

RESEARCH ARTICLE

Evaluation of the bacterial ocular surface microbiome in clinically normal cats before and after treatment with topical erythromycin

Joshua E. Darden¹, Erin M. Scott^{1*}, Carolyn Arnold², Elizabeth M. Scallan¹, Bradley T. Simon¹, Jan S. Suchodolski¹

1 Department of Small Animal Clinical Sciences, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, Texas, United States of America, **2** Department of Large Animal Clinical Sciences, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, Texas, United States of America

* escott@cvm.tamu.edu



OPEN ACCESS

Citation: Darden JE, Scott EM, Arnold C, Scallan EM, Simon BT, Suchodolski JS (2019) Evaluation of the bacterial ocular surface microbiome in clinically normal cats before and after treatment with topical erythromycin. *PLoS ONE* 14(10): e0223859. <https://doi.org/10.1371/journal.pone.0223859>

Editor: Brian B. Oakley, Western University of Health Sciences, United States of America

Received: April 25, 2019

Accepted: September 30, 2019

Published: October 11, 2019

Copyright: © 2019 Darden et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: Data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP161480.

Funding: The authors received no external funding for this work. Internal funding from Texas A&M in the form of start-up for Dr. Erin Scott and Ginn funds for resident research (JED) were received.

Competing interests: The authors have declared that no competing interests exist.

Abstract

The ocular surface microbiome of veterinary species has not been thoroughly characterized using next generation sequencing. Furthermore, alterations in the feline ocular surface microbiome over time or following topical antibiotic treatment are unknown. Aims of this study were to further characterize the ocular surface microbiome of healthy cats and to identify whether there are microbial community changes over time and following topical antibiotic use. Twenty-four eyes from twelve adult, research-bred, female spayed domestic short-haired cats were evaluated. Erythromycin ophthalmic ointment (0.5%) was applied to the ocular surface of one randomly assigned eye per cat three times daily for 7 days, while the fellow eye served as an untreated control. The ocular surface was sampled by swabbing the inferior conjunctival fornix of both eyes prior to initiating treatment (day 0), after 1 week of treatment (day 7), and 4 weeks after concluding treatment (day 35). Genomic DNA was extracted from the swabs and sequenced using primers that target the V4 region of bacterial 16S rRNA genes. At baseline, the most common bacterial phyla identified were Proteobacteria (42.4%), Firmicutes (30.0%), Actinobacteria (15.6%), and Bacteroidetes (8.1%). The most abundant bacterial families sequenced were Corynebacteriaceae (7.8%), Helicobacteraceae (7.5%), Moraxellaceae (6.1%), and Comamonadaceae (5.6%). Alpha and beta diversity measurements were largely unchanged in both treatment and control eyes over time. However, univariate and linear discriminant analyses revealed significant and similar changes in the abundance of some bacterial taxa over time in both treatment and control eyes. Overall, the feline ocular surface microbiome remained stable over time and following topical antibiotic therapy.

Introduction

The ocular surface consists of the corneal epithelium along with the most prominently exposed mucous membrane of the body, the conjunctiva. Furthermore, it represents a predominately open system that is subject to a barrage of pathogenic and non-pathogenic organisms as it is constantly in contact with its environment [1–3]. This open exposure relies on adaptive and innate immunologic constructs to prevent pathogenic colonization of the ocular surface [4, 5]. However, there likely is a component of harmonious interaction between microbiota acting in a commensal and symbiotic nature against overgrowth or colonization of pathogenic microorganisms [2, 6–17]. This mucosal tolerance allows normal ocular surface microbiota to occupy its environment yet remain non-stimulatory [5].

Infectious conjunctivitis in cats is associated with viral or bacterial etiologies and is a frequent cause of ocular problems in veterinary medicine, where both pathogenic and opportunistic bacterial microorganisms contribute to the disease process [1, 18–21]. Standard of care for the treatment of feline conjunctivitis and keratitis consists of topical ophthalmic antibiotics, such as erythromycin [1–3, 22]. Antibiotics may negatively alter the microbial community of the ocular surface [11], potentially contributing to opportunistic invasion of pathogenic species and ocular disease [12, 13].

The microorganisms inhabiting the ocular surface of veterinary species have been evaluated traditionally using standard culture-based techniques from corneal and conjunctival swabs [1–3, 18–19, 21–35]. Additionally, biochemical tests and mass spectrometry have been utilized to identify microbes that were cultivated through the aforementioned techniques [1–3, 19, 23–27]. The percent of total positive cultures from healthy feline eyes is low, with gram positive bacteria such as *Staphylococcus*, *Streptococcus*, and *Corynebacterium* spp. representing the most commonly cultivated microorganisms [1–3, 18–22]. Due to the inability of several studies to culture bacteria from the eyes of healthy cats [2, 3, 21], the ocular surface was speculated to be sterile [2, 19, 22]. It is now known that limitations exist for standard culture-based techniques as many bacteria are not easily cultivable [36]. With this shortcoming of accurately depicting the complete bacterial composition of an environment, terminology describing the once mysterious population of microbiota has been erected giving rise to the phrase the “uncultured microbial majority” [36]. To identify and characterize this enigmatic population of microorganisms, molecular-based methods that target specific DNA markers allow microbes to be defined by their genomes, giving rise to the microbiome [6–10, 13, 37–41].

