Original Article

Utilizing Microbiome Approaches for Antibiotic Resistance Analysis; an Ocular Case Evaluation

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Abstract:

Background: Metaorganism or microbial communities of eukaryotic organisms provide an inclusive set of functions related to immunity, host metabolism, and stress tolerance. Ocular microbiota refers to pathogenic and commensal microorganisms in or on the eye. On the one hand, antibiotic treatment can give rise to pathogen overgrowth due to an imbalance of microbiota and cause various ophthalmic diseases. On the other, antibiotic therapy is considered the leading cause of antibiotic resistance. The present study aimed to describe the bacterial community changes following antibiotic treatment in the ocular surface microbiome.
Material and Methods: In this scenario, we evaluated the composition of thirteen canine ocular microbiomes during treatment with a typical mixture of antibiotics, neomycin-polymyxin-bacitracin. Microbiome taxonomy and downstream bacterial richness and evenness were analyzed using microbiome bioinformatics platforms.
Results: Accordingly, bacterial taxonomy at the level of phyla and genus was mapped, and alter of antibiotic resistance genes werereported. An increase in the Staphylococcus genus traced during the time and one month following antibiotic treatment. Bacterial network, alpha, and beta diversity indicated a significant microbiota change at the genus level.
Conclusion: This study highlights the effect of commonly used ocular antibiotics on commensal microbiota and the emergence of the antibiotic-resistant genus.
Keywords: Microbiota; Antibiotic Resistance; Ocular Microbiome; Ophthalmic Diseases.

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Introduction

Microbiomes are the genetic material of bacteria, fungi, viruses, protozoa, and eukaryotes in specific tissues. The microbiota, on the other hand, refers to a community of microorganisms colonizing a particular tissue (Aragona et al., n.d.). is a community of microorganisms colonizing is a community of microorganisms inhabiting Ocular surface microbiota consists of all resident organisms in the cornea, conjunctiva, and eye tear film [1]. Overall, animal body organs were colonized by different bacterial phyla dominated by Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. The microbial communities are essential for regulating immune response and reciprocally pathogen n resistance [2]. Antibiotics will eradicate indigenous commensal bacteria sensitive to the antibiotic, so they vastly alter the microbiome. Moreover, the effect on the structure and function of the eyes, this issue is vital regarding antimicrobial resistance. It rises from the Antibiotic Resistance Genes (ARGs) of commensal bacteria or acquired antibiotic resistance via horizontal gene transfer [3]. Ocular surfaces are susceptible to severe diseases such as infectious ulcerative keratitis due to their exposure to the environment. A typically utilized antibiotic is neomycin-polymyxin-bacitracin [4]. Neomycin binds to the 30S ribosomal subunit and interrupts translation mechanisms in Gram-negative and positive bacteria. Polymyxins affect lipopolysaccharide (LPS) stabilization as a Gram-positive bacteria outer membrane substance. Bacitracin obtained from Bacillus subtilis can inhibit peptidoglycan synthesis at Gram-positive cell walls. So, this mixture of antibiotics will interfere with both Gram types of bacteria at two-level, and affection for commensal microbiota should be investigated [5].

Shotgun metagenomic sequencing identifies the total genomic DNA of all organisms in a sample, avoiding the need to isolate and cultivate microorganisms. Most microorganisms cannot be cultivated in the laboratory, which is crucial. Next-generation sequencing (NGS) technology provides information not only on the taxonomic annotations of each organism but also on the functional profiling, gene prediction, and microbial interaction of the entire community, unlike the targeted approach used in 16S/18S/ITS amplicon sequencing. Shotgun metagenomic sequencing combines shotgun cloning and shotgun metagenomic sequencing to sequence DNA in a way that evenly fragments (fragments) DNA into many tiny pieces. The DNA sequences can be identified by sequencing fragmented DNA, then stitching them back together with bioinformatics tools. By examining every part of genomic DNA in a sample, shotgun metagenomic sequencing explores every region of DNA instead of focusing on a single area, such as 16S rRNA. Microbiome research can benefit significantly from shotgun sequencing, which can simultaneously identify and profile bacteria, fungi, viruses, and other microorganisms. Furthermore, identifying and profiling microbial genes can provide additional information about the functional potential of microbiomes over and above what can be determined by sequencing a genome.

