral |
| *denovo22674* | *Burkholderia- Paraburkholderia* | 1.51 | *Burkholderia cenocepacia* EF634151.1 | 2 | 100 | *Burkholderia cepacia* 571_7530 | 2 | 99.4 | environment |
| *denovo893* | *Paracoccus* | 0.73 | *Paracoccus yeei* CP031078.1 | 2 | 100 | *Paracoccus yeei* 104N072A | 2 | 100 | environment/not fully defined |
| *denovo16824* | *Capnocytophaga* | 0.59 | *Capnocytophaga sputigena* CP031078.1 | 2 | 99.6 | *Capnocytophaga sputigena* 775_4920 | 2 | 99.6 | oral |

**Table 1.** List of the most abundant OTUs, aligned against two different databases for species affiliation. The most abundant OTUs were aligned against two different databases (NCBI and eHOMD) to identify the closest known bacterial representative on species level. Only taxa with a relative abundance of >0.5% are displayed. *RG = risk group classification according to German TRBA 466; N/D = no data available.

for 85% of the total sequences of all spectacles, with a prevalence of more than 1% relative abundance. The most relatively abundant taxon was *Propionibacterium* with an overall average relative abundance of 57%, followed by *Corynebacterium* (5%), *Staphylococcus* (4%), *Pseudomonas* (3%), *Sphingomonas* (3%), *Lawsonella* (3%), *Paracoccus* (2%), *Haemophilus* (2%), *Burkholderia-Paraburkholderia* (2%) and *Capnocytophaga* (2%).

Due to the used sequencing technology, it was not possible to analyze complete 16S rRNA genes. Therefore, the taxonomic identification is limited. In order to classify the most abundant OTUs on species level, we performed BLAST (Basic Local Alignment Search Tool) analyses against two databases (Table 1). The majority of the identified taxa belonged to risk group 2 (classification according to German technical rules for biological agents (TRBA) #466)[4], i.e. represent potential pathogens.

## Discussion

In this study, we identified the bacteriota on different parts of worn spectacles, their community structure and diversity. We found statistically significant differences within the alpha and beta diversity indices between the three sample sites (nosepads, earclips and glasses). Therefore, it is safe to assume that the sampled site plays a significant role for bacterial community composition. In particular the glasses tended to differ from the other sample sites, as they carried the most diverse bacterial community.

We assume that bacteria are transferred easily from human skin to the earclips and nosepads, whereas glasses are in a more remote position to the skin and exposed to other microbial sources, such as air and dust. Regular cleaning measures[3] and the lens material, as previously reported for contact lenses[5,6] or water pipe materials[7], might also contribute to a different taxonomic composition here. Factors such as the age of the spectacle wearer, gender, material of frames and different lens coatings may influence the bacterial community composition as well[8,9]. We evaluated the influence of age and gender, but for now we could not detect any statistical significant association with alpha and beta diversity. With respect to age, this may be due to the low spread of age data in our subject group. Shibagaki and colleagues[8] showed a change in community composition of skin bacteria with age, but only between younger (about 30 a old) and older (about 70 a old) subjects.

Regarding the taxonomic composition, we found relatively high shares of propionibacteria at genus level. In 2016, the cutaneous species of the genus *Propionibacterium* were renamed to *Cutibacterium*[10]. In our case, the SILVA database 128 release returned *Propionibacterium*, as identification result, while searching against the NCBI (National Center for Biotechnology Information) and eHOMD (Human Oral Microbiome Database) 16S databases revealed the respective sequences to be affiliated with *Cutibacterium*. Nevertheless, following the SILVA database outputs, the respective data are presented as *Propionibacterium* here.

Clearly, the majority of bacterial microorganisms found on the investigated spectacles seem to originate from human skin. Propionibacteria, corynebacteria and to a lesser extent staphylococci dominate sebaceous sites as found behind the ears (retroauricular crease) and on the sides of the nostrils (ala of nose)[11–13], where close contact to earclips and nosepads occurs. Propionibacteria, mainly *P. acnes*, are predominant members of the human skin microbiome[14]. This matches our finding that propionibacteria are the most frequent bacteria on worn spectacles, along with corynebacteria. Propionibacteria are aerotolerant anaerobes, reside in pilosebaceous glands, carry a variety of virulence factors, and therefore are involved in diseases, such as Acne vulgaris[15]. These bacteria were also found in infected eyes suffering from endolphtalmitis[16].

Staphylococci and corynebacteria colonize moist habitats, such as the palms of the hands[15,17], and might find their way onto spectacles during cleaning or touching these devices. Previous cultivation-based analyses from our group revealed *S. epidermidis* as the most frequent bacterium on worn spectacles[3]. It is known that *S. epidermidis* normally colonizes human skin without being harmful, but rather being benign or mutualistic for its host[16]. However, many staphylococci include antibiotic resistant strains[18], such as MRSA (Methicillin-resistant *Staphylococcus aureus*) or MRSE (Methicillin-resistant *Staphylococcus epidermidis*). Although the relative abundance of staphylococci on spectacles might be lower than previously expected from our cultivation-based study[3], further investigations should nevertheless examine spectacles as potential carriers of antibiotic resistant bacteria in more detail. This issue could be of high hygienic relevance, especially in clinical environments.

The genus *Pseudomonas* also comprises many species known to cause opportunistic (skin-) infections, and has the potential to be problematic, particularly in clinical environments. Specifically, *P. aeruginosa* can cause severe infections at different anatomic sites[19]. With *Lawsonella*, we identified a just recently described novel genus. BLAST analysis revealed our sequences to represent *Lawsonella clevelandensis* (Table 1), presumably an anaerobic bacterium, affiliated with the genus *Corynebacterium*, which are typical colonizers of sebaceous skin[20]. *L. clevenlandensis* was first isolated from human abscesses, mainly from immunocompromised patients. The authors assumed this bacterium to be of environmental origin or as a member of the human skin microbiota and as a potential pathogen[21,22]. Escapa and colleagues[23] described *L. clevelandensis* to be rather common on oily skin sites, particularly at the alar crease, the glabella and occiput, but also to be present in human nostrils. Apparently, it also occurs on spectacles in rather high shares.

Figure 3 indicates that bacterial genera from the human aerodigestive tract were also frequently detected on the investigated spectacles, such as *Moraxella, Haemophilus, Actinomyces, Capnocytophaga* or *Lautropia*[24–27]. Coughing, sneezing or cleaning with clothes after breathing on the lenses might promote this diversity. Other bacteria that were identified on the spectacles represent ubiquitous or environmental taxa, such as *Skermanella, Paracoccus* or *Aeronmonas*. Notably, many of the identified genera contain species known to cause skin and eye diseases[20,28–30].

Additional database searches (Table 1) classified the most abundant OTUs on species level. OTUs classified as *Enhydrobacter* rather seem to be affiliated with *Moraxella osloensis*. which is a member of the respiratory tract and nasophyryngeal microbiota that is also known to cause malodor on washed laundry[24,31,32]. The majority of the taxa displayed in Table 1 belong to risk group 2, i.e. they represent potential pathogens. They may be harmless to healthy people but may cause infections in newborns, immunocompromised patients, pregnant women or elderly persons.

## Conclusions and Outlook

Spectacles - widely used devices that aid human vision - carry a significant and highly diverse bacterial load. Our study provides first, cultivation-independent insights into this spectacle bacteriota, which is mainly comprised of bacteria of human skin and epithelia origin. The community was dominated by bacteria typical for the skin areas that are in physical contact with the spectacle frames. The bacteriota on the lenses differed significantly from the other sample sites and showed the highest diversity. As many of the identified genera comprise potentially pathogenic species that may cause skin and eye diseases, spectacles clearly must be regarded as fomites. This is of particular importance in clinical environments, but also for people daily working with worn spectacles, such as opticians.

Future studies should address the role spectacles play as fomites in more detail, e.g. regarding the role as carriers and vectors of multi-resistant bacteria in clinical environments or as reservoirs for microorganisms that can cause recurring eye [space] infections. Clearly, such investigation should also consider less easy accessible parts of spectacles, such as the hinges. Due to the use of *Bacteria*-specific primers we could not detect any fungal or viral species on the spectacles investigated here. However, this would be of additional interest, as there are several fungal and viral taxa known to be involved in severe eye infections[33–35], such as *Candida albicans, Fusarium solani, Aspergillus flavus*, Herpes simplex and Varizella zoster.

In addition, the protocols and data published here might serve as a basis to study the surfaces of other devices with close contact to human eyes and facial skin, such as microscopes, slit lamps or surgeon´s eyeglasses, in order to gain a deeper understanding of their hygienic relevance, too. Finally, the bacterial taxa identified here as being prominent on spectacles might serve as practically very relevant organisms for the testing of antimicrobial coatings and/or cleaning strategies for spectacles.

## Material and methods

**Ethics statement.** In this study, no human samples but swab samples obtained from worn spectacles were investigated. All swab samples were collected at Furtwangen University. Spectacles and usage data of the spectacle wearers were provided voluntarily. Informed consent to use the obtained data for scientific purposes was obtained orally. Personal data of the participants were not recorded, rendering it impossible to assign a spectacle microbiota to a specific wearer. Moreover, the spectacle wearers neither provided directly health-related data, nor were the analyses aimed at detecting directly health-related bacteria, such as obligate pathogens. Therefore, we believe that the study was performed in an ethically appropriate manner.

**Spectacle sampling.** Spectacles for swab-sampling were kindly provided by 30 students and employees (mean age 24 ± 6.6 years, (mean ± SD), 12 males and 18 females) of Furtwangen University, Campus Villingen-Schwenningen. All collected metadata, such as age, gender or frame material are included in supplementary file 1.

Standardized sampling was performed from October to December 2018 in a university laboratory. Each spectacle was sampled in a meandering pattern, at 3 sites each: lenses (left and right, front and back, respectively), ear clips (left and right side, inside and outside, respectively) and nose pads. One swab sample was obtained per sampled site using dry, sterile Puritan Hydra Flock Swabs (Puritan Diagnostics LLC, Maine, USA). Swabs were broken off into sterile 1.5 ml microfuge tubes, stored at −20 °C, and processed within one week.

**DNA extraction.** DNA was extracted and purified from the swab heads using the PureLink Microbiome DNA Purification Kit (Life Technologies GmbH, Darmstadt, Germany) with slight modifications to the manufacturer's 'buccal, vaginal or skin swab samples' protocol. Samples were incubated at 75 °C for 10 min at 850 rpm, followed by five rounds of bead beating in a FastPrep 24 instrument (MP Biomedicals LCC, Santa Ana, USA) for 1 min at 6.5 m/s and then placed on ice for 1 min. After 2 min of incubation at room temperature, the DNA was eluted with 40 µl of elution buffer. The flow through was reloaded onto the same filter, and again incubated for 2 min. After centrifugation, additional 10 µl of elution buffer was added onto the same filter, incubated for 1 min and centrifuged. The purified DNA was stored at −20 °C until further analyses.

**Library preparation.** The V1 and V3 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primer pair 63F (5′-CAGGCCTAACACATGCAAGTC-3′)[36] and 511R (5′-GCGGCTGCTGGCACRKAGT-3′)[37] (Eurofins Genomics GmbH, Ebersberg, Germany), added to an overhang adapter sequence tail (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′), yielding a PCR product of ~545 bp. The V1–V3 primer pair covers a typical region widely used for skin microbiome studies[38], but it's also recommended for nasopharyngeal areas[39]. We assume, that this region provides an accurate insight into the human skin and nasopharyngeal microbiota, which we expected to dominate on spectacles.

All extracted samples were amplified in duplicates. PCR setup and cycling conditions for the primary amplification were as follows: 3 µl of template DNA, 15.05 µl of nuclease and DNA free water (VWR International, Darmstadt, Germany), 5 µl of 5× KAPA High Fidelity Buffer (KAPA Biosystems, Wilmington, USA), 0.6 µl of 10 mM KAPA dNTP Mix, 0.25 µl of 20 mg/ml BSA (Life Technologies GmbH), 0.5 µl of KAPA High Fidelity Hot Start Polymerase, 0.3 µl of forward (10 µM) and 0.3 µl of reverse primer (10 µM).

The PCR profile was as follows: 98 °C initial denaturation for 3 min, followed by 35 cycles of 98 °C for 30 s, 63 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 2 min. PCR products were verified by standard 0.8% agarose gel electrophoresis using Midori Green as DNA-dye (Biozym, Olderndorf, Germany). With each batch, water template control reactions were included. As additional negative controls, sterile, unused swabs were prepared as described above. No PCR background contamination from either reagents and/or collection procedures was discovered. As positive controls, we used diluted (1:1000) DNA from overnight cultures of *Escherichia coli* K12, extracted with the same DNA purification kit.

Two replicates of each sample were pooled and cleaned up using Agencourt AMPure XP Beads (BeckmanCoulter Inc., Krefeld, Germany) according to the Illumina library preparation protocol with an adapted bead to sample ratio of 0.7 : 1[40].

Subsequently, a second amplification step was carried out to anneal dual-index barcodes. The Illumina Nextera XT Index Kit v3 and Nextera XT Index Kit v2 Set B adapters (Illumina Inc., San Diego, USA) with different dual indices were combined to allow multiplexing and good performance of all samples. Two unique indices were attached to each amplicon sample, while performing a second PCR reaction.

We used 5 µl of cleaned amplicon PCR product, with 4 µl index primer i7xxx and 4 µl index primer i5xxx, respectively. 25.6 µl of nuclease and DNA free water, 10 µl of 5 × KAPA High Fidelity Buffer (KAPA Biosystems), 1.2 µl of dNTP Mix (10 mM) and 0.2 µl of KAPA High Fidelity Hot Start DNA Polymerase were added. Cycling started at 98 °C initial denaturation for 3 min, followed by 8 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. Index PCR products were verified by standard 0.8% agarose gel electrophoresis and cleaned up as described above, with a bead to sample ratio of 0.8 : 1. Post PCR quality checks on a Bioanalyzer 2100 Instrument with the DNA High Sensitivity Kit (both Agilent Technologies Deutschland GmbH, Waldbronn, Germany) revealed the exact amplicon size (bp) of each sample. After quantification using a Qubit 2.0 Fluorometer (Life Technologies GmbH), equimolar concentrations were calculated.

**Sequencing.** The library was adjusted to 3 nM (with 10 mM Tris buffer, pH 8.5), pooled, combined with 30% PhiX control (Illumina Inc.) and finally diluted to 5 pM. The sequencing was run on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycle) (Illumina Inc.) with a quality score ≥30 and default settings. Sequence files were deposited at the European Nucleotide Archive (ENA) under the accession number PRJEB32211.

**Bioinformatics.** Sequences were processed in QIIME 1.9.1[41]. Paired end reads were joined using the "join_paired_ends.py" script with default settings. Chimeras from the demultiplexed sequences were removed using "vsearch"[42]. Operational Taxonomic Units (OTUs) were clustered de novo with a 97% similarity threshold using "uclust"[43]. Taxonomy was assigned to representative OTUs against the SILVA database, release 128[44]. Parallel sequence alignment was performed via PyNAST[45]. Chloroplast and mitochondrial OTUs were removed.

To identify the relatively most abundant genera down to species level, their 16S rRNA amplicon sequences were aligned against two different databases to identify the closest known bacterial representative using the standard (nucleotide) BLAST at NCBI (National Center for Biotechnology Information) and eHOMD (Human Oral Microbiome Database; www.ehomd.org)[23]. eHOMD is a database providing 16S rRNA gene sequences from different body sites, especially the human aerodigestive tract. The metadata, the unrarefied OTU table and the taxonomic assignments are provided in the supplementary file 1.

**Statistical analyses.** All statistical analyses and graphical visualizations were performed in R 3.5.3 using the "phyloseq"[46], "vegan"[47], "coin"[48] and "microbiome"[49] packages. Figures were created in R using "ggplot2"[50] and MS Excel 2016. The analysis-report was created with R-studio (version 1.1.463)[51] and can be found in the supplementary file 2.

We only kept taxa with a prevalence of more than one. The 85 samples were rarefied to a level of 21416 sequences for even sampling depth (seed: 1121983).

To determine which surfaces hosted the most diverse communities, alpha diversity metrics (Observed, Chao1, Shannon and Simpson) were calculated. For comparative analysis of the diversity indices among the different factors (e.g. sample site), one-way ANOVAs (Analysis of Variance) with Holm-adjusted p-values were performed[52]. For factor-specific categories, pairwise multiple comparisons between the sample sites were calculated using Tukey's honest significant differences (HSD) as post-hoc test[53].

In order to measure beta diversity, principle coordinate analysis ordinations (PCoA) were generated based on weighted and unweighted UniFrac distance matrix[54] while using abundance information of OTUs and phylogeny.

ANOSIM (Analysis of Similarities) calculations on UniFrac distance matrices, using 9999 permutations, were performed as non-parametric tests for similarity between groups using the "vegan" package, version 2.5–5. The ANOSIM statistic variable R ranges from −1 to +1 with a value of 0 indicating no difference between the groups[55].

All tests were two-sided, and p-values, respectively Holm-adjusted p-values below 0.05 were regarded as statistically significant.

## Data availability

The sequences supporting the conclusions of this article are available at the European Nucleotide Archive (ENA - https://www.ebi.ac.uk/ena) under the accession number PRJEB32211. All data generated or analysed during this study are included in this published article (and its Supplementary Information files). A full record of all statistical analysis is included as supplementary file 2 and was created using the knitr package in R[56].

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## Author contributions
B.F. designed the experiments, sequenced the samples and analyzed the data. M.M. collected the spectacle samples and prepared sequencing. Se.W. assisted with sequencing and data analyses. Si.W. and F.Z. performed project administration and supervised the work. B.F. and M.E. wrote the manuscript. M.E. supervised and conceptualized the work. All authors edited and approved the final manuscript.

## Competing interests
The authors declare no competing interests. The affiliation of one author (Si.W.) with Carl Zeiss Vision International GmbH did not play any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of all authors are articulated in the 'Author' contributions' section.

## Additional information
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## 2.3 Publication III: Eye-catching microbes – polyphasic analysis of the microbiota on microscope oculars verifies their role as fomites

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# Eye-Catching Microbes—Polyphasic Analysis of the Microbiota on Microscope Oculars Verifies Their Role as Fomites

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**Abstract:** Microscopes are used in virtually every biological and medical laboratory. Previous cultivation-based studies have suggested that direct contact with microscope eyepieces increases the risk of eye infections. To obtain a deeper insight into the microbiota on oculars, we analysed 10 recently used university microscopes. Their left oculars were used for a cultivation-based approach, while the right oculars served for massive gene sequencing. After cleaning with isopropyl alcohol, the oculars were re-sampled and analysed again. All oculars were found to be contaminated with bacteria, with a maximum load of $1.7 \times 10^3$ CFU $cm^{-2}$. MALDI Biotyping revealed mainly *Cutibacterium* (68%), *Staphylococcus* (14%) and *Brevibacterium* (10%), with the most abundant species being *Cutibacterium acnes* (13%) and *Staphylococcus capitis* (6%). Cleaning reduced the microbial load by up to 2 log scales. Within 10 uncleaned and 5 cleaned samples, 1480 ASVs were assigned to 10 phyla and 262 genera. The dominant genera before cleaning were *Cutibacterium* (78%), *Paracoccus* (13%), *Pseudomonas* (2%) and *Acinetobacter* (1%). The bacteriota composition on the cleaned oculars was similar; however, it probably largely represented dead bacteria. In summary, used oculars were significantly contaminated with skin and environmental bacteria, including potential pathogens. Regular cleaning is highly recommended to prevent eye and skin infections.