The arrival of molecular-based methods, such as 16S rRNA gene sequencing, has allowed in-depth and detailed species identification of the bacterial microbiota residing on the ocular surface in humans [6, 8, 12, 13, 37–40, 42, 43], while limited preliminary studies have been published in veterinary species such as cats [41], horses [44], and dogs [45]. Presently, there are no published studies evaluating the ocular surface microbiome of cats using molecular-based techniques that evaluate temporal stability and the impact of antibiotic usage. Knowledge of these microbial populations, how to sample them, and how they change over time and with treatment may one day lead to an improved understanding of ocular diseases in both veterinary and physician ophthalmology. This study was designed to examine the ocular surface microbiome of healthy cats using next-generation sequencing. The aims of the study were, 1) to further describe the resident ocular microbiota in healthy research-bred cats at baseline, 2) to assess the temporal stability the ocular microbiota at three distinct time points, and 3) to evaluate the influence of antimicrobial therapy before, immediately after 1 week of therapy, and after 1-month hiatus of antimicrobial use.

Materials and methods

Participants

The study was approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocol #2017–0313) and performed in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research (<https://www.arvo.org/About/policies/statement-for-the-use-of-animals-in-ophthalmic-and-vision-research/>). Twelve healthy research-bred (Liberty Research Inc., Waverly, NY) adult domestic shorthair cats were included in the study. All twelve cats were spayed females with ages ranging from 1 to 1.5 years old. Cats were housed together in groups of 2 to 4 in adjacent free-ranging enclosures of 2.4 m L x 2.4 m W x 3.0 m H. The facility provided controlled light cycle (12/12-hour light/dark), temperature (21–22° C) and humidity (55–60%) conditions. Environmental enrichment consisted of toys, scratch posts, raised condos, and bedding. One wall of each enclosure consisted of large windows that provided natural light. Cats were well accustomed to handling and acclimated to ophthalmic examinations for two weeks before the study was initiated. Water and food were available at all times.

Sample collection

All cats had a complete ophthalmic examination performed by a board-certified veterinary ophthalmologist (EMS) and ophthalmology resident in training (JED) to ensure they were free of ocular disease. As described in detail previously [44], this included evaluation of the anterior segment of the eye by slit-lamp biomicroscopy (SL-17, Kowa Optimed Inc., Torrance, CA), and the posterior segment of the eye by indirect ophthalmoscopy (Vantage Plus Wireless Headset, Keeler Instruments Inc., Malvern, PA). A routine minimal ophthalmic database that included fluorescein staining (Amcon Laboratories Inc., St. Louis, MO) and tonometry (Tono-Vet, Icare VET, Jorgensen Laboratories Inc., Loveland, CO) was performed.

Baseline conjunctival samples were collected before fluorescein staining in order to prevent contamination or dilution of the sample. Sample collection was performed in awake cats using gentle manual restraint following the application of one drop 0.5% proparacaine (Bausch & Lomb Inc., Bridgewater, NJ) to the ocular surface of each eye to provide topical analgesia. As described previously [44], swabs of the inferior conjunctival fornix were collected from both eyes of every cat at three separate time points. Two Isohelix buccal swabs (Boca Scientific Inc., Westwood, MA) were used per eye and each side of the swab was rubbed in the inferior conjunctival fornix 10 times. Swabs were immediately transferred into DNeasy PowerBead tubes with 750- μ l buffer containing guanidine thiocyanate (QIAGEN Inc., Germantown, MD). To control for environmental contamination, an unused swab containing one drop of 0.5% proparacaine was collected at the same time and place as the conjunctival swabs. All samples were immediately stored for no longer than 24 hours at 4° C until the extractions were performed.

Once baseline samples (day 0) were collected, one eye of each cat was randomly selected for treatment with a topical broad-spectrum antibiotic ointment, 0.5% erythromycin (Bausch & Lomb Inc., Bridgewater, NJ), commonly used for the treatment of feline conjunctivitis and ulcerative keratitis. Utilizing online software (<https://www.randomizer.org>), randomization of eyes into treatment and control groups for each cat was established. One quarter-inch strip of erythromycin ophthalmic ointment was applied directly to the ocular surface of the randomly selected eye of each cat three times daily for 7 days, while the fellow eye served as an untreated control. Care was taken to avoid contact of the ointment with the outer surface of the eyelids or periocular skin, and handlers wore nitrile gloves while administering

the ophthalmic medication. Repeat inferior conjunctival fornix swabs were collected on day 7 (after completion of antibiotic therapy) and day 35 (one month after antibiotic therapy ended).

DNA extraction and sequencing

Genomic DNA was extracted from the conjunctival swabs and negative controls using a single 100 tube DNeasy Powersoil DNA isolation kit (Cat ID 12888–100, Lot number 157049640, QIAGEN, Inc., Germantown, MD) following the manufacturer's instructions. Negative controls consisting of one unused swab and one drop 0.5% proparacaine were collected at each timepoint. All three negative controls did not show amplification on PCR and, therefore, were not sequenced with the conjunctival swabs. Sequencing of the 16S rRNA gene V4 variable region was performed at MR DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA) to produce 2x300 paired-end reads using 515F (5' -GTGYCAGCMGCCGCGGTAA- 3') and 806R (5' -GGACTACNVGG GTWCTAAT- 3') primers, as described previously [44].