According to the 16S ribosomal RNA (rRNA) sequencing approach, the microbiota profiling has become the most popular for determining the taxonomic composition in various environments. 16s rRNA details are shown in Figure 1. Bacterial 16S rRNA has nine hypervariable parts used for evolutionary relationship studies, flanked by highly conserved regions typically utilized for polymerase chain reaction (PCR) primers

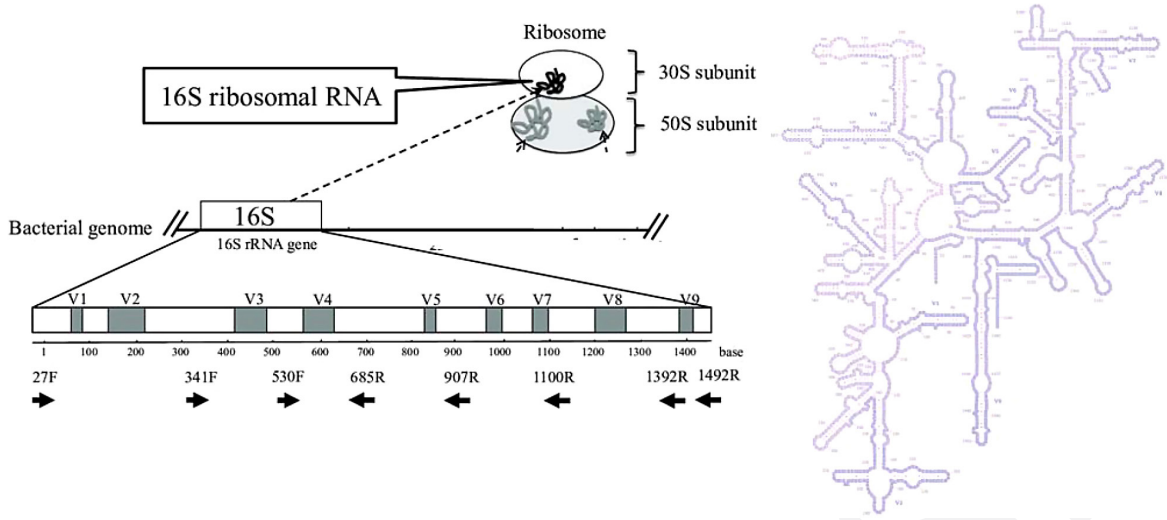

Figure 1: 16SrRNA gene

design [6]. The 16S rRNA-related profiling includes sample collections, DNA isolation, 16S primer design, PCRs, libraries indexing, DNA sequencing, pipeline construction and choosing software, OTU (Operational Taxonomic Unit) or ASV (Amplicon Sequence Variant) selection, database picking, downstream diversity investigation, and statistical study. Some bioinformatics pipelines have been developed, such as QIIME and Mothur [7], using several databases, including SILVA, Greengenes [8], Ribosomal Database Project [9], etc.

In genetics, richness is defined as many genetically related or functionally related individuals. It is usually called species richness in most vegetation surveys when richness is expressed as the number of species. The proportion of species or functional groups present on a site is called evenness. Shannon's Entropy measures how much information is present in a variable. This corresponds to the amount of storage (e.g. number of bits) required to store the variable, which intuitively corresponds to its degree of information. When calculating distance, gaps are either ignored or counted as mismatches at aligned positions. In distance methods, an all-to-all matrix describing the distance between each pair of sequences is constructed from the sequence query set.

So, at present, performing microbiome studies has been facilitated by the advent of Next Generation Sequencing (NGS), eliminating traditional laboratory culture limitations [10]. Recently the importance of microbiome research is more apparent regarding essential roles in regulating the host immune system and physiology; this has led to the launch of the Human Microbiome Project or chartering of federally supported microbiome research in 2015 by the National Science and Technology Council Committee of US government [11]. On the other hand, the US national intelligence council introduced the antibiotic resistance issue as one of the most global challenge trends up to 2040 [12].

Comprehending the composition of the ocular surface microbiota and related commensal, opportunistic, or pathogenic microorganisms will better understand the pathogenic mechanisms underlying some visual diseases.

In the present study, we sought to investigate ocular bacterial microbiota Unbalanceness following a routine antibiotic mixture administration.

Material and Methods

Data files

The main input files of the BioProject were downloaded from EBI, consisting of reading counts features tables and related describing metadata files. The 16S rRNA gene sequences retrieved in the present study are available under EBI project number PRJNA488106 and Sequence Read Archive (SRA) OF SRA771229. (https://www.ebi.ac.uk/ena/browser/view/PRJNA488106)

Study design

Thirteen dogs, healthy in terms of ocular disease, were recruited following appropriate animal care, aged 1–12 years in different breeds. This sample size would be adequate for experimental robustness and applying gaussian population issues. Animals remained healthy, under control, and housed in separateindoor stalls during three steps: before antibiotic treatment, a week of neomycin-polymyxin-bacitracin antibiotics administration, and four weeks following treatment.

Sequence data processing

The metagenome data were retrieved from the National Center for Biotechnology Information (NCBI) BioProject section with a Sequence Read Archive (SRA) of SRP161472. Ocular surface microbiome data was obtained via ILLUMINA (Illumina MiSeq) technique. The Quantitative Insights into Microbial Ecology package 2 (QIIME 2, 2022.2) software was used to process and analyze sequences [13]. Demultiplexed single-end sequencing reads were checked for quality control, minimum acceptable read depth, and error correction. Besides low-quality sequence filtering, the chimeric sequences were detected using the DADA2 plugin and removed before analysis [14].

