Original Article

Ocular Surface Microbiome Analysis: Exploring Dry Eye Disease

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Abstract

Background: Dry eye disease (DED) is an ocular medical condition affecting people worldwide, and there is a growing concern over its high prevalence. Due to the human body's microbiome having a powerful impact on many diseases, it was necessary to study the relationship between DED and the ocular microbiome, and the purpose of this study is to examine the existence of this connection.
Material and Methods: Two datasets of the ocular surface microbiome in dry eye patients were used for this research, one with data of patients before and after treatment with intense pulsed light (IPL), while the other only holds information on cases suffering from a dry eye condition. Both are available on molecular data resources under specific study accessions. After pre-processing both datasets, they were analyzed, and phylogenic tree, alpha diversity, and beta diversity plots were produced.
Results: The first dataset of both eyes of 20 patients with dry eye symptoms before and after IPL therapy was analyzed entirely. Bacteroidales (in 61 percent of the patients), Actinomycetales (in 60 percent of patients), Lactobacillales (in 61 percent of patients), and Erysipelotrichales (in 61 percent of patients) declined after the treatment. Still, the total difference between patient populations and treatment was not statistically proven (P value > 0.05). The second dataset contained data from 87 patients with dry eye disease, and it demonstrated that Burkholderiales, Actinomycetales, Pseudomonadales, and Clostridiales are among the most abundant bacteria in this group, in contrast to the first dataset, which was occupied by Clostridiales, Burkholderiales, Actinomycetales, Bacteroidales, Bacillales, and Lactobacillales.
Conclusion: This study showed there are multiple bacterial orders that have increased or decreased after the patients received their treatment with IPL, stating a potential connection between the mentioned orders and DED. More research is necessary to indicate a solid relationship between these two.

Keywords: Conjunctival Sac Microbiome; Dry Eye Disease (DED); Intense Pulsed Light Therapy (IPL); Microbiome Analysis; Ocular Surface; Tear Microbiome.

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Introduction

Dry eye disease (DED) is a widespread chronic ophthalmic disorder affecting people worldwide in aspects such as physical condition, quality of life, productivity, and financial issues [1–3] ”id”:”ITEM-1”,”issue”:”2”,”issued”:{“date-parts”:[[“2021”]]},”page”:”207”,”publisher”:”MDPI”,”title”:”Formulation considerations for the management of dry eye disease”,”type”:”article-journal”,”volume”:”13”},”uris”:[“http://www.mendeley.com/documents/?uuid=3fdf8230-bb3c-4e00-8d50-cd23cd17ca5a”]},{“id”:”ITEM-2”,”itemData”:{“DOI”:”10.1016/j.clae.2021.01.004”,”ISSN”:”1476-5411 (Electronic. It is estimated that 5 to 30 percent of people are suffering from DED globally [3] observational study to characterize the ocular surface microbiome (OSM. The most important characteristic of this condition is inflammation of the ocular surface [4], followed by hyperosmolarity and tear film instability, caused by homeostatic perturbation of the outer layer of the eye and the tear’s abnormal quality or quantity. The mentioned process is self-repeating and leads to blurry vision and general discomfort [2,3] 136 males; mean ± SD age, 41 ± 22 years. Despite the fact that DED has attracted researchers’ interest over the past decade since it is among the most common eye illnesses, numerous of its risk factors and pathogenesis are still unclear.

Multiple factors contribute to dry eye conditions. Age is one of the most commonly accepted ones, as most of the patients are from the adult population. Furthermore, females and east Asians are at increased risk of suffering from dry eye [2,5]. Also, symptoms of DED can appear in response to many diseases and disturbances, namely autoimmune disorders, surgery, and even psychiatric issues. Dry eye symptoms are within many diagnostic criteria, one important example being Sjögren’s syndrome which has a strong effect on lacrimal glands [5].

Environmental conditions dramatically affect this disorder, particularly air pollutants, such as PM10, NO2, and CO [5]. In addition, in June 2020, the first case of mask-associated dry eye (MADE) was observed and mask is now considered one of the many risk factors among regular mask users [6]. Lastly, changes in the ocular microbiome might lead to chronic DED, as analyzed and discussed later in this article.

Microbiota is specific for each organ. The microbiome colonized in different tissues of the human body is thought to have an essential role in maintaining the equilibrium of that tissue [7] limbal stem cell deficiency, etc. Furthermore, ocular microbiome may also be altered in SJS. This is prospective, age and sex matched analytical study which including 20 chronic SJS patients and 20 healthy subjects for specimen collection from inferior conjunctiva for microbiome analysis by conventional cultures and Next-Generation Sequencing (NGS. The complex microbiome of humans has many functions, such as regulating vital processes like immune tolerance, epithelial barrier, and metabolism [8] but no study has directly compared different sampling methods applied to the same eyes to one another or to a reference standard of corneal epithelial biopsy. We addressed this lack by comparing the microbiome from three conjunctival swabs with those of corneal epithelial biopsy. METHODS: Twelve eyes [11] patients. The most acknowledged mechanism of microbiome resistance to infection is that microbiota and pathogenic bacteria compete for metabolites needed for their maintenance and nourishment [9] because the microbiome, composed of the genetic material of bacteria, fungi, viruses, protozoa, and eukaryotes on

