Original Article

Assessing the Impact of Sodium Hyaluronate Eye Drops on the Ocular Surface Microbiome: Implications for Dry Eye Management and Ocular Health

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Abstract:

Background: The ocular surface microbiome regulates immunity, maintains integrity, and affects eye disease. Dry eye is treated with sodium hyaluronate eye drops, but its impact on the microbiome is unclear. We studied bacterial diversity before and after using hyaluronate eye drops with or without preservatives, to understand its effects on the ocular surface microbiome. By comparing the results with a previous study, we aimed to gain insight into the potential effects of this treatment on the ocular surface microbiome, which may have implications for the management of dry eye and other ocular diseases.
Material and Methods: In our study, we analyzed 64 samples of 16 adults from a public cohort comprising 632 features, and a total frequency of 113,647. We used a method of randomly dividing 16 healthy adults into two groups. To classify the bacteria at the genus level, we utilized the GreenGenes dataset. Our objective in selecting this dataset for microbiome analysis was twofold: firstly, to compare our results with those of a recent study, and secondly, to understand how hardware limitations in the analysis process can impact the identification of bacteria in the eye microbiome.
Results: We successfully identified 103 distinct genera belonging to 21 different phyla. Sediminibacterium, Achromobacter, and Turneriella were more abundant in participants who did not use sodium hyaluronate eye drops. In contrast, Comamonas, Corynebacterium, Flarobacterium, Pigmentiphaga, Zeogloea, and Meiothermus have high abundance in participants who did use sodium hyaluronate eye drops.
Conclusion: Sodium hyaluronate drops can alter the bacterial community at the surface of the eye, regardless of the content of benzalkonium chloride (BAC).The results obtained in our study were confirmed by some previous studies, with the explanation that in this study, due to the use of the GreenGenes database, more complete results were obtained compared to some previous studies.

Keywords: Ocular Surface Microbiota; Preservatives; Sodium Hyaluronate Eye Drops.

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Introduction

Bacteria play a key role in preserving the health of the human body. These microorganisms are ten times more numerous than human cells [1, 2]. According to new research, it has been discovered that there are more microorganisms on the surface of the eye than on other parts of the body, but they are relatively low in number when compared to other parts of the body. On the other hand, from a biodiversity point of view, there are more species on the surface of the eye [3-7]. Different populations of people possess different microbiomes on their ocular surfaces, and these microbiomes are influenced by factors such as genetics, age, and the type of living environment in which they live [8-11]. A person's risk of developing an ocular surface disorder increases as they age, and women are more likely to be affected than men [1].

Dry eye is one of the most prevalent eye diseases. When a person suffers from this disease, the amount of moisture and tears in the eyes decreases or the quality and quantity of tears are inadequate. In the past, dry eye disease was a problem in the older adult, but today, with the increasing use of computers and mobile phones, this problem is seen in different ages. Tears are a complex mixture of water, fatty oil, protein, and electrolytes. Dry eye is associated with several complications and symptoms, one or more of these symptoms may observe in dry eye patients. These symptoms include burning in the eyes, redness in the eyes, and tiredness after a short study period, and so on. Several studies have examined the microbiota of dry eye patients [12- 18].

Artificial teardrops are used to temporarily raise the moisture in the eyes. The composition of artificial tears comprises a wide variety of active ingredients, and comes in preservative-free- single-dose units or multi-dose vials with preservatives [29].

Sodium hyaluronate eye drops are water-soluble polymers that become an option for artificial tear therapy in the recent decade [30].

Sodium hyaluronate (SH) is an aminoglycan derived from glucose and composed of repeating units of N-acetyl-D-glucosamine and sodium D-glucuronate, which have properties that are viscoelastic and rheological. This substance improves the stability of the tear film layer on the surface of the eye and reduces the process of tear washing during blinking. In addition, sodium hyaluronate binds to water in the tear and prevents the tear layer from evaporating and drying out.

Preservatives used in artificial tears include benzalkonium chloride (BAC), which is among the most common. To prevent the growth of microorganisms in artificial tears and to ensure the product's shelf life, preservatives are added to the product. The result of previous studies has shown that toxicity of BAC associated with abundancy, concentration, extent of lacrimation, and severity of ocular surface disease [19, 22].

In order to colonize the ocular surface epithelium and the tear layer, there is a high possibility that sodium hyaluronate eye drops will affect bacterial microbiota colonization. The effects of BAC on the bacterial community of the ocular surface are also unclear at the moment. To answer these questions, the present study aimed to evaluate the diversity of bacterial microbiota on the surface of the ocular surface before and after the use of sodium hyaluronate eye drops.

