Article

The Effect of Topical Anesthetics on 16S Ribosomal Ribonucleic Acid Amplicon Sequencing Results in Ocular Surface Microbiome Research

Heleen Delbeke[1,2], Ingele Casteels[1,2], and Marie Joossens[3]

[1] Department of Ophthalmology, University Hospitals Leuven, Leuven, Belgium
[2] KU Leuven, Biomedical Sciences Group, Department of Neurosciences, Research Group Ophthalmology, Leuven, Belgium
[3] Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

Correspondence: Heleen Delbeke, Herestraat 49, 3000 Leuven, Belgium. e-mail: heleen.delbeke@uzleuven.be

Purpose: To clarify the short-term effect of topical anesthetics on 16S ribosomal ribonucleic acid amplicon sequencing results in ocular surface microbiome research.

Methods: Both eyes of 24 eligible volunteers undergoing general anesthesia were sampled. Before sampling, a drop of artificial tears or a drop of topical anesthetic was applied in a randomized way. By using artificial tears as a control, we assured blinding of the executer and took a potential diluting effect into account. Bacterial DNA was extracted using the QIAGEN RNeasy PowerMicrobiome Kit with specific adaptations. Amplified DNA was sequenced with the Illumina MiSeq sequencing platform.

Results: Four sample pairs were excluded due to low yield of bacterial DNA. In the remaining 20 sample pairs, no differences were observed with topical anesthetics at the levels of amplicon sequence variants (ASVs), phylum, genera, or alpha and beta diversity. Weighted UniFrac distance confirmed that the intraindividual distance between the right and left eye was smaller than the effect of the topical anesthetic. Interestingly, however, we identified *Cutibacterium* as a potential discriminative biomarker for topical anesthetic use. Overall, a significantly higher number of observed reads were assigned to genera with Gram-positive characteristics.

Conclusions: Based on our targeted, double-blinded, within-subject study, topical anesthetics do not affect the overall sequencing results but display a specific effect on *Cutibacterium*. When comparing research results, the impact of topical anesthetics on prevalence and abundance of *Cutibacterium* should be considered.

Translational Relevance: Understanding and standardization of sampling techniques are indispensable to properly execute clinical microbiome research.

Introduction

Research on the ocular surface microbiome is an emerging field with evident publication growth from 2016 onward.[1] To understand the potential role of the microbiome in ocular pathology, knowledge of the ocular surface microbiome in the absence of pathology is mandatory. The currently published cohort sizes of healthy ocular surface samples vary between four[2] and 105[3] individuals. Meta-analyses of currently published data are hampered by the lack of standardization of sampling, extraction, and sequencing procedures in the field. Evidence-based choices of sampling and processing methods will make amplicon sequencing results more robust and make comparisons more precise, resulting in improved insights into the ocular surface microbiome.

Sampling is the first element prone to bias. Different swabbing techniques (dry vs. wet and firm vs. gentle) have resulted in significant differences in the proportions of retrieved genera.[2] In addition to the swabbing technique, the effect of anesthetic drops may be of importance. The antibacterial activity of topical anesthetics has been demonstrated in several studies.[4,5] It has been suggested that the inhibitory effect of local anesthetics on bacterial growth results from the disruption of the bacterial cell wall or cytoplasmic

membrane,[6,7] leading to leakage of cellular components and subsequent cell lysis. Interestingly, bacterial cell wall disruption is also the first step of every molecular extraction protocol. It is also well-known that Gram-positive bacteria are more likely to be affected by extraction methods due to their bacterial cell wall properties.[8] When bacterial cell wall disruption has already taken place as a result of anesthetics use, a significant impact on the outcome of molecular analyses could be anticipated. Indeed, Shin et al.[9] reported a significantly different microbial community composition in samples collected with or without the use of topical anesthetics. However, the link between microbial community compositions and confounding factors was not made, although different subject groups were used per treatment arm. These results have not been validated yet, resulting in some studies using topical anesthetics[10,11] and others not.[12,13]

In this paper, we investigated the short-term effect of topical preservative-free anesthetics, with the goal of improving the consistency of 16S ribosomal ribonucleic acid (rRNA) amplicon sequencing results. In a cohort of patients requiring surgery under general anesthesia, we performed a double-blinded study sampling both eyes of volunteers after sedation.

Materials and Methods

Study Population

This prospective study was approved by the research ethics committee UZ/KU Leuven, Belgium, in accordance with the tenets of the Declaration of Helsinki. This project is registered on ClinicalTrials.gov (NCT04193774). The study was performed in adult patients (minimum age 18 years).

Patients undergoing strabismus, ear, or throat surgery under general anesthesia at the University Hospitals Leuven, Belgium, were informed about the study and consent of participating volunteers was obtained before enrollment. Aforementioned indications for surgery were chosen based on no need for pretreatment with antibiotics or antiseptics before surgery. Exclusion criteria were allergy to oxybuprocaine hydrochloride, the use of topical antibiotics in the month before sampling, usage of eye drops in one eye only, and ocular surface disease.