the ocular surface, differs from the microbiota, which are the community of microorganisms that colonize the ocular surface. The observed composition of the ocular surface flora depends on harvesting and examining methods, whether with traditional culture or with more refined genetic analysis based on rRNA and DNA sequencing. Environment, diet, sex, and age influence the microbial flora composition, thus complicating the analysis of the baseline status. Moreover, potentially pathogenic organisms can affect its composition, as do various disorders, including chronic inflammation, and therapies applied to the ocular surface. A better understanding of the composition and function of microbial communities at the ocular surface could bring new insights and clarify the epidemiology and pathology of ocular surface dynamics in health and disease. The purpose of this review is to provide an up-to-date overview of knowledge about this topic.","author":[{"dropping-particle":"","family":"Aragona","given":"Pasquale","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Baudouin","given":"Christophe","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Benitez del Castillo","given":"Jose M.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Messmer","given":"Elisabeth","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Barabino","given":"Stefano","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Merayo-Lloves","given":"Jesus","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Brignole-Baudouin","given":"Francoise","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Inferrera","given":"Leandro","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Rolando","given":"Maurizio","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Mencucci","given":"Rita","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Rescigno","given":"Maria","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Bonini","given":"Stefano","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Labetoulle","given":"Marc","non-dropping-particle":"","parse-names":false,"suffix":""}],"container-title":"Survey of Ophthalmology", "id": "ITEM-1","issue":"6","issued":{"date-parts":[["2021"]]},"page":"907-925","publisher":"Elsevier Inc.","title":"The ocular microbiome and microbiota and their effects on ocular surface pathophysiology and disorders","type":"article-journal","volume":"66"},"uris":["http://www.mendeley.com/documents/?uuid=960f9e0d-acf1-4037-b3f9-595f79c3af00"]}],"mendeley":{"formattedCitation":" [9]. A commonly used molecular method to study and analyze microbiome is 16S ribosomal ribonucleic acid (rRNA) gene sequencing. 16S rRNA has both conserved and variable regions, and the latter is different in each specific bacterium. The gut microbiome is well studied and recent experiments showed that mammals' normal development and homeostasis are dependent on the microbial community in the intestines [10]. Although the brain and the eye are two organs that are

considered distant from the intestines, the gut microbiome's role in general well-being may subsequently affect eye health. If there happens to be an interim ostium in the blood-retina barrier, the microbes that originated from the gut microbiome can enter the eye and disturb its equilibrium and potentially result in a disease or discomfort [11] the microbial community that possibly impacts ocular disease has been identified. This review provides an overview of the literature on approaches to microbiota analysis and the roles of commensal microbes in ophthalmic diseases, including autoimmune uveitis, age-related macular degeneration, glaucoma, and other ocular disorders. In addition, this review discusses the hypothesis of the \"gut-eye axis\" and evaluates the therapeutic potential of targeting commensal microbiota to alleviate ocular inflammation.","author":[{"dropping-particle":"","family":"Xue","given":"Wei","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Li","given":"Jing Jing","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Zou","given":"Yanli","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Zou","given":"Bin","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Wei","given":"Lai","non-dropping-particle":"","parse-names":false,"suffix":""}],"container-title":"Frontiers in cellular and infection microbiology","id":"ITEM-1","issued":{"date-parts":[["2021"]]},"language":"eng","page":"759333","publisher-place":"Switzerland","title":"Microbiota and Ocular Diseases.","type":"article-journal","volume":"11"},"uris":["http://www.mendeley.com/documents/?uuid=2286f706-8f92-457e-aa07-d8c20c21d389"]}],"mendeley":{"formattedCitation":" [11].

The ocular microbiome is an extraordinary microbial community in the human body, since many conditions can have a conspicuous impact on its content. The eye's outer layer has consummated exposure to external surroundings, so it can be modified under the influence of new microbes, especially under immune imbalance. a specific kind of microbiota in tears, also kenned as closed-eye tears, can modify even when a person is slumbering [4]. In a study conducted recently, it was shown that only 50 % of individuals shared the same microbiome in both eyes [3] observational study to characterize the ocular surface microbiome (OSM. Age, adequate control, and sampling procedures all have an impact on investigations and data analysis of the ocular microbiome. Because of the low biomass of the ocular surface, DNA contamination can change the outcome of the research remarkably [8] but no study has directly compared different sampling methods applied to the same eyes to one another or to a reference standard of corneal epithelial biopsy. We addressed this lack by comparing the microbiome from three conjunctival swabs with those of corneal epithelial biopsy. METHODS: Twelve eyes (11 patients.