Material and Methods

The data were downloaded from EBI databases with Accession number: PRJNA720296 with a total of 16 healthy volunteers including 10 females and 6 males (aged 28.25 3.51 years)

that divided into 2 groups randomly, each group consisted of five women and three men. The data used in this study were obtained by comparing the differences in taxonomy and diversity between different groups before and after the intervention with sodium hyaluronate eye drops (with or without preservatives), and then sequencing the 16S rRNA gene region from the V3 to V4 region within the samples.

Subjects in this study lived in the same city, worked at the Xiamen Eye Center as medical staff, and did not exhibit any signs of superficial eye disease. An overview of the study population is shown in Table S1, along with a description of the categorization and distribution within each group. In this study, the sampling has been done in two stages. Immediately following the eye examination, the first sampling was conducted in the morning after the eye examination had been completed. Afterwards, the eyes were treated with artificial tears, and a second sample was collected two weeks after the artificial tears were applied, following the application of artificial tears. The first group of subjects received 0.3 % sodium hyaluronate eye drops with preservatives (Santen) twice a day for two weeks, two times a day for a period of two weeks each, for a period of two weeks each. As part of the second group, 3% sodium hyaluronate eye drops without preservatives (Santen, Osaka, Japan) were administered with the same frequency and duration as the first group of patients. Table S2 describes the population information used in the samples. The sampling process is very sensitive and accurate. For more data on the sampling method, see the article [23].

Preprocessing and data analysis

During the preparation of the metadata information about the samples, the following tools were used. A complete set of metadata information has been extracted from the available information (including "grouping and date of testing time," "experimental group," "left and right eye," etc.). A confirmation of the created metadata was performed by using the QIIME 2 plugin.

Denoising and QC filtering

o model and correct errors associated with Illumina sequence domains for sample quality evaluation, the software package DADA2 [23] was utilized. The DADA2 algorithm generates sample sequences with a fine granularity in the OTUs rather than a coarse granularity. It is of interest to note that the DADA2 tool is used in QIIME 2 [24], which is a powerful package for analyzing the microbiome that emphasizes transparency and data. As input to the QIIME 2 tool, paired FASTQ files are received as pairs. After merging and filtering the readings at the end of the pair, quality values were calculated based on the merged readings.

DADA2 was used to analyze the quality values of 10000 base pairs read/sequence for more clarification. A noise filter can be used based on demultiplexer output results. By considering the output quality, DADA2 performed significant quality control measures by removing noise, resulting in better discrimination between actual sequence variation and sequence errors. Next, the QIIME 2 attribute table function was used to display the filtered/extracted data. To illustrate the results, the (*.qza) files were converted to qzv files then https://view.qiime2.org/ was used for viewing.

According to the observation results, we check the quality of the nucleotide reads and for the denoising step, according to the quality score, we select the desired nucleotide number for truncate. This number was 210 for forward

sequences and 220 for reverse sequences

Taxonomic Classification and Diversity

After removing the noise with DADA2, some of the readings were erased. The information from the table summary was displayed using the QIIME View website. This platform generates a visual representation that effectively showcases the sequencing depth in the provided examples. The lower bound of the samples was established through visualization. This was accomplished by filtering the samples using a command with a sequence depth of five, as depicted in the figure below. The execution of this command resulted in the deletion of two samples.

Sequences were classified using the Scikit-learn method and the GreenGenes database [25]. QIIME 2 plugin builds an interactive bar chart for each species in the samples using the taxonomic profiles of each sample and illustrates the taxonomic profiles of each sample with the QIIME 2 bar plot. The frequency is sorted by a specific classification group or based on metadata grouping (figure 1). For the investigation of phylogenetically based alpha diversity criteria and construction of a phylogenetic tree, a multi-sequence alignment was performed using Mafft alignment. On the basis of the Diversity Core-Metrics-Phylogenetic, we calculated phylogenetic and non-phylogenetic diversity metrics as well as alpha and beta diversity metrics.