16S rRNA gene-based microbiome analysis

Taxonomically annotated 16S rRNA sequences were utilized to map bacterial community composition (BCC). Related Operational taxonomic units (OTUs) were investigated and clustered using the QIIME2 open-reference OTU picking protocol versus a compartment with the Silva database (silva-138-99) [15]. Existence phyla were visualized and drawn by q2view tools of QIIME web server, at the first time-step or before antibiotic treatment. The ocular microbiome community was monitored in the next two steps, immediately following one week of antibiotic therapy and four weeks after that.

Downstream analysis

As a downstream calculation; the total bacteria genus and the cumulative number of bacteria in this taxonomy level were counted at three-time points at the ocular surface. Prevalence diagrams were plotted using the GraphPad PRISM software (version 9.0). Alpha diversity as an index of both species' evenness and richness was estimated using the OTUs. Phylogenic Distribution (PD) and Shannon entropy metrics were calculated over time in this term. Beta diversity was calculated using QIIME2 Bray-Curtis metrics and visualized with Principle Coordinate Analysis (PCoA) plots. Precise Bacterial community composition differences were indicated with Beta bray distance analysis over time steps.

Statistical investigation

the effect of antibiotics on microbiome community structuring was assessed.

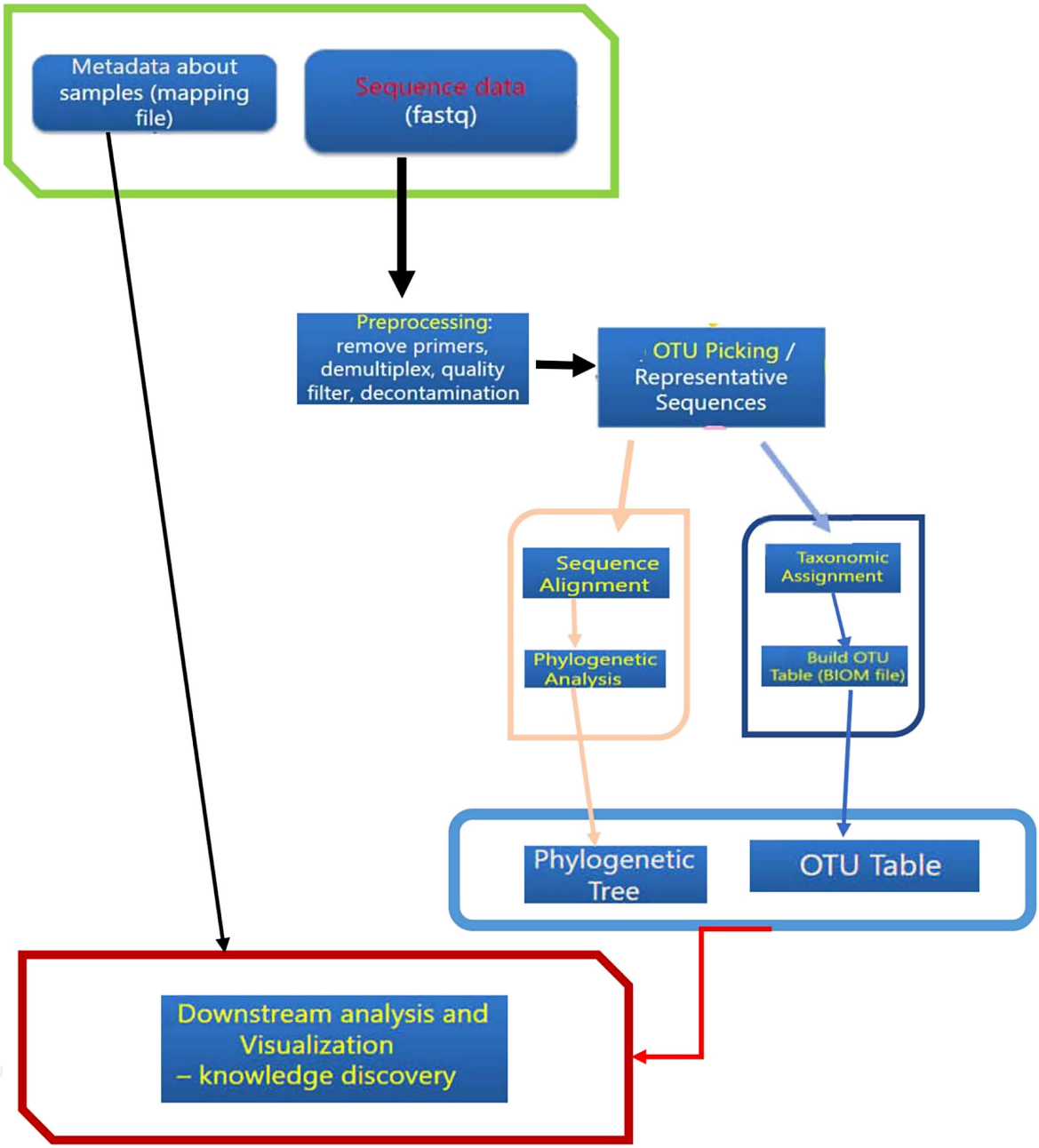

Figure 2: Flowchart

Differences in the assemblage of bacterial Amplicon Sequence Variants (ASVs) were analyzed using statistics of two-way permutational multivariate analysis of variance (PERMANOVA). A Kruskal- Wallis test was applied to investigate the prevalence and the cumulative number of bacteria because of the assumed non-Gaussian distribution. A comparison among all-time steps was performed.

Figure 2 shows the flowchart of the above steps.

Results

Sequence Retrieve

Briefly, Sequence samples had an acceptable quality. They had a minimum depth of 17170

Table 1: Sequence SRA features in different time points

Dataset	Number of samples	Number of features	Total frequency	Frequency per sample			Frequency per feature		
				Minimum	Maximum	Mean	Minimum	Maximum	Mean
Time Point 1	26	1981	1371655	17170	87120	52755.9	2	200103	692.4
Time Point 2	26	2376	1620150	26453	107190	62313.4	2	315417	681.8
Time Point 3	26	1649	1636012	28330	127303	62923.5	2	358615	992.1