Eligible participants filled out a short questionnaire concerning the most obvious confounding factors (age, gender, ethnical background, medication usage, ophthalmic history, sleeping side, eye rubbing). After patients were put under general anesthesia, a drop of artificial tears (Thealoz Duo; Théa Pharma, Clermont-Ferrand, France) in one eye and a drop of topical anesthetics (Minims Oxybuprocaine Hydrochloride 0.4%; Bausch + Lomb, Aubenas, France) in the other eye were applied to each subject in a randomized way by a pre-informed nurse. A timed 2 to 3 minutes passed between application of the drops and the sampling. A drop of artificial tears was applied to prevent unblinding of the executer and to mimic any diluting effect of the anesthetic drops in the control eye. Both types of drops were preservative free. By using volunteers under general anesthesia, we assured similar pressure with or without topical anesthetic because the executer was not influenced by the patient's reaction. The left and the right eye were sampled in a standardized way by a blinded medical student or resident. The student was trained before sampling and supervised by an author (HD) during the first execution. A step-by-step protocol was available as a mnemonic device for subsequent sampling moments. Sampling was performed before any presurgical disinfection of the skin and conjunctival sac. A single, sterile, nylon, flocked swab (FLOQSwabs; Copan, Brescia, Italy) was rubbed from the nasal to temporal inferior conjunctival sac and simultaneously swirled in the opposite direction of the sampling itself. The swab was placed in an Eppendorf tube and stored for less than 2 weeks at –18°C before being transferred to a −80°C freezer until further processing.

DNA Extraction and Amplicon Sequencing

Microbial deoxyribonucleic acid (DNA) was extracted from frozen samples using the RNeasy PowerMicrobiome Kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines with specific adaptations for DNA extraction and higher yield.[14] In short, the DNase and β-mercaptoethanol steps were omitted. After vortexing the PowerBead Tubes for 10 minutes, the tubes were placed at 90°C for 10 minutes as a heat lysis step. Final DNA was dissolved in 50 µL DNase-Free Water instead of the standard 100 µL. Before final centrifugation, the collection tubes with filter membrane were incubated at room temperature for 5 minutes instead of 1 minute. DNA was quantified via fluorometry (Life Technologies Qubit dsDNA High Sensitivity Kit; Thermo Fisher Scientific, Waltham, MA). Library preparation and sequencing were performed at BaseClear, Leiden, The Netherlands. The hypervariable V3–V4 region of the 16S rRNA gene was amplified with polymerase chain reaction (PCR; 341F/785R primer set). The amplified DNA was sequenced with the Illumina MiSeq sequencing platform (Illumina, San Diego, CA) to generate 2×300 base-pair (bp) paired-end reads.

Table 1.   ASV Count Relative to Included Data

	ASV Count, n
Raw data	2034
After removal of eukaryotes, chloroplasts, mitochondria, not assigned phyla, and technical singletons	377
After removal of biological singletons	295
After removal of ASVs < 0.1%	60

Our initial dataset showed 2034 ASVs. After removing eukaryotes, chloroplasts, mitochondria, not assigned phyla, and technical singletons (ASVs present only once in our dataset), we retained 377 ASVs. After removal of specific ASVs only present in one individual (biological singletons), 295 ASVs were preserved belonging to 99 genera. When excluding ASVs with an abundance below 0.1%, we retrieved 60 ASVs belonging to 23 genera.

Data Analysis

The paired-end fastq files were analyzed using DADA2 pipeline version 1.12 in R (open-source version 1.2.5033) according to the tutorial for quality profiling, filtering (maximum expected error of 2) and trimming reads, sequence variants interference, removal of Chimeric sequences, and taxonomic assignment.[15,16] The primers were removed during filtering and trimming and the forward reads were trimmed at 290 bp; reverse reads were cut at the 240-bp position based on the quality profile and to maintain a minimal of 20 bp nucleotides for overlapping.[17] Taxonomical assignment was done using the SILVA database version 132.[18] After calculating alpha diversity measures, all amplicon sequence variants (ASVs) detected only once in the whole dataset and all ASVs belonging to only one single volunteer (i.e., ASVs detected in both eyes of only one individual) were removed, excluding both technical as well as biological singletons. Next analyses were performed with either all ASVs or with only ASVs with an abundance > 0.1% as specified in the results section (Table 1). All stated comparisons were calculated with relative abundances.

Alpha diversity measures such as the observed number of ASVs (i.e., total number of ASVs detected per sample[19]), Shannon's diversity index (i.e., number of taxa and evenness of their distribution, more influenced by richness and rare species[20,21]), and Simpson's diversity index (i.e., number of taxa and evenness of their distribution, more influenced by evenness and commons species[19,21]) were compared. Bray–Curtis dissimilarity was used to visualize beta diversity in order to examine the difference in microbial composition between both of the sampled groups. Alpha and beta diversity were calculated using Phyloseq 1.24.0. The principal component analysis (PCA) was visualized in R via fviz_pca(), after downloading the factoextra library. The weighted UniFrac distance was calculated to determine the UniFrac distance between sample pairs. Via the Galaxy web application, linear discriminant analysis (LDA) effect size (LEfSe) (LDA threshold > 2, $P < 0.05$) was executed to identify potential biomarkers differentiating both bacterial communities.[22–24]

Statistical Analyses

The data were analyzed using R 1.2.5033 statistical software.[16] The Shapiro–Wilks normality test was used to test the data for normality. When normally distributed, the mean percent and standard deviation (SD) were used; when not normally distributed, the median percent and interquartile range (IQR) Q1 to Q3 were noted. Normally distributed paired samples were compared using Welch two-sample t-test. Paired samples not normally distributed were compared with the Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant. Relative abundance was used for normality testing of samples. When results were statistically significant, the Benjamini–Hochberg procedure for multiple hypothesis testing was applied.

Results

Twenty-four eligible patients volunteered to participate in the study. Upon DNA extraction of the samples, the DNA yield was too low for sequencing (≤0.5 ng/mL) in seven samples, resulting in the exclusion of four sample pairs. The four excluded volunteers with unexplained low yield were all females between the age of 40 and 59 years old; one volunteer was a contact lens wearer. Forty samples from 20 patients (three females, 17 males) were sequenced and further analyzed. In these patients, the mean age was 48.5 ± 18 years (range, 19–81). Two of the included volunteers were contact lens wearers.