**Keywords:** microscope; ocular; 16S rRNA gene; sequencing; Illumina; eye; hygiene; microbiota

## 1. Introduction

Surfaces regularly touched by humans become easily contaminated with microorganisms. Many recent studies have addressed the microbial load and associated health risks of frequently used objects, such as smartphones or money [1–3], transportation vehicles [4], restrooms [5] or hospital surfaces [6]. All of them were found to be colonized by a broad variety of bacteria of mainly human skin and epithelia origin, depending on how they are used and/or the respective human body parts they get in contact with. Transmission of pathogens is likely to occur and especially surfaces regularly touched with human hands must consequently be regarded as fomites [7]. Pathogenic and/or potentially

pathogenic microorganisms may cause infections, particularly if there is close contact to the skin, mouth and eyes, and if devices are used by different persons.

To elucidate the bacterial load and hygienic relevance of optical devices, which are in physical proximity to the eyes, we recently performed an aerobic, cultivation-based study on used spectacles, which are remarkably widespread devices in the population [8]. We found significant amounts of bacteria, dominated by staphylococci, whereby many of the identified taxa represented potential pathogens that may cause skin and eye infections. Using a molecular approach, based on high-throughput 16S rRNA gene sequencing, we recently showed that the spectacle community is dominated by bacteria typical for the skin areas that are in physical contact with the spectacle frames [9]. These studies allowed a first insight into the bacteriota of personal ophthalmic objects in close contact to human skin and eyes. Even though the observed bacterial colonization may be problematic in clinical environments or for infection-susceptible people, the majority of the identified bacteria were assumed to be part of the normal, personal skin microbiota, and therefore unlikely to cause severe infections in healthy individuals.

However, sharing optical devices may be more problematic. Previous, cultivation-based studies [10] suggested that direct contact with microscope eye-pieces significantly increases the risk of reoccurring eye infections, such as conjunctivitis. 26% of the investigated oculars carried bacteria known to be pathogenic or potentially pathogenic, such as *Staphylococcus aureus* [10].

To come to a more comprehensive insight into the microbial community of shared ophthalmic objects, we examined the microbiota on used microscope oculars with a polyphasic approach, using gene sequencing and cultivation-based techniques. Our study represents the first comprehensive analysis of the microbial contamination on microscope oculars and we believe it provides a solid basis for a deeper understanding of the hygienic relevance of these optical devices, which are used in virtually every laboratory.

## 2. Materials and Methods

### *2.1. Cultivation-Based Analyses*

The 10 light microscopes (Motic BA 310, and Leica DME, both Wetzlar, Germany) used for swab-sampling were taken from a security level 1 microbiology laboratory at Furtwangen University, Campus Villingen-Schwenningen. They are used for basic courses in practical microbiology, but not for specific research analyses. Sampling was performed in May 2019, immediately after a student laboratory course. These microscopes were selected because they were stored in the same room and were mainly used for the same purpose (teaching) and by similar users (students).

Preliminary analyses showed that separate sampling of lenses and plastic eyecups did not yield enough material for downstream analyses. Therefore, lenses and plastic eyecups of each single ocular were sampled with one swab, respectively.

Each left ocular (lens and plastic eyecup) was sampled for the cultivation-based analysis (Figure 1a).

The sampled area was calculated by measuring the geometry of the ocular. Microbial loads were determined according to DIN 10113–1:1997–07—Part 1 [11]. Standardized sampling was performed in the university laboratory as described elsewhere [8], with a modified sample area to wetting medium ratio of 1.5:1 (1.5 mL medium was used per 1 $cm^2$) in order to increase the cell concentrations.

Germ numbers were determined from that suspension by plating 50 µL, each on Tryptic Soy Agar (TSA; Carl Roth, Karlsruhe, Germany) as a non-selective medium for bacterial cultivation and Thioglycolate Agar (Merck KGaA, Darmstadt, Germany), which enhances the growth of non-stringent anaerobic/aerotolerant microbes, especially if applying prolonged cultivation times [12,13]. To detect fungi, 50 µL of suspension were plated on Malt Extract (Merck KGaA) and Sabouraud-4%-Glucose Agar (Carl Roth), respectively.

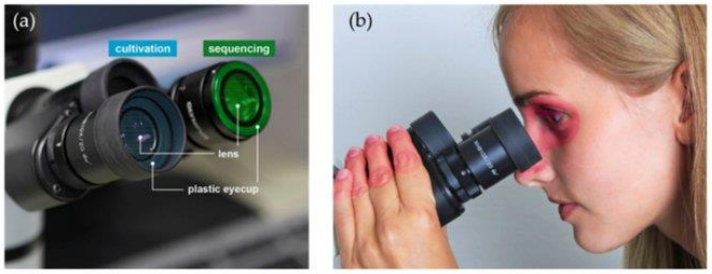


**Figure 1.** Sampled parts of the microscope oculars and skin areas that act as source for microbial contamination: (**a**) Each right ocular (lens and plastic eyecup) was sampled for sequencing-based analysis, each left ocular (lens and plastic eyecup) was sampled for cultivation-based analysis; (**b**) Skin and eye areas (highlighted in red) with probable contact to microscope oculars. Photographs with permission of Furtwangen University.

Aerobic cultivation conditions were as follows: 3 d for TSA Agar and 10 d for Thioglycolate Agar at 37 °C, respectively. 7 d at 30 °C for Malt Extract and Sabouraud-4%-Glucose Agar. Anaerobic cultivation was performed in an anaerobic jar using Anaerocult with indicators (Merck KGaA) for 7 d for TSA Agar and 10 d for Thioglycolate Agar at 37 °C, respectively. Germ numbers were determined after incubation, referred to the sampled area, and expressed as colony-forming units (CFU) $cm^{-2}$. No anaerobic incubation was performed for the fungal growth media.

### *2.2. Identification of Microbial Isolates by MALDI Biotyping*

From each agar plate showing microbial growth, a representative of each morphotype was subcultured and controlled for purity. A colony of each pure culture was suspended in 300 µL ultrapure water and stored at −80 °C until further processing. Samples were extracted and identified using a MALDI Biotyper system (MALDI Biotyper Microflex LT, Bruker Daltonics, Bremen, Germany) following the protocol for ethanol-formic acid extraction [14]. The volumes of formic acid and acetonitrile (both Carl Roth) were adapted as specified in the protocol for single, small colonies. The obtained protein spectral profiles were matched against the MALDI Biotyper reference database (software version 4.1.90, 8936 entries) and expressed as score values ranging from 0 to 3.0. According to the manufacturer, scores >1.7 indicate a reliable genus identification, scores >2.0 a reliable genus and probable species identification, and scores >2.3 a highly probable species identification. Detailed germ numbers and MALDI Biotyping results are provided in the Supplementary Table S1.

### *2.3. Sequencing-Based Analyses*

Each right ocular (lens and plastic eyecup, Figure 1a) of the microscopes was sampled in a meandering pattern using dry, sterile Puritan Hydra Flock Swabs (Puritan Diagnostics LLC, Guilford, ME, USA). Swabs were broken off into RNA/DNA shield tubes (Zymo Research, Freiburg, Germany) with beads and stored at room temperature until further processing.

### *2.4. DNA Extraction*

DNA was extracted and purified from the swab heads using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research) following the manufacturer's instructions with slight modifications. The samples within the shield were incubated at 50 °C for 20 min at 600 rpm, followed by five rounds of bead beating in a FastPrep 24 instrument (MP Biomedicals LCC, Santa Ana, CA, USA) for 1 min at 6.5 $ms^{-1}$ and then placed on ice for 1 min.

After 2 min of incubation at room temperature, the DNA was eluted with 40 µL of 60 °C warm, DNA-free water. The flow-through was reloaded onto the same filter, and again incubated for 2 min.

After centrifugation, an additional 10 µL of elution buffer were added onto the same filter, incubated for 1 min and centrifuged. The purified DNA was stored at −20 °C until further analysis.

### 2.5. Library Preparation

The V1–V3 region of the 16S rRNA gene was amplified using primers 63F (5′-CAGGCCTAA CACATGCAAGTC-3′) [15] and 511R (5′-GCGGCTGCTGGCACRKAGT-3′) [16] (Eurofins Genomics GmbH, Ebersberg, Germany), with Illumina flow cell adapters (5′-TCGTCGGCAGCGTCAGAT GTGTATAAGAGACAG-3′), yielding a PCR product of ~545 bp. We chose these primers to ensure data comparability with a previous study about the spectacle microbiota [9]. Moreover, these primers did not yield many unspecific PCR products. Most of the extracted samples were processed in duplicates. Triplicates were performed if the gel electrophoresis showed only weak bands. All samples were amplified on a Bio-Rad T 1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total reaction volume of 25 µL, containing 3 µL of template DNA, 15.05 µL of nuclease and DNA free water, 5 µL of 5 × KAPA High Fidelity Buffer (KAPA Biosystems, Wilmington, MA, USA), 0.6 µL of 10 mM KAPA dNTP Mix, 0.25 µL of 20 mg/mL BSA (Thermo Fisher Scientific, Darmstadt, Germany), 0.5 µL of KAPA High Fidelity Hot Start Polymerase, 0.3 µL of forward (10 µM) and 0.3 µL of reverse primer (10 µM).

The PCR profile was run as follows: 98 °C initial denaturation for 3 min, followed by 35 cycles of 98 °C for 30 s, 63 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 2 min. The DNA amplicons were verified by standard 0.8% agarose gel electrophoresis using Midori Green as DNA-dye (Biozym, Olderndorf, Germany). With each batch, water template control reactions were included. No PCR background contamination from either reagents and/or collection procedures was discovered. As positive controls, we used diluted (1:100) DNA from overnight cultures of *Escherichia coli* K12, extracted with the same DNA purification kit.

Clean-up of two or three pooled replicates of each PCR sample was performed using Agencourt AMPure XP Beads (BeckmanCoulter Inc., Krefeld, Germany) according to the Illumina library preparation protocol with changes in the bead to sample ratio of 0.7:1 [17].

For the following annealing step of the dual-index barcodes, we used the Nextera XT Index Kit v2 Set B and Nextera XT Index Kit v2 Set C adapters (Illumina Inc., San Diego, CA, USA) and followed the Illumina library preparation protocol with slight modifications. We used 5 µL of cleaned amplicon PCR product, with a unique combination of 4 µL index primer, each, and performed a 25 µL PCR reaction with eight cycles. Index PCR products were verified by standard 0.8% agarose gel electrophoresis and cleaned up as described above, with a bead to sample ratio of 0.8:1. The Bioanalyzer 2100 Instrument with the DNA High Sensitivity Kit (both Agilent Technologies Deutschland GmbH, Waldbronn, Germany) was used for the final PCR quality check. Subsequently, the DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

### 2.6. Sequencing

The library was adjusted to 3 nM (with 10 mM Tris buffer, pH 8.5), combined with 30% PhiX control (Illumina Inc.), and finally diluted to 4 pM. Sequencing was performed on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycle) (Illumina Inc.) with a quality score ≥30 and default settings. Sequence files were deposited at the European Nucleotide Archive (ENA) under the accession number PRJEB37105.

### 2.7. Cleaning Tests

To evaluate the efficacy in reducing the microbial load, oculars for both the cultivation-based and sequencing analyses were cleaned directly after sampling and re-sampled after 30 s residence time, as described above. The oculars were rubbed with sterile cotton swabs (Deltalab, Barcelona, Spain) wetted with 70% isopropyl alcohol, following the recommendations for microscope maintenance [18].

### 2.8. Bioinformatics

Sequences were processed with QIIME 2-2019.7 [19]. Raw sequence data were imported and demultiplexed using the cassava 1.8 paired-end and demultiplexed fastq format. The data were quality filtered, denoised and chimera-checked using the paired-end dada2 pipeline (–p-trunc-len-f 301 –p-trunc-len-r 257 trim-left-f 0 –p-trim-left-r 0) [20,21]. Referring to this pipeline, identified amplicon sequence variants are denoted as ASVs (Amplicon Sequence Variants). Taxonomic classification was performed with the feature-classifier plugin, trained with scikit-learn 0.19.1. [22] by the 63F/511R region using the SILVA 132 99% reference database [23]. This was followed by taxonomy-based filtering to remove mitochondrial and chloroplast sequences. Sequence alignment was created using mafft [24] with the phylogeny pipeline 'align-to-tree-mafft-fasttree'. Following taxonomic classification, ASVs classified as mitochondria or chloroplasts were removed.

The EzTaxon database (16S-based ID, January 2020; https://www.ezbiocloud.net/) [25] was used for further identification of the relatively most abundant ASV sequences. Additionally, they were classified into risk groups according to the German Technical Rules for Biological Agents (TRBA) 466 [26].

Alpha- and beta-diversity analysis was carried out within QIIME 2 using an even sampling depth of 19250 sequences per sample.

For diversity metrics and generation of principal coordination analysis (PCoA) plots, we used the 'diversity core-metrics-phylogenetic'. Alpha rarefaction curves, alpha-diversity metrics ('observed', 'shannon', 'evenness' and 'faith's phylogenetic diversity') and beta-diversity (unweighted and weighted UniFrac distances) were analysed using the 'alpha-rarefaction', 'alpha-group-significance' and 'beta-group-significance' functions.

Significant associations between alpha-diversity metrics (within the metadata group 'Cleaning' were calculated within QIIME 2, using a non-parametric Kruskal-Wallis-Test with Benjamini-Hochberg multiple test correction. Pairwise comparison of beta diversity distances between the factor 'Cleaning' was performed employing permutational multivariate analysis of variance (PERMANOVA, 999 permutations).

All metadata, the unrarefied ASV table, and the taxonomic assignments are provided in Supplementary Table S2.

### 2.9. Statistical Analyses

Statistical analyses and graphical visualizations for the cultivation and sequencing analyses were performed in R 3.6.3 using the packages 'phyloseq' [27], 'vegan (version 2.5-6)' [28], 'coin' [29], 'tidyverse' [30] and 'qiime2R' [31]. Figures were created in R using 'ggplot2' [30] and 'ggpubr' [32]. For differences within the microbial counts, between anaerobic and aerobic cultivation and cleaned and uncleaned oculars, we used the Wilcoxon Signed Rank Test for differences between paired samples.

For sequencing, we processed 20 samples, whereby 15 samples (10 uncleaned and 5 cleaned) yielded sufficient sequences for downstream analyses. The 15 samples were rarefied using R to a level of 19250 sequences for even sampling depth (seed: 1121983).

## 3. Results

### 3.1. Cultivation-Based Results

To quantify and identify the cultivable, living microorganisms on microscope oculars, we performed a cultivation-based approach. While no fungi were detected, we found all investigated oculars to be significantly contaminated with bacteria.

Averaged over all cultivation media showing bacterial growth, we determined a median bacterial count prior to cleaning of 235 ± 485 CFU $cm^{-2}$ (median ± SD) for aerobic cultivation and 575 ± 727 CFU $cm^{-2}$ for anaerobic cultivation. Cleaning reduced the bacterial load by ~2 log scales leaving 0 ± 9 CFU $cm^{-2}$ for aerobic cultivation and 0 ± 230 CFU $cm^{-2}$ for anaerobic cultivation. Differences between the cleaned and uncleaned oculars were significant ($p = 3.05 \times 10^{-5}$, aerobic cultivation; $p = 3.82 \times 10^{-6}$,

anaerobic cultivation). Differences between the bacterial load of uncleaned oculars for the two cultivation conditions were also found to be significant ($p = 0.009$; Figure 2a). Bacterial contaminants on Malt Extract Agar were excluded from the evaluation. After cleaning, only 5 out of 10 oculars still showed microbial growth. The differences between aerobic and anaerobic cultivation were not significant ($p = 0.125$).

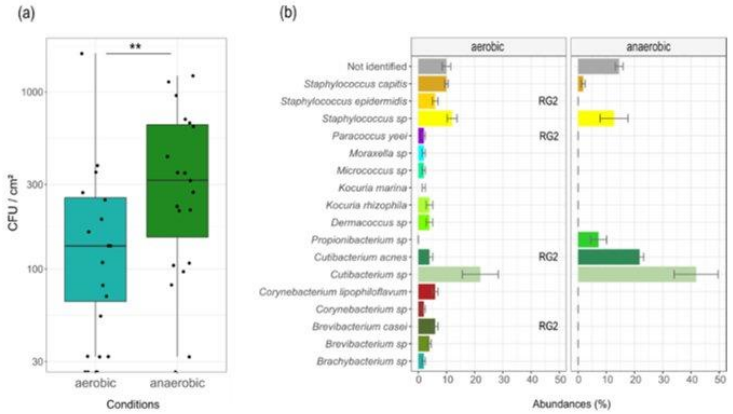


**Figure 2.** Microbial load and relative taxonomic abundances of bacteria isolated from ten uncleaned microscope oculars under two cultivation conditions: (**a**) Box-whisker plot showing the microbial counts (CFU $cm^{-2}$) under two cultivation conditions and from two cultivation media ($n = 10$ oculars, each) before cleaning. Displayed are median, 25% and 75% quartiles, and outliers. Whiskers represent the lowest and highest microbial counts within the 1.5-fold of the interquartile range (IQR) (the 25% and 75% quartile). Asterisks mark a statistically significant difference between cleaned and uncleaned oculars (** $p = 0.009$), based on Wilcoxon Signed Rank Test; (**b**) Barplot of identified bacterial taxa isolated from the oculars before cleaning. Bars show the relative abundance for aerobic cultivation (n = 50 isolates) and anaerobic cultivation ($n = 55$ isolates). 'Not identified' indicates a MALDI identification score <1.7. 'RG2' indicates a risk group 2 classification according to German TRBA. Data are expressed as median ± standard deviation.

MALDI-TOF fingerprints of 114 bacterial isolates (105 obtained before, 9 after cleaning) were used for identification at species or genus level. Ninety-two isolates (uncleaned) were reliably assigned on species or genus level (Figure 2b). All 9 isolates obtained after cleaning were reliably identified as cutibacteria.

In general, we found higher germ numbers but a lower number of genera under anaerobic conditions (11 genera for aerobic cultivation, 3 genera for anaerobic cultivation, Figure 2b).

The bacterial community was dominated by cutibacteria/propionibacteria among all anaerobically cultivated samples, before cleaning (71% on genus level; Figure 2b).

The next most common genera were staphylococci (aerobic: 28%, anaerobic: 15% on genus level) and brevibacteria (aerobic: 10% on genus level), followed by corynebacteria (aerobic, 8% on genus level). Further abundant taxa were *Kocuria* (aerobic: 6% on genus level) and *Dermacoccus* (aerobic: 4%). The remaining bacteria were all found with a frequency of 2%. Notably, four identified species are categorized as biosafety risk group 2 (Figure 2b).

### 3.2. Sequencing Results

Out of 1,983,441 raw sequences, we obtained 1,080,020 sequences after the dada2 pipeline. Five 'cleaned' samples did not yield enough sequences for downstream analyses and were excluded from further analyses. A total of 1,037,731 sequences were retained in the remaining 15 samples, with a mean of 72,912 (min. 19,250, max. 96,303) sequences per sample. After removal of singleton taxa and

rarefication to 19,250 reads per sample (rngseed = 1,121,983) using R, we identified 1480 ASVs from 15 samples of uncleaned and cleaned microscope oculars (10 uncleaned, 5 cleaned). The taxonomic assignment of the ASVs revealed 10 bacterial phyla, 22 classes, 60 orders, 117 families and 262 genera.