(at the first-time step), and approximately 95 % of samples had a sufficient depth of >30000. The dataset comprises 4,627,817 reads obtained from 78 samples (26 samples for each time point), and a total of 6006 recognized OUT (Table 1). DADA2 pipeline was utilized to filter, denoise, and remove chimera samples from obtained samples and generate related ASVs.

Composition of microbiome

In the QIIME procedure, each time step, samples were filtered based on minimum sequencing depth, 17170. All of the existing taxa of these ASVs were mapped by comparing them with the Silva rRNA database (SSU 138). The samples were associated with 25 phyla and 78 classes (Figure 3). At the baseline, the most abundant phyla across all models were the Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidota in order of prevalence.

As shown in Figure 4a, these phyla prevalence percentages altered following antibiotic treatment. Approximately 73 % of the OTUs belonged to a genus, and 20 % were annotated at the species level. The remaining 7 % could not be recognized based on the type of mapping performed. There are three main phyla that make up the majority of our population: Proteobacteria, Actinobacteria, and Firmicutes. Bacterial taxa comprised 163 new bacterial genera following antibiotic administration (44 genera) and 119 new genera one month after antibiotic treatment compared with baseline microbiota. We found 20 % (44 genera) of core microbiome change, retrieved again after one month (Figure 4b).

Microbiota diversity analysis

Bacterial evenness and richness were estimated in every animal's eye at three-time points. It is shown in figure 5 on behalf of the total counted bacterial genus and the presence of different genera.

Also, in the case of bacterial evenness, alpha diversity was estimated by Shannon entropy, phylogenetic distribution, and Pielou's evenness indices (Figure 6a). The variety of the given sample was calculated with these indices at different time points (Figure 6b).

Kruskal determined the Phylogenetic diversity P-values–Wallis statistics and related q-values were considered at a cutoff of <0.1. Accordingly, bacterial abundance was significantly different between baseline flora and 28 days after antibiotic treatment (P-value = 0.029, Q-value = 0.087). Microbial community structure was assessed during the time as beta diversity analysis. The PCoA plot of Jaccard analysis as an index of sample similarity over time indicates in Figure 7.

Based on the Jaccard method, there were significant changes in the microbial community at each time step (verdict package, R = 0.008, Sig = 0.049). Also, the Beta bray distance method indicated significant