The microbiome is proven to change in response to many diseases; as an example, a recent study showed that the ocular microbiome had altered in patients with chronic Stevens-Johnson syndrome, with an increase in opportunistic pathogens and a higher microbiome verity [7] limbal stem cell deficiency, etc. Furthermore, ocular microbiome may also be altered in SJS. This is prospective, age and sex matched analytical study which including 20 chronic SJS patients and 20 healthy subjects for specimen collection from inferior conjunctiva

for microbiome analysis by conventional cultures and Next-Generation Sequencing (NGS.

Many DED risk factors also influence the microbiome and alter it in unique ways. Aside from this, multiple other pieces of evidence indicate a relationship between the ocular microbiome and eye diseases. Following the previous statements, this study aims to test the hypothesis that DED and bacteria, and other unicellular organisms located in the eye might be related to each other. Due to the significant number of people with dry eye disease and the growing vigilance regarding the paramountcy of microbiome effects on the human body, it had been imperative to investigate the connection between the microbiota and DED.

Material and Methods

1) Data: two datasets were analyzed in this paper. A dataset containing information on patients before and after treatment of DED is the primary dataset and more analysis was done on it. The other dataset was chosen to analyze the eye microbiome further and compare both datasets to provide more substantial evidence for powerful statements and conclusions.

1-1) First dataset (patient/treatment dataset): The 16s ribosomal sequences used as the first data set were provided by Sagaser, S., et al. [12] treated, and administered the ocular surface disease index (OSDI and were accessible on the European Nucleotide Archive (ENA) at EMBL-EBI under study accession number PRJNA693337 (https://www.ebi.ac.uk/ena/browser/view/PRJNA693337). The sequences were obtained from teardrops of twenty patients with ocular rosacea with dry eye symptoms. Ten patients were randomly assigned to receive intense pulsed light with meibomian gland expression (IPL-MGX) or MGX treatment. Their tear samples were taken at the first and final visits following four therapy sessions. 16s rRNA amplicon sequencing of the ocular microbiome was done. The detailed methodology for this part is described in the paper published by Sagaser, S. as previously cited. A metadata file was built with certain adjustments regarding what was important in clustering the samples.

1-2) Second dataset (Only dry eye patient dataset): Sequences analyzed in the second part of the project were supplied by The First Affiliated Hospital of Xian Jiaotong University located in China by Qi, Y., et al. [13] dry eye group and the data was submitted on the European Nucleotide Archive (ENA) at EMBL-EB under study accession number PRJNA743593 (https://www.ebi.ac.uk/ena/browser/view/PRJNA743593). Swab samples from the inferior fornix of the conjunctival sac were collected from 49 patients without autoimmune diseases and 38 patients with autoimmune diseases. Dry eye symptoms were observed in both groups. Bacterial DNAs were isolated from swabs and analyzed with 16S rRNA amplicon sequencing. The method used in this experiment can be accessed through Qi, Y. paper in greater detail.

2) Methods: First, the data was imported into the analysis tool. Next, the quality score of 10,000 random samples of reads without replacement was analyzed, and the Q scores boxplot was produced.

Then, to achieve the optimal merging of the forward and reverse reads by disregarding the inferior quality portions of the read, denoising with DADA2 wrapped in QIIME2 was completed, truncation was performed at position 150 in forward reads and at position 140 throughout reverse reads, and trimming was carried out at position 19 in forward reads and position 20 in reverse reads. Visualizing and filtering came after the previous stage.

DADA2 is more efficient and easier to use in this analysis rather than methods such as Deblur, as it includes paired-end read joining. Next, the data was classified against naive Bayes using the GreenGenes 99 % phylogenetic tree most recent version (v13.8) under the name of gg-13-8-99-nb-classifier, which is a frequently used 16S rRNA reference database contains classified sequences from Archaea and Bacteria (2006, http://greengenes.lbl.gov and also accessible at qiime2 data resources). Taxonomy bar plots were produced using the metadata file that was built in the first step. Taxonomy plots are necessary for diversity analysis [14].

From this step towards the end, the mentioned methods were only performed for the first data set. Sequences were aligned using MAFFT, a multiple-sequence alignment program. The alignments that did not provide useful information were removed and as a result, masked alignments were provided. Both rooted and unrooted phylogenetic trees were generated subsequently. As a final step, obtained bacterial rooted phylogeny tree was tested for between group differences and alpha diversity at 4000 sequences. Alpha diversity indicates microbiome diversity in a single sample, while beta diversity measures the similarity or differences between two communities. Principal Coordinates Analysis (PCoA) plot is produced in this step. Multiple plots are produced in this step, the most important ones being alpha PD significance which represents a diversity analysis based on phylogenic distance within categories, alpha Shannon significance as an indicator of evenness and richness diversity within categories, and Bray Curtis significance which is a beta diversity indicator, as it visualizes between categories differences. The Shannon index (H) was used to describe global species diversity, as it considers both present species and their abundance. Higher H represents higher diversity. Kruskal-Wallis was employed to detect differences between groups. The tool used in this analysis is Qiime2, a powerful and efficient microbiome analysis program that turns raw sequences into a form analyzable for researchers. This package is accessible at https://qiime2.org/ (2019).