Using the Emperor tool, a powerful tool for exploring scattered charts, the PCoA chart was constructed to determine the criteria for beta diversity. A number of quantitative and beta diversity criteria were calculated on the basis of taxon abundance (e.g., Bray-Curtis criteria). We calculated two types of phylogenetic beta diversity criteria, namely qualitative (non-weighted) and quantitative (weighted). The difference between the metadata groups of different samples was also tested for significant differences between them. A Kruskal-Wallis test was used to assess whether there were significant differences between the groups on the basis of bar plots of alpha values. A beta diversity boxplot was created to illustrate how the distance between samples in the metadata table file was derived from the distance between samples from the specific groups in the metadata file. An analysis of PERMANOVA was used to assess whether there were significant differences between the groups.

Results

The used data in this study included 64 samples from 16 participants. There were 6870617 reads with an average of 107353.39 reads in each sample. A total of 634 features with a total frequency of 113650 were identified. The maximum observed frequency was 57814.0 for sample SRR14162133. The mean frequency in each attribute was 10.5. More information like the properties of data before filtering has been summarized in Table 1.

DADA2 is a technique used to deduce sample sequences from a collection of amplitude sequences. In this study, the quality assessment plot of both the forward and reverse data was

Table 1: Sample information summary. 64 samples were included in both groups before and after the intervention. After the noise removal operation, the number of samples reached 62

Metric	Sample	Filtered sample
Number of samples	64	62
Number of features	634	632
Total frequency	113,650	113,647

Table 2: Kruskal- Wallis test for PC PC (Preservative- Containing) and PF (Preservative- Free) groups

Group 1	Group 2	H	P value	q-value
PC-D0 (n = 15)	PC-D14 (n = 16)	0.535846	0.464159	0.93831
PC-D0 (n = 15)	PF-D0 (n = 16)	0.000391	0.984216	0.984216
PC-D0 (n = 15)	PF-D14 (n = 15)	0.228544	0.632606	0.93831
PC-D14 (n = 16)	PF-D0 (n = 16)	0.56839	0.450899	0.93831
PC-D14 (n = 16)	PF-D14 (n = 15)	0.076624	0.781925	0.93831
PF-D0 (n = 16)	PF-D14 (n = 15)	0.264276	0.607198	0.93831

analyzed using DADA2 to generate a table of features and representative sequences. The findings of this analysis are presented in Figure 1 and table 2.

The number of samples reached to 62 and 2 samples (SRR14162121 and SRR14162110) were removed from the analysis. The latest version of the GreenGenes database was used as the reference database for Classify. The output of this classification bar plot is shown in phyla level (Level 2) (Figure 2) and genus level (Level 6) (Figure 4).

The results of each sample before and after the use of artificial tears were plotted (Figure 4). Comamonas, Corynebacterium, Flarobacterium, Pigmentiphaga, Zeogloea, and Meiothermus have high abundance in both groups (PC and PF). Participants who used eye drops without preservatives had more Sediminibacterium, Achromobacter, and Turneriella. Contrary to this, and Megamonas were more abundant in participants who used sodium hyaluronate eye drops with preservatives.

Alpha diversity measures was used to identify the richness and uniformity of individual taxa.