Before quality filtering, the 2,050,659 reads had a mean of $100,366 \pm 19,371$ per sample. After quality filtering, the total amount dropped to 955,823

Table 2. Alpha Diversity Measures in the Control Group and the Topical Anesthetic Group

Measure	Topical Anesthetic	Artificial Tears	*P*
Observed ASVs			*P* > 0.05, Wilcoxon rank-sum test
Median	61	46	
IQR, Q1–Q3	21–75	23–66	
Shannon diversity index			*P* > 0.05, Welch two-sample *t*-test
Mean	2.6	2.5	
SD	0.7	0.7	
Simpson diversity index			*P* > 0.05, Wilcoxon rank-sum test
Median	0.8	0.8	
IQR, Q1-Q3	0.8–0.9	0.8–0.9	
Aerobic genera			*P* > 0.05, Wilcoxon rank-sum test
Median	1140	3884	
IQR, Q1–Q3	314–3879	1191–3402	
Anaerobic genera			*P* > 0.05, Wilcoxon rank-sum test
Median	445	372	
IQR, Q1–Q3	151–963	42–1677	
Facultative anaerobic genera			*P* > 0.05, Wilcoxon rank-sum test
Median	926	1889	
IQR, Q1–Q3	256–1507	389–4039	

No significant differences were observed for ASVs, Shannon diversity index, or Simpson diversity index between the two examined groups. Alpha diversity measures were calculated before removing singletons.
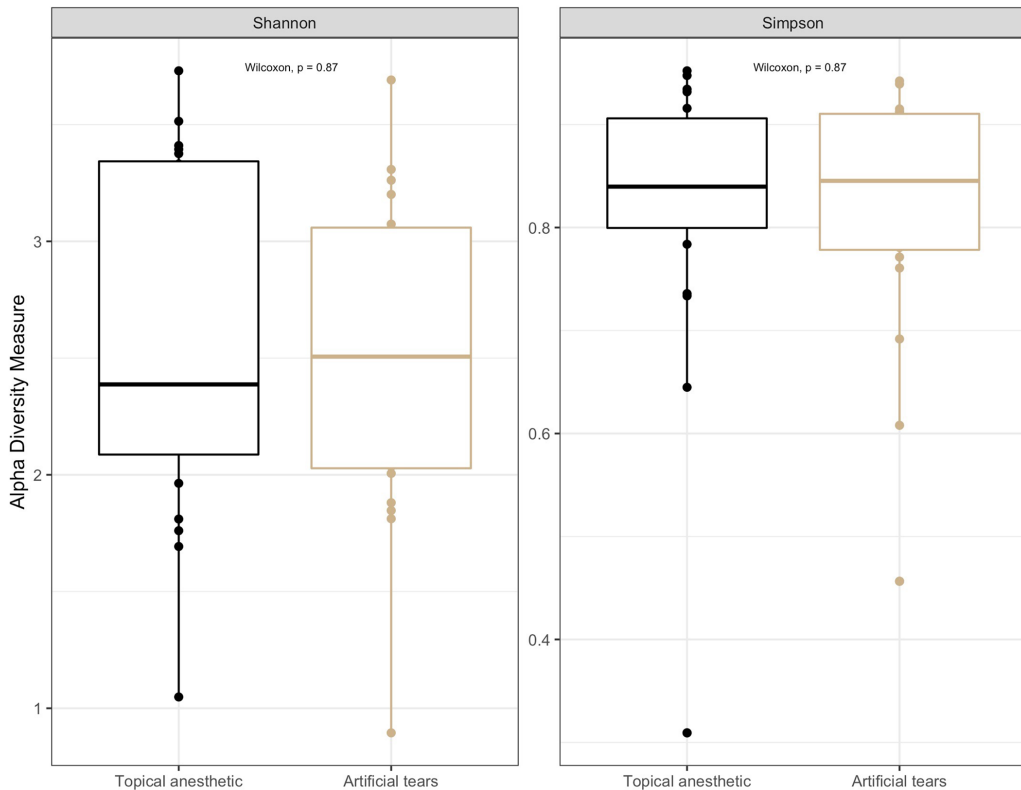