### 3.3. Community Composition and Diversity

According to the phylogenetic classification, most of the reads were affiliated with the genus *Cutibacterium* (78% uncleaned, 71% cleaned, Figure 3a). ExTaxon analysis revealed the most abundant sequences to be *Cutibacterium acnes* subsp. *defendens* (99% similarity). Other frequent genera were *Paracoccus* (13% uncleaned, 5% cleaned) and *Pseudomonas* (2% uncleaned, 9% cleaned), followed by *Acinetobacter* (1% uncleaned, 2% cleaned) and *Corynebacterium* (1% uncleaned, 3% cleaned). These top 5 genera comprised 91% (cleaned) to 94% (uncleaned) of all identified taxa. Figure 3 shows the relatively most abundant bacterial genera within the different samples. Less cutibacteria and more bacteria of the genus *Pseudomonas* were present on the cleaned oculars. However, this finding is strongly influenced by one of five samples, therefore it may not be representative.

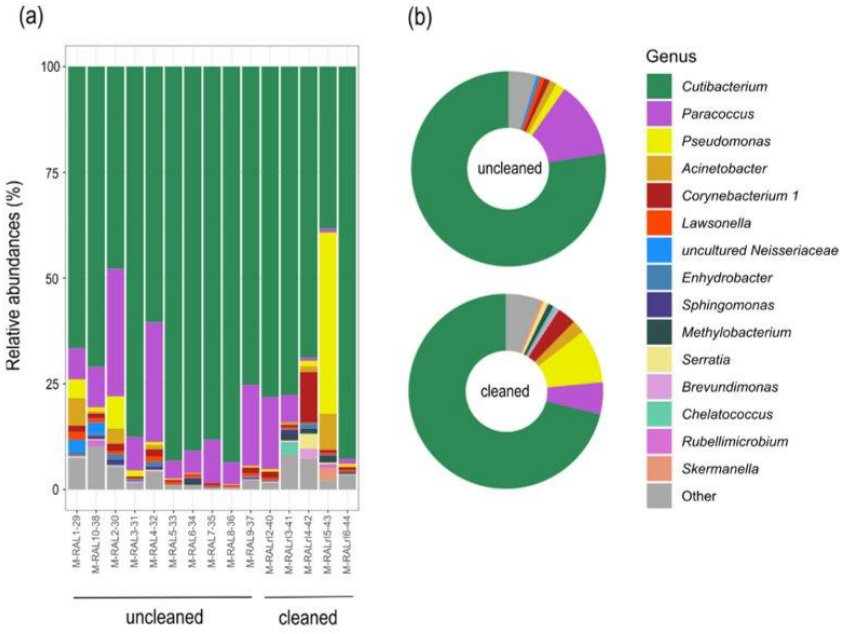


**Figure 3.** Stacked barplots of the relative abundances on genus level of uncleaned ($n$ = 10) and cleaned ($n$ = 5) microscope oculars: (**a**) Each bar represents one ocular sample; (**b**) Samples merged to pie charts for the factor 'uncleaned' and 'cleaned', representing the uncleaned and cleaned oculars. To facilitate comparison, only taxa with a relative abundance of >0.5% are displayed, the remaining taxa were summarized as 'Other'.

In addition to *C. acnes*, ExTaxon analyses abundantly assigned sequences to *Paracoccus yeei* (100% similarity) and *Pseudomonas panacis* (100% similarity). The sequence of the most abundant ASVs within the genus *Acinetobacter* could not be classified down to species level, whereas *Corynebacterium* was affiliated with *Coynebacterium kroppenstedtii.*

When comparing the cultivation- and sequencing-based results, we found cutibacteria in similar ratios. However, we identified 21% staphylococci using the cultivation-based approach, but only 0.2% using molecular methods.

Only faith's phylogenetic diversity (faith pd) of the calculated Alpha-Diversity indices (Figure 4) showed a statistically significant difference in community composition between cleaned and uncleaned samples (Kruskal-Wallis, Benjamini-Hochberg corrected, $p = 0.04$). To assess beta-diversity, we calculated structural similarity and variation between the microbiota from cleaned and uncleaned microscopes using weighted and unweighted UniFrac-distances. No significant differences between cleaned and uncleaned oculars were detected ($p > 0.05$, PERMANOVA, 999 permutations).

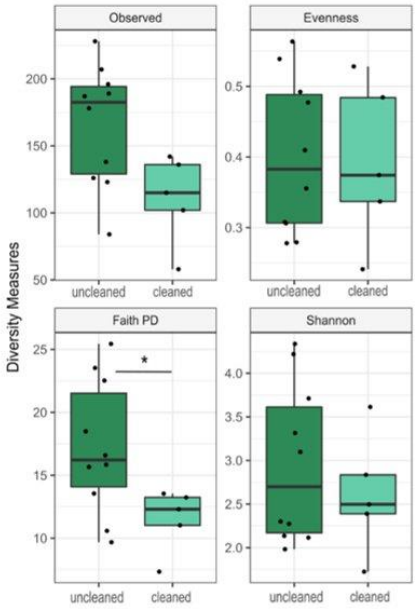


**Figure 4.** Comparison of alpha diversity measures between uncleaned and cleaned microscope oculars. Differences are shown by four indices (observed, Pielou's evenness, Faith's phylogenetic diversity and Shannon diversity). Points represent individual samples. Displayed are the median, the 25% and 75% quartiles and outliers. Whiskers represent the lowest and highest microbial counts within the 1.5-fold of the interquartile range (IQR) (the 25% and 75% quartile). Asterisks mark a statistically significant difference between cleaned and uncleaned oculars (* $p$-adjust = 0.04), based on Kruskal-Wallis Test with Benjamini-Hochberg multiple test correction.

## 4. Discussion

Frequently used objects and other regularly touched surfaces often carry a significant bacterial load and therefore represent fomites. This microbial contamination might lead to cross-contamination, if surfaces and devices are touched or used by different persons.

Our data provide evidence for significant microbial contaminations of microscope oculars as well, which are widely used optical devices in clinical or biological laboratories. Up to now, data on the microbial contamination of microscope oculars have been scarce, although there is a suggested relationship between their use and eye diseases [10]. Moreover, oculars are permanently exposed to the environment, so a diverse bacterial community was likely. As microscopes are touched regularly by hand, and the oculars are also likely to have direct skin contact, it was also safe to assume that typical dermal taxa would occur here (Figure 1b).

Our cultivation-based and molecular results were largely congruent. Indeed, we identified typical colonizers of human skin and mucous membranes as being dominant on the used oculars, such as staphylococci, corynebacteria, micrococci [33], and mainly cutibacteria [34]. In particular, the detected

cutibacteria, which are slow-growing, aerotolerant anaerobes, are known to reside predominantly on facial skin and sebaceous glands [35], but are also found on the hands [36], and can therefore be easily transferred onto any touched surface [3,7].

Compared to other frequently touched surfaces, the detected numbers of cutibacteria were high [37,38]. This might be explained through the cultivation conditions that were used (aerobic and anaerobic, Thioglycolate Medium in addition to Tryptic Soy Agar, incubation time up to 10 d) [12], as well as the fact that the microscopes were sampled immediately after use.

Furthermore, some cutibacteria are known to develop biofilms, even on steel or silicone [39], which may lead to a better adherence to and/or persistence on surfaces, compared to other bacteria.

As expected, the molecular approach allowed a more comprehensive insight, i.e., it unravelled a higher microbial diversity. Based on gene sequencing, the next most frequent genera alongside the cutibacteria were *Pseudomonas, Paracoccus* and *Acinetobacter.* ExTaxon analyses assigned the most frequent sequences to *Paracoccus yeei*, recently isolated from contact lenses and proposed to cause keratitis [40], and *Pseudomonas panacis*, an environmental species, recently isolated from rusty ginseng roots [41] and raw milk [42]. The sequence of the most abundant ASV within the genus *Acinetobacter* could not be classified at species level, whereas *Corynebacterium* was affiliated with *Coynebacterium kroppenstedtii*, a potentially opportunistic human pathogen [43].

In addition, cultures obtained from uncleaned oculars, and from both media, were identified as *Kocuria* and as brevibacteria, more specifically as *Brevibacterium casei*, which are typically associated with human skin [33,44,45]. Other bacteria that were found on the oculars represent ubiquitous or environmental taxa, such as *Paracoccus* [46] or *Brachybacterium* [47].

Many of the detected bacterial taxa are commonly found in the indoor and built environment [48]. They are associated with the human skin microbiome, comprising species also known to cause skin and eye infections [40,49,50]. Although we used media selective for fungi, no fungal growth was detected. This may be due to a shorter persistence of some fungi on surfaces, compared to bacteria [51].

Interestingly, using the cultivation-based approach we identified 21% staphylococci (on uncleaned oculars over all cultivation conditions), but only 0.2% using molecular methods. Staphylococci are known to thrive under a broad range of aerobic and anaerobic cultivation conditions [52], whereas, for instance, the optimal length of cultivation for *Cutibacterium acnes* is proposed to be around 7 to 10 days [13]. Therefore, staphylococci might have outcompeted other species during cultivation, leading to an overrepresentation in the cultivation results.

On the other hand, it is well known [53] that molecular methods can also discriminate certain groups of microorganisms, e.g., due to primer selectivity. In this study, we used primers targeting the V1–V3 region of the 16S rRNA gene that we had previously used for the analysis of the bacteriota on worn spectacles [9]. In that study, higher proportions of staphylococci were detected than here, demonstrating the potential of the used primers to amplify this group of bacteria. Clearly, future studies with other molecular methods and/or other primer combinations will be needed to corroborate or correct the results presented here and to help answer the question whether microscope surfaces select for certain microbial species. Nevertheless, our results strongly suggest that besides staphylococci, cutibacteria are an abundant bacterial genus on surfaces of microscope oculars. We recently reported a very similar trend for worn spectacles [9].

Notably, all isolated bacteria represented viable cells, i.e., they can potentially cause infections. To evaluate a probable pathogenic potential, the identified bacteria were categorized into biosafety risk groups. With *S. epidermidis*, *P. yeei*, *C. acnes*, and *B. casei* we found four potentially pathogenic bacterial species on the investigated oculars, i.e., species classified as risk group 2, which implies a probable infectious risk to humans.

*Propionibacterium* (*Cutibacterium*), *Staphylococcus* (especially *S. epidermidis)*, and Corynebacterium are part of the normal ocular microbiota and have previously been observed on eyelashes, eyelids and in tears [54,55]. Nevertheless, they are also known to be associated with blepharitis and bacterial

keratitis [56,57]. We assume that bacteria are transferred easily from the skin, the area around the eyes, or the eyelashes to the oculars, and vice versa.

Importantly, many staphylococci comprise antibiotic resistant strains [58], such as MRSA (Methicillin-resistant *Staphylococcus aureus*) or MRSE (Methicillin-resistant *Staphylococcus epidermidis)*. A study by Gerba and colleagues [38] showed that antibiotic resistant strains are typically present on frequently used and shared devices such as computer touchscreens. Other devices, such as the phones of health care workers, also carried nosocomial bacteria and antibiotic resistance strains [59]. Furthermore, frequently touched hospital and non-hospital surfaces were shown to carry a high proportion of multidrug resistant bacteria, mainly staphylococci [60]. Therefore, oculars should be considered as a potential reservoir for antibiotic resistant strains, too, which is of special importance in clinical environments, especially as it is known that many pathogens are persistent on surfaces for days or even months [51,61].

Cleaning with isopropanol had no notable effect on the taxonomic composition on the investigated oculars when considering the molecular data. Nonetheless, we found a lower Faiths phylogenetic diversity [62] on cleaned oculars, indicating more phylogenetic different taxa on uncleaned oculars, which matches the cultivation-based results. However, it is safe to assume that most of the detected sequences after cleaning stemmed from dead cells, because cultivation showed an ~2 log scale reduction of viable cells. These results strengthen the use of a biphasic analysis approach, combining cultivation-based and molecular methods. Future studies might also involve metagenomic approaches or the use of specific qPCR methods, which allow for a cultivation-independent detection of fungi, protozoa, such as acanthamoeba, or viruses. Detection of viruses, such as Herpes simplex or Varizella zoster, would be of particular interest, as many viruses cause severe eye infections [63–65]. A significant viral load on microscope oculars is likely, as studies showed that they can remain infectious on environmental surfaces for considerable time periods [51,66]. Even the (enveloped) new human coronavirus SARS-CoV-2 is detectable on plastic and steel surfaces for about 72 h [67].

Our findings corroborate and extend the findings by Olcerst [10] that microscopes carry potentially pathogenic bacteria and therefore may be associated with eye diseases of microscope users. After cleaning with 70% isopropyl alcohol and a 30 s residence time, only low numbers of cutibacteria were still detected on the oculars, which might be compensated by longer residence times. Clearly, cleaning reduced the microbial load significantly and therefore should be applied in a regular manner.

## 5. Conclusions

Microscope oculars carry a diverse bacterial load. Our study significantly extends previous findings about the bacterial load on microscope oculars by applying cultivation-based and cultivation-independent techniques. It provides a solid and comprehensive basis for a deeper understanding of the hygienic relevance of these widely used laboratory devices. We identified many viable taxa of human skin or mucosa origin, many of which are known to cause skin and eye infections. Due to the close skin and eyelash contact, microscope oculars must be regarded as fomites, especially when they are used by different individuals and in clinical environments. Cleaning with isopropyl alcohol reduced the microbial load significantly and should be performed on a regular basis. The dominant bacteria identified in our study appear as ideal test bacteria for antimicrobial efficacy testing of building materials and/or cleaning agents and strategies for microscope surfaces.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2077-0383/9/5/1572/s1, Table S1: Rawdata Cultivation, Table S2: Rawdata Sequencing.

**Author Contributions:** Conceptualization, M.E.; methodology, B.F.; formal analysis, B.F.; investigation, B.F., K.S. and M.M.; resources, M.E.; data curation, B.F.; writing—original draft preparation, B.F.; writing—review and editing, M.E.; visualization, B.F. and F.Z.; supervision, M.E., F.Z. and S.W.; project administration, S.W. and F.Z., funding acquisition, M.E. and S.W. All authors have read and agreed to the published version of the manuscript.

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## 2.4 Publication IV: Comprehensive compositional analysis of the slit lamp bacteriota

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# Comprehensive Compositional Analysis of the Slit Lamp Bacteriota

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Slit lamps are routinely used to examine large numbers of patients every day due to high throughput. Previous, cultivation-based results suggested slit lamps to be contaminated with bacteria, mostly coagulase-negative staphylococci, followed by micrococci, bacilli, but also *Staphylococcus aureus*. Our study aimed at obtaining a much more comprehensive, cultivation-independent view of the slit lamp bacteriota and its hygienic relevance, as regularly touched surfaces usually represent fomites, particularly if used by different persons. We performed extensive 16S rRNA gene sequencing to analyse the bacteriota, of 46 slit lamps from two tertiary care centers at two sampling sites, respectively. 82 samples yielded enough sequences for downstream analyses and revealed contamination with bacteria of mostly human skin, mucosa and probably eye origin, predominantly cutibacteria, staphylococci and corynebacteria. The taxonomic assignment of 3369 ASVs (amplicon sequence variants) revealed 19 bacterial phyla and 468 genera across all samples. As antibiotic resistances are of major concern, we screened all samples for methicillin-resistant *Staphylococcus aureus* (MRSA) using qPCR, however, no signals above the detection limit were detected. Our study provides first comprehensive insight into the slit lamp microbiota. It underlines that slit lamps carry a highly diverse, skin-like bacterial microbiota and that thorough cleaning and disinfection after use is highly recommendable to prevent eye and skin infections.

**Keywords: eye, hygiene, MRSA, Illumina MiSeq ®, microbiota (16S), 16S rRNA gene amplicon sequencing**

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## INTRODUCTION

Surfaces with regular contact to the human body are usually contaminated with microorganisms. Most of them belong to the resident commensal skin and mucosa microbiota, but can nevertheless carry a pathogenic potential. Many studies deal with the bacterial load on daily used devices or frequently touched surfaces, which may also carry antibiotic resistant bacteria (Brady et al., 2007; Anderson and Palombo, 2009; Di Lodovico et al., 2018; Cave et al., 2019; Gohli et al., 2019).

**Abbreviations:** ASV, Amplicon Sequence Variant; HAI, hospital acquired infections; TC, Tertiary Center; SCCmec, staphylococcal cassette chromosome; PCA, principal component analysis; ANCOM-BC, analysis of compositions of microbes with bias correction; qPCR, quantitative PCR; MRSA, methicillin-resistant Staphylococcus aureus; PERMANOVA, permutational multivariate analysis of variance; QIIME, Quantitative Insights Into Microbial Ecology.

Such surfaces usually represent fomites. Fomites are of particular concern in clinical environments, as bacteria on surfaces can be transferred easily from one person to another (Weber et al., 2010; Christoff et al., 2019), promoting the spread of infectious diseases, which is particular problematic for ill or otherwise immunocompromised persons. In 2017, 8.3% of all European patients in an intensive care unit suffered from hospital acquired infections (HAI) (EDC - European Centre for Disease Prevention and Control, 2019). For surgical site infections, the percentage varied between 0.5% and 10% (EDC - European Centre for Disease Prevention and Control, 2019). It is estimated that about 20% to 40% of the HAI in intensive care units are caused by hand-to-hand transmissions (Weinstein, 1991) and that 10% of acute care patients acquire multi-drug resistant microorganisms during their stay (Cao et al., 2016).

During routine diagnostics in eye clinics, many patients are examined in a short time and often suffer from highly contagious eye infectious (Watson et al., 2018). Common HAI in ophthalmology are acute (viral and bacterial) conjunctivitis, keratitis and endophthalmitis (Wang et al., 2006), while frequent and increasing infections by multi-resistant bacteria are caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Schulte et al., 2020). Therefore, special hygienic attention is required for optical surfaces. Previous studies revealed a significant and diverse bacterial load on optical devices, such as microscopes (Fritz et al., 2020b), surgeons loups or surgeons eyeglasses (Graham et al., 2019; Butt et al., 2021) and reusable tonometer tips (Hillier and Kumar, 2008). They all contained significant amounts of bacteria, including many species known to cause skin and eye infections and with a potential to carry antibiotic resistances.

Slit lamps count among the most important and most often used ophthalmological devices, demanding close contact between examiner, many different patients and device surfaces. Previous studies revealed their relevant surfaces to be contaminated with bacteria, mostly coagulase-negative staphylococci, micrococci, bacilli and also *Staphylococcus aureus* (Graham et al., 2008; Sobolewska et al., 2018). However, these examinations were performed with cultivation-dependent techniques, which provide only a very limited overview on the present microbiota, as the cultivation conditions for most microorganisms are still unknown. Here, we used the cultivation-independent, molecular approach of 16S rRNA gene sequencing to analyse the bacteriota on different slit lamp surfaces in detail. Our study represents the first comprehensive analysis of the microbial contamination on slit lamps and we assume it provides a solid basis for a deeper understanding of the hygienic relevance of these widely used optical devices.