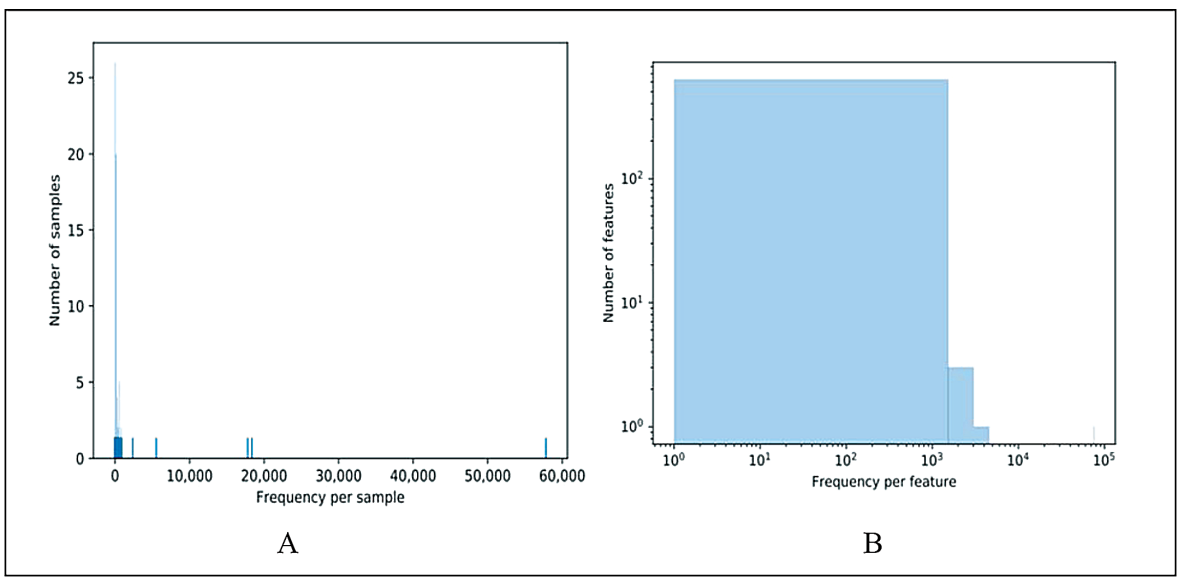

Figure 1: (A) The frequency per sample is plotted as a bar graph. (B) Frequency per feature bar plot

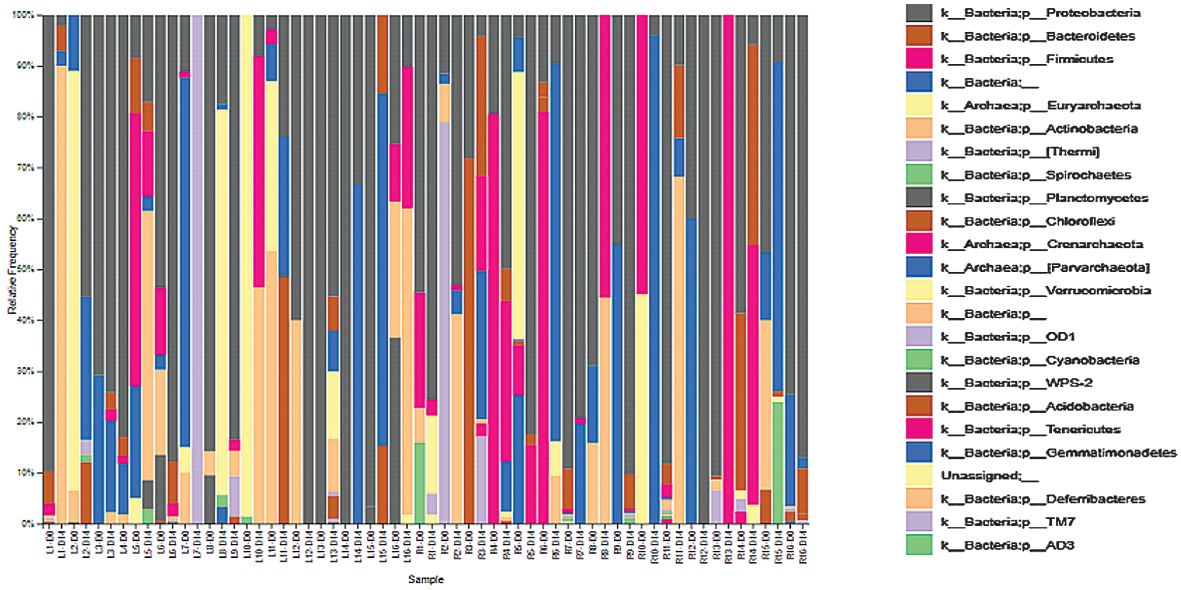

Figure 2: taxa-bar-plots- Groups of left and right eyes for phyla level of the taxonomy bar chart

These indicators do not consider the phylogeny of the identified species in the sequencing. Beta diversity measures differences between samples from different groups to determine differences in the overall community composition and structure.

Before and after the intervention, alpha variability was analyzed for each group. A comparison was also made between the groups regarding the differences of significant indicators between alpha PD and Shannon before and after intervention. Figures 3, 5 show the results of analysis within and between groups of individuals using different excretion rates. As Table 3 shows, alpha variability has not been significantly changed (P value> 0.05) through using sodium hyaluronate eye drops with or without BAC (Table 3).

All groups showed no significant change in alpha diversity when Kruskal-Wallis was used (Table S3, S4). As shown in Figure S1, there is no significant difference between groups when taking unpreserved and preservative hyaluronic acid eye drops.

The beta variability in two different groups using the PERMANOVA method and the statistical pseudo-F-test has been shown in Table 4. In the second column, are the results for PC (Preservative-Containing) or PF (Preservative-Free) and third groups in eight different categories (PC or PF group and left or right eye and before or after eye drops) are not significant in both columns (Tables 4, P value > 0.05). The obtained results show that the changes in the ocular microbiome are not significant in the case of using or not using preservatives, or in the left and right eyes.