Figure 1. Alpha diversity measures (Shannon and Simpson diversity indexes) for both the control group ($n = 20$) and the topical anesthetic group ($n = 20$). We observed no significant difference in the Shannon and Simpson diversity indexes between the control group and the topical anesthetic group. The Shannon diversity index (mean $\pm$ SD) in the topical anesthetic group was 2.6 $\pm$ 0.7, with similar values being found in the control group (2.5 $\pm$ 0.7). The Simpson diversity index was equal in the two groups, with a median of 0.8 and an interquartile range of 0.8 to 0.9.
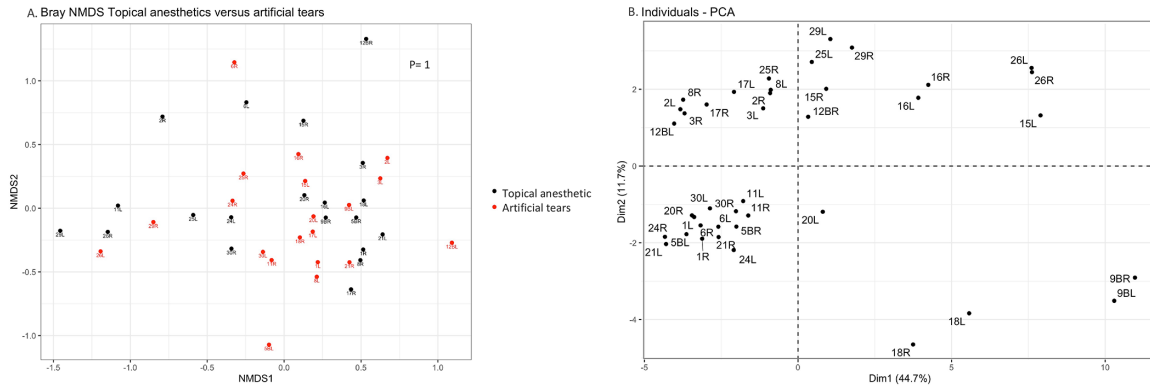
Figure 2. Bray–Curtis plots comparing the control group versus the topical anesthetic group. (A) Ordination diagram of nonmetric multidimensional scaling (NMDS), calculated based on the Bray–Curtis dissimilarity index (and square-rooted to make NMDS metric). (B) Ordination diagram of the PCA, calculated based on the Bray–Curtis dissimilarity index.
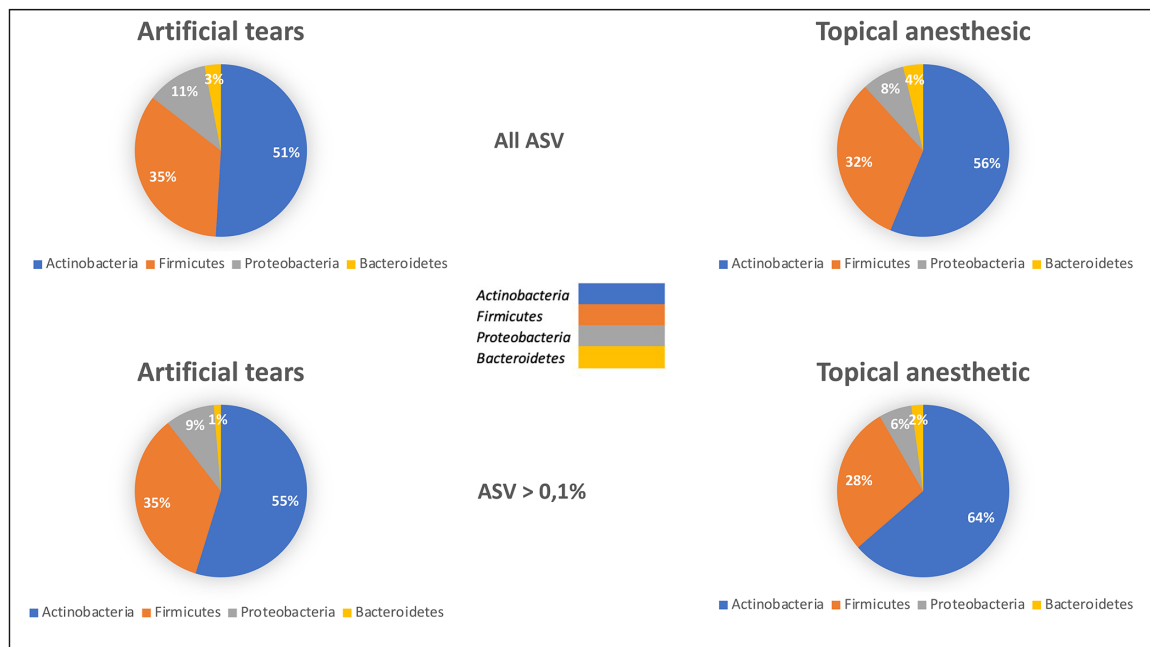

Figure 3. Relative abundances at the phylum level of the control group and the topical anesthetic group. Actinobacteria is the most prevalent phylum (all ASVs, control 51% and anesthetic 56%; ASVs > 0.1%, 55%–64%), followed by Firmicutes (all ASVs, 35%–32%; ASVs > 0.1%, 35%–28%) and Proteobacteria (all ASVs, 11%–8%; ASVs > 0.1%, 9%–6%). Bacteroidetes accounted for only 3% and 4%, respectively, when looking at all ASVs after singleton removal and 1% and 2%, respectively, when taking into account ASVs with abundance > 0.1%. The differences between the two groups were not significant.

paired-end reads with a mean of $23,896 \pm 18,153$ per sample, belonging to 2034 ASVs. After the removal of biological and technical singletons, the reads of both groups belonged to 295 ASVs (Table 1). When considering only ASVs with an abundance > 0.1%, 60 ASVs remained.

None of the alpha diversity measures (observed ASV, Shannon and Simpson diversity) was significantly different between the control group and the topical anesthetic group (Table 2, Fig. 1). No difference in beta diversity (Fig. 2) was observed between the control and the anesthetic group in either the dataset with all ASVs or the dataset with ASVs > 0.1%.

Looking at the relative abundance at the phylum level in the control and topical anesthetic groups, respectively, Actinobacteria was the most prevalent (all ASVs, 51%–56%; ASVs > 0.1%, 55%–64%), followed by Firmicutes (all ASVs, 35%–32%; ASV > 0.1%, 35%–28%), and Proteobacteria (all ASV, 11%–8%; ASV > 0.1%, 9%–6%). Bacteroidetes accounted for only 3% and 4% (all ASVs) and 1% and 2% (ASV > 0.1%), respectively. No significant difference was found between the two treatment groups at the phylum level (Fig. 3).