Results

Note that the second half of this section contains plots generated from data of only dry eye patients, up to taxonomy bar plots. The results are as follows:

The first dataset (patient/treatment dataset) has 31959 as the minimum sequence count, 265997 as the maximum, 100947.86 as the

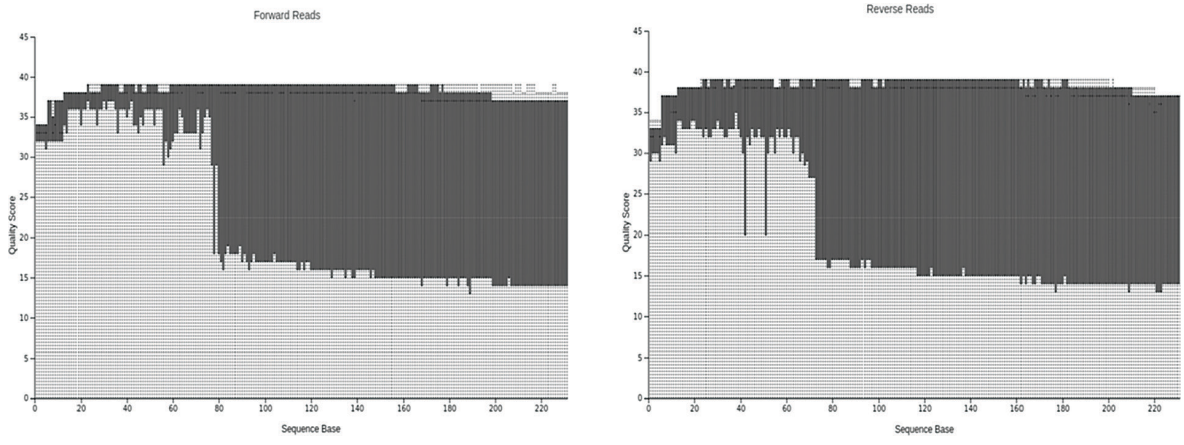

Figure 1-1: Quality plot for forward reads(left) and quality plot for reverse reads(right)

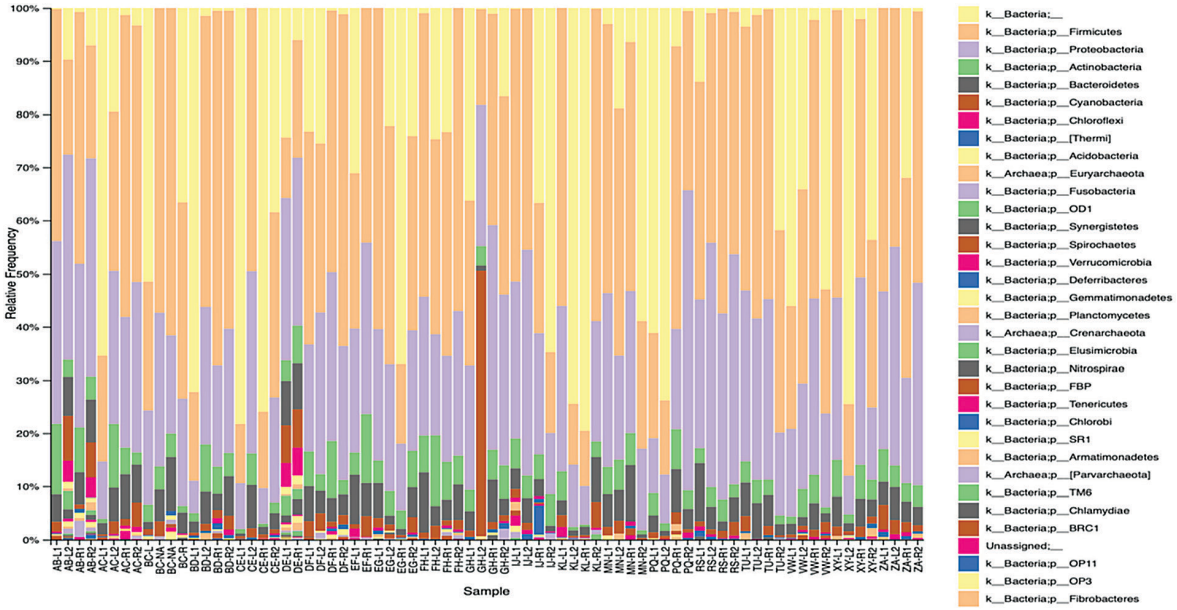

Figure 1-2: Taxonomic bar plots on phyla level, sorted by labID (E.g. AB), eye (L for left or R for right), and disease(1) or treatment(2)

mean, and a total of 7672037 counts. The second dataset (Only dry eye patient dataset) has 45985, 79997, 69585.76, and 6035547 respectively. For both datasets, data was the same before and after denoising and filtering, with 7,327 features with a total frequency of 5,172,832 for the first data and 36,162 features with a total frequency of 5,878,927.