Data analysis

Statistical analysis was performed as previously described [44]. Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME 2) [46]. Raw sequence data were de-multiplexed and low-quality reads were filtered using default parameters for QIIME. Chimeric sequences were detected and removed using DADA2 prior to analysis [47]. Operational taxonomic units (OTUs) were assigned and clustered using an open-reference protocol in QIIME and defined as having at least 97% similarity against the Greengenes reference database [48,49]. For downstream analysis, unassigned contaminant sequences and those assigned as mitochondria, chloroplasts, or the phylum Cyanobacterium, were excluded from further analysis. Data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP161480.

Alpha diversity metrics (observed OTUs, Shannon, and Chao1) were used to compare species richness and evenness between eyes at baseline and among control and treatment eyes over time. Data were assumed to follow a non-normal distribution. Therefore, a non-parametric Wilcoxon matched-pairs signed-ranks test was used for statistical comparison between treatment and control eyes at baseline. A non-parametric Friedman test, followed by a Dunn's multiple comparison post-test were performed to assess differences in treatment and control eyes over three time points [49]. Statistical analysis was performed using the software package PRISM (PRISM 7, GraphPad Software Inc., San Diego, CA).

Beta diversity, which assesses bacterial community composition, was determined using both weighted and unweighted UniFrac metrics to measure similarity between samples, and evaluated for clustering with Principle Coordinate Analysis (PCoA) plots. An Analysis of Similarity test (ANOSIM) within PRIMER 6 (PRIMER-E Ltd. Luton, UK) was used to assess differences in bacterial community composition between samples.

Differences in the relative abundance of bacterial taxa between eyes at baseline, and among control and treatment eyes over time, were examined. Most datasets did not meet the assumption of normality using the Shapiro-Wilk test (JMP Pro 14, SAS, Marlow, Buckinghamshire). Therefore, a non-parametric Mann-Whitney U test was used to compare treatment and control eyes at baseline. A non-parametric Friedman test was applied to assess differences in treatment and control eyes over three time points (PRISM 7, GraphPad Software Inc., San Diego, CA). A Dunn's multiple comparison post-test was then used to determine which time points

were significantly different. P-values were adjusted for multiple comparisons and corrected for false discovery rate [50]. P- and q-values <0.05 were considered statistically significant.

To analyze the abundance of bacterial taxa in treatment and control eyes and their associations with each time point, linear discriminant analysis effect size (LEfSe) was performed using Calypso [51, 52].

Results

Sequence analysis

Initial DNA quantities extracted from the conjunctival swabs are reported in S1 Table. All sequences were rarified to an even sequencing depth of 15,999 sequences per sample to correct for unevenness between samples. A total of 72 samples were collected from 24 eyes at three time points, and 3,350,060 sequences were amplified (min: 15,999, max: 87,824, median: 45,005, mean: 46,528, standard deviation: 16,666). For each individual sample, the relative abundance of bacteria was defined.

Healthy feline eyes at baseline

Species richness and diversity. Baseline samples from treatment and control eyes were compared prior to antibiotic treatment on Day 0 (S2 Table). Three alpha diversity metrics were analyzed including observed OTUs, Shannon, and Chao1 to examine taxonomic diversity within a sample. Wilcoxon match-pairs signed-ranks test revealed no difference in alpha diversity between control eyes and treatment eyes at baseline. Thus, there was no difference in species richness, evenness, or abundance between eyes at baseline (Fig 1).

Microbial community structure. Two beta diversity metrics, weighted UniFrac and unweighted UniFrac, were analyzed to examine taxonomic diversity between samples. There was no difference in community structure between treatment and control eyes at baseline ($R = -0.037$, $R = -0.052$, respectively, $p > 0.05$). Treatment eyes did not cluster differently from control eyes at baseline (Fig 2).

Microbial community composition. Bacterial taxa abundance did not differ between treatment and control eyes at baseline. Data from all 24 eyes were averaged to describe the bacterial taxa composition of the healthy feline ocular surface. A total of 5 bacterial phyla were detected and 4 phyla were present in all 24 eyes (Table 1). The most common phyla were



Fig 1. Scatter plots of 16S rRNA gene sequences obtained from 12 healthy cats (24 eyes), comparing treatment and control groups at baseline (day 0). Each dot represents one eye. There is no difference in alpha diversity between eyes at baseline (Wilcoxon match-pairs signed-ranks test).