At the genus level, the 295 ASVs could be attributed to 98 genera; the 60 ASV with an abundance > 0.1%
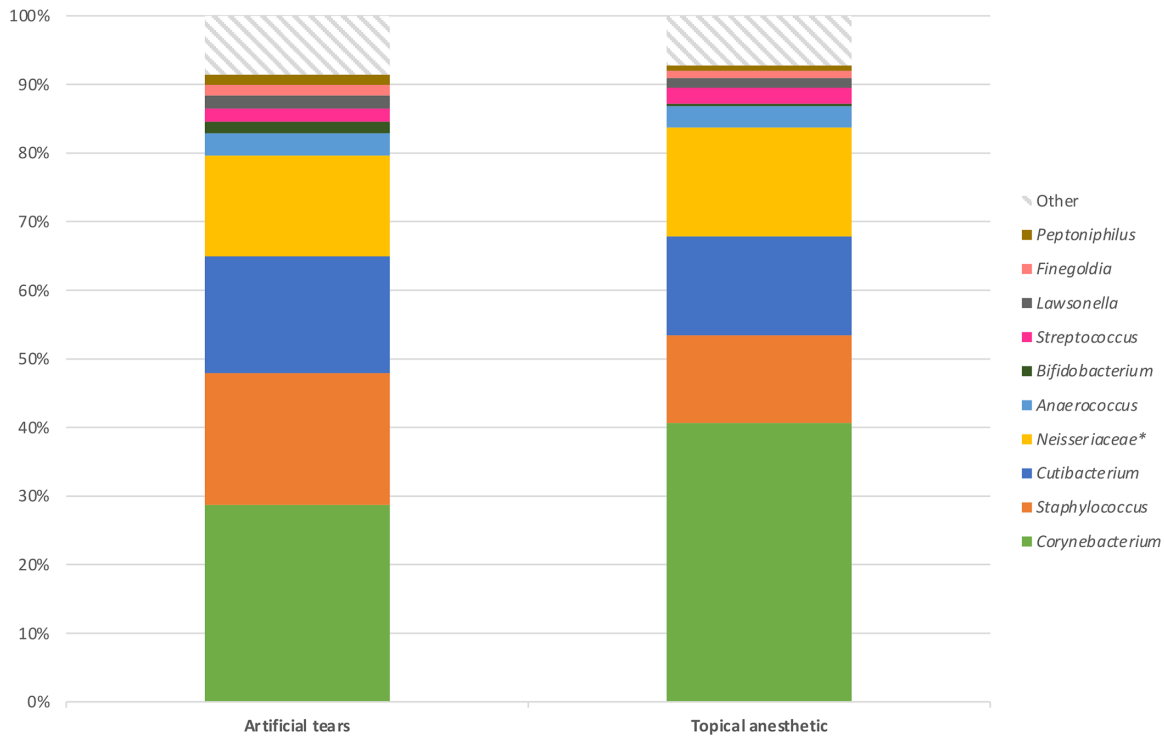
Figure 4. Genera with a relative abundance ≥ 1%, control group versus the topical anesthetic group (retrieved from dataset with all ASVs). In the control group, the genera with relative abundance ≥ 1% ($n = 10$) were *Corynebacterium* (29%), *Staphylococcus* (19%), *Cutibacterium* (17%), Neisseriaceae (15%), *Anaerococcus* (3%), *Bifidobacterium* (2%), *Streptococcus* (2%), *Lawsonella* (2%), *Finegoldia* (1%), and *Peptoniphilus* (1%). In the topical anesthetic group, those genera ($n = 8$) were *Corynebacterium* (40%), *Cutibacterium* (14%), *Staphylococcus* (13%), Neisseriaceae (16%), *Anaerococcus* (3%), *Streptococcus* (2%), *Lawsonella* (1%), and *Finegoldia* (1%). Note that the family Neisseriaceae has not been specified up to genus level.

could be attributed to 24 genera. When looking at the dataset for all ASVs, 10 genera had a relative abundance of ≥1%. In the artificial tears group, the same 10 genera remained, whereas in the anesthetic group, two genera dropped below the abundance threshold of 1% (*Bifidobacterium* and *Peptoniphilus*) (Fig. 4).

The comparison of weighted UniFrac distances between samples (Fig. 5) revealed that within-subject similarity overruled treatment effect in this cohort ($P < 0.05$). No impact was observed for the distance of ocular samples after the application of a drop of topical anesthetics or artificial tears.

LEfSe analysis (on the data including all ASVs) identified *Cutibacterium* as a potential discriminative genus to differentiate samples based on the presampling drop used, and *Cutibacterium* was more abundant in the control group ($P < 0.05$). Seven different ASVs in this dataset belonged to the genus *Cutibacterium* (ASV 2, ASV 20, ASV 65, ASV 138, ASV 609, ASV 710, and ASV 1059).

Next, we analyzed differences related to bacterial cell wall properties. Looking at the dataset with

the most abundant ASVs (ASVs with abundance > 0.1%; $n = 60$ ASVs), we analyzed and compared genera with Gram-positive and genera with Gram-negative characteristics. As expected, all samples had a large prevalence of Gram-positive bacteria. Of the 60 ASVs, 46 belonged to genera with Gram-positive characteristics and 11 to genera with Gram-negative characteristics (three ASVs were not assigned at the genus level). Moreover, Gram-positive genera were significantly more abundant as compared with Gram-negative genera in both the control and the topical anesthetic groups. With our chosen extraction protocol, eight samples had no Gram-negative bacteria. Of those eight samples, five belonged to the anesthetic group and three to the artificial tears group. Two of the samples without Gram-negative bacteria were the right and left eye from the same volunteer.

When comparing the control group with the topical anesthetic group, there was no significant difference in relative abundance of Gram-positive and Gram-negative bacteria ($P > 0.05$).