The effectiveness of sodium hyaluronate eye

Table 3: P value, q-value and alpha diversity before and after taking hyaluronic acid eye drops without and with preservatives are not significant

Group 1	Group 2	H	P value	q-value
D0 (n = 31)	D14 (n = 31)	0.787811	0.374763	0.374763

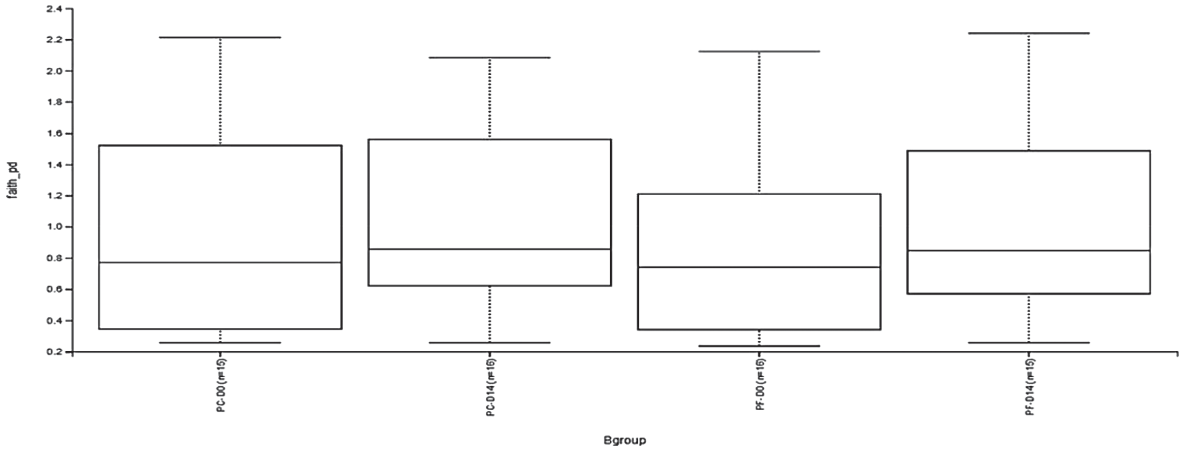

Figure 3: Box plot of alpha PD significance for PC (Preservative-Containing) and PF (Preservative-Free) groups before intervention

drops for specific bacterial strains has been studied in different ways. A number of common and significant differences were observed in the results of the index analysis, whether they were observed between individuals or between individuals belonging to different groups of individuals. There is also evidence that the use of sodium hyaluronate eye drops, with or without BAC, can affect the bacterial microbiota of the ocular surface as a result of the use of these eye drops (Supplementary file 1).

Table 4: The beta variability of sodium hyaluronate eye drops (whether or not containing BAC) was not significantly altered with the PERMANOVA method and pseudo-F statistical test in two different groups

method name	PERMANOVA	PERMANOVA
test statistic name	pseudo-F	pseudo-F
sample size	62	62
number of groups	2 (PC, PF)	8 (G-LR-D)
test statistic	1.06285	1.04071
p-value	0.311	0.349
number of permutations	999	999

Zilliox et al. [13], suggested the two samples have a high degree of similarity. In our study beta variability comparing the left and right eye microbiome showed similarities in the post-intervention groups on samples in the ocular microbiome (Figure 6, subject 14).

According to UniFrac's weight metrics of diversity, PCoA Emperor provides plots that are based on the UniFrac weight metrics. The Uni FRAC distance metric can be used to compare the biological communities based on their distance from each other. Using the weighted UniFrac distance index as the basis for a PCoA plot in the present study, it was discovered that after intervention with sodium hyaluronate eye drops (with or without BAC), the bacterial population on the ocular surface of the eye changed, either within or between individuals within a group (Figure 7).