<https://doi.org/10.1371/journal.pone.0223859.g001>

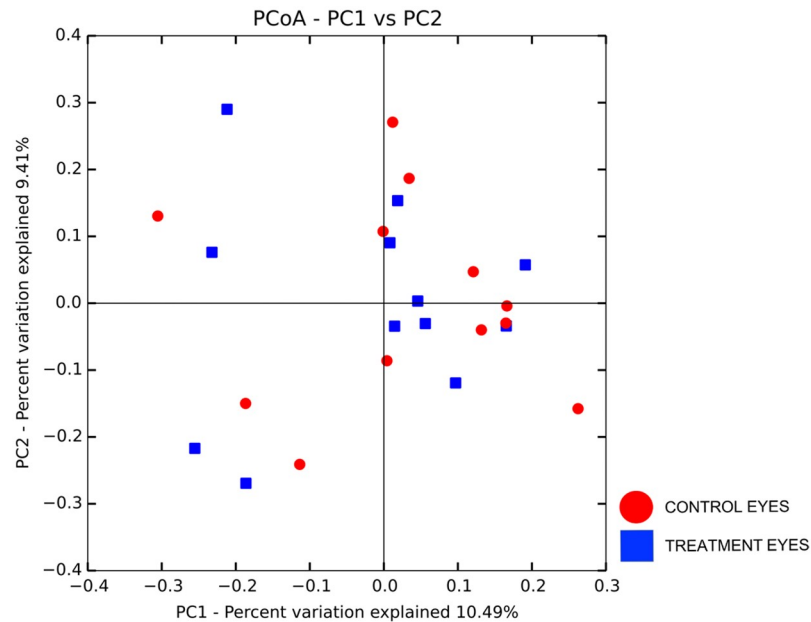


Fig 2. Principle coordinate analysis plot (PCoA) of unweighted UniFrac distance matrices between treatment and control eyes at baseline (day 0). Each dot represents the microbial composition of one eye. Clustering was not observed indicating no difference in beta diversity between eyes at baseline.

<https://doi.org/10.1371/journal.pone.0223859.g002>

Proteobacteria (42.4%), followed by Firmicutes (30.0%), Actinobacteria (15.6%), and Bacteroidetes (8.1%) (Fig 3).

Thirty-four bacterial families at >1% relative abundance were detected and three families were present in all 24 eyes (Table 1). The most common bacterial families sequenced were Corynebacteriaceae (7.8%), Helicobacteraceae (7.5%), Moraxellaceae (6.1%), and Comamonadaceae (5.6%). Other commonly identified families present in most eyes included Pseudomonadaceae (5.4%), Staphylococcaceae (5.3%), and Weeksellaceae (4.7%) (Fig 4). Streptococcaceae, Bacillaceae, and Micrococcaceae represented 4.4%, 4.4%, and 1.9% of the bacterial families sequenced, respectively. The relative abundances of bacterial taxa varied both between eyes and between cats; however, the overall composition remained consistent (Figs 3 and 4). An average of 353 different OTUs were detected throughout all samples.

Temporal variability of ocular surface microbiome in control eyes

Two additional samples were collected from control eyes one week (day 7) and five weeks (day 35) after the baseline collection (day 0), in order to investigate the temporal stability of the ocular surface microbiome in healthy cats.

Species richness and diversity. Alpha diversity was largely unchanged in control eyes over time (S3 Table and Fig 5). There was no difference in observed OTUs and Chao1 based on the sampling time point. However, Shannon diversity was lower on day 35 compared to day 7 ($p = 0.013$).

Microbial community structure. Beta diversity did not differ in control eyes sampled over time as visible by the lack of clustering in the PCoA plot (Fig 6). No difference in microbial communities was detected with ANOSIM (unweighted UniFrac, $R = 0.088$, $R = 0.109$, $R = 0.125$ for day 0 vs. 7, day 0 vs. 35, and day 7 vs. 35, respectively, $p > 0.05$); (weighted UniFrac, $R = 0.085$, $R = 0.262$, $R = 0.159$ for day 0 vs. 7, day 0 vs. 35, and day 7 vs. 35, respectively, $p > 0.05$).

Table 1. Taxa present at $\geq 1\%$ mean relative abundance in healthy cats. Mean percentages and standard deviation of bacteria present at baseline annotated to the level of phylum, family, and genus, based on sequencing of 16S rRNA genes.

Taxon	Healthy Cats at Baseline		
	Mean %	SD %	Number of eyes with positive detection (n = 24)
Phylum			
Family			
-Genus			
Proteobacteria	42.4	14.4	24
Helicobacteraceae	7.5	1.4	23
-Unclassified Helicobacteraceae	7.5	1.4	20
Moraxellaceae	6.1	3.5	23
-Acinetobacter spp.	4.9	3.1	23
Comamonadaceae	5.6	4.4	23
-Unclassified Comamonadaceae	1.9	1.8	18
-Delftia spp.	1.6	2.1	14
Pseudomonadaceae	5.4	3.7	24
-Pseudomonas spp.	4.7	3.3	24
Pasteurellaceae	3.3	3.7	19
-Unclassified Pasteurellaceae	2.8	3.0	17
Halomonadaceae	1.7	1.9	22
-Halomonas spp.	1.7	1.9	22
Enterobacteriaceae	1.8	1.9	17
-Unclassified Enterobacteriaceae	1.4	1.6	16
Neisseriaceae	1.4	1.6	17
-Unclassified Neisseriaceae	1.0	1.0	10
Xanthomonadaceae	1.4	2.9	14
Rhodobacteraceae	1.0	1.3	13
Oxalobacteraceae	1.0	1.0	13
Sphingomonadaceae	1.0	1.0	11
Firmicutes	30.0	8.3	24
Staphylococcaceae	5.3	5.2	24
-Staphylococcus spp.	4.8	5.3	23
Streptococcaceae	4.4	2.6	24
-Streptococcus spp.	4.3	2.7	24
Bacillaceae	4.4	4.5	18
-Bacillus spp.	3.0	3.4	16
-Anoxybacillus spp.	1.0	1.9	6
-Geobacillus spp.	1.0	1.4	7
Aerococcaceae	2.5	2.1	21
-Unclassified Aerococcaceae	1.7	2.0	20
Ruminococcaceae	1.7	2.3	15
-Unclassified Ruminococcaceae	1.0	1.3	12
Tissierellaceae	1.4	4.6	11
-Anaerococcus spp	1.1	4.6	6
Lactobacillaceae	1.1	2.1	10
-Lactobacillus spp.	1.2	2.3	10
Enterococcaceae	1.1	1.9	10
-Unclassified Enterococcaceae	1.0	1.8	8
Exiguobacteraceae	1.1	1.3	15
-Unclassified Exiguobacteraceae	1.0	1.3	13
Planococcaceae	1.0	1.5	11