When looking at oxygen tolerance, all samples had facultative anaerobic genera. The facultative
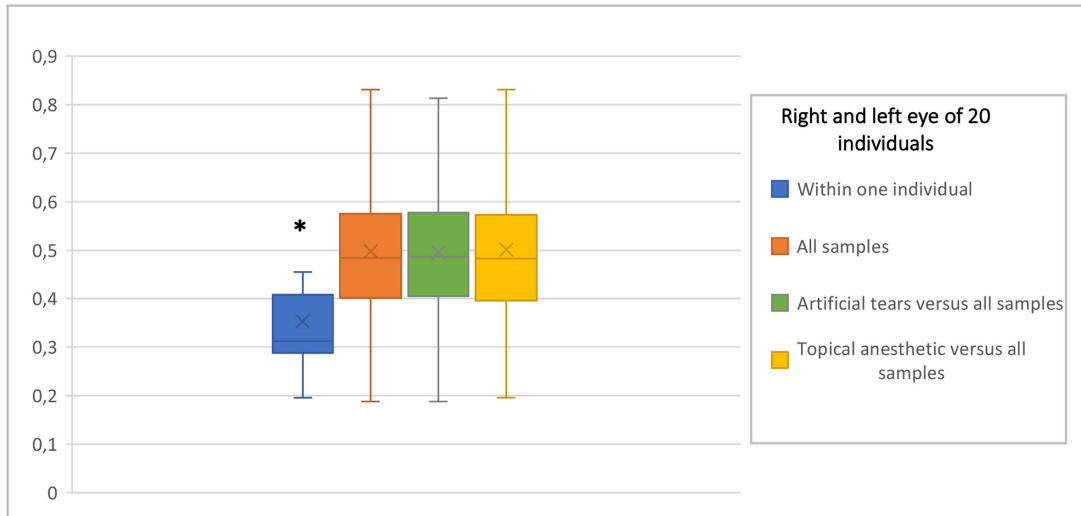
Figure 5. The weighted UniFrac distance (calculated after technical and biological singleton removal). The distance within an individual (right vs. left eye) is smaller than the effect of the topical anesthetic used before sampling ($P = 1.014 \times 10^{-5}$). The distance between all samples was similar to the distance between the artificial tears/topical anesthetic versus all samples. Asterisks indicate significant *P* values. Within one individual, $n = 20$; all samples, $n = 40$; artificial tears versus all samples and topical anesthetic versus all samples, $n = 20$ versus $n = 40$.

anaerobic genera in our dataset were *Staphylococcus*, *Streptococcus*, *Dolosigranulum*, *Enhydrobacter*, *Escherichia/Shigella*, and *Gemella*. Five samples did not have any anaerobic bacteria (two samples from the artificial tears group and three samples from the anesthetic group), and three samples had no aerobic genera (one sample from the artificial tears group and two samples from the anesthetic group). Interestingly, four samples (out of the five) without anaerobic bacteria also had no Gram-negative bacteria. There was no difference in abundance ($P > 0.05$) of (facultative) anaerobic and aerobic bacteria between the two groups.

Discussion

This comparative project aimed to assess the immediate effect of topical anesthetic drops on the 16S rRNA sequencing results of ocular surface samples. We set up a prospective research project with strong confounder monitoring. Artificial tears were used as a control, to differentiate the effect of the active component (oxybuprocaine hydrochloride 0.4%) from the diluting effect of the drop itself and to prevent unblinding of the executer. Moreover, preservative-free drops were used to avoid an additional confounding factor. Also, by sampling under general anesthesia and in a blinded way, we ensured similar pressure when swabbing with or without topical anesthesia. Furthermore, both eyes of one person were compared based

on a study by Cavuoto et al.,[13] which reported that the microbial composition of the right and left eye is similar, in order to minimize compositional differences due to a different immunological and/or genetic background. Finally, the effect of lifestyle factors, such as sleeping side preference, was minimalized by randomization.

Four of the 24 included volunteers were excluded due to low bacterial yields in seven of the eight samples. Interestingly, they were all females between the ages of 40 and 59 years. This unexpected result, obtained with our currently used extraction protocol, might indicate quantitative differences between individuals, with certain females having a very low bacterial load on their ocular surface. Validation of our results will allow further assessment of whether this low bacterial load is of clinical relevance, as is the case for the gut microbiome,[25] and searching for other correlations between potential biomarker genera and certain confounding factors (such as age or hormonal status).

When performing our subsequent analyses to identify differences between the samples collected after topical anesthetic or those after artificial tears, we were not able to discriminate at the level of relative abundances of the different phyla and genera in our cohort of both eyes from 20 volunteers. Furthermore, diversity measures such as alpha and beta diversity were not significantly different between both groups. These results are in line with the narrow intra-individual distance of samples pairs (weighted UniFrac distance) (Fig. 5), revealing a larger within-

subject similarity compared with the treatment effect ($P < 0.05$).