1) patient/treatment dataset

In the pre-processing steps, figure 1 was produced to check the quality of forward reads as well as reverse reads. Quality boxplot visualizes the distribution of quality scores of each sequence. All samples were significant to be used in the analysis

The relative frequency of each phylum against each sample is visualized in figure 1-2. Every sample is labeled by a combination of two words, alongside L or R to indicate left or right eye respectively, and 1 or 2 to show the patient's state (1 for the first visit and 2 for the visit after treatment). In addition, each color indicates a distinct phylum. The most prevalent known bacteria phylum in all samples is Firmicutes, followed by Proteobacteria, Actinobacteria, and Bacteroidetes. Also, there are sequences in some samples belonging to the Archaea kingdom, namely Euryarchaeota and Crenarchaeota. A minority of the sequences were unassigned, which means that they don't belong to the Bacteria kingdom or Archaea kingdom.

The initial explanation for figure 1-3 is similar to the previous figure, only instead of phylum, it is the order of the bacteria. In these bar plots, the ocular microbiome is dominated by Clostridiales, Burkholderiales, Actinomycetales, and Bacteroidales. Also, from the Archaea kingdom, Methanobacteriales and Nitrososphaerales have the highest percentage in this sample.

P value and H index to compare between diversities within all groups or pairwise comparison between each eye during DED and after the treatment was done. It is not statistically proven that there is a significant difference in pairwise comparison before and

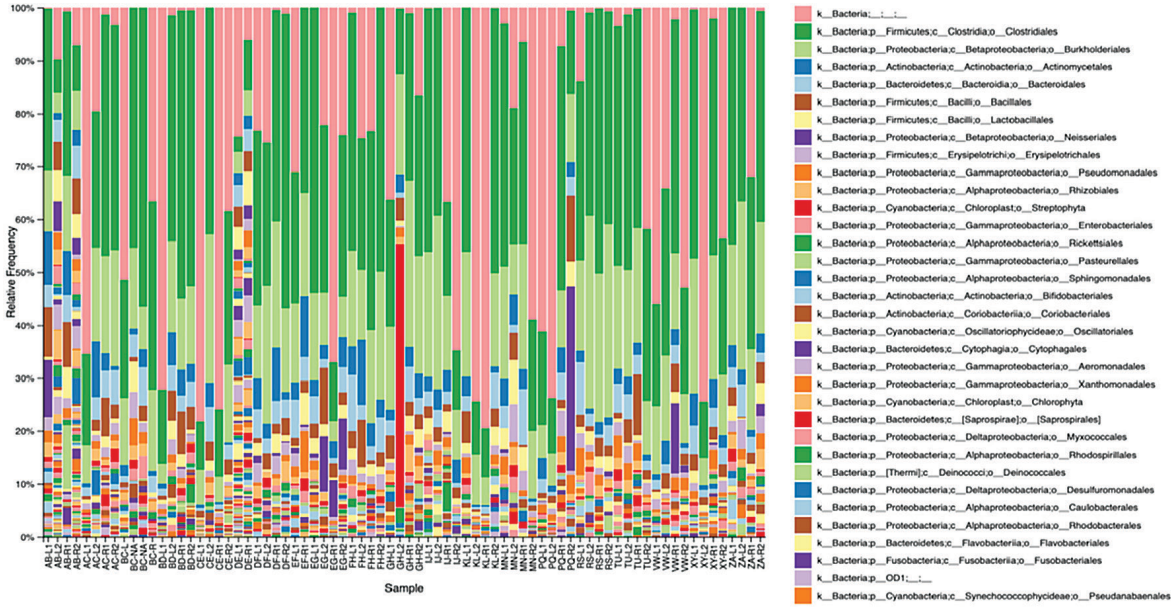

Figure 1-3: Taxonomic bar plots on order level, sorted by labID (E.g. AB), eye (L for left or R for right) and disease (1) or treatment (2)

after treatment (p = 0.24 for the left eye and p = 0.67 for the right eye), and for the right eye, the H index is relatively low, indicating a low diversity before and after treatment.

Bray Curtis emperor shows between groups differences. 5 distinguished categories are present in this plot and are visualized by different colors, as the list on the right indicates (L1 for left eye before treatment or R2 for right eye after treatment). There are two points left unassigned to left or right eye, so they are labeled as NA.