(Continued)

Table 1. (Continued)

Taxon	Healthy Cats at Baseline		
	Mean %	SD %	Number of eyes with positive detection (n = 24)
Phylum			
Family			
-Genus			
-Unclassified Planococcaceae	1.0	1.3	9
Gemellaceae	1.0	1.5	11
-Unclassified Gemellaceae	1.0	1.5	11
Lachnospiraceae	1.0	1.4	12
Erysipelotrichaceae	1.0	1.1	14
Clostridiaceae	1.0	1.0	12
Actinobacteria	15.6	8.8	24
Corynebacteriaceae	7.8	6.7	23
-Corynebacterium spp.	7.8	6.7	23
Microbacteriaceae	2.2	2.2	18
-Unclassified Microbacteriaceae	2.0	2.1	16
Micrococcaceae	1.9	1.9	19
-Micrococcus spp.	1.0	1.3	12
Bifidobacteriaceae	1.0	1.1	13
-Bifidobacterium spp.	1.0	1.1	13
Bacteroidetes	8.1	5.7	24
Weeksellaceae	4.7	5.7	20
-Cloacibacterium spp.	4.4	5.6	19
Bacteroidaceae	1.0	1.0	11
-Bacteroides spp.	1.0	1.0	11
Porphyromonadaceae	1.0	1.0	18
-Porphyromonas spp.	1.0	1.0	15
Fusobacteria	1.6	2.6	17
Fusobacteriaceae	1.2	2.1	14
-Fusobacterium spp.	1.2	2.0	14

<https://doi.org/10.1371/journal.pone.0223859.t001>

Microbial community composition. The relative abundance of bacteria in control eyes sampled over time is illustrated in Fig 7. Four taxa were differentially abundant on the ocular surface of control eyes at each timepoint (Table 2). At the phylum level, Proteobacteria were increased on day 35 compared to day 0 and day 7 ($p < 0.001$, $q = 0.001$). At the genus level, *Burkholderia* spp. were enriched in control eyes on day 35 compared to baseline (day 0) and day 7 ($p < 0.001$, $q = 0.004$).

Based on LEfSe analysis, the relative abundance of several bacterial taxa were altered over time (Table 3). As noted with the analysis of bacterial groups using univariate testing (Friedman and Dunn’s multiple comparison tests), *Burkholderia* spp. and their associated family, Burkholderiaceae, and phylum, Proteobacteria, were amplified on day 35 among control eyes (Tables 2 and 3).

Temporal variability of ocular surface microbiome in eyes treated with erythromycin antibiotic ointment

Two additional samples were obtained from treatment eyes following baseline (day 0) in order to observe the temporal stability of the ocular surface microbiome in healthy cats following topical antibiotic use. Sampling occurred after one week of antibiotic therapy was