## MATERIAL AND METHODS

### 16S rRNA Gene Amplicon Sequencing-Based Analyses

46 slit lamps (various manufacturers) stemming from the Center of Ophthalmology, University Hospital Tuebingen (hereinafter called Tertiary Center 1 – TC1), Germany (n = 29) and the Eye Center, Medical Center, University of Freiburg (hereinafter called, Tertiary Center 2 – TC2), Germany (n = 17) were swab-sampled in October 2020, during routine patients examinations within an unannounced audit. TC1 and 2 were chosen as they are among the largest tertiary care facilities for specialized, consultative, ophthalmological health care in our region and allowed for sufficient sample material.

Patient throughput ranged between 4 and 200 patients per day within the respective rooms (median ± SD: 81 ± 47.1). Predominately rooms with a high patient throughput were chosen, as a high occupancy rate was suspected to lead to higher bacterial load and diversity. Rooms with a low patient throughput were sampled as reference. The examination rooms were shared between 1 and 20 physicians per day (median ± SD: 6 ± 4.6). To ensure comparability with previous studies (Sobolewska et al., 2018; Fritz et al., 2020b), the following regions of slit lamps were sampled, resulting in two samples per device: The 'oculars' (lens and plastic eyecup) as regions in close proximity to the physicians eyes and the surfaces with direct skin contact, such as the joystick, the handrail, the headrest and the headband (pooled as 'contact area', **Figure 1**). In both clinics, all slit lamp contact areas were claimed to be wipe disinfected between different patients. More comprehensive cleaning data were obtained from TC1. Here, slit lamps were in addition cleaned carefully either three times a day, once a day or weekly. All relevant metadata details are provided in the **Supplementary Table 1**.

All surfaces were sampled in a meandering pattern using dry, sterile Puritan Hydra Flock Swabs (Puritan Diagnostics LLC, Maine, USA). After sampling, swab heads were broken off into RNA/DNA shield tubes with beads (Zymo Research, Freiburg, Germany) and stored at room temperature until further processing.

### DNA Extraction

For cell disruption, collected swab heads were treated in a FastPrep 24 instrument (MP Biomedicals LCC, Santa Ana, CA, USA) by five rounds of bead beating for 1 min at 6.5 $ms^{-1}$ and then placed on ice for 1 min. DNA was then extracted and purified with the ZymoBIOMICS DNA Miniprep Kit (Zymo Research) following the manufacturer's instructions with slight modifications: After 2 min of incubation at room temperature, the DNA was eluted with 50 µl of 60°C warm, DNA-free water. The flow through was reloaded onto the same filter, incubated for 1 min and centrifuged again. The purified DNA was stored at -20°C until further analyses.

### Preparation of Controls

To better evaluate the community composition analysis process and probable contaminations, positive (mocks) and negative controls were carried out along the experiment. The Skin Microbiome Whole Cell Mix (ATCC MSA-2005, LGC Standards GmbH, Wesel, Germany) was used as a mock community standard, covering a typical part of the human skin bacterial community. The mock community consisted of 6 typical skin bacterial species in equal total cell abundances (*Acinetobacter johnstonii* ATCC 17090, *Corynebacterium striatum* ATCC 6940, *Micrococcus luteus* ATCC 4698,

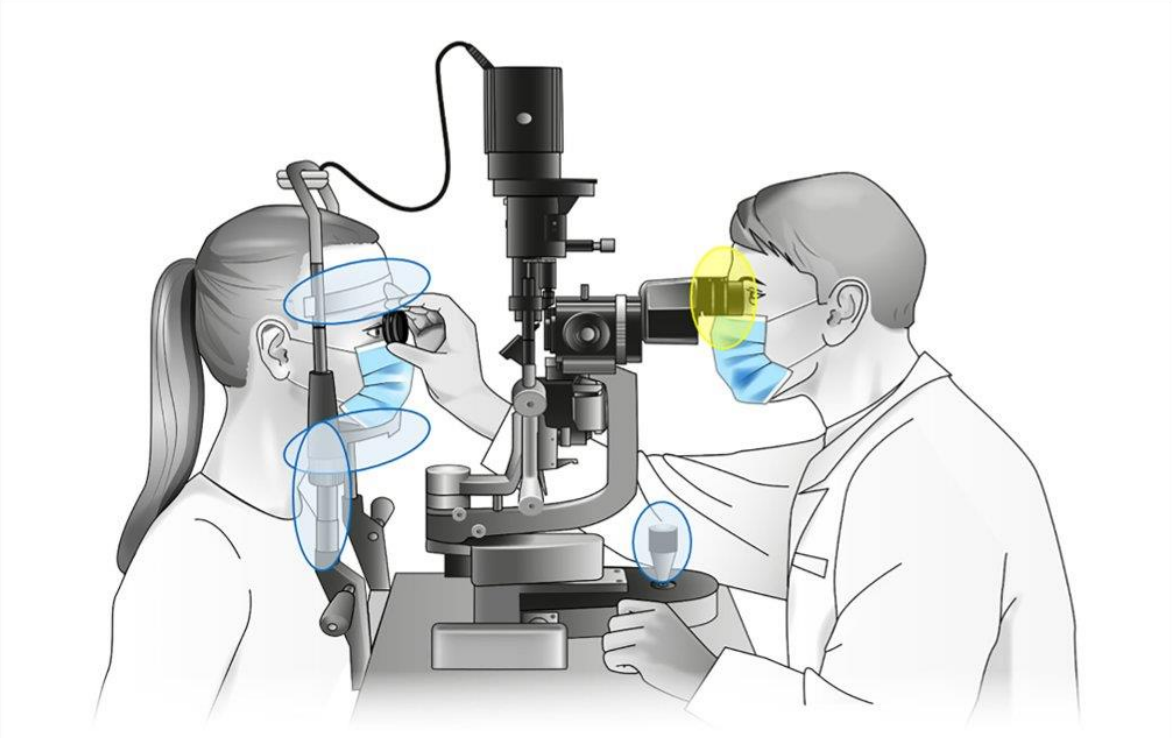

**FIGURE 1** | Sampled parts of the investigated slit lamps. Samples designated "contact area" were taken from headrest, headband, joystick and handholders (marked blue), with probable microbial transmission between patient and physician *via* the handrails. Samples designated "oculars" were taken from the lenses and plastic eyecups (marked yellow). Slit lamp graphic: Own illustration.

*Cutibacterium acnes* ATCC 11828, *Staphylococcus epidermidis* ATCC 12228 and *Streptococcus mitis* ATCC 49456, 16.7% each). The standard was prepared according to the manufacturer´s recommendations (American Type Culture Collection, 2018), so that the final suspension contained about 1.2 x $10^8$ cells/vial (± 1 log). As negative (blank) control, two sterile swabs were processed independently as described above.

## Library Preparation

For construction of amplicon libraries, primers Bact-0341f (5'-CCTACGGGNGGCWGCAG-3') and Bact-0785r (5'-GACTACHVGGGTATCTAATCC-3'), covering the V3-V4 region of the bacterial 16S rRNA gene, were used. We chose this primer pair as it is widely used in many microbiome studies (Klindworth et al., 2013; Thijs et al., 2017; Illumina, 2019; Mancabelli et al., 2020) also with regard to skin (Castelino et al., 2017) and oral microbiota (Zheng et al., 2015), however sometimes with slight modifications. All primers contained an additional adapter sequence tail (Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG), yielding a final PCR product of ~ 529 bp.

Samples were processed in duplicates. Triplicates were performed, if the gel electrophoresis showed only weak bands.

All samples were amplified in a total reaction volume of 25 µl using 3 µl of template DNA as specified elsewhere (Fritz et al., 2020b). The PCR conditions were as follows: 98°C (3 min) initial denaturation, followed by 98°C (30 s), 55°C (30 s), 72°C (45 s), and a final extension at 72°C for 2 min using 35 cycles. DNA amplicons were checked by standard 0.8% agarose gel electrophoresis. With each batch, no-template control reactions were included. Diluted (1:100) DNA from overnight cultures of *Escherichia coli* K12, extracted as described above, was used as template for the positive controls. Clean-up of the PCR products using Agencourt AMPure XP Beads (BeckmanCoulter Inc., Krefeld, Germany), followed by annealing of the dual-index barcodes from the Nextera XT Index Kit v2 Set B (Illumina Inc., San Diego, USA), was performed as described previously (Fritz et al., 2020b). The cleaned libraries were quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Karlsruhe, Germany), while the final quality check was performed with a Bioanalyzer 2100 Instrument with the DNA High Sensitivity Kit (both Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

## Sequencing

The libraries were adjusted to 4 nM (with 10 mM Tris buffer, pH 8.5), combined with 30% PhiX control (Illumina Inc.) and finally diluted to 4 pM. Sequencing was performed on an Illumina

MiSeq platform using the MiSeq Reagent Kit v3 (600 cycle) (Illumina Inc.) with a quality score ≥ 30 and default settings.

## Quantitative Real-Time PCR Detection of MRSA

The obtained DNA extracts were used for a quantitative real-time PCR (qPCR) approach targeting the *mecA* gene of MRSA as described by Huletsky and collegues (Huletsky et al., 2004). The *mecA* gene causes methicillin resistance in *S. aureus* and is part of the staphylococcal cassette chromosome *mec* element (SCCmec), an important mobile genetic element of staphylococci.

The *S. aureus*-specific primer Xsau325f (*5'*- GG ATCAAACGGCCTGCACA-3') and the resistance specific primer SSCmec_mecii574r (*5'*- GTCAAAAATCATG AACCTCATTACTTATG-3') were used at 0.4 µM with 1x Light Cycler 480 SYBR Green Master mix I (Roche Molecular Systems Inc., Mannheim) and 1 U Uracil-DNA Glycosylase (Thermo Fisher Scientific) in a final volume of 20 µl. The mixture was amplified on an Roche LightCycler 480 instrument (Hoffmann-La Roche Ltd, Basel, Switzerland) using the following thermal profile: 50°C (2 min), 95°C (5 min), 45 cycles of 95°C (10 sec), 60°C (20 sec), 72°C (30 sec).

Absolute quantification analysis using the 2nd derivative maximum method was performed followed by a melting curve, applying the '*Tm*-calling' method with default settings. The limit of detection (LOD) was set to a *cp* = 33 using a standard logarithmic serial dilution from Methicillin-resistant *Staphylococcus aureus* EDCC 5246 (DSM 28766). As negative controls, water-template controls were included, as well as the antibiotic sensitive strains *S. aureus* 209 (DSM 799) and *S. aureus* Wichita (DSM 2569). The latter shows only limited antibiotic resistance. Control DNA was extracted from 48 h bacterial cultures as described above and diluted 1:10. To control, if the used staphylococci indeed show antibiotic sensitivity, all strains were plated on Tryptic Soy Agar (TSA; Carl Roth) and Oxoid Brilliance MRSA 2 Agar (Thermo Fisher Scientific). The Brilliance MRSA 2 Agar contains an antibiotic cocktail including Cephalosporin, whereby MRSA grows as blue colonies (Veenemans et al., 2013).

## Bioinformatics and Statistics

All sequences were processed with QIIME 2 – 2020.6 (Quantitative Insights Into Microbial Ecology) (Bolyen et al., 2019). Raw sequence data were imported and demultiplexed using the cassava 1.8 paired-end and demultiplexed FASTQ format. The paired end sequences were joined, quality filtered, denoised and chimera-checked using the q2-dada2 pipeline (–p-trunc-len-f 300 –p-trunc-len-r 257 trim-left-f 0 –p-trim-left-r 0) (McDonald et al., 2012; Callahan et al., 2016). Sequence variant data, resulting from the q2-dada2 pipeline, were then referred to as amplicon sequence variants (ASVs).

For taxonomic assignment, the machine-learning based q2-feature-classifier was trained at a similarity threshold of 99% with q2-scikit-learn (Pedregosa et al., 2011; Bokulich et al., 2018) by the Bact-0341f/Bact-0785r region (V3-V4) of the SILVA 132 database (Quast et al., 2013), followed by taxonomy based-filtering of ASVs classified as mitochondria or chloroplasts.

Due to the sparse compositional nature of microbiome data, beta diversity metric was analysed using robust Aitchinson distances *via* the q2-deicode plugin (Aitchison et al., 2000; Martino et al., 2019). Calculations were performed on the raw count table. Samples with less than 10 features and less than 1000 reads were removed. Statistical differences between the factors 'location' (TC1/TC2) and 'type' (oculars/contact areas) were performed using the 'beta-group-significance' function employing (pairwise) permutational multivariate analysis of variance (PERMANOVA, 999 permutations) with Benjamini-Hochberg p-value correction. For visualisation, compositional principal component analysis (PCA) biplots were created using the emperor biplot function.

Further statistical analyses and graphical visualizations for the sequencing analyses were performed in R 4.0.5 using the packages 'phyloseq' (McMurdie and Holmes, 2013), 'vegan (version 2.5-7)' (Oksanen et al.), 'coin' (Hothorn et al., 2008), 'tidyverse' (Wickham, 2009) and 'qiime2R' (Bisanz, 2019). Figures were created in R using 'ggplot2' (Wickham, 2009).

Correlation of microbial composition between the sample sites at TC1 and TC2 were assessed by procrustes rotation analysis comparing PCA scores, using the 'procrustes' function from the 'vegan' package for R (Peres-Neto and Jackson, 2001; Mardia et al., 2003). The significance of the correlation between samples sites was analysed using the 'protest' function from 'vegan', using 10.000 permutations.

To identify differentially abundant taxa between the covariates 'location' and 'type', analysis of compositions of microbes with bias correction (ANCOM-BC; R-package 'ancombc') (Lin and Peddada, 2020; Edslev et al., 2021) was performed on untransformed and unrarefied counts, including only the 30 most abundant taxa. As there is a bias within the sequence fractions of all samples, this method estimates the unknown sampling fractions and corrects their bias, while normalizing the observed microbial abundance data (Lin and Peddada, 2020). Results are p-values for multiple testing with Benjamini-Hochberg adjustment.

To determine any differences between the communities on the respective surfaces and between the locations, alpha diversity metrics (observed, evenness, faith's phylogenetic diversity and shannon) were calculated using the R-packages 'phyloseq', 'microbiome' (Lahti et al., 2017) and 'picante' (Kembel et al., 2010). For comparative analysis of the diversity indices among the different sample sites, a non-parametric Kruskal-Wallis-Test (Hollander et al., 2014) was performed. A Wilcoxon-rank-sum-test (Hollander et al., 2014) for unpaired samples was calculated to evaluate statistical differences between the two locations TC1 and TC2. Both tests were performed with Benjamini-Hochberg multiple test correction. All metadata are provided in the **Supplementary Table 1**. Further data, such as the unrarefied ASV table and the taxonomic assignments, can be obtained from the corresponding author upon reasonable request.

# RESULTS

## Sequencing Results

High throughput sequencing from 96 samples (91 slit lamp samples, 3 mock samples, 2 negative controls) yielded 467842 chimera-filtered

sequences after the dada2 pipeline, with a mean of 5377 sequences per sample. 9 samples (2 TC1 slit lamp samples, 6 TC2 slit lamp samples and 1 blank control sample) did not yield enough sequences and were removed after the q2-dada2 pipeline for downstream analyses. The remaining samples were rarefied for the calculation of relative abundances using R to a level of 1056 sequences for even sampling depth (seed: 1121983). After removal of singleton taxa, we identified 3369 ASVs from all slit lamp samples. The taxonomic assignment of these ASVs revealed 19 bacterial phyla, 42 classes, 105 orders, 210 families and 468 genera across all slit lamp samples.

## Taxonomic Composition at Different Locations

**Figure 2A** (and **Supplementary Figure 5**) provide relative abundances to get an overview of the community composition. However, for the comprehensive downstream analyses, we used methods that are not based on relative abundances, due to the compositional nature of sequencing data (Gloor et al., 2017).

According to the phylogenetic classification, most of the reads were affiliated with only 10 genera accounting for about 80% of all taxa, with *Cutibacterium* (TC1: 51%; TC2: 38%), *Corynebacterium* (TC1: 15%; TC2: 19%) and *Staphylococcus* (TC1: 8%; TC2: 7%) being the most frequent representatives.

Using the V3-V4 region specific Bact-0341f/Bact-0785r primers, all genera from the mock community were identified correctly, but in slightly varying relative abundances (**Figure 2B**). *Corynebacterium* and *Cutibacterium* were rather underrepresented, while *Acinetobacter* and *Staphylocoocus* seem to be slightly overestimated in their relative abundance. In case of the negative controls, only one out of two unused swabs yielded enough sequences for downstream analysis, which suggests contamination predominantly with *Pseudomonas* species.

## Diversity Analyses

As the relative abundances of bacterial taxa differed between the two sampling sites (contact area, oculars) and the two locations (TC1, TC2), alpha diversity metrics were calculated. Diversity tended to be higher in TC2 with regard to the observed, shannon and faith-fd metrics, but no statistically significant differences could be detected, neither between TC1 and TC2 nor between contact areas and oculars (**Supplementary Figure 1**) or the cleaning intervals, three times a day, once a day or weekly, within TC1 (**Supplementary Figure 2**) or occupancy of physicians (**Supplementary Figure 4**).

Compositional beta diversity analysis also revealed no statistical significant differences between the locations (PERMANOVA $p_{adjust}$-value > 0.05) and the contact areas (PERMANOVA $p_{adjust}$-value > 0.05). However, some samples from TC1 tended to cluster apart from TC2.

The biplot (**Figure 3**) highlights the taxa which drive the placement of the samples in the plot and strongly influence the PCA-axes. Some separation of samples from TC1 and TC2 are presumably driven by differences within the taxonomic composition, especially by corynebacteria.

No statistically significant influence of the patient throughput and the occupancy of physicians for the different rooms was

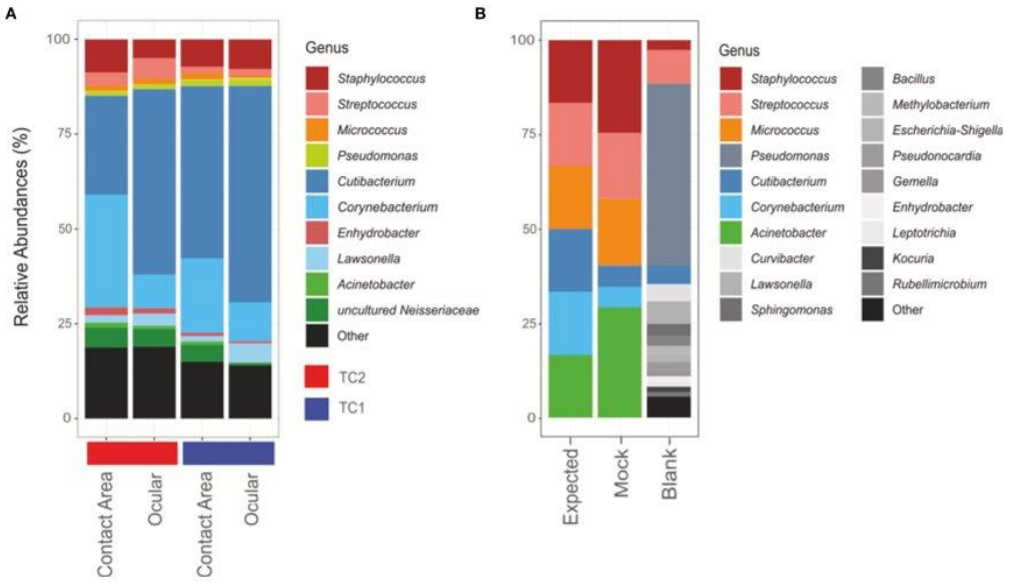


**FIGURE 2** | Bar chart of relative abundances of ASVs classified on genus level. Taxa with relative abundance of less than 1% were collectively summarized as 'Other'. **(A)** Taxonomic composition of the slit lamp bacteriota from TC1 (Tertiary Center) and TC2 respectively and the respective sample sites (contact area – TC1: n = 28; ocular – TC1: n = 28; contact area - TC2: n = 14; ocular – TC2: n = 13). **(B)** Composition of mock taxa and controls (expected = expected mock abundances; mock = mock standard using Bact-0341f/Bact-0785r primers; blank = blank negative control).

detected (**Supplementary Figures 3**, **4**, **5B, C**), although alpha diversity measures suggest a slightly higher diversity on slit lamps in rooms with low occupancy rates at TC1.