2) Only dry eye patients dataset

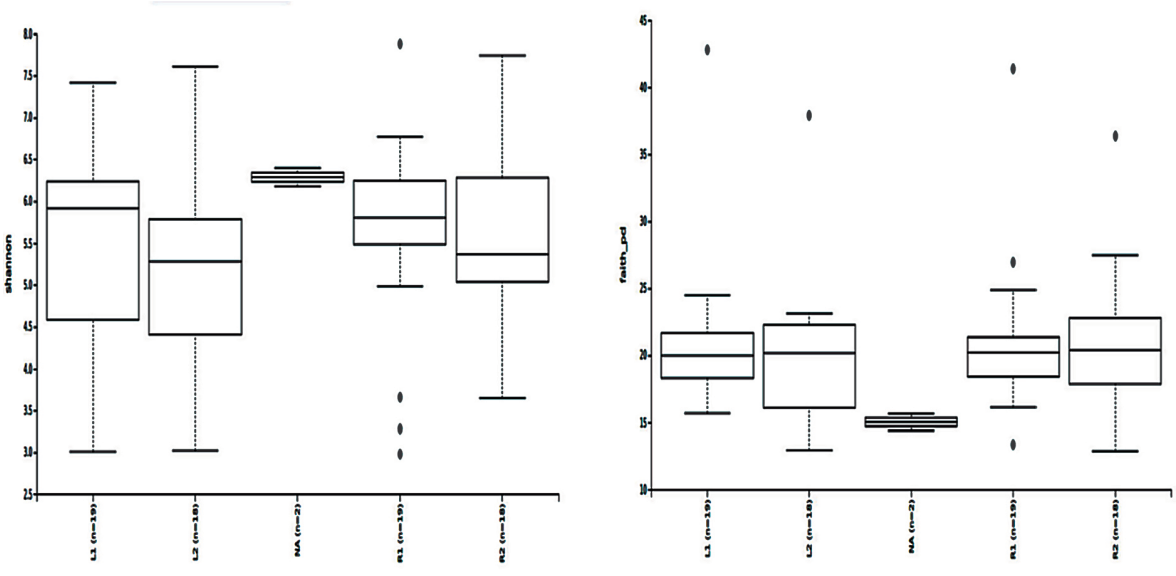

Figure 1-4: Alpha Shannon significance(left) and Alpha PD significance(right), both categorized by eye (L for left or R for right) and disease(1) or treatment(2) (E.g. L1 means left eye, disease)

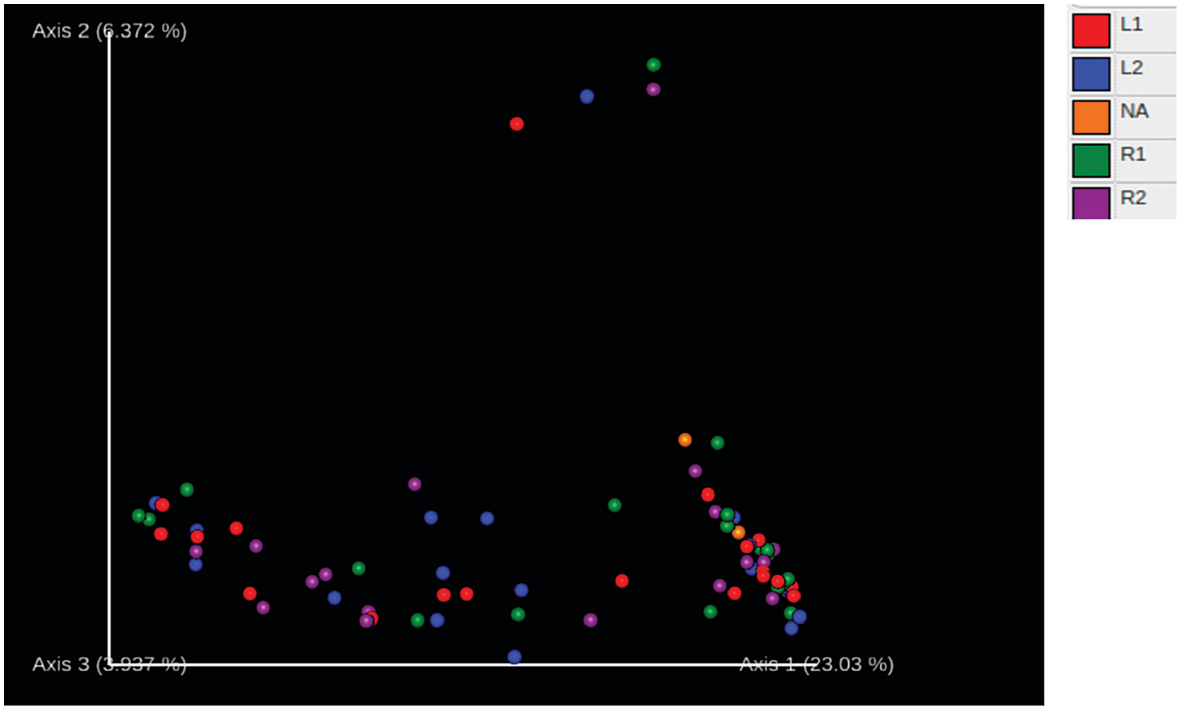

Figure 1-5: Beta diversity by employing Bray Curtis emperor indicator, categorized by eye (L for left or R for right) and and the state of treatment (disease(1) or treatment(2))