Discussion

Among the available eye drops for dry eyes, the most commonly used artificial tears are sodium hyaluronate eye drops, accounting

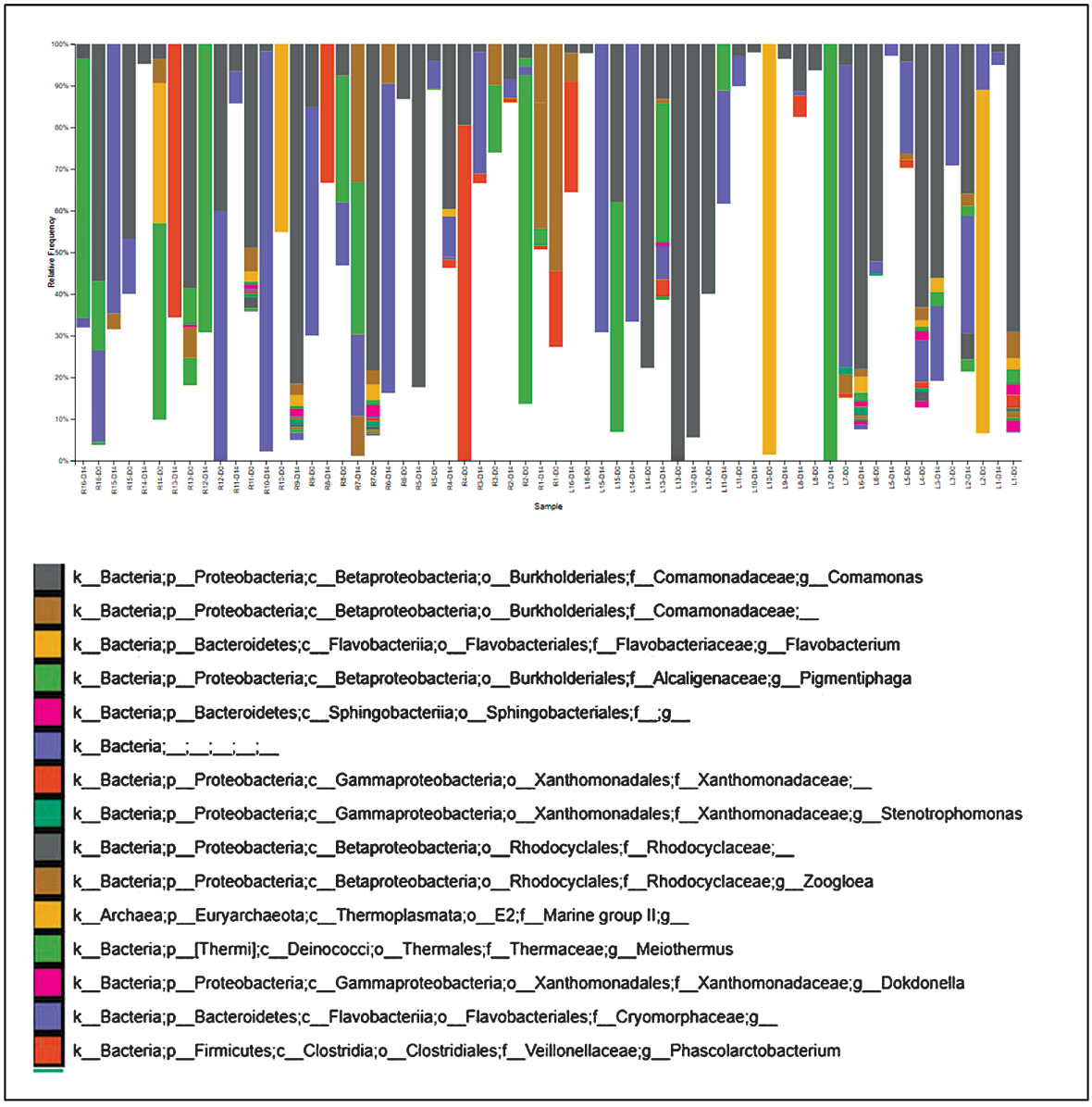

Figure 4: The results of each sample before using artificial tears and after using artificial tears for the genus level

for a significant market share. A number of different packaging models are available for these drops, including those for single doses and multi-doses. Preservatives should be added to artificial tears that are regularly administered in multiple doses to prevent microbial growth. An important role played by surface bacteria on the surface of the eye is that they are responsible for the health of the eye. In this study, the effects of sodium hyaluronate eye drop in various doses (with or without a preservative) were evaluated on ocular surface bacteria. According to the study's results, sodium hyaluronate eye drops (with or without BAC) can alter the bacterial population of the ocular surface after treatment with sodium hyaluronate eye drops.