To determine the community congruency between the ocular and contact area samples, we used procrustes analysis, which compares the microbiome on the two sample sites of each slit lamp. However, no statistically significant association could be detected. Nevertheless, a correlation between microbial compositions at the two sampling sites from the same slit lamp instrument is suggested, since many sample pairs stemming from the same instrument are located in relatively close proximity within the PCA plot (**Figure 4**), especially at TC1.

However, differential abundance analysis (**Figure 5**) revealed the taxa with significant differences between the two locations: Two taxa were more abundant in TC2 (*Enhydrobacter*, *Chryseobacterium*; $p_{adj} < 0.05$), whereas five groups/genera (unclassified bacteria, *Cutibacterium*, *Turicella*, *Methylobacterium*, *Staphylococcus*, $p_{adj} < 0.05$) were enriched at the slit lamps at TC1. At TC1, four genera showed significant differences between the two sample sites ($p_{adj} < 0.05$): *Unclassified Neisseriaceae*, *Lawsonella*, *Corynebacterium* and *Acinetobacter*. Except *Lawsonella*, all genera were enriched at the contact areas. For the slit lamps at TC2, no significant difference in community composition between oculars and contact areas were detected.

## Quantitative Real-Time PCR Results for MRSA

MRSA strain *S. aureus* EDCC 5246 grew as blue colonies on Brilliance Oxoid MRSA2 agar after 48 h at 37°C, whereas the other *S. aureus* strains did not thrive on the respective plates.

Genomic DNA from *S. aureus* EDCC 5246 was used as standard and positive control in the assay. DNA was prepared and 1:10 diluted from 48 h old cultures that yielded a DNA concentration of 1.94 ng/µl resulting in a mean *cp*-value of 17 and a melting temperature ($T_m$) of 79.8°C. The detection limit (LOD) was determined at a *cp* -value of 33, corresponding to a 1:1000 dilution. Although higher dilutions also showed *cp*-values, these curves were in too close proximity to signals from the non-MRSA-strains.

The antibiotic sensitive *S. aureus* 209 showed *cp* -values > 36 and the antibiotic susceptible *S. aureus* Wichita *cp* -values > 35. Water-template controls resulted in *cp* values > 40 or showed no signals. Only one sample from TC2 (contact area) showed relevant *cp*-values of 35 and a melt-peak at 80°C, which is close, but still below the detection limit, respectively close to the MRSA *S. aureus* Wichita. In conclusion, MRSA DNA was below the detection limit for all investigated samples.

## DISCUSSION

Many regularly touched surfaces represent fomites (Rusin et al., 2002; Gerba et al., 2016; Di Lodovico et al., 2018). Their microbial load is problematic especially in clinical environments (Weber et al., 2010; Graham et al., 2019), where it contributes to hospital acquired infections and particularly threatens immunocompromised patients. This study focuses on slit lamps, optometric devices widely used in medical eye facilities, characterized by surfaces with close contact to the examiner and many different patients. Although relations between shared optical devices and eye infections have long

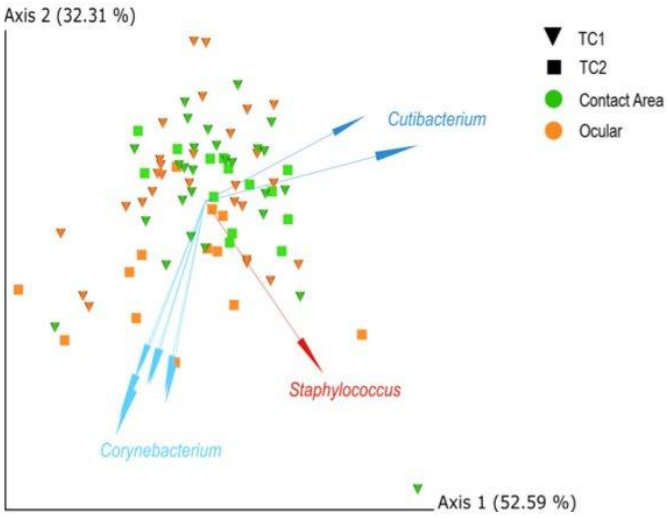


**FIGURE 3** | PCA biplots of centred-log-ratio (clr)-transformed data for Location and Type, based on robust Aitchinson distances. Arrows indicate important log-ratios between features that strongly influenced the principal component axis. Squares = TC1, cones = TC2, orange = ocular samples and green = contact area samples; TC, Tertiary Center.

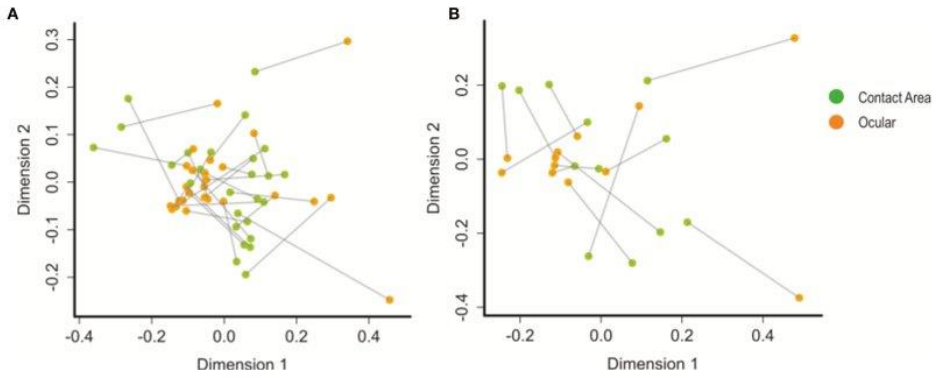


**FIGURE 4** | PCA plots by procrustes analysis between the ocular and contact area data sets using Bray-Curtis distances and 10.000 iterations. At **(A)** TC1 (contact area: n =28; ocular: n = 28) and **(B)** TC2 (contact area: n = 14; ocular: n = 13). Every data point represents a single slit lamp sample coloured by sample site (orange = ocular samples, green = contact area samples). Lines connect the same slit lamp samples (Contact area – TC1: n = 28 ocular – TC1: n = 28; contact area - TC2: n = 14; ocular – TC2: n = 13). TC, Tertiary Center.

been suspected (Olcerst, 1987), knowledge about the bacterial contamination of slit lamps is scarce. Previous cultivation-based examinations, (Graham et al., 2008; Sobolewska et al., 2018) of slit lamps proved the presence of human skin bacteria, however with max. amounts of 3 CFU/24 $cm^2$ at a relatively low concentration, which is at least 2 to 3 log scales less, than the bacterial load on similar devices, such as spectacles (Fritz et al., 2018) and microscope oculars (Olcerst, 1987; Fritz et al., 2020b). Recent molecular studies (Fritz et al., 2020b; Fritz et al., 2020a) of the microbiota on such surfaces, revealed a high bacterial diversity stemming from human skin, mucosa and the environment, and including potential pathogens. Our study significantly increases knowledge about the bacterially diverse communities on slit lamp surfaces and their hygienic relevance. It completes the picture, when evaluating the microbial contamination of slit lamps, as previous studies have been

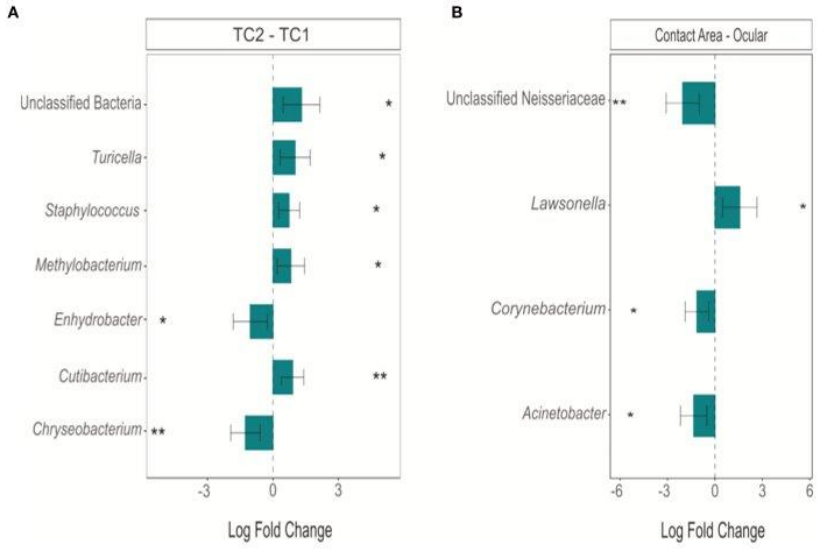


**FIGURE 5** | Differentially abundant genera between **(A)** the two locations (TC2 – TC1) and **(B)** the sample types at TC1 (contact area – ocular), calculated using ANCOM-BC. Bars represent the estimated log-fold change (natural log) of genera between the selected conditions, error bars represent the 95% confidence intervals. Asterisks mark a statistically significant difference between the conditions (*$p_{adj} < 0.05$; **$p_{adj} < 0.01$), based on two-sided Chi-square tests using W with Benjamini-Hochberg correction; TC, Tertiary Center.

limited to cultural-dependent analyses alone. Molecular methods better account for anaerobic, slow-growing or yet uncultured bacteria and therefore provide a more comprehensive insight. Following recent suggestions (Gloor et al., 2017; Martino et al., 2019), our bioinformatic analyses also considers the compositional nature of sequencing data.

Clearly, also sequencing-based methods can be biased, e.g. by discriminating certain groups of organisms due to primer selectivity (Kong, 2016; Kong and Segre, 2017; Zeeuwen et al., 2017; Escapa et al., 2018; Knight et al., 2018). Therefore, we evaluated the used primer set on a standard skin-mock community (**Figure 2B**). It became apparent, that all typical skin bacteria were covered comprehensively, while the primers also produced good quality PCR products (data not shown). However, as all sequencing data presented here are DNA-based, they do not reveal if the detected bacterial taxa were alive or dead.

As negative controls, unused swabs (blank) were processed to identify potential contaminants. In one out of two swabs, DNA from bacteria probably stemming from water, environmental and human sources could be detected, dominated by *Pseudomonas*. It is known that even small amounts of contaminating DNA can become overrepresented using PCR-based methods and influence the community composition of a sample (Karstens et al., 2019). Generally, the identified genera in the blank control were reported before as typical contaminants from analysis kits, PCR reagents or water in NGS-experiments (Salter et al., 2014), or as representing just sequencing errors. Therefore, it is recommended to remove all sequences below a defined relative abundance threshold from further analysis (Karstens et al., 2019). In accordance with the results from the mock samples, we set this threshold at 1% relative abundance. Therefore, it is safe to assume that contaminants from exogenous sources are negligible here.

Our results show bacterial contaminations on 83 out of 91 investigated slit lamp samples, as these yielded enough sequences for downstream analyses. Based on the relative abundances of the bacterial taxa and alpha diversity measures, the bacteriota composition was largely similar between the two investigated locations and the two different sample sites. A few taxa differed in read abundance between TC1 and TC2, which may reflect the individuality of the patient´s and/or doctor´s skin microbiota.

Predominantly, we identified cutibacteria, corynebacteria and staphylococci on the slit lamps. This is in line with previous findings for spectacles and microscope oculars, where we found largely the same dominant taxa. We could also prove the presence of staphylococci, which matches the cultivation-based detection of coagulase-negative staphylococci by Sobolewska et al. (Sobolewska et al., 2018). While they identified staphylococci as the most frequent bacteria on slit lamps, we found this genus in lower proportions, which may be due to the use of different methods. Nevertheless, their presence is important, as staphylococci, along with streptococci, are among the most common bacteria to cause bacterial conjunctivitis and keratitis (Watson et al., 2018).

In general, many of the identified bacterial taxa are common colonizers of the eye surface and the lid margins, such as *Staphylococcus*, *Cutibacterium*, *Corynebacterium* and *Pseudomonas* (Grzybowski et al., 2017; Delbeke et al., 2021). Under normal conditions, these commensals may also contribute to eye surface health and immunity (Kugadas and Gadjeva, 2016; Cavuoto et al., 2019), however, if in dysbiosis they may lead to severe eye infections. In addition to the previously mentioned taxa, the relatively most abundant identified genera also included *Streptococcus, Neisseria* and *Enhydrobacter*. All these genera are known to comprise several species with the potential to cause eye infections (cf. **Supplementary Table 2**). Although our sequencing data do not allow a reliable identification on species or strain level, they nevertheless suggest a considerable pathogenic potential of the investigated surfaces.

All frequently identified genera are also associated with human skin, mucosa or the environment (Seifert et al., 1997; Fernández-Natal et al., 2008; Humbert and Christodoulides, 2019; Delbeke et al., 2021) and have been reported in the context of eye and nosocomial infections (Kuriyan et al., 2017; Wong et al., 2017; Humbert and Christodoulides, 2019).

While the oculars of slit lamps are predominantly used by only a few physicians, surfaces such as headrest, headband and handholders are touched by many different patients, as well as by the examiner. However, alpha diversity analysis did not detect any statistical significant differences of the bacteriota composition on the two sampled sites, nor between samples with different patient and physician occupancies or cleaning intervals.

Procrustes analysis suggested a relatedness of microbial community compositions at the two sampling sites from the same slit lamp instrument. However, this association was not found statistically significant, which may be a function of the relatively low number of sampled instruments. Nevertheless, our data allow careful speculations that bacteria between ocular and contact areas are exchanged, putting emphasis on hygienic cleaning of the ocular area after use.

Evaluation of the different cleaning intervals in TC1 did not reveal statistically significant differences in bacterial community composition. As the contact areas were wipe disinfected on a regular basis, frequent contact by different persons obviously could not contribute to an increased bacterially diverse community for both locations and sampling sites. Notably, our sampling took place during the COVID-19 pandemic, which was accompanied by special hygienic measures, such as more frequent cleaning and disinfection as well as rigorous wearing of face-masks. This might have influenced bacterial load and diversity on the investigated surfaces. Nevertheless, microbial transfer between skin, eyes and slit lamp surfaces might take place by touching, but also breathing or direct eye or eyelashes contact. In view of the overall great bacterial diversity detected here, strict hygiene measures are definitely required.

Multi-drug resistances are of particular hygiene concern. Methicillin-resistant *Staphylococcus aureus* (MRSA) is known to be highly prevalent in hospital environments (Boucher and Corey, 2008), but also on daily used and shared devices (Gerba et al., 2016), showing a high transmission efficiency (Del Campo et al., 2019). Previous 16S rRNA gene sequencing studies (Fritz et al., 2020b; Fritz et al., 2020a) identified many

staphylococci on optical surfaces, while its known, that several staphylococci comprise antibiotic resistant strains (Ventola, 2015), such as MRSA. Our qPCR analysis did not reveal the presence of MRSA in any sample. We used a single primer pair published by Huletsky et al. (Huletsky et al., 2004), which covers a variety of MRSA strains. A multiplex assay would expand the detection spectrum and could be considered for further studies. However, since many of the identified taxa comprise species known to carry (multiple) antibiotic resistances, this topic remains challenging. Further studies might also include a metagenomic approach, such as whole genome shotgun sequencing (WGS) to allow a more comprehensive detection of resistant genes and virulence factors. Furthermore, if enough reads are generated, WGS can provide a broader and more accurate resolution of microbial diversity, especially for less abundant taxa (Ranjan et al., 2016). However, as WGS does not rely on PCR amplification, it requires a larger amount of input DNA compared to 16S rRNA amplicon sequencing, which often turns out to be a limiting factor. Finally, polyphasic studies (such as (Fritz et al., 2020b), involving cultivation-based as well as molecular techniques might be useful to better discriminate living from dead taxa, the latter of which are surely less important from a hygienic point of view.

Beside bacteria, viruses, such as Adenovirus or Herpes simplex, would be interesting study subjects, as many viruses cause severe eye infections (Yoshikawa et al., 2001; Azher et al., 2017; OYong et al., 2018). A significant viral load on slit lamps may be possible, as studies showed that they can remain infectious on environmental surfaces for considerable time periods (Kramer et al., 2006; Ganime et al., 2014).

## CONCLUSION

We were able to show that slit lamps carry a broad diversity of bacteria and therefore might be associated with ophthalmic diseases. Our study significantly extends previous findings about their bacterial load by applying molecular, cultivation-independent techniques. It provides a solid and comprehensive basis for a deeper understanding of the hygienic relevance of these widely used medical devices. We identified many bacterial genera of human skin, eye or mucosa origin, known to comprise species to cause skin and eye infections, such as staphylococci or streptococci. Even if wipe cleaning is performed regularly between each patient, also the disinfection of the oculars, mostly used by the physicians, should be considered. Clearly, slit lamps represent fomites and proper disinfection of all contact surfaces is important to secure the health of patients and examiners.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB45031.

## AUTHOR CONTRIBUTIONS

BF designed the experiments, took the samples, performed qPCR analyses, sequenced the samples and analyzed the data. EP prepared sequencing. FZ and DB provided access to the samples. SW, FZ, and ME performed project administration and supervised the work. BF and ME wrote the manuscript. ME supervised and conceptualized the work. WY assisted with the ancom-bc and procrustes analysis. All authors edited and approved the final manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.745653/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## 2.5 Establishment of a qPCR-based assay to quantify human adenovirus.

### 2.5.1 Introduction

Ocular infections are also commonly caused by a variety of different viruses, wherein any part of the eye can be involved, even leading to vison loss.

Known and frequent pathogens of viral eye infections are diverse herpes viruses, such as herpes simplex virus (HSV) or varicella zoster virus (VZV), causing (reoccurring) keratoconjunctivitis, conjunctivitis or blepharitis, for instance, but cytomegalovirus (CMV) is also a common cause of eye inflammation (Ritterband and Friedberg 1998).

However, most viral eye infections are assumed to be of human adenovirus (HAdV) origin and may lead to an extremely contagious epidemic keratoconjunctivitis. This virus is reported as being responsible for several outbreaks in hospitals and schools within recent decades (King et al. 2013; OYong et al. 2018), particularly caused by the human adenovirus species A, D and E (Jonas et al. 2020). Furthermore, viral transmission is considered to occur mostly from contaminated surfaces and instruments (Ganime et al. 2014).

The seven species (A - G) of human adenoviruses belong to the family *Adenoviridae* and are medium sized, non-enveloped, dsDNA viruses. The capsid consists of several proteins, whereas the gene coding for the highly conserved hexon protein is most frequently used for diagnosis (Jonas et al. 2020). Figure 3 gives an overview of the different HAdV species, types and associated diseases.

As no universal marker for overall virus identification exists, every PCR-based viral detection assay requires a target of a sufficiently conserved DNA section. The study presented here aimed at establishing a robust qPCR assay for the detection of human adenovirus in swab samples obtained from ophthalmologically relevant surfaces.