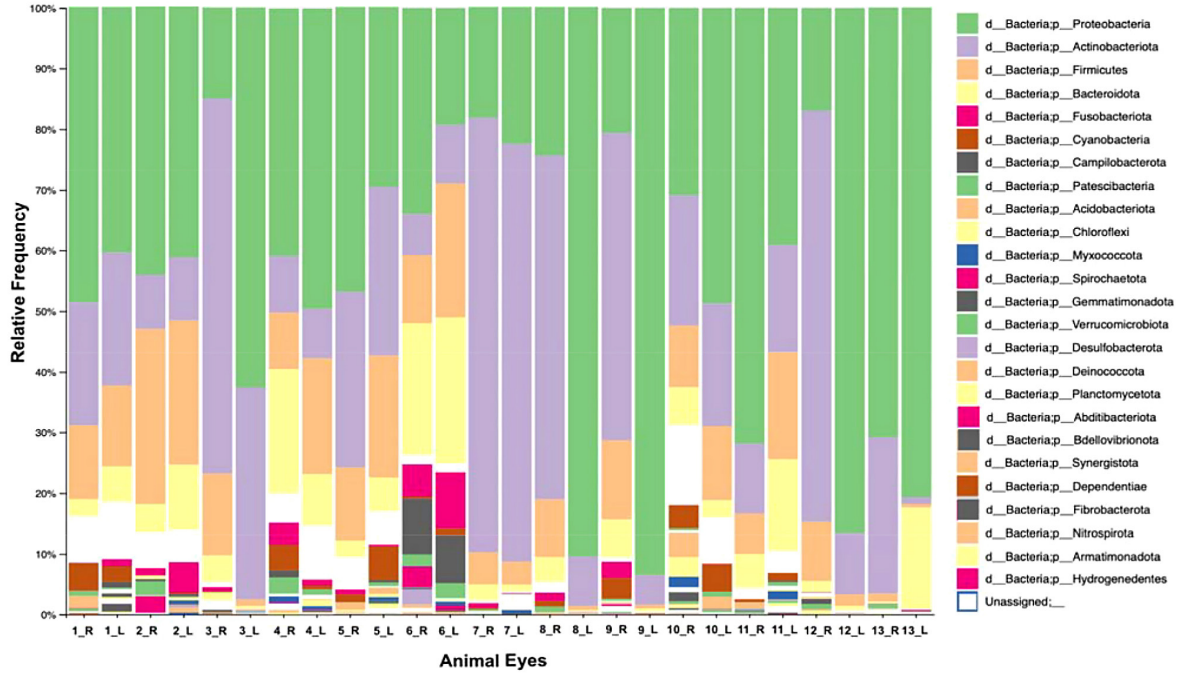

Figure 3: predicted bacterial phyla based on the filtered operational taxonomic unit (OTU). Twenty-five phyla were mapped by comparing with the silva (138.1) database. *R(right eye), L(left eye), 1-R means Sample 1 right eye

differences among first and second-time points and between 1st and 3rd-time steps (PERMANOVA, $p < .0001$). The SpiecEasi package drew the microbiome network clusters. The modules and isolate nodes are annotated over time (Figure 8).

Discussion

The intensity of ocular infections varies from mild inflammation by self-limiting bacteria to severe, sight-threatening conditions [16]. In the current decade, there is an estimated more than forty million blind people with approximately

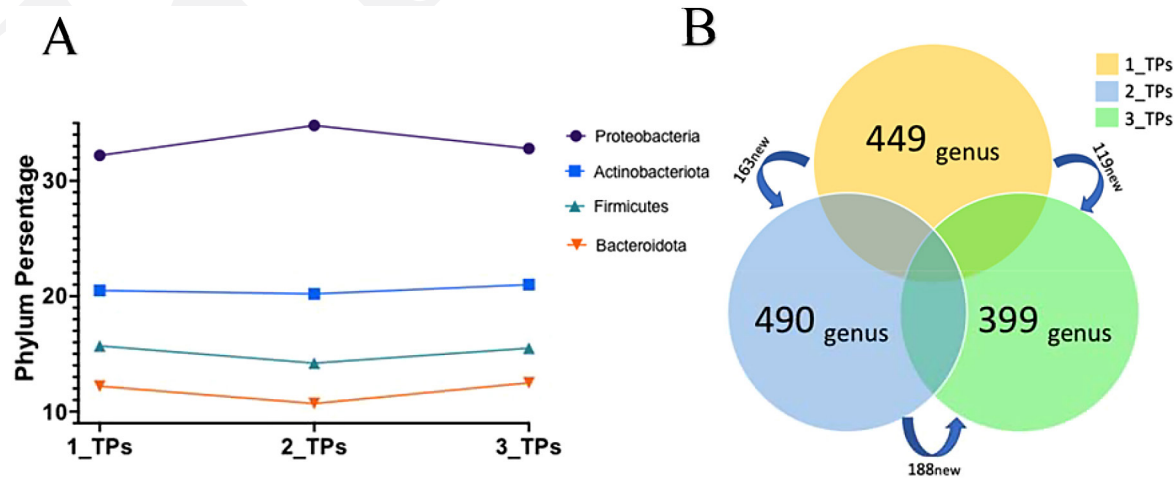

Figure 4: Phyla prevalence changes during the time and following antibiotic treatment (A). Bacterial taxa comprised 163 new bacterial genera following antibiotic administration (44 genera) and 119 new genera one month after antibiotic treatment compared with baseline microbiota (B)