No significant difference in alpha diversity was found in the right/left eyes in the results of previous studies that compared the ocular

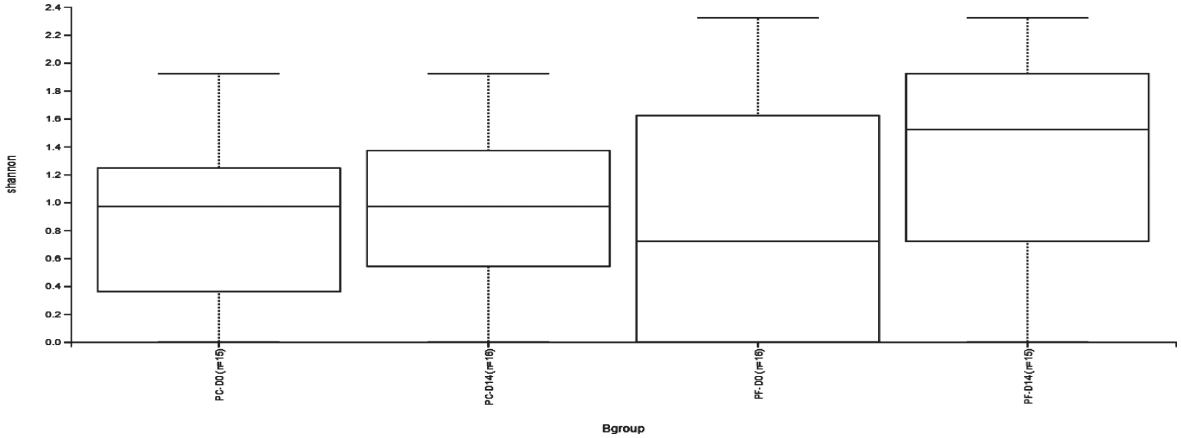

Figure 5: PC(Preservative-Containing) and PF(Preservative-Free) groups' alpha Shannon significance before intervention

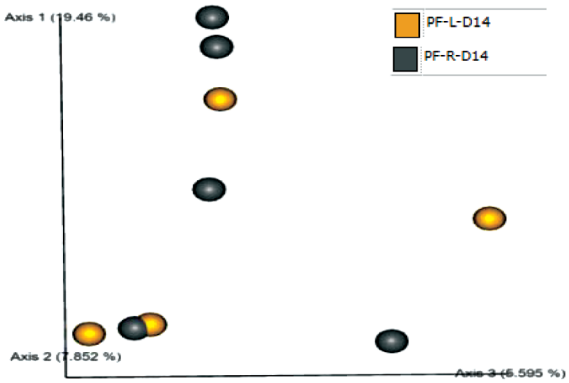

Figure 6: Beta variability comparing the left and right eye microbiome similarities in the post-intervention groups

surface microbiota in patients with dry eye disease [1, 9, 13]. In spite of this, the study [23] found that there was a significant difference between genders as well as relative abundances of species. Despite differences in the surface bacterial communities on the surface of the eyes before and after the intervention, alpha diversity was not significantly different in this study between before and after the intervention. Some of the commonly known bacteria at the genus level in Preservative-Free are Corynebacterium, Faecalibacterium, Alteromonas, and Bacteroides, which were also mentioned in the study of Zhong et al [28]. Considering that in this research, the GreenGeens database was used to identify bacteria. Other bacteria were also identified at the genus level, which were more abundant, such as Camamonas, Pigmentiphaga, Phascolarctobacterium, Zeogloea, Meiothermus, Stenotrophomonas and Flarobacterium.

The distances between the alpha and beta variables in different groups of the sample were analyzed in this study as well, in which the distances between the groups of the sample were indicative of the different types of society in these different kinds of groups. A number of confounding factors, such as age and region, contributed to the observed differences in the microbial communities between individuals. As compared to previous studies, the limitations of this study and previous studies were the lack of gold standard data processing methods (tests) and the large sample sizes of the data. There is a way to solve this problem by conducting research with larger sample sizes and using well-known data research methods.

There is evidence from a study conducted by Zilliox et al. [13] suggests that half of the patients with ocular surface disorders and healthy individuals have a variety of