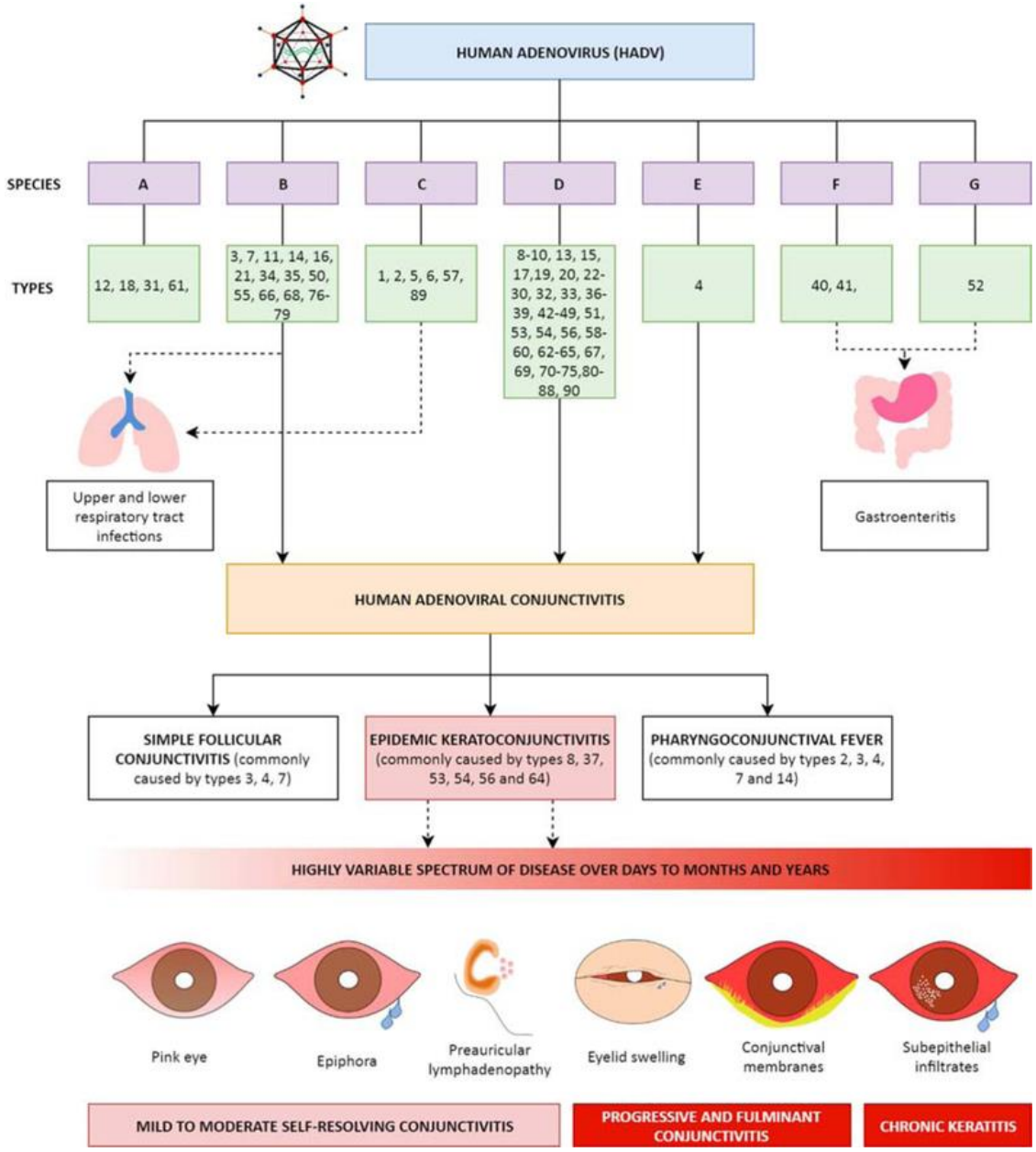


***Figure 3:*** *Overview of HAdV (human adenovirus) species, types and associated clinical syndromes. Reprinted from: Mystery eye: Human adenovirus and the enigma of epidemic keratoconjunctivitis, Vol. 76 by Jonas et al., 2020, with permission from Elsevier.*

### 2.5.2 Material and Methods

For the spectacle samples, the entire surface of seven spectacles from university staff and students was swab sampled using dry, sterile Puritan Hydra Flock Swabs (Puritan Diagnostics LLC, Maine, USA). Spectacles and usage data of the spectacle wearers were provided voluntarily. Informed consent to use the obtained data for scientific purposes

was obtained orally. The personal or health-related data of the participants were not recorded.

After sampling, swab heads were broken off into a tube with DNase-free water and incubated at 37 °C for 3 h at 400 RPM, followed by 10 s of rigorous shaking.

Additionally, DNA extracts from the slit lamp study (Fritz et al. 2021) were analysed. However, unlike the freshly prepared spectacle samples for explicit virus detection, DNA extraction here was performed using a FastPrep 24 instrument (MP Biomedicals LCC, Santa Ana, CA, USA) and the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Freiburg, Germany), as described elsewhere (Fritz et al. 2021), wherein the basic intention was downstream 16S rRNA gene amplicon sequencing for bacterial identification.

**Samples and controls**

An artificially synthetized DNA fragment (Invitrogen by Thermo Fisher Scientific, Passau, Germany) was used as a highly concentrated standard (1.0 x $10^{10}$ copies/µl), representing the amplicon generated by the qPCR primer pair (hex1deg and hex2deg) as described below. Starting from this initial concentration, a standard 1:10 dilution series for quantification was prepared in DNase-free water.

As a positive control, a commercially available, inactivated "Adenovirus PCR control" (Mikrogen GmbH, Neuried, Germany) was obtained, consisting of virus particles of the supernatant of human adenovirus-infected Vero cells. This control represents a weak positive clinical sample and contains ~100 copies DNA/µl with a mean cp value of 31, according to the manufacturer's specifications. As negative (no template) controls, water-template controls were included.

As a control to proof whether viral DNA can be extracted from swabs, two microscope slides were each covered with 30 µl of the "Adenovirus PCR control" suspension, air-dried for 5 min and sampled in a meandering pattern with a single, dry, sterile Puritan Hydra Flock Swab (Puritan Diagnostics LLC) and processed as described for the spectacle samples above.

**DNA-extraction**

The viral DNA from controls and spectacle swabs was extracted and purified with the "PureLink Viral RNA/DNA Mini Kit" (Invitrogen by Thermo Fisher Scientific) following the manufacturer's instructions. The purified DNA was stored at -20°C until further analyses.

**Amplification of samples and controls using qPCR**

The established qPCR assay was based on a study by Allard et al. (2001) for rapid typing of human adenoviruses, targeting a part of the hexon gene. Amplification was performed using the primer pair hex1deg (5'-GCCSCARTGGKCWTACATGCACATC-3') and hex2deg (5'-CAGCACSCCICGRATGTCAAA-3'), yielding a qPCR product of 301bp. The target sequence of the hexon gene is of sufficient heterogeneity to allow discrimination between subgenera and even between serotypes of HAdV.

All extracted samples were amplified in duplicates, using the LightCycler480 SYBR Green I Master on a LightCycler 480 system (both Roche, Basel, Switzerland). In order to eliminate carryover-contaminations of previous runs, Uracil-DNA-Glycosylation was performed prior to the actual qPCR reaction. The qPCR setup and cycling conditions for the amplification were as follows: 1 U Uracil-DNA Glycosidase (Thermo Fisher Scientific), 4.5 µl of DNase-free water, 12.5 µl Mastermix, 1 µl of forward (10 µM) and reverse primer (10 µM), respectively, and 5 µl of template DNA, in a final volume of 25 µl. The PCR profile was as follows: UDG reaction at 50 °C for 2 min, followed by 95 °C initial denaturation for 10 min and 50 cycles of 95 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s. Subsequently, the melting curve thermal profile was 95 °C (5 s), 65 °C (1 min) and final denaturation starting at 97 °C at a ramp rate of 0.11 °C/s. Figure 4 provides an overview of the complete workflow.

Absolute quantification analysis using the 2$^{nd}$ derivative maximum method was performed followed by a melting curve, applying the "Tm-calling" method with default settings. The limit of detection (LOD) was set to a cp = 39 using a standard logarithmic serial dilution of the artificial amplicon generated by the two given primers.

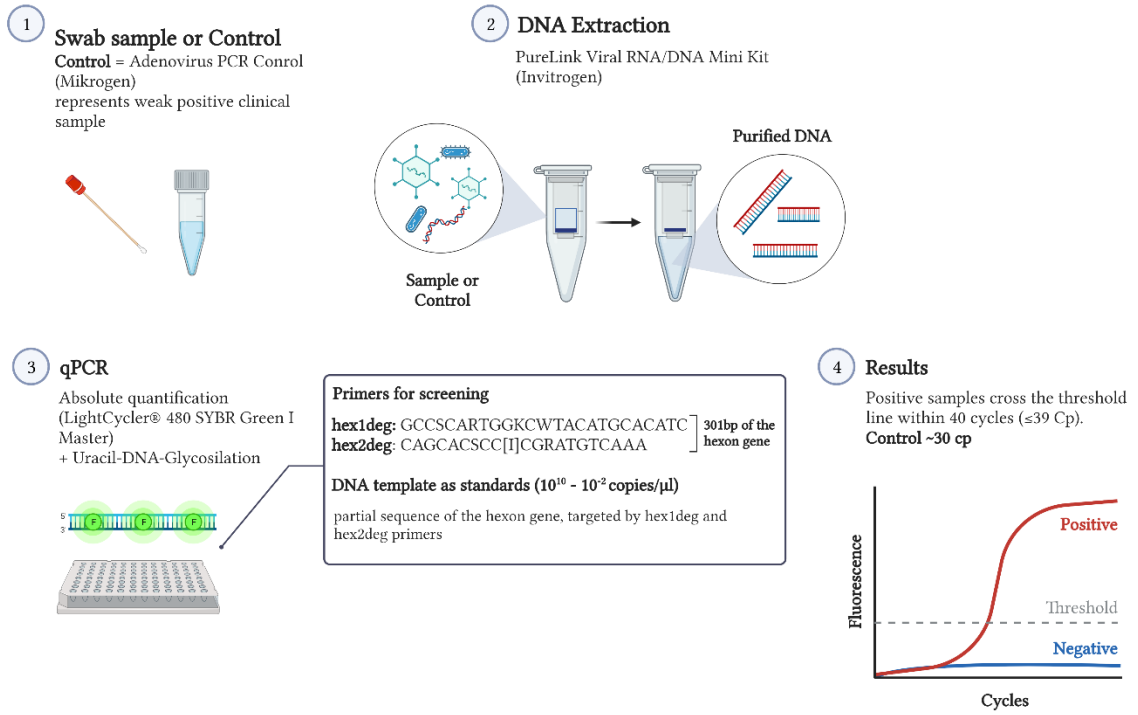


***Figure 4:*** *Adenovirus-detection assay. Workflow using qPCR with absolute quantification. Own illustration using biorender.com.*

### 2.5.3 Results

A standard curve from the artificial amplicons was created from $10^6$ - $10^{-2}$ copies/µl, with a slope of -3.54 and an efficiency of 1.91 at a mean Tm (melting temperature) of 88.3°C and saved as an external standard curve (figure 5). No signals could be detected below $10^0$ copies/µl, at a mean cp of 39, therefore determining the limit of detection (LOD).

The control swabs from the microscope slides, covered with the "Adenovirus PCR control", revealed signals at a mean cp of 32.6 and a Tm of ~88.3°C. The "Adenovirus PCR control" revealed signals at a mean cp of 30.5, resulting in a Tm of 88.3°C. All spectacle samples, as well as 5 out of 69 slit lamp-samples showed signals at a Tm of ~88 °C, but below the detection limit (data not shown).

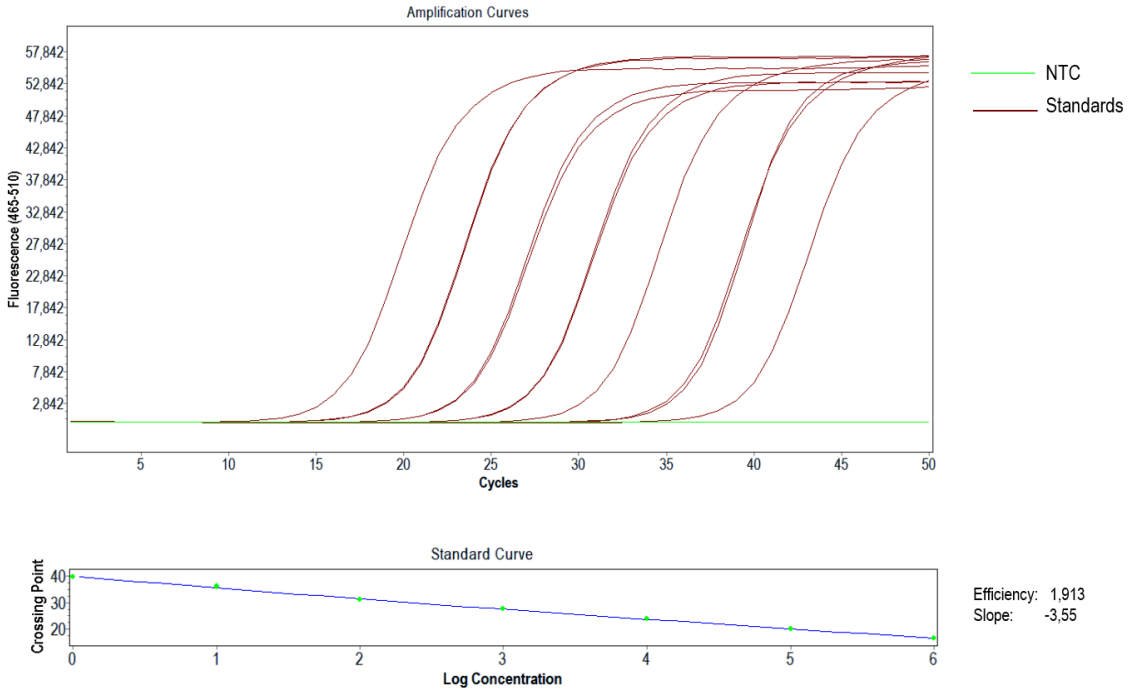


***Figure 5:*** *Adenovirus amplification- and standard curves (ranging from $10^6$ - $10^0$ copies/µl; Standards with $10^7$, $10^8$, and <$10^0$ copies/µl were excluded from calculation due to insufficient quality or lacking signals). Curves were amplified from an adenovirus artificial template. Only standard curves that yielded signals are displayed.*

### 2.5.4 Discussion

Adenovirus contaminations are of special importance within hospitals, as they may be particularly contagious and a source of severe eye infections, even though the vast majority are non-fatal infections (Lynch and Kajon 2016). Additionally, these viruses are remarkably resistant to chemical or physical agents and extreme pH conditions, allowing them to stay infectious on surfaces for prolonged periods of time, even weeks (Gordon et al. 1993). Especially in clinical environments, up to 44% of all evaluated surfaces were found to be contaminated with HAdV (Ganime et al. 2014).

It has been shown that a viral DNA amplicon content of more than 1 - 10 copies/µl can be detected from the swabs. Neither from the spectacle samples nor from the slit lamps could clear qPCR signals be detected. Since the sample size of seven spectacles is small, follow-up studies should focus on more items, probably with a broader subject variety. For instance, spectacles from children (≤4 years) would be interesting, as HAdV respiratory tract infections are reported to be recurrent within this group. HAdV8 genotype mostly causing ophthalmic infections is prevalent among persons older than 20

years (Akello et al. 2020). Spectacle evaluations among this group would therefore be interesting, too.

As many human adenovirus infections in eye clinics are supposed to originate from contaminated ophthalmic instruments or eye drops (OYong et al. 2018), evaluation of clinical devices would be of special interest.

The reason that no or only weak HAdV signals were detected from the slit lamp samples here, suggests no or little HAdV contamination. However, it's also possible that the DNA extraction method used for the slit lamp samples was not suitable for viral extraction, as these DNA samples were initially prepared for 16S rRNA gene sequencing analysis and DNA extraction was optimized for bacteria. Preliminary experiments (data not shown) revealed that the "Adenovirus PCR control", extracted with the Zymo-Kit applied for bacteria, indeed showed a significant increase of 8 cycles (from a cp ~31 to a cp ~39).

In addition, the use of SYBR green as an intercalating DNA dye might lead to the detection of non-specific PCR signals, which can lead to false positive results, even though melting curves were performed. Further projects should therefore consider the use of specific DNA probes, especially if a distinction between different virus subtypes is intended.

### 2.5.5 Conclusion

Rapid detection of human adenovirus (HAdV) from surface samples with clinical relevance might be useful to prevent outbreaks of highly infectious viral diseases. This study aimed at establishing a robust qPCR assay for rapid detection of HAdV from liquid and swab samples to analyse surface contamination of ophthalmic objects. However, although the assay per se worked reliably, no signals above the LOD were obtained from the sampled devices. Further studies should include a larger sample size as well as clinical samples from spectacle-wearing patients suffering from acute eye diseases caused by HAdV.

# 3 Discussion

Spectacles, microscope oculars and slit lamps are frequently and widely used technical devices that are prone to contamination with microorganisms of hygienic relevance. Knowledge about microbial load is scarce, if available at all. Despite the known limitations, microbial surface contaminations on these devices have so far been studied with cultivation-based methods only. For the first time a cultivation independent method, high throughput 16S rRNA gene amplicon sequencing, was applied, to thoroughly analyse the bacterial community composition of ophthalmologically relevant devices.

## 3.1 Ambient bacterial load of frames and lenses of spectacles and evaluation of different cleaning methods

In publication I (Fritz et al. 2018) the microbial load and community composition of 11 spectacles obtained from university members and 10 spectacles from inhabitants of a nursing home for elderly people were determined by aerobic cultivation.

All spectacles were found to be contaminated with bacteria, with sites undergoing direct skin contact (nosepads and earclips) showing the highest densities. The median bacterial load of the spectacles from both environments did not differ significantly (university spectacles: $1.4 \pm 10.7 \times 10^3$ CFU $cm^{-2}$; nursing home spectacles: $20.8 \pm 39.9 \times 10^3$ CFU $cm^{-2}$). There were 182 isolates that could be assigned to 10 different bacterial genera, with staphylococci dominating the spectacles of both groups of persons. Generally, well-known colonizers of the human skin were observed, such as staphylococci, micrococci, corynebacteria, brevibacteria or *Acinetobacter* sp. (Gao et al. 2006). Obviously, spectacles reflect the skin community where direct skin contact occurs, such as the area behind the ears (retroauricular crease) or the sides of the nose (alar crease). Given that spectacles represent an aerobic environment, the study was performed under aerobic cultivation conditions. Aerotolerant anaerobes will likely not thrive well under such conditions. Consequently, no propionibacteria (cutibacteria) and only one single isolate of *Corynebacterium* sp. were detected, although these taxa are known to colonize skin sites that experience direct contact with spectacles.

The diversity was found to be 5 times higher on the spectacles of elderly wearers, since the skin is subjected to age-related changes, affecting hormone balances, pH value, and sebum production. Such changes consequently influence the skin's bacterial community

composition and increase its diversity (Leyden et al. 1975; Shibagaki et al. 2017). Obviously, this age-related effect also affects bacteria on spectacles.