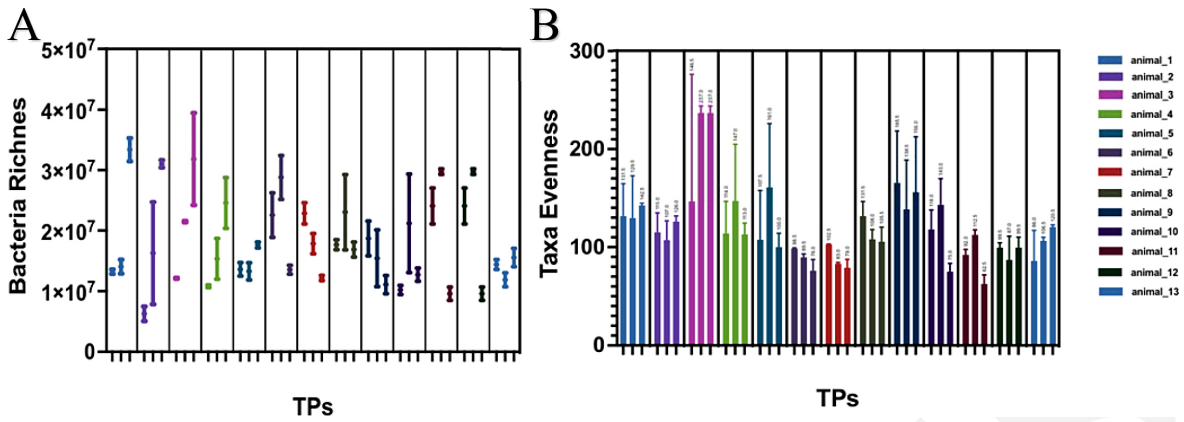

Figure 5: Total estimated bacterial count at three-time points in the animal's eyes (A). Bacterial genera present at different time steps at animal's ocular (B)

1.1 billion vision problems [17]. On the other hand, Anti-Microbial Resistance (AMR) is a significant cause of death globally. Up to 2020, about five million people have been confronted with drug-resistant bacterial infections (1·27 million deaths annually), which will cause 10 million deaths annually up to 2050 [18]. Antimicrobial resistance occurrence can alter treatment conditions in ophthalmic infections. This study also reports microbiome exchange, knowing that some rehabilitated bacteria, even the Escherichia genus, can be fatal. Escherichia coli and methicillin-resistant Staphylococcus aureus are more accountable bacterial species regarding flora exchange and causing death [19]. Here, the ocular bacterial communities of thirteen dogs were investigated because of the challenges of human eye sampling due to low

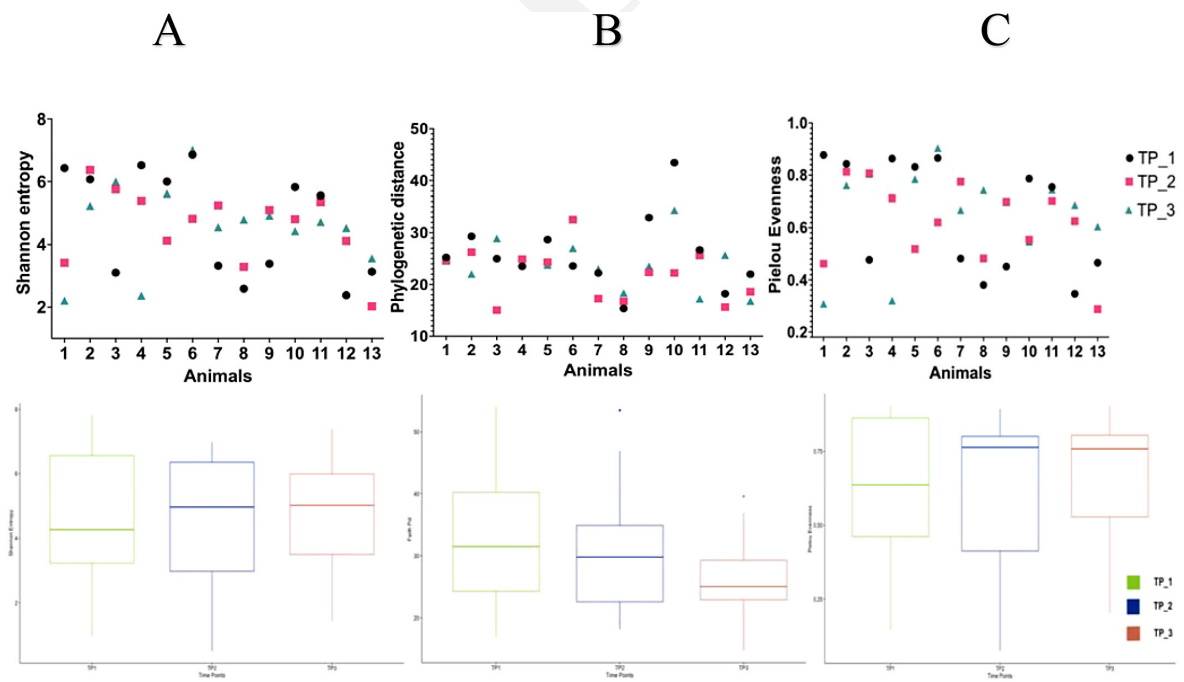

Figure 6: Shannon entropy (A), Phylogenetic distance (B), and Pielou's evenness indices of ocular microbiota at various time steps, before antibiotic treatment (1st), immediately following antibiotic treatment (2nd), and four weeks after antibiotic treatment (3rd)