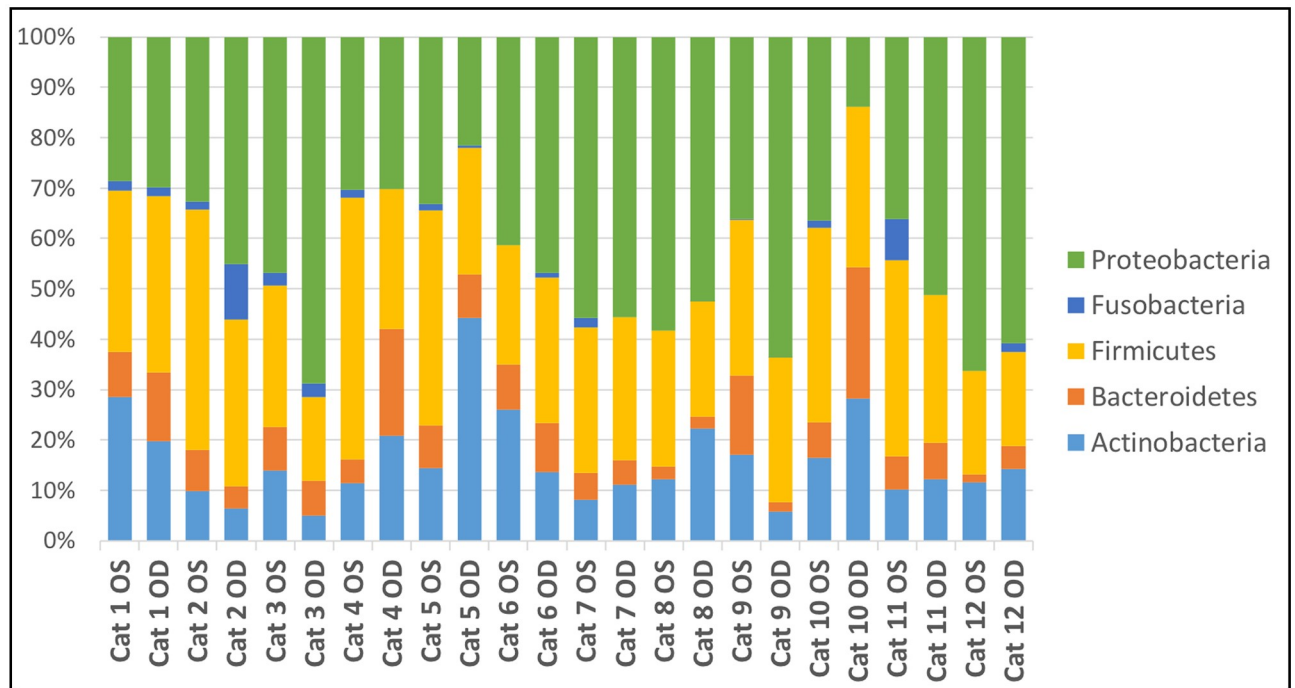


Fig 3. Ocular surface microbiome composition in healthy cats. Relative abundance of taxa present at >1% and annotated to the level of bacterial phylum at baseline (day 0). Each bar chart represents the left (OS) or right (OD) eyes of 12 cats.

<https://doi.org/10.1371/journal.pone.0223859.g003>

applied to the eye three times daily (day 7), and four weeks after discontinuing antibiotic therapy (day 35).

Species richness and diversity. Alpha diversity did not differ in treatment eyes based on the sampling time point (S4 Table and Fig 8).

Microbial community structure. Beta diversity did not differ in treatment eyes sampled over time. This is apparent by the lack of clustering in the PCoA plot (Fig 9). No difference in microbial communities was detected with ANOSIM (unweighted UniFrac, $R = 0.112$, $R = 0.187$, $R = 0.058$ for day 0 vs. 7, day 0 vs. 35, and day 7 vs. 35, respectively, $p > 0.05$); (weighted UniFrac, $R = 0.181$, $R = 0.359$, $R = 0.131$ for day 0 vs. 7, day 0 vs. 35, and day 7 vs. 35, respectively, $p > 0.05$).

Microbial community composition. The relative abundance of bacteria from treatment eyes sampled over time is demonstrated in Fig 10. Seven taxa were differentially abundant on the ocular surface of treatment eyes over time (Table 4). At the phylum level, Proteobacteria were increased on day 35 compared to day 0 ($p = 0.009$, $q = 0.037$). Firmicutes were decreased on day 35 compared to day 0 and day 7 ($p = 0.018$, $q = 0.037$), and Actinobacteria were decreased on day 35 compared to day 0 ($p = 0.017$, $q = 0.037$). At the family level, Burkholderiaceae were increased on day 35 compared to day 0 ($p = 0.001$, $q = 0.025$). Lachnospiraceae were increased on day 7 compared to day 0 ($p < 0.001$, $q = 0.018$), and Microbacteriaceae were decreased on day 35 compared to day 0 ($p = 0.002$, $q = 0.037$). At the genus level, *Burkholderia* were enriched in treatment eyes on day 35 compared to day 0 ($p < 0.001$, $q = 0.004$).

Differences in the relative abundance of bacterial phyla, families, and genera over time was discovered with LEfSe (Table 5). As noted with the analysis of bacterial groups using univariate testing (Friedman and Dunn's multiple comparison tests), *Burkholderia* spp. and their associated family, Burkholderiaceae, and phylum, Proteobacteria, were increased on day 35 among



Fig 4. Ocular surface microbiome composition in healthy cats. Relative abundance of taxa present at >1% and annotated to the level of bacterial family at baseline (day 0). Each bar chart represents the left (OS) or right (OD) eyes of 12 cats.

<https://doi.org/10.1371/journal.pone.0223859.g004>

treatment eyes (Tables 4 and 5). Additionally, the phyla Firmicutes and Actinobacteria, as well as the family Microbacteriaceae, were most abundant on day 0, while Lachnospiraceae were enriched on day 7 (Tables 4 and 5).

A direct comparison of taxa between control eyes and treatment eyes on day 7 and day 35 did not reveal a difference in either alpha or beta diversity (S5 Table) (unweighted UniFrac, $R = -0.06$, $R = -0.07$, for day 7 and day 35, respectively, $p > 0.05$); (weighted UniFrac, $R = 0.05$, $R = -0.06$ for day 7 and 35, respectively, $p > 0.05$). The feline ocular surface microbiome displays an overall lack of clustering when comparing treatment and control eyes throughout the experimental design (S1 Fig).