Notably, all isolated bacteria clearly represent viable cells that could potentially cause infections. Up to 64% of the identified bacteria represented potential pathogens, mainly affiliated with the genus *Staphylococcus*. Some of them, such as *Staphylococcus epidermidis* or *S. aureus*, are associated with skin diseases and eye infections (see chapter 1.3, table 1). Additionally, these species are known to comprise antibiotic resistant strains, such as MRSA (Methicillin-resistant *S. aureus*) or MRSE (Methicillin-resistant *S. epidermidis*) (Ventola 2015).

Multidrug resistant organisms are a particular problem in modern medicine. Therefore, one of the goals of the World Health Organization's "Global Action Plan" (WHO 2015) is to reduce the risk of infection through effective sanitation, hygiene and infection prevention measures. Resistant bacteria circulate in communities and can be transmitted via skin-to-surface contact and vice versa, which can be particularly problematic in health-related and public areas. One method of control is to prevent bacterial transmission. For ophthalmologically relevant surfaces, this can be achieved, for instance, by attending to hand hygiene, proper cleaning measures and/or the use of antimicrobial coatings.

Hence, the antibacterial efficacy of four widespread spectacle cleaning methods was investigated by using test bacteria that had been identified as being dominant on spectacles (Fritz et al., 2018) and smartphone touchscreens (Egert et al. 2015), representing a similar surface. Best cleaning results with a germ reduction of up to two log scales were obtained with impregnated wipes, with and without an alcoholic formulation; dry cleaning was less effective (mean germ reduction of 85% - 90%, compared to 99% - 100% using wet wipes). In order to verify the measured germ reduction for naturally contaminated spectacles, worn spectacles from university members were thoroughly cleaned using wet wipes. Following cleaning, the spectacles showed no or only slight bacterial contamination (94% less bacteria). Clearly, spectacle surface cleaning should be performed regularly (Fritz et al. 2018).

## 3.2 Site-specific molecular analysis of worn spectacles

This study represents the first molecular, cultivation-independent analysis of the bacterial community on spectacles. Compared to the 10 genera found with the cultivation-based approach (Fritz et al. 2018), 665 genera were identified here, which underlines the power of molecular analyses to unravel microbial diversity.

At genus level, 13 genera accounted for 84% of the total sequences of all spectacles, with a prevalence of more than 1% relative abundance. *Propionibacterium (Cutibacterium)* (57%), *Corynebacterium* (5%), *Staphylococcus* (4%), *Pseudomonas*, *Sphingomonas* and *Lawsonella* (3% each) were the dominant taxa.

Unlike the cultivation-based study (Fritz et al. 2018), propionibacteria were the dominant taxon, probably due to the aerobic cultivation conditions applied in publication I. The majority of bacterial microorganisms likely originate from human skin and the aero-digestive tract. From skin studies it is known that especially propionibacteria (cutibacteria), corynebacteria and to a lesser extent staphylococci dominate sebaceous sites, as found at the retroauricular crease and on the alar crease, but also on the palms of the hands, as shown in chapter 1.2, figure 1 (Grice and Segre 2012; The Human Microbiome Project Consortium 2012; Scholz and Kilian 2016). These bacteria might also find their way onto spectacles during cleaning or touching these devices.

In order to evaluate the spatial distribution of microorganisms across spectacles, the community structure and diversity of three different sample sites were analysed. In particular the glasses tended to differ from the other sample sites, as they carried the most diverse bacterial community. Presumably, bacteria are transferred easily from human skin to the earclips and nosepads, whereas the glasses are in a more remote position to the skin and are therefore exposed to additional microbial sources, such as (breathing) air and dust.

Furthermore, bacterial species with a risk group 2 classification according to the German TRBA (BAUA – Bundesanstalt für Arbeitsschutz und Arbeitsmedizin 2015) were identified on the investigated spectacles. This implies a probable infection risk to humans. Additionally, genera were detected that are known to comprise species with antibiotic resistances (such as staphylococci), as mentioned earlier. Although the relative abundance of staphylococci on spectacles might be lower than previously expected from the cultivation-based study, further investigations should nevertheless examine spectacles as potential carriers of antibiotic-resistant bacteria in more detail.

## 3.3 Polyphasic analyses of the microbiota on microscope oculars

Bacterial transmission between human beings is more likely if surfaces are touched by different persons. Light microscopes are extremely widespread analytical instruments (see chapter 1.5), present in virtually any clinical and/or biological laboratory, and are usually used by more than one person. A previous, cultivation-based study (Olcerst 1987) indeed suggested that direct contact with microscope eyepieces significantly increases the risk of eye infections and 26% of the investigated oculars carried bacteria known to be pathogenic or potentially pathogenic, such as *Staphylococcus aureus* (Olcerst 1987).

In order to obtain a comprehensive view of the microbial community profile on microscope oculars, and its potential hygienic relevance, publication III was a polyphasic study (Fritz et al. 2020b) using cultivation-based and molecular analyses in parallel. Samples of 10 left oculars were used for cultivation, quantification and MALDI-biotyping (MALDI-TOF MS) of representative isolates. Samples from the respective 10 right oculars were used for a 16S rRNA gene sequencing approach. After sampling, all tested surfaces were carefully cleaned with 70% isopropanol, re-sampled and analyzed again.

All oculars were found to be contaminated with bacteria. Cell counts ranged between a mean of 235 and 575 CFU $cm^{-2}$, which is approximately one log scale less than the bacterial load on spectacles (Fritz et al. 2018), but higher compared to the bacterial load on other frequently touched items, such as smartphone touchscreens and similar to computer keyboards (Messina et al. 2011; Fritz et al. 2018). Although selective media for fungal detection were used, no fungal colonies were identified. 114 morphologically different isolates were assigned to 64 genera and 34 species, mainly *Cutibacterium*, *Staphylococcus* and *Brevibacterium*, with the most abundant species being *Cutibacterium acnes* and *Staphylococcus capitis*. Cleaning reduced the microbial load up to 98%, leaving mainly cutibacteria. Based on sequencing results, 227 genera were identified. The dominant genera before cleaning were *Cutibacterium* and *Paracoccus, Pseudomonas* and *Acinetobacter*. The post-cleaning bacteriota composition was very similar, though probably largely representing dead bacteria. Generally, cultivation-based and molecular results were rather congruent. Used oculars were notably contaminated with skin and environmental bacteria, including potential pathogens, while many of the identified taxa are noted to be associated with blepharitis, endophthalmitis or bacterial conjunctivitis (O'Callaghan 2018; Watson et al. 2018).

A polyphasic approach was chosen as DNA-based analysis provides a deeper insight into the community composition. However, it does not discriminate between dead and viable cells. Nevertheless, the amount of viable cells and identification down to species level are very important to evaluate the hygienic relevance of contaminated surfaces. The study underlines that regular cleaning of oculars after use is highly recommended to prevent transmission of bacteria between users and associated eye and skin infections (Fritz et al. 2020b).

## 3.4 Compositional analysis of the slit lamp bacteriota

Slit lamps count among the most important and most often used ophthalmological devices, demanding close contact between the device surface, the examiner and many different patients. Previous cultivation-based studies reported a notable contamination of slit lamps with bacteria, mostly coagulase-negative staphylococci, followed by micrococci, bacilli, but also *Staphylococcus aureus* (Sobolewska et al. 2018). Using the previously established molecular approach for spectacles and oculars, the presented study (Fritz et al. 2021) aimed at obtaining a comprehensive, cultivation-independent overview of the bacteriota on different slit lamp surfaces. The bacteriota of 46 slit lamps from two tertiary care centers (Center of Ophthalmology, University Hospital Tuebingen and the Eye Center, Medical Center, University of Freiburg) were analyzed during routine patient examinations within an unannounced audit. Two sampling sites were chosen in order to investigate probable bacterial transmission from contact areas to the examiners' oculars. In both clinics, all slit lamp contact areas were claimed to be wipe disinfected between different patients.

Sequencing results disclosed contaminations with bacteria originating mostly from human skin, mucosa and probably the eyes, predominantly cutibacteria, staphylococci and corynebacteria. The taxonomic assignment of 3369 ASVs (amplicon sequence variants) revealed 19 bacterial phyla and 468 genera across all samples, which is broadly similar to the other surfaces investigated in publications II and III.

A transfer of pathogens between patients and examiners is likely to occur, which might be of particular concern, as the throughput of patients, partially suffering from severe and contagious eye infections, is usually high.

As staphylococci were among the most abundant taxa on all analyzed optical devices including slit lamps and as antibiotic resistances pose major health problems (Talebi Bezmin Abadi et al. 2019), MRSA (Methicillin-resistant *Staphylococcus aureus*) was searched for by means of qPCR. However, no MRSA signals above the detection limit were detected. As MRSA is highly prevalent in hospital environments, this is a favorable result from a hygienic point of view. However, the results should be verified with a more sensitive, multiplexed qPCR assay, which enables the detection of more than one target at the same time (Henegariu et al. 1997).

The study underlines that slit lamps carry a highly diverse, skin-like bacterial microbiota and that thorough cleaning and disinfection of the patient's and examiner's sites after use are highly recommended to prevent the transmission of microorganisms and associated eye and skin infections.

## 3.5 Strengths and limitations of the methods used

Many and especially former studies have analysed microbial communities using cultivation-based techniques. Due to factors, such as growth conditions, symbiotic dependencies, dormancy or low abundance, cultivation discriminates against all groups of microorganisms that are difficult or not yet cultivable and thus results in a cultivation bias (Lewis et al. 2021). Nevertheless, cultivation-based methods are still reasonable, as they make it possible to determine the number of viable cells in different environments. Since living cells might have pathogenic potential, statements can also be made about their hygienic significance and possible threat to humans. Cultivation was used in this work to get an insight not only into the bacterial load (publication I), but also the fungal load (publication III), however, no fungi were detected. From a practical perspective, the identification of the most abundant living microbial isolates can be used to develop efficacy tests for cleaning methods or antimicrobial coatings with a high practical relevance.

Furthermore, microbial isolates can be identified down to species and even strain or subspecies level and screenings for antimicrobial resistances can be carried out. Besides classical biochemical reactions, classification and identification of bacterial isolates can be performed via MALDI-biotyping using MALDI-TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry). This technique was used in this

thesis (publications I and III), and allows for very rapid identification based on profiles of the ribosomal protein (Wieser et al. 2012). MALDI biotyping requires fresh cultures or protein extracts and a comprehensive database, then it allows for fast and simple identification of cultivable bacteria and fungi.

Although cultivation-based methods provide an insight into microbial communities, state-of-the-art molecular methods offer the opportunity to target yet-uncultured, low abundant or even unknown taxa. The current gold-standard for molecular analyses of prokaryotic community composition is based on "next-generation" sequencing technologies (NGS). Here, the bacterial 16S rRNA gene serves as a stable phylogenetic marker, since it consists of nine hypervariable regions (V1 - V9) that provide sufficient sequence diversity among distinct species (Clarridge 2004).

For the first time, this method was applied here to thoroughly analyse the bacterial community composition of ophthalmologically relevant devices (publications II, III IV). However, as no method is optimal, this PCR amplification-based technique has both advantages and disadvantages. Since it's not yet possible to analyze the ribosomal genes in full-length with the applied Illumina technique (Illumina 2019), taxonomic classification is limited to genus or even family level. Nevertheless, species identification is particularly relevant in a clinical context, since many genera comprise taxa with pathogenic potential. To circumvent this problem, the most abundant sequences obtained in the presented studies, were preliminary classified at species level against specified databases to get an idea of the present species.

The selected regions of the 16s rRNA gene are highly variable and as a consequence, primers for PCR amplification discriminate between different bacterial groups. Additionally, the choice of the variable region may influence the accuracy and specificity of the phylogenetic assignment (Yang et al. 2016). To counteract this discrimination, the respective primer pairs used here were chosen following the suggestions of Ying et al. (2015); Meisel et al. (2016); Castelino et al. (2017) and Zeeuwen et al. (2017) in order to cover the skin and mucosal bacterial community as comprehensively as possible. Generally, it was expected that touching, coughing, sneezing or cleaning with clothes after breathing on lenses, oculars or slit lamp surfaces might influence the composition of the surface microbiota, stemming both from the human skin as well as from the aerodigestive tract or even the human eye. Publication I (Fritz et al. 2018) has already

corroborated this expectation and publications II, III and IV (Fritz et al. 2020b; Fritz et al. 2020a; Fritz et al. 2021) further verified it.

Molecular skin microbiota analyses are challenging, as the microbial load on skin is comparatively low, for instance in comparison to stool samples. In the case of the spectacle analyses, even fewer bacteria were found on surfaces undergoing skin contact and least of all on the lenses. In addition, the sampled surfaces were smooth and limited in size. Therefore the workflow presented here (PCR conditions, primer pairs, subsequent clean-up steps) was optimized for use with samples with low DNA content.

Besides primers and their target region, many other factors may cause aberrations in the resulting microbial community composition compared to the real community composition, determined by "next-generation" sequencing. For instance, the number of PCR cycles (McGovern et al. 2018) and the use of low concentrated DNA samples (Salter et al. 2014). To account for such biases, it is recommended to include mock communities (bacterial samples with defined compositions) as external standards in microbial community analyses (Knight et al. 2018), as done in publication IV (Fritz et al. 2021).

Additionally, the vast amount of sequencing data requires extensive bioinformatics and statistical analyses, which is often challenging and a rapidly changing field. This work uses several methods, such as those described in publication II (Fritz et al. 2020a) and III (Fritz et al. 2020b). Following recent suggestions, publication IV (Fritz et al. 2021) placed a special emphasis on contemporary data analysis considering the sparse compositional nature of microbiome data (Gloor et al. 2017; Knight et al. 2018).

## 3.6 Outlook

The studies presented here provide a solid basis for several future projects, especially with a focus on the analysis of different microbes and additional hygiene/health aspects.

### 3.6.1 Viruses

Until now, optical devices were studied with a focus on bacteria. However, especially for infectious eye diseases, viruses play a major role, too. For instance, human adenoviruses are predominant (O'Brien et al. 2009) and known pathogens for highly contagious and severe keratoconjunctivitis, and have been responsible for several major outbreaks within hospitals and schools (King et al. 2013; Lamson Bs et al. 2018). But

various herpes viruses (herpes simplex virus, varicella zoster virus, cytomegalovirus) may also cause severe eye infections, such as keratitis, uveitis and retinitis, eventually leading to severe eye damage (Ritterband and Friedberg 1998).

Comprehensive virus detection is more challenging than bacterial community analyses. Studies usually focus on selected taxa, as universal marker genes are lacking. Moreover, viral cultivation is complex or even not possible at all (Hodinka 2013). Future projects should include targeted qPCR experiments or comprehensive metagenomic sequencing analyses, to better account for (certain) viruses.

### 3.6.2 Antibiotic resistances

Antibiotic resistances are of special concern. A recent study estimated that in 2019 approximately 225,000 deaths in Western Europe and 1.27 Mio deaths worldwide were attributed to or associated with bacterial antibiotic resistances. Six pathogens were most responsible for these severe infections: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, ordered by number of deaths (Murray et al. 2022).

In addition, Katzenberger and colleagues (2021) reported *S. aureus*, *K. pneumonia*, *P. aeruginosa*, *A. baumanii* and *E.coli* to be mainly associated with hospital-acquired infections. The authors also showed that these bacteria may be persistent on inanimate surfaces for days or even weeks, if not cleaned appropriately. Within the relatively most abundant genera, *Acinetobacter*, *Pseudomonas*, *Staphylococcu*s and *Streptococcus,* were also identified on the optical surfaces investigated in this thesis. However, due to the applied methods, identification down to species level was not always possible.

Presumably spectacles can serve as a reservoir for pathogens. Further research, for instance, should also target conjunctiva samples from patients with infectious eye diseases compared to bacteria on the respective spectacles, in order to determine the risk of reoccurring eye infections, especially with MDROs.

### 3.6.3 Eukaryotes

Even though fungi were examined in one cultivation-based study (publication III) of this thesis, none were detected. Nevertheless, fungal eye infections by *Candida, Fusarium* or *Aspergillus* species have been reported, although they are generally less common or

predominantly occur in immunocompromised patients or are introduced from trauma or surgery (Klotz et al. 2000).

In addition, especially while using contact lenses, *Acanthamoeba* spp. eye infections were reported (Klotz et al. 2000; Szentmáry et al. 2019). It would be interesting to evaluate whether the thophozoites and cysts of these protozoans also occur on spectacles or other ophthalmic objects, and whether transmission to the eye is likely, for example while using both contact lenses and spectacles.

### 3.6.4 Methodological considerations

All methods have their strengths and limitations. Cultivation-based approaches discriminate against groups of microorganisms that are difficult or not yet cultivable, however it's possible to differentiate between dead and viable cells. Along with the identification down to species level, statements about the pathogenic potential and the hygienic relevance can be made. In contrast, 16S rRNA gene amplicon sequencing can be biased due to PCR amplification, the choice of primers and their targeted region, which may cause aberrations in the resulting microbial community composition. Nevertheless, this technique provides comprehensive insight into the bacterial community also from low concentrated DNA samples, even though species identification might not always be possible.

Establishing a metagenomic approach, based on DNA shotgun sequencing, can provide a broader and more accurate resolution of microbial diversity in a given habitat, as it is possible to detect prokaryotes, viruses and eukaryotes all at once. Since this method is not based on PCR amplification, no PCR bias would occur. Furthermore, bacterial resistant genes and virulence factors can be detected (Quince et al. 2017).

By extending these studies to "metatranscriptomics", also the activity of the bacterial genes (gene expression) could be analyzed, which gives an idea about the active taxa and functions of the microbial community (Aguiar-Pulido et al. 2016).

However, harvesting sufficient non-host template DNA (or even RNA) for such analyses is challenging, especially since most ophthalmic surfaces are smooth and relatively small. That leads to the fact that within a sample containing a lot of host DNA the targeted microbial and/or viral DNA is probably largely underrepresented. Therefore deep sequencing will be required in order to detect these taxa.

As we expect more comprehensive insights into the microbiome of ophthalmological devices, we are currently working on establishing a metagenomic approach.

### 3.6.5 Cleaning recommendations and antimicrobial coatings

Finally, the comprehensive findings on microbial community composition on spectacles and related surfaces will help to improve the design of customized cleaning and efficacy tests, in particular regarding the choice of specific test organisms with high practice relevance. Indeed, smart coatings appear to be an attractive strategy to prevent and reduce microbial contamination of ophthalmologically relevant surfaces. These coatings are based on the idea of surface-contact microbial inactivation or ion diffusion through the microbial membrane, based, for instance, on nanoengineered particles (Rtimi 2021).

Lastly, there are many other ophthalmologically relevant devices that could be examined, such as phoropter glasses, tonometer or testing spectacles, which are closer to the patient's eye and are used throughout the day with a high throughput. Also, spectacles used by surgeons, healthcare professionals or protective eyeglasses/face shields could be analyzed even more comprehensively.

This work partially took place during the SARS-CoV2 pandemic and therefore it was more difficult to access clinical samples. In addition, the stricter hygiene and access rules may have altered the results compared to non-pandemic times. This should especially be considered for the slit lamp examinations in publication IV (Fritz et al. 2021). On the other hand, the hypothesized influence of the sepctacles as a protective shield against SARS-CoV2 infections should be examined more comprehensively, as studies suggested such an effect (Lehrer and Rheinstein 2021).