Dong et al.[2] reported that different swabbing techniques led to a significant change in the proportions of retrieved genera. In their cohort, the use of a dry cotton swab applied with firm pressure resulted in higher abundance of Proteobacteria (*Bradyrhizobium* spp., *Delftia*, and *Sphingomonas* spp.), whereas an increased abundance of *Staphylococcus* spp. and *Corynebacterium* spp. was observed in samples collected through the use of moist cotton swabs applied with minimal pressure. These results might be explained, on the one hand, by a diluting effect caused by the moist swab or, on the other hand, by a difference in swabbing depth, revealing a more transient superficial microbiome (when swabbed with minimal pressure) and a deeper resident microbiome (when swabbed with firm pressure). Shin et al.[9] found a different microbial community in the group sampled after applying a drop of topical anesthetic. The use of a topical anesthetic can elicit an unintentional difference in applied pressure while swabbing, explaining these sequencing differences. To exclude this confounder, volunteers in our project were sampled under general anesthesia in a blinded way. Furthermore, Shin et al.[9] used different cohorts in their study (subjects from the ophthalmology practice vs. subjects working in the laboratory). The difference (currently attributed to the use of topical anesthetics) seen between both groups might also be attributed to confounding factors specific to the volunteers sampled at the laboratory (e.g., younger, healthier, in contact with chemicals). Finally, as mentioned above, this difference might also be due to a diluting or flushing effect of rinsing away the superficial (transient) layer of bacteria.[26]

Lysis of the bacterial cell wall is the first step of every molecular extraction protocol, as it is necessary to gain access to the bacterial DNA. In our extraction protocol, this lysis was executed by the beads present in the QIAGEN RNeasy PowerMicrobiome tubes (mechanical lysis) and an additional heat lysis step. Reports suggest that topical anesthesia also has an effect on the bacterial cell wall.[6,7] When the bacterial cell wall disruption had already taken place during the process of numbing the ocular surface, the beads might have affected the bacterial DNA itself instead of the cell wall. Furthermore, we know from previous publications that Gram-positive bacteria are more resistant to cellular lysis due to the high concentration of peptidoglycan within their cell walls.[27,28] In light of the aforementioned, we hypothesized that the use of topical anesthetics might have a different effect on Gram-positive and Gram-negative bacteria. With the currently used extraction protocol, we could not find a difference between the two groups when comparing genera with Gram-positive and Gram-negative characteristics. There was a significantly higher abundance of genera with Gram-positive characteristics in both the control and anesthetic groups. This observation is in line with our previously described core ocular surface microbiome where four out of the six core genera were Gram-positive.[1]

When looking at the necessity of oxygen for their metabolism, there was no difference between the two groups in (facultative) anaerobic and aerobic bacteria. Interestingly, all samples had facultative anaerobic bacteria.

Although the aforementioned analyses were not able to discriminate samples based on the use of topical anesthetics, LEfSe analysis (on the data including all ASVs) indicated that the Gram-positive bacteria *Cutibacterium* could serve as a potential discriminative biomarker. LEfSe analysis is a bioinformatics tool used to perform very specific comparisons taking into account both biological consistency and effect size, thus alleviating possible artifacts and the statistical inhomogeneity known to be common in metagenomic data. LEfSe analysis has been shown to have a lower false-positive rate compared with standard statistical tests such as Kruskal–Wallis.[29] Overall, pretreatment with a topical anesthetic has had only a minor effect on overall sequencing results, but when comparing papers it is important to take into account the specific effect of anesthetics on the abundance of *Cutibacterium*. In previously published ocular surface microbiome research, *Cutibacterium* was not mentioned. This can be explained by the reclassification of 10 species from the genus *Propionibacterium* to, among others, *Cutibacterium*.[30,31]

Our cohort is also the first European ($n = 20$) population cohort published to date, to the best of our knowledge. Based on earlier published data, an ocular core microbiome was defined at the phylum and genus levels.[1] The weighted core ocular surface microbiome at the phylum level was calculated taking into account the frequency of the respective genera for the different publications having raw data available.

When comparing our control cohort to the core phyla described there, we observed a lower proportion of Proteobacteria (11% vs. 41%) and a higher proportion of Firmicutes (35% vs. 17%). This might be due to differences in the DNA extraction protocols used[8] but potentially also by geographical differences, among other confounding factors.

At the genus level, there was quite some overlap with the previously described core ocular surface microbiome: *Corynebacterium*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Propionibacterium*, and *Streptococ-*

cus.[1] *Corynebacterium*, *Cutibacterium* (formerly classified under *Propionibacterium*[30,31]), and *Staphylococcus* were present in more than 95% of the control samples. *Pseudomonas* and *Acinetobacter* had an abundance below 1% in our current dataset. *Bacillus* (although strictly not part of the core ocular surface microbiome) could not be retrieved from our whole cohort.

The major limitations of our study include that we performed a within-subject comparison, based on the publication of Cavuoto et al.[13] stating that the microbial composition is similar between both eyes, albeit this observation requires validation. To counter this shortcoming, we randomized the right and the left eye and looked at general trends. By comparing weighted UniFrac distances between samples (Fig. 5), we confirmed that there seems to be a within-subject similarity. If the latter is disproven by future research, our results would have to be reassessed. Second, we only investigated the immediate effect of topical anesthetics on the bacterial cell wall and its subsequent effect on DNA extraction and sequencing. We did not investigate the effect of the product on the microbial composition. A longitudinal study is necessary to gain more insight into this long-term effect of topical anesthetics on the microbial composition. Third, we only looked at the microbial composition based on 16S rRNA gene analysis and the inferred genetic potential based on that information. We did not perform functional analysis such as research on metabolomics or proteomics. The subanalyses on the Gram-positive and Gram-negative bacteria, as well as anaerobic versus aerobic bacteria, were conducted purely to gain more insight into our data and should not be extrapolated as having any functional value. Next, our sample size was rather limited; specifically, the number of female subjects with sufficient bacterial DNA for sequencing was unexpectedly low. Finally, as the DNA extraction protocol has a high impact on the observed composition, other extraction protocols might reveal different results.[8]

In conclusion, our comparative double-blinded study allowed a targeted search for the effects of topical anesthetics on the eye microbiome, taking into account the most obvious confounding factors. In our cohort, the topical anesthetics did not affect the overall sequencing results (at the levels of ASV, phylum, genera, or alpha and beta diversity). However, *Cutibacterium* was identified as a specific discriminative biomarker for topical anesthetics. Hence, it is of importance to report the use of a topical anesthetic when publishing 16S rRNA sequencing results of ocular surface samples, as the effect of this anesthetic on the prevalence and abundance of *Cutibacterium* should be taken into account when comparing papers.