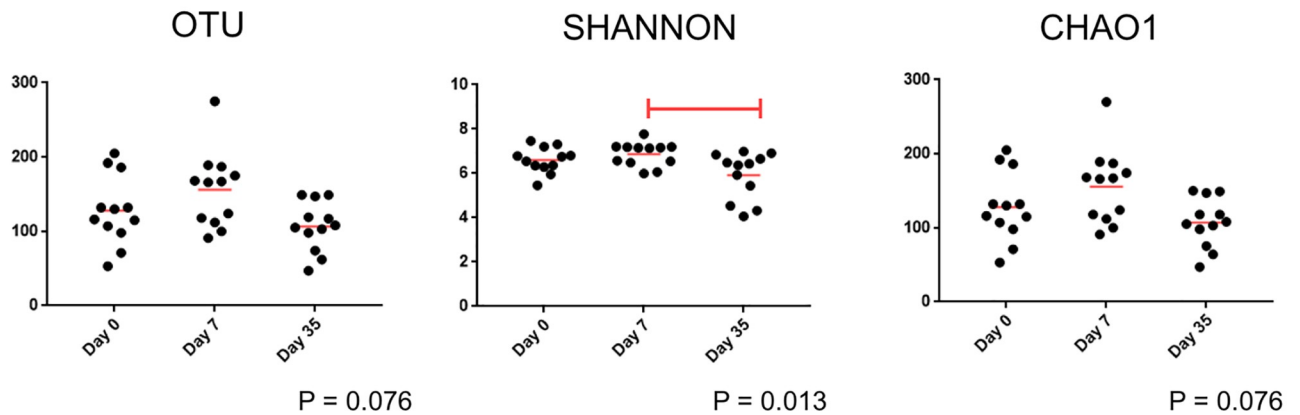


Fig 5. Scatter plots of 16S rRNA gene sequences obtained from 12 control eyes of 12 healthy cats at 3 time points: Day 0, day 7, day 35. There is no difference in observed OTUs and Chao1 in control eyes over time (Friedman test and Dunn’s post-test). Shannon diversity, which evaluates species richness and evenness, is lower on day 35 compared to day 7.

<https://doi.org/10.1371/journal.pone.0223859.g005>

Discussion

Advancements in sequencing technologies have enhanced our understanding of microbial composition and diversity in humans and animals. The present study reveals the feline ocular surface consists of a more complex and diverse bacterial community than previously detected using standard culture-based techniques. Five bacterial phyla and 34 bacterial families were detected at >1% relative abundance (Table 1). Throughout all samples, an average of 353 observed species were detected on the feline eye.

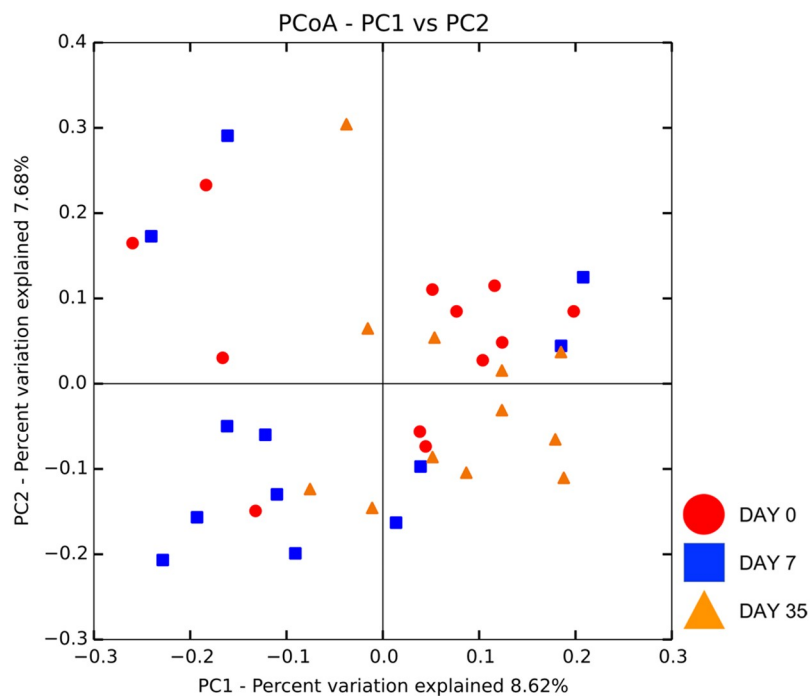


Fig 6. Principle coordinate analysis plot (PCoA) of unweighted UniFrac distance matrices of 12 control eyes from 12 healthy cats at three time points: Day 0, day 7, day 35. Clustering was not observed indicating no difference in beta diversity in control eyes over time.