## 3.7 Main conclusions

Inanimate surfaces that come into contact with the human body are usually contaminated with microorganisms and might be considered as fomites. The same applies for spectacles, but little was known about their microbial contamination. By applying a polyphasic approach to analysing the bacterial load in relevant ophthalmological devices, the scientific contribution of this thesis can be summarized as follows:

i) New and extensive insight into the spectacle bacteriota was unveiled. It was shown that all spectacles in this research were found to be contaminated with bacteria, while spectacles of older wearers contained a broader diversity, presumably due to skin characteristics changing with age, resulting in a broader bacterial diversity on the skin. The NGS approach revealed 67 times more bacterial genera, than the cultivation-based study. Generally, spectacles are contaminated with bacteria originating from human skin and mucosa, as they are also affected by coughing and breathing. The main cultivated genera were staphylococci, while propionibacteria (cutibacteria) were the dominant taxa within the molecular approach.

ii) The effect of sampling sites was investigated. It was shown that all three tested spectacle sites showed a different bacterial diversity. Lenses were the most diverse sites.

iii) Cleaning recommendations were made, as four cleaning methods were investigated using lenses artificially contaminated with test bacteria. They included *S. epidermidis*, the dominant isolate of the cultivation-based study. Best cleaning results (germ reduction of up to two log scales) were obtained using impregnated wipes. Dry cleaning was less effective.

iv) Spectacles and other ophthalmologically relevant surfaces, should be seen as fomites. This could have possible impacts on shared objects such as microscopes or optometric examination devices in eye clinics, which are shared by doctors and patients. These studies revealed that these objects harbour a broad diversity of bacteria. All frequently identified genera could be associated with human skin, mucosa or the environment, such as staphylococci, corynebacteria, micrococci, and mainly cutibacteria. Many of the identified taxa are known to comprise potentially pathogenic species, and species that can carry antibiotic resistances. This could be a particular problem in clinics and other health-related areas. Notably, for the cultivation-based analysis of the microscopes, all isolated bacteria clearly represented viable cells that could potentially be infectious. Cleaning with isopropyl alcohol reduced the bacterial load about two log scales. In order to

prevent bacterial transmission, applying proper cleaning measures or the use of antimicrobial coatings is recommended.

v) Overall, this research represents the first comprehensive analysis of microbial contamination of spectacles and other ophthalmologically relevant devices and provides a new and solid basis for a deeper understanding of their hygienic relevance. These findings might be relevant not only in clinical or laboratory environments, but also for opticians, for instance, who have contact with many spectacles from different persons. Especially in the light of the SARS-CoV2 pandemic, these items should be disinfected regularly, as this virus could be transmitted via aerosols and smears, while being persistent on surfaces for several hours.

# 4 Summary

The aim of this thesis was to provide a solid basis for a deeper understanding of the hygienic relevance of spectacles and related ophthalmologically important surfaces. To do so, comprehensive cultivation-dependent and -independent studies on the microbial community composition of worn spectacles, microscope oculars and slit lamps were conducted. Using spectacles from university staff (n = 11) and inhabitants of a nursing home (n = 10) for elderly people, it was shown that all spectacles were contaminated with bacteria, with nosepads and earclips showing the highest density. In particular sites undergoing direct skin contact showed high germ counts and were dominated by staphylococci. The microbial load of the university spectacles was similar to the nursing home ones. However, the latter showed a higher diversity (10 genera, compared to 2 genera at the university environment), presumably due to skin factors changing with age. Using a collection of gram-negative and gram-positive test bacteria (including *Staphylococcus epidermidis* as the dominant isolate of the study) it was shown that wet cleaning wipes reduced the microbial load on spectacle lenses by about 2 log scales, while dry cleaning was less effective. These results were corroborated in a cleaning experiment with naturally contaminated, worn spectacles. Here, the average bacterial load was significantly (94%) lower compared to the uncleaned university spectacles investigated before. To account for the well-known bias of cultivation, a molecular analysis pipeline based on NGS of 16S rRNA gene amplicons was established. Using this protocol on 30 worn spectacles at three different sampling sites, a remarkable bacterial diversity of 665 bacterial genera was unravelled. In addition, significant differences in community composition between the sampling sites were detected, with the highest bacterial diversity on the lenses. On all spectacle sites, only a few taxa dominated the bacteriota: *Staphylococcus*, *Propionibacterium (Cutibacterium)*, *Corynebacterium*, *Lawsonella* and *Streptococcus*, in decreasing order. The taxa identified as dominant on spectacle surfaces can be used a test organisms with a high relevance for practice in the development of novel cleaning and coating strategies for spectacles. Bacterial transmission between inanimate surfaces and human beings is more likely if surfaces are touched by different persons. Therefore, 10 microscope oculars from a university laboratory and 46 slit lamps from two eye clinics were included in this thesis as reference surfaces to the previously analysed spectacle surfaces. In the case of the microscope oculars, both cultivation-based

and molecular analyses of the microscope microbiota were performed. All oculars were found to be contaminated with bacteria, with a maximum load of 1.7 x $10^3$ CFU $cm^{-2}$. Although selective media for fungal detection were also used, no fungi could be isolated. 64 bacterial genera were detected with cultivation, compared to 227 when based on the sequencing results. The dominant bacterial genera were *Cutibacterium* (*C. acnes*), *Staphylococcus (S. capitis), Brevibacterium, Paracoccus, Pseudomonas* and *Acinetobacter.* Wet cleaning of microscope oculars with isopropyl alcohol reduced the microbial load by up to two log scales. All investigated slit lamp samples also showed contamination with bacteria originating mostly from human skin, mucosa and probably from eyes. Across all samples, 268 genera were identified, predominantly cutibacteria, staphylococci and corynebacteria. Statistical analysis suggested an exchange of bacteria between the patients' and examiners' sites, presumably including a potential pathogen transfer. As staphylococci were among the most abundant taxa on all analyzed optical devices including slit lamps, MRSA (Methicillin-resistant *Staphylococcus aureus*) was searched for by means of qPCR. However, no MRSA signals above the detection limit were detected. As MRSA is highly prevalent in hospital environments, this is a favorable result from a hygienic point of view.

In summary, the studies conducted in the course of this thesis clearly showed that spectacles, microscope oculars and slit lamp surfaces are colonized by a diverse bacterial community, mostly originating from human skin, epithelia and the environment. Many of the detected genera are known to comprise potential pathogens. Successful cultivation of bacteria from the investigated surfaces clearly indicated the presence of viable cells, i.e. cells that can potentially cause infections. Transmission of potential pathogens is more likely to occur if spectacles, microscopes and slit lamps are regularly touched or used by different persons (e.g. ophthalmologists, healthcare workers, opticians, etc.). Consequently, these devices must be regarded as fomites. Regular cleaning significantly reduces the bacterial load and is therefore highly recommended to prevent eye and skin infections. Future research will address more deeply whether spectacles might serve as a reservoir for pathogens in recurring eye infections or function as vehicles to spread antibiotic resistance genes in healthcare environments. To do so, stronger function-oriented analysis methods will be established, such as shotgun metagenomic sequencing. This technique provides a broader and more accurate resolution of microbial diversity

and can include non-bacterial microorganisms, in particular viruses. Establishing a metagenomic approach will also provide for more comprehensive detection of bacterial resistance genes and virulence factors on ophthalmologically relevant surfaces.

# 5 German summary

Ziel der vorliegenden Doktorarbeit war es, die mikrobielle Gemeinschaft auf getragenen Brillen und ähnlich ophthalmologisch relevanten Objekten mittels kultureller und kultivierungsunabhängiger Methoden umfassend zu untersuchen, um so die hygienische Bedeutung dieser Oberflächen besser zu verstehen.

Bei der Analyse von Brillen von Mitgliedern einer Hochschule (n=11) und Bewohnern eines Altenheims (n=10), zeigte sich, dass Brillen stark mikrobiell besiedelt sind. Für beide Personengruppen wurden hohe Keimzahlen auf Ohrbügeln und Nasenpads nachgewiesen (vorwiegend Staphylokokken), d.h. auf Stellen mit direktem Hautkontakt.

Die Brillen der älteren Personen zeigten jedoch eine deutlich höhere bakterielle Diversität (zehn verschiedene Bakteriengattungen, im Vergleich zu zwei Gattungen auf den Brillen der jüngeren Personen), was vermutlich auf altersbedingte Hautveränderungen zurückzuführen ist.

Standardisierte Reinigungstests mit künstlich kontaminierten Glasrohlingen wurden mit gram-positiven und gram-negativen Testbakterien durchgeführt, darunter auch *Staphylococcus epidermidis* als dominierendes Isolat der Studie. Hier zeigte sich, dass eine Reinigung mit feuchten Reinigungstüchern eine Keimzahlreduktion um bis zu zwei log-Stufen erreichen konnte, eine trockene Reinigung war weniger wirksam. Diese Ergebnisse wurden mit getragenen Brillen bestätigt. Auch hier war die bakterielle Belastung nach der Reinigung signifikant (94%) niedriger.

Da mittels kultivierungsbasierter Verfahren immer nur ein Bruchteil einer mikrobiellen Gemeinschaft erfasst wird, wurde eine molekularbiologische Analysepipeline etabliert, die auf der Hochdurchsatzsequenzierung von Amplifikaten des bakteriellen 16S rRNA Gens basiert. Unter Verwendung dieses Protokolls wurden je drei verschiedene Probestellen von 30 getragenen Brillen untersucht. Es zeigte sich eine bemerkenswerte bakterielle Vielfalt, mit signifikanten Unterschieden zwischen den jeweiligen Probestellen, wobei die Gläser die höchste bakterielle Diversität zeigten. Insgesamt wurden 665 verschiedene Bakteriengattungen identifiziert, dominiert von wenigen Taxa, darunter die Gattungen *Staphylokokkus*, *Propionibakterium* (*Cutibakterium*),

*Corynebakterium*, *Lawsonella* und *Streptokokkus*, in abnehmender Reihenfolge. Die auf den Brillenoberflächen dominierenden bakteriellen Gattungen bieten sich als sehr praxisnahe Testorganismen bei der Entwicklung neuer, praxisrelevanter Reinigungs- und Beschichtungsverfahren von Brillen an.

Ein Transfer von Bakterien zwischen Oberflächen und Menschen ist wahrscheinlicher, wenn die Oberflächen von mehreren Personen berührt werden. Deshalb wurden in dieser Arbeit auch Okulare von 10 Mikroskopen aus einem Hochschullabor und 46 Spaltlampen aus zwei Augenkliniken als Referenzoberflächen zu den zuvor untersuchten Brillen mit einbezogen. Für die Okulare wurden sowohl kulturelle als auch molekulare Analysen durchgeführt. Auf allen Mikroskopokularen fanden sich Bakterien, mit einer maximalen Keimzahl von 1,7 x $10^3$ KBE $cm^{-2}$ (Kolonien bildende Einheiten). Obwohl zusätzlich Selektivmedien zur Isolation von Pilzen verwendet wurden, ließen sich keine Pilze auf den getesteten Okularen nachweisen. Bei der Kultivierung fanden sich 64 verschiedene Bakteriengattungen, im Vergleich zu 227 bei der Sequenzierung, wobei *Cutibakterium* (*C. acnes*), *Staphylokokkus* (*S. epidermidis*), *Brevibakterium*, *Parakokkus*, *Pseudomonas* und *Acinetobakter* zu den häufigsten Gattungen (Arten) zählten. Auch hier zeigte eine Reinigung der Okulare mit Isopropanol eine Keimzahlreduktion um zwei log-Stufen.

Auf den Spaltlampen konnten ebenfalls auf allen Probestellen Bakterien nachgewiesen werden, die meist von der Haut, den Schleimhäuten und wahrscheinlich aus dem Auge stammten. Dabei wurden 268 Gattungen identifiziert, vorwiegend Cutibakterien, Staphylokokken und Corynebakterien. Statistische Analysen legen zudem nahe, dass es zu einem Austausch von Bakterien, und damit auch von Pathogenen, zwischen der Patienten- und der Arztseite kommen kann. Da Staphylokokken zu den am häufigsten vorkommenden Taxa auf allen untersuchten optischen Geräten, einschließlich Spaltlampen, gehörten, wurde mittels qPCR zusätzlich nach MRSA (Methicillin-resistenter *Staphylokokkus aureus*) gesucht. Es konnten jedoch keine MRSA-Signale über der Nachweisgrenze festgestellt werden konnten. Da MRSA im klinischen Umfeld weit verbreitet ist, ist dies aus hygienischer Sicht ein positives Ergebnis.

Zusammenfassend zeigen die durchgeführten Studien eindeutig, dass die Oberflächen von Brillen, Mikroskopokularen und Spaltlampen von einer vielfältigen bakteriellen Gemeinschaft besiedelt sind, die zumeist von der Haut, der Schleimhaut oder aus der Umwelt stammt. Viele der identifizierten Gattungen beinhalten potentiell pathogene

Arten. Die erfolgreiche Kultivierung von Bakterien von den unterschiedlichen Oberflächen zeigt eindeutig, dass lebende Zellen vorhanden sind, welche in der Lage sind, Krankheiten auszulösen.

Ein Transfer von Pathogenen ist umso wahrscheinlicher, wenn Brillen, Mikroskope und Spaltlampen häufig von verschiedenen Personen (z. B. Augenärzten, medizinischem Personal, Optikern usw.) berührt oder benutzt werden. Deshalb müssen diese Gegenstände als Keim(über)träger angesehen werden. Eine regelmäßige Reinigung reduziert die bakterielle Belastung erheblich und ist daher empfehlenswert, um Augen- oder Hautinfektionen vorzubeugen.

Zukünftige Studien sollten sich noch umfassender mit der Rolle von Brillen als Reservoir für wiederkehrende Augenerkrankungen oder Vektoren für die Verbreitung von Antibiotikaresistenzen, speziell im Gesundheitswesen, beschäftigen. Das kann mit einem funktionelleren Ansatz geschehen, wie es die sog. Shotgun-Metagenomsequenzierung ermöglicht. Mit Hilfe dieser Technik lässt sich ein tieferer und umfassenderer Einblick in die komplette mikrobielle Gemeinschaft gewinnen, welche auch nicht-bakterielle Mikroorganismen, insbesondere Viren, beinhaltet. Zudem können mit diesem Ansatz auch bakterielle Resistenz- und Virulenzgene auf ophthalmologisch relevanten Oberflächen umfassend analysiert werden.

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# 7 Declaration of contribution of others

This dissertation work was carried out at Furtwangen University of Applied Sciences, Institute of Precision Medicine, Microbiology and Hygiene Group, Villingen-Schwenningen under the supervision of Prof. Markus Egert. Prof. Dr. Focke Ziemssen supervised the dissertation work at the Center for Ophthalmology at Eberhard-Karls-University Tuebingen and Prof. Dr. Siegfried Wahl from the Institute for Ophthalmic Research at the Eberhard-Karls-University Tuebingen mentored the work.

I confirm that I wrote the doctoral dissertation submitted with the title: "The spectacle microbiota and its hygienic relevance" by myself (under the mentioned supervision) and that any additional sources of information have been duly cited.
The respective authors' contributions in each of the four publications are as follows:

**Publication I: A view to a kill? – Ambient bacterial load of frames and lenses of spectacles and evaluation of different cleaning methods**

Fritz, B.*, Jenner, A.*, Wahl, S., Lappe, C., Zehender, A., Horn, C., Blessing, F., Kohl, M., Ziemssen, F. and Egert, M., *PLoS ONE* 13 (11), e0207238, 2018

* authors contributed equally to this work

Birgit Fritz, Prof. Dr. Siegfried Wahl, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert created the research concept and designed the study. The majority of selection of methods was done by Birgit Fritz, with support in equal parts by Anne Jenner, Prof. Dr. Frithjof Blessing and Prof. Dr. Markus Egert. Study subjects were mainly acquired by Anne Jenner, with the support of Prof. Dr. Siegfried Wahl, Dr. Christian Lappe and Achim Zehender. Christian Horn supervised MALDI-biotyping. Data were acquired mainly by Anne Jenner and Birgit Fritz, with the support of Prof. Dr. Frithjof Blessing and Prof. Dr. Matthias Kohl. The majority of data analysis, interpretation of results and preparation of the manuscript were conducted by Birgit Fritz, and to a lesser extent by Anne Jenner and with support from Prof. Dr. Siegfried Wahl, Prof. Dr. Matthias Kohl, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. Prof. Dr. Focke Ziemssen, Prof. Dr. Markus Egert and Prof. Dr. Siegfried Wahl supervised the study.

**Publication II: Site-specific molecular analysis of the bacteriota on worn spectacles**

Fritz, B., März, M., Weis, S., Wahl, S., Ziemssen, F. and Egert, M., *Scientfic Reports*. 10, 5577, 2020a

Birgit Fritz created the research concept and the study with support from Prof. Dr. Siegfried Wahl, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. The methods were selected by Birgit Fritz, with support from Dr. Severin Weis, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. Subjects were recruited mainly by Melanie März, with the support of Birgit Fritz and Prof. Dr. Markus Egert. Data were acquired mainly by Birgit Fritz, with the support of Melanie März and Dr. Severin Weis. The majority of data analysis and interpretation was conducted by Birgit Fritz, with the support of Melanie März and Dr. Severin Weis. The majority of the preparation of the manuscript was conducted by Birgit Fritz. Prof. Dr. Focke Ziemssen, Prof. Dr. Siegfried Wahl and Prof. Dr. Markus Egert supervised the study and supported interpretation of the results and preparation of the manuscript.

**Publication III: Eye-catching microbes – polyphasic analysis of the microbiota on microscope oculars verifies their role as fomites**

Fritz, B., Schäfer, K., März, M., Wahl, S., Ziemssen, F. and Egert, M., *Journal of Clinical Medicine*. 9 (5) 1572, 2020b)

Birgit Fritz created the research concept and the study with support from Prof. Dr. Siegfried Wahl, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. The methods were selected for the most part by Birgit Fritz, with support from Prof. Dr. Markus Egert. Subjects were recruited in equal parts by Karin Schäfer and Melanie März, with support from Birgit Fritz and Prof. Dr. Markus Egert. Data were acquired mainly by Karin Schäfer, with the support of Birgit Fritz and Melanie März. The data analysis was completely conducted by Birgit Fritz. Interpretation of data was conducted mainly by Birgit Fritz, with support in equal parts by Karin Schäfer, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. The majority of the preparation of the manuscript was conducted by Birgit Fritz. Prof. Dr. Focke Ziemssen, Prof. Dr. Siegfried Wahl and Prof. Dr. Markus Egert supervised the study, supported interpretation of the results and preparation of the manuscript.

**Publication IV: Comprehensive compositional analysis of the slit lamp bacteriota**

Fritz, B., Paschko, E., Young, W., Böhringer, D., Wahl, S., Ziemssen, F. and Egert, M. *Frontiers in Cellular and Infection Microbiology*, 11.745653, 2021

Birgit Fritz created the research concept and the study with support from Prof. Dr. Siegfried Wahl, Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. The methods were selected for the most part by Birgit Fritz, with support from Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert. Subjects were recruited by Prof. Dr. Focke Ziemssen, Birgit Fritz and Prof. Dr. Daniel Böhringer. Data was acquired in equal parts by Birgit Fritz and Edita Paschko. The majority of data analysis and interpretation of data was conducted by Birgit Fritz with the support of Dr. Wayne Young. The majority of the preparation of the manuscript was conducted by Birgit Fritz. Prof. Dr. Focke Ziemssen and Prof. Dr. Markus Egert supervised the study, supported interpretation of the results and preparation of the manuscript.

Signed _____

on July 26, 2022 in Tuebingen

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