A quality control plot was illustrated in pre-processing of the second dataset sequences to examine the data quality

In the second data set analyzed, each patient is labeled with a number, and each phylum has a distinguished color. The plot indicates that the most widespread bacterial phylum is Proteobacteria. Next are Actinobacteria, Firmicutes and Bacteroidetes. There are many sequences unassigned and some represent Archaea in this sample. Many sequences belonging to the Bacteria kingdom, but having unidentified phylum, are visualized in light green and are fairly wide-speared in samples

On order level, Burkholderiales, Actinomycetales, Pseudomonadales and Clostridiales have the highest prevalence. Archaea at this level is not considerable, and many are still unassigned

Discussion

Studying microbiomes in dry eye patients is a subject that has attracted researchers' attention in recent years. But the uniqueness of this study comes from the samples. Samples used as material to compare disease and healthy (control) conditions are from the same person for each group. As mentioned before, each person's microbiome content can differ from another, as it is easily affected by many factors

Table 1-1: All groups and pairwise Kruskal-Wallis for Alpha Shannon plot

Group 1	Group 2	H	P value
All groups		4.99019678382561	0.2883048463369179
L1	L2	1.404432	0.235983
R1	R2	0.180979	0.670533

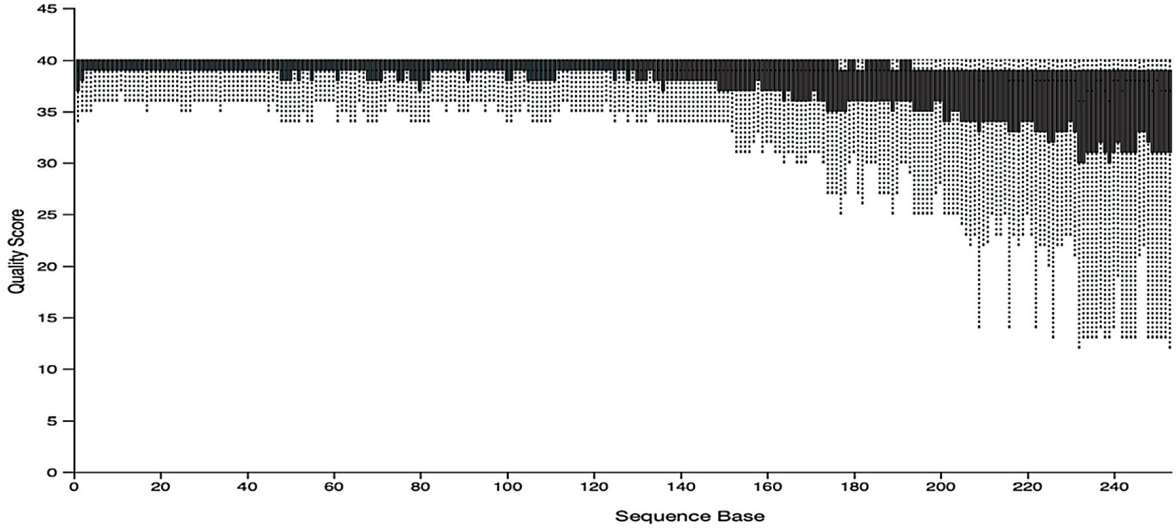

Figure 2-1: Quality plot for reads

such as age. In addition, both eyes were sampled for each person, which will power up the analysis, as many people have a different microbiome in each eye.

Microbiome analysis was performed using specialized tools and was carried out in several steps, from quality control and denoising to producing taxonomic bar plots and studying alpha and beta diversity. In both patients and treatment groups, the most abundant bacteria phyla are Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes which is in an agreement with the findings of Andersson et al. [15] we used RNA-sequencing (RNA-Seq. From the dataset featuring only DED patients, the four dominant phyla are the same, but in a different order, with Proteobacteria being the most prevalent.

According to the study of the patient dataset, the Burkholderiales, Actinomycetales,