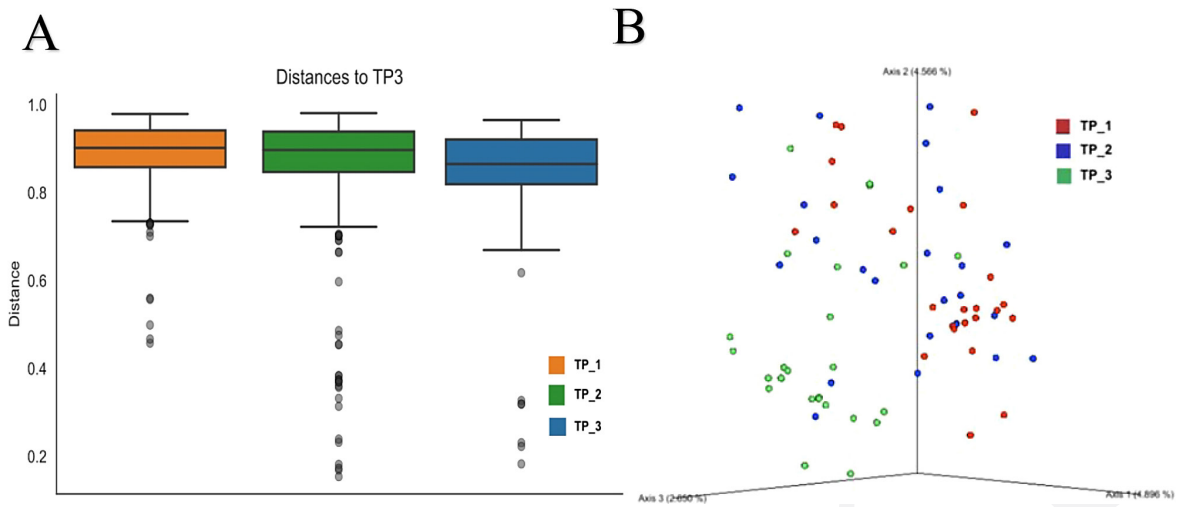

Figure 7: Beta bray distance analysis of beta diversity in different time steps (A). It indicates significant differences between time points 1-2 and 1-3 (PERMANOVA, $p < .0001$). PCoA plot of Jaccard analysis as an index of sample similarity over time (B)

microbial biomass [20]. Following the mapping of obtained SRAs with the Silva database, the samples belonged to 25 phyla. However, mitochondrial contamination can map with cyanobacterium phylum, as mentioned earlier. There was no significant difference in phylum-level during the experiment time, while colonization of new bacterial genus was traced. Results indicated that there were 119 novel genera compared to normal flora at the third time point. Knowing that Escherichia, Staphylococcus, Klebsiella, Streptococcus, Acinetobacter, and Pseudomonas are the main genus regarding antimicrobial resistance is crucial. In this context, a significant increase was observed in the Staphylococcus genus (70 %) and an increase in the Acinetobacter population. After the Escherichia genus, Staphylococcus leads the most antibiotic resistance globally.

Over three distinct time points: day 0, day 7, and day 35, the relative abundances of bacterial taxa, alpha diversity metric, and beta diversity metric did not differ in control eyes. Canine ocular surface microbiomes have not changed significantly over time in terms of species diversity, community structure, or community composition.

Up to date, various studies have illustrated the effects of antibiotics in microbial-resistance models, and some specifically covered their affection on ocular diseases [19]. Ozkan et al. assessed the effect of a tobramycin antibiotic treatment on the eye microbiota. Their results implied a decrease in the commensal flora, specifically Gram-positive bacteria. Identical results were observed following moxifloxacin treatment, i.e., a reduction of Gram-positive bacterial [21]. In this term, Sarita et al. evaluated azithromycin, gatifloxacin, moxifloxacin, and ofloxacin effect on the eye microbiota, especially Staphylococcus epidermidis and Staphylococcus aureus. They investigated 24 patients who objected to these ocular antibiotic treatments and reported reducing Gram-negative species [22]. Toillustrate, Kaspar et al. demonstrated that endophthalmitis inflammation caused by AMR Staphylococcus epidermidis causes more severe inflammation with eye tissue distraction than antibiotic-sensitive strains [23]. Several studies have proposed utilizing