References

1. Delbeke H, Younas S, Casteels I, Joossens M. Current knowledge on the human eye microbiome: a systematic review of available amplicon and metagenomic sequencing data. *Acta Ophthalmol.* 2021;99(1):16–25.
2. Dong Q, Brulc JM, Iovieno A, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci.* 2011;52(8):5408–5413.
3. Zhou Y, Holland MJ, Makalo P, et al. The conjunctival microbiome in health and trachomatous disease: a case control study. *Genome Med.* 2014;6(11):99.
4. Celebi OO, Celebi ARC. The effect of topical ocular anesthetic proparacaine on conjunctival and nasal mucosal flora in dry eye disease patients. *J Clin Med.* 2018;7(4):73.
5. Pelosini L, Treffene S, Hollick EJ. Antibacterial activity of preservative-free topical anesthetic drops in current use in ophthalmology departments. *Cornea.* 2009;28(1):58–61.
6. Leung YW, Rawal BD. Mechanism of action of tetracaine hydrochloride against *Pseudomonas aeruginosa. J Infect Dis.* 1977;136(5):679–683.
7. Silva MT, Sousa JC, Polónia JJ, Macedo PM. Effects of local anesthetics on bacterial cells. *J Bacteriol.* 1979;137(1):461–468.
8. Costea PI, Zeller G, Sunagawa S, et al. Towards standards for human fecal sample processing in metagenomic studies. *Nat Biotechnol.* 2017;35(11):1069–1076.
9. Shin H, Price K, Albert L, Dodick J, Park L, Dominguez-Bello MG. Changes in the eye microbiota associated with contact lens wearing. *mBio.* 2016;7(2):e00198.

10. Li Z, Gong Y, Chen S, et al. Comparative portrayal of ocular surface microbe with and without dry eye. *J Microbiol*. 2019;57(11):1025–1032.

11. Chao C, Akileswaran L, Bailey JNC, et al. Potential role of ocular microbiome, host genotype, tear cytokines, and environmental factors in corneal infiltrative events in contact lens wearers. *Invest Ophthalmol Vis Sci*. 2018;59(15):5752–5761.

12. Butcher RMR, Sokana O, Jack K, et al. Active trachoma cases in the Solomon Islands have varied polymicrobial community structures but do not associate with individual non-chlamydial pathogens of the eye. *Front Med (Lausanne)*. 2017;4:251.

13. Cavuoto KM, Banerjee S, Miller D, Galor A. Composition and comparison of the ocular surface microbiome in infants and older children. *Transl Vis Sci Technol*. 2018;7(6):16.

14. Falony G, Joossens M, Vieira-Silva S, et al. Population-level analysis of gut microbiome variation. *Science*. 2016;352(6285):560–564.

15. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13(7):581–583.

16. R Core Team . *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing; 2014.

17. Yau JWK, Hou J, Tsui SKW, et al. Characterization of ocular and nasopharyngeal microbiome in allergic rhinoconjunctivitis. *Pediatr Allergy Immunol*. 2019;30(6):624–631.

18. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41(database issue):D590–D596.

19. Reynders T, Devolder L, Valles-Colomer M, et al. Gut microbiome variation is associated to multiple sclerosis phenotypic subtypes. *Ann Clin Transl Neurol*. 2020;7(4):406–419.

20. Ozkan J, Diez-Vives C, Nielsen S, Thomas T, Coroneo MT, Willcox M. The temporal stability of the ocular surface microbiome. *Invest Ophthalmol Vis Sci*. 2017;58(8).

21. Fedor P. Answer to "What is the deference [sic] between Shannon Wiener diversity index and Simpson diversity index?" 2014. Available at: https://www.researchgate.net/post/what_is_the_deference_between_Shannon_Wiener_diversity_Index_and_Simpson_diversity_Index. Accessed February 11, 2022.

22. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013;8(4):e61217.

23. Oksanen J, Blanchet FG, Friendly M, et al. Package "vegan," version 2.5-7. 2020. Available at: https://cran.r-project.org/web/packages/vegan/vegan.pdf. Accessed February 11, 2022.

24. Afgan E, Baker D, Batut B, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res*. 2018;46(W1):W537–W544.

25. Vandeputte D, Kathagen G, D'Hoe K, et al. Quantitative microbiome profiling links gut community variation to microbial load. *Nature*. 2017;551(7681):507–511.

26. Baim AD, Movahedan A, Farooq AV, Skondra D. The microbiome and ophthalmic disease. *Exp Biol Med*. 2019;244(6):419–429.

27. Farhan H. Evaluation three methods of the extraction and purification of bacterial DNA of Gram positive and Gram negative bacteria. *World J Exp Biosci*. 2016;4(1):62–65.

28. Sambrook JF, en Russell DW. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press; 2001.

29. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12(6):R60.

30. Scholz CFP, Kilian M. The natural history of cutaneous propionibacteria, and reclassification of selected species within the genus *Propionibacterium* to the proposed novel genera *Acidipropionibacterium* gen. nov., *Cutibacterium* gen. nov. and *Pseudopropionibacterium* gen. nov. *Int J Syst Evol Microbiol*. 2016;66(11):4422–4432.

31. Parte AC, Sardà Carbasse J, Meier-Kolthoff JP, Reimer LC, Göker M. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol*. 2020;70(11):5607–5612.

translational vision science & technology