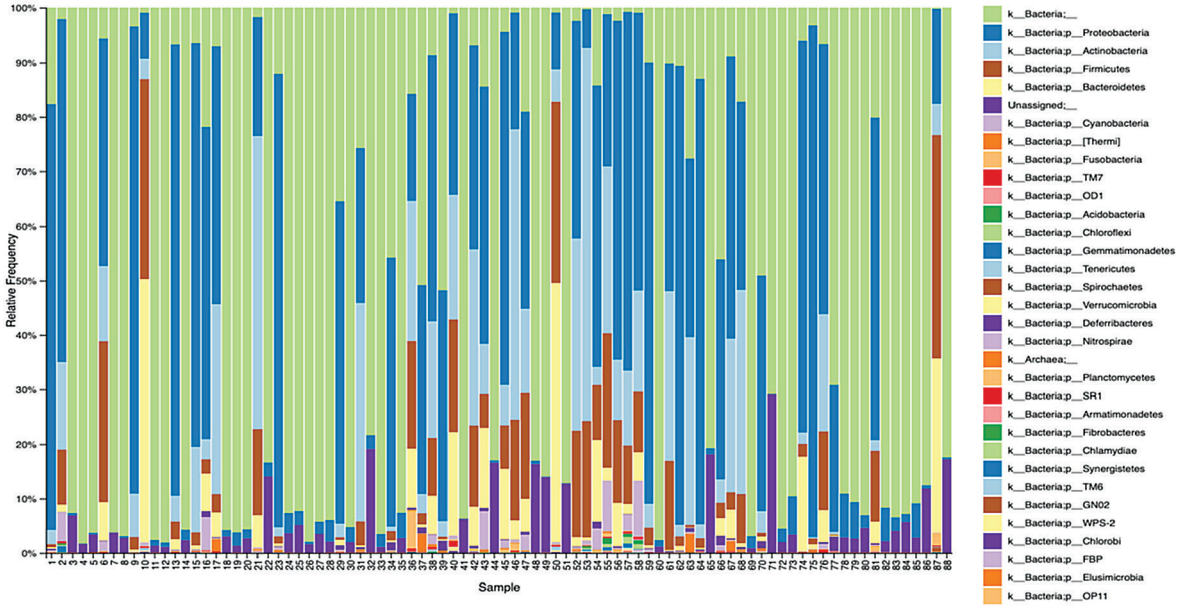

Figure 2-2: Taxonomic bar plots on phyla level, sorted by bee type

Figure 2-3: Taxa bar plots on order level, sorted by bee type

Pseudomonadales, and Clostridiales phyla dominate the ocular microbiome in the aforementioned illness. In a previous study by Ozkan et al., Pseudomonadales dominancy in fornix samples (only patient dataset) was discovered [16] clustered into operational taxonomic units (OTUs. But for patient/ treatment, it is not the case, as Clostridiales, Burkholderiales, Actinomycetales, Bacteroidales, Bacillales and Lactobacillales are the most prevalent. These findings are partially similar to what was observed by Zilliox et al. [3] observational study to characterize the ocular surface microbiome (OSM. This difference might emanate from several reasons, the most important one is the combination of healthy ocular microbiome and disease-associated ocular microbiome. That said, it can also happen because of sampling, differences between individuals and geographic location, similar to what was declared by Katzka et al. [8] but no study has directly compared different sampling methods applied to the same eyes to one another or to a reference standard of corneal epithelial biopsy. We addressed this lack by comparing the microbiome from three conjunctival swabs with those of corneal epithelial biopsy. METHODS: Twelve eyes (11 patients.

By a one-by-one analysis, for each eye of each person before and after treatment in patient/ treatment dataset, it is indicated that in 60 percent of the cases, Burkholderiales, and in the majority of cases Neisseriales, Streptophyta and Aeromonadales have increased after the treatment, while in 61 percent of patients Bacteroidales has declined. Also in most cases, Actinomycetales, Lactobacillales and Erysipelotrichales have decreased. Also, there seems to be a correlation in enhancement and reduction between Actinomycetales and Clostridiales, and between Clostridiales and Burkholderiales. Furthermore, Erysipelotrichales and Actinomycetales showed signs of an alliance as they often increase and decrease in accord with one another.

Alpha Shannon diversity indicator performed on patient/treatment dataset demonstrated a change in the richness and evenness of features

present in each eye, before and after treatment. For both left and right eye, it pointed out a decrease in diversity but a broader range, but considering the calculated P value, the changes seem statistically insignificant. Diversity based on the phylogenic distance, illustrated by Alpha PD plot, indicates that there is no considerable difference in the phylogeny of features before and after treatment. In addition, the beta diversity plot doesn't show a remarkable clustering for each category.

Archaea phyla, such as Euryarchaeota and Crenarchaeota, are more witnessed inpatien/ treatment samples than only patients group. It is hypothesized that this phenomenon is due to the sampling method. As was observed by Matysiak et al. [15] we used RNA-sequencing (RNA-Seq, there is a higher probability to encounter archaeal sequences when sampling from the corneal tissue, which tears are closely linked to this part of the eye. But samples gathered from the inferior fornix of the conjunctival sac, are more likely to be more sterile and fewer archaeal elements can be found there.

Conclusion

This study focused on a prevalent ocular condition affecting millions of people worldwide, dry eye disease (DED), and how ocular microbiome alteration affects this disease. Although many pieces of evidence suggest a relationship between the eye's medical conditions and the microbiome in this organ, determining valid proof and details for it needs more solid and variable studies revolving around these subjects. Results from this study showed multiple bacterial orders increased or decreased after treatment, indicating a possible connection between them and DED.

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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.