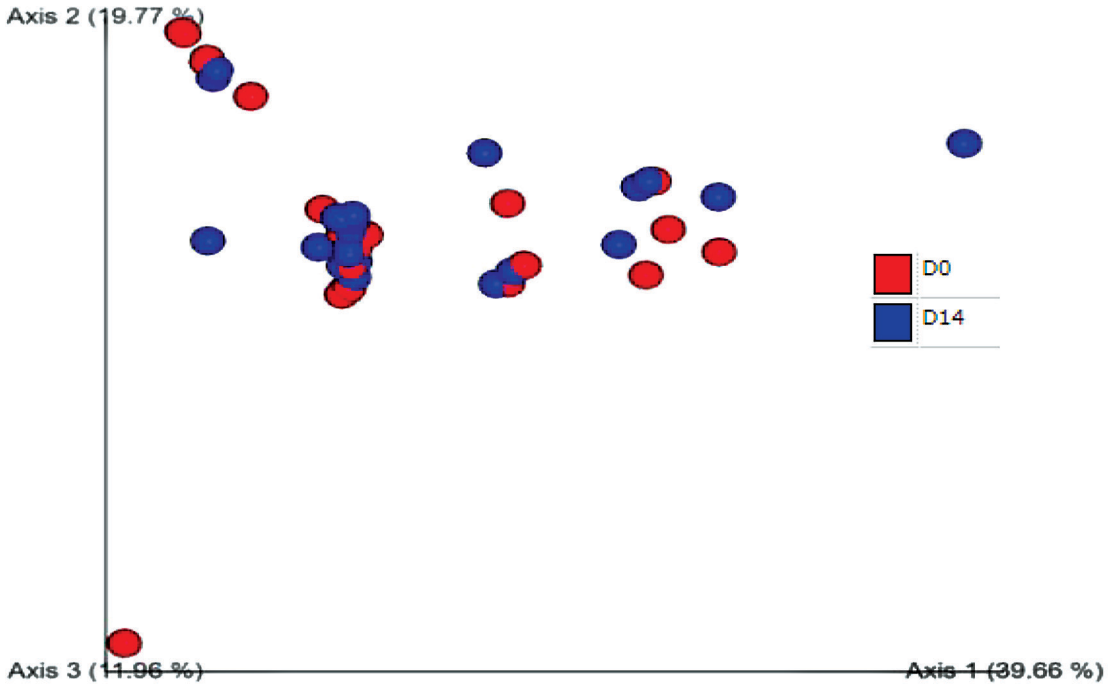

Figure 7: PCoA analysis between right (R) and left (L) eyes based on UniFrac weight variation criteria between the two groups before and after artificial tear use

microbiota compositions between their eyes, which is consistent with the findings of our study. Although there are some differences between the results of the present study and previous reports, there are also some similarities. In their study of the microbial community composition in the left and right eyes of a volunteer, Van et al. [9] did not find any significant difference between the two eyes. In addition, when it comes to the relative abundances of the species within the core, no significant differences were found between the species. As a result of several factors, there could be a significant difference in the results of our study as compared to other studies. There are a number of reasons for this, starting with the use of different methods. There may be some explanation for the high abundance of most retrieved OTUs in part due to the varying depth of sequencing. As a result, the OTU filter strategy used in our study was different from that used in other studies.

The present study has several limitations such as the small sample size of the dataset. The results can be more generalizable and the differences in society can be better shown with a larger sample size with different age groups. In addition, the samples in this database are for Xiamen Eye Center medical staff that only were assigned to one location. Future studies with expanded patient recruitment and sample collection will allow for a more detailed study of the ocular surface and adjacent microbiomes. The human core microbiome is a set of genes of all microorganisms present in a given habitat in all or the vast majority of humans [26]. In a systematic review, Delbek et al summarized the data on the microbiome of the ocular surface [27]. In a study conducted by Ozkan et al. [7] they evaluated the three time points over which 43 participants were evaluated for their ocular surface microbiota. All participants were not covered by a single OTU at any given time, or any time when they were participating in the study.

It is important to note that people of different

ages were not compared in this study. As a result, it would be worthwhile to investigate the effect of the same intervention on infants and older adults on a more detailed level. There is no clear understanding of which factors within the ocular surface microenvironment are altered by sodium hyaluronate eye drops, and how they are affected. Additionally, it is not possible to predict how the body will respond to sodium hyaluronate drops if you stop using them or continue to use them for an extended period of time.

Conclusion

The 16S rDNA sequencing of healthy eyes revealed a rich diversity of bacterial microbiota on their surface, which is consistent with the presence of diverse bacterial microbiota. Despite the fact that there were slight differences in the bacterial communities between the eyes. With or without the use of BAC as a preservative, sodium hyaluronate drops can alter the bacterial community on the surface of the eye by altering the growth of bacteria. As a result of these findings, we may be able to better understand how bacteria grow on the surface of the eye and make better decisions about how to use medications on the eye surface.

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Footnotes and Financial Disclosures

Conflict of interest:

The authors have no conflict of interest with the subject matter of the present manuscript.