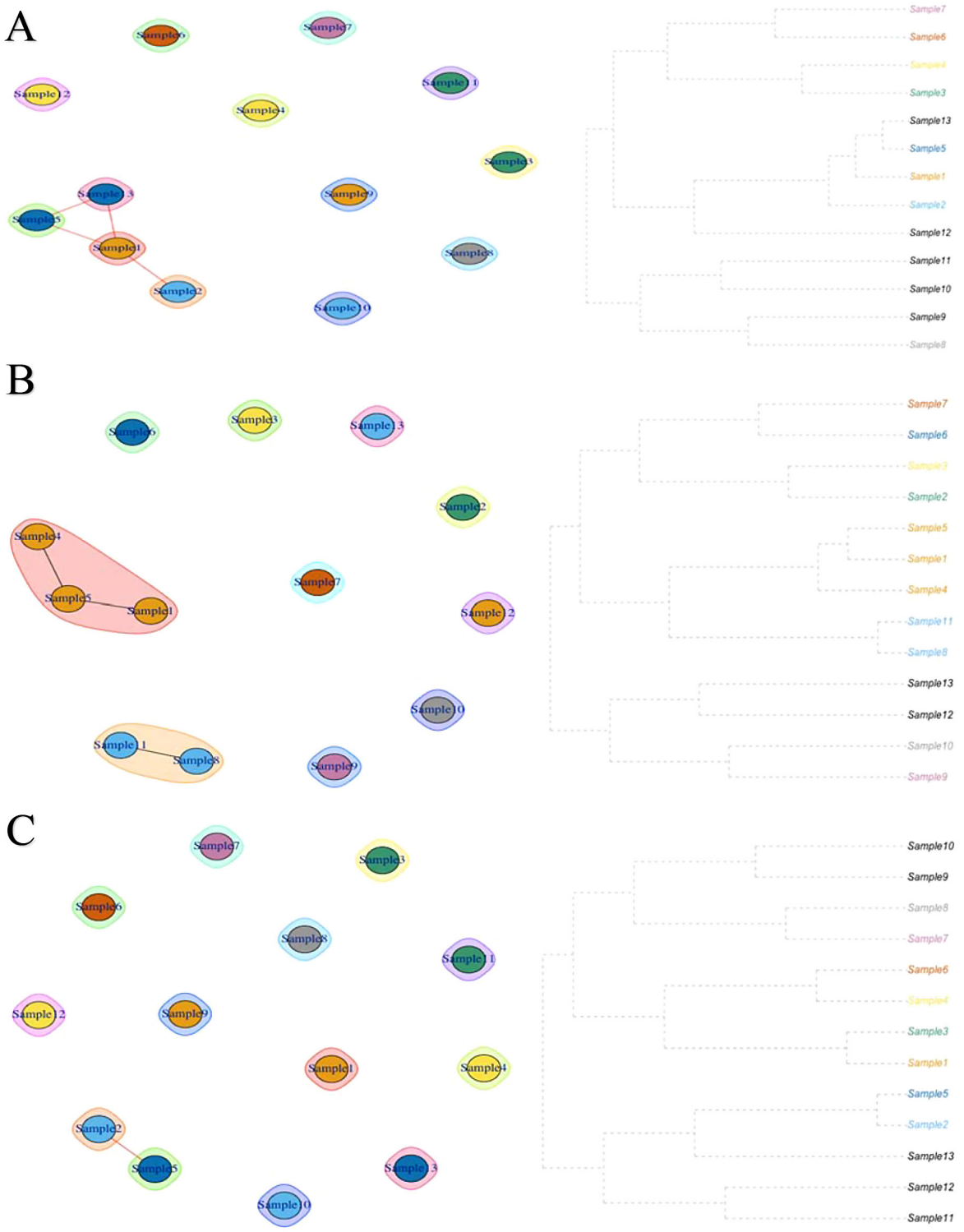

Figure 8: Microbiome network clusters during three-time points

probiotics, Prebiotics, or bacteriophage therapy instead of antibiotic treatment against pathogens in recent years. Probiotics that almost belong to the Bifidobacterium and Lactobacillus are live bacteria with many advantages to host health [24,25]. Also, Prebiotics are dietary carbohydrates the body can not digest, such as galactooligosaccharide

and fructooligosaccharide. Bacteriophages utilized is a novel strategy against pathogenic bacteria, which can be used as an alternative to antibiotics [26]. The main finding of this study is an increase of Staphylococcus spp. as a primary antibiotic resistance candidate following antibiotic administration.

Conclusion

Nowadays, next-generation sequencing techniques allowed the surface and intraocular microbiota to be a former black box. The ocular microbiome is essential, which influences ophthalmic homeostasis and ocular health. The present study has highlighted how antibiotic treatment can alter the ocular microbiota and leads to later ophthalmic pathogenicity.

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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.