<https://doi.org/10.1371/journal.pone.0223859.g006>

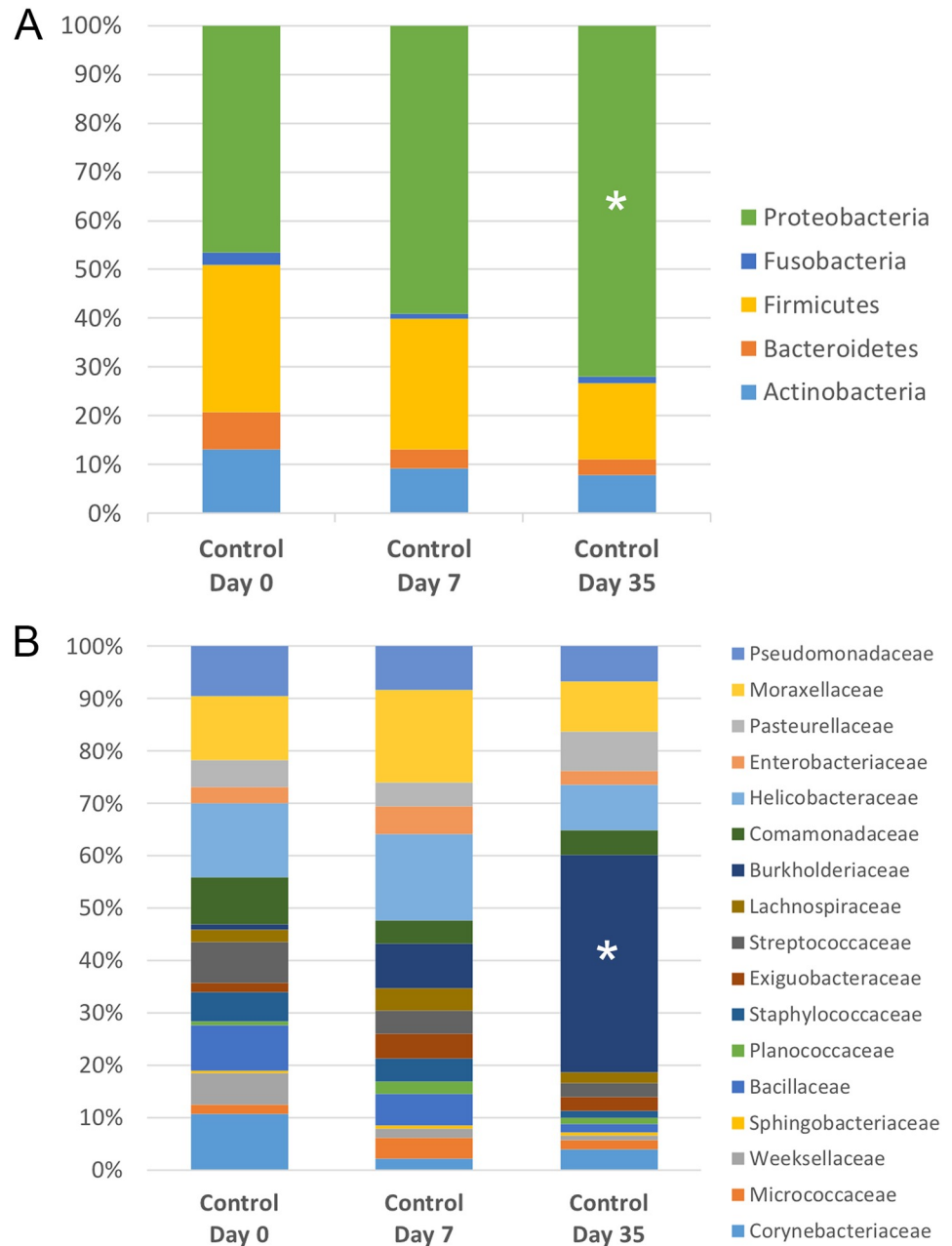


Fig 7. Temporal composition by bacterial phyla (A) and families (B) in control eyes. Bars represent mean percentage of taxa present at $\geq 3\%$ mean relative abundance. (A) Note the relative abundance of Proteobacteria (*) is increased on day 35, and (B) the relative abundance of Burkholderiaceae (*) is increased on day 35.

<https://doi.org/10.1371/journal.pone.0223859.g007>

The most common phyla and their relative proportions colonizing the feline ocular surface, Proteobacteria (42.4%), Firmicutes (30.0%), Actinobacteria (15.6%), and Bacteroidetes (8.1%), are similar to investigations of the human [38, 39, 42] and equine [44] ocular surface microbiome. Preliminary studies describing the ocular surface microbiome of cats and dogs utilizing NGS also identified Proteobacteria and Firmicutes as the two most common bacterial phyla, though at different proportions [41, 45]. For example, Firmicutes (43%) had the highest relative abundance followed by Proteobacteria (30%) across all samples from 14 healthy cats [41].

Table 2. Temporal variation of bacterial taxa isolated from the ocular surface of control eyes of healthy cats at three time points. Median relative percentages and ranges of bacterial groups, annotated to level of phylum, family and genus, based on sequencing of 16S rRNA genes.

Taxa	Day 0		Day 7		Day 35		p-value*	q-value**
	Median %	Range %	Median %	Range %	Median %	Range %		
Proteobacteria	42.1 ^a	28.9–67.8	58.5 ^a	41.5–79.4	64.1 ^b	48.2–93.5	<0.001	0.001
Burkholderiaceae	0 ^a	0–3.1	0.8 ^a	0–28.3	3.7 ^b	0.8–86.5	<0.001	0.005
- <i>Burkholderia</i>	0 ^a	0–3.1	0.8 ^a	0–28.3	3.7 ^b	0.8–86.4	<0.001	0.004
Firmicutes	28.3 ^a	16.2–42.6	26.7 ^a	2–43.8	13.9 ^b	4.9–32.5	0.018	0.055
Staphylococcaceae	2.8 ^a	0.3–8.9	2.5 ^a	0.1–9.9	0.4 ^b	0–4	0.005	0.080
- <i>Staphylococcus</i>	2.0 ^a	0–8.9	2.3 ^{a,b}	0.1–9.9	0.4 ^b	0–4	0.026	0.131
Actinobacteria	12.6 ^a	4.5–24.1	8.6 ^{a,b}	2–15.1	5.6 ^b	0.5–31.3	0.050	0.075
Corynebacteriaceae	7 ^a	0.2–14.2	0.5 ^b	0–5.7	1.4 ^{a,b}	0–20.6	0.003	0.074
- <i>Corynebacterium</i>	7 ^a	0.2–14.2	0.5 ^b	0–5.7	1.4 ^{a,b}	0–20.6	0.003	0.070
Bacteroidetes	6.6 ^a	2–20.7	3.8 ^{a,b}	0.5–6.4	2.0 ^b	0.4–11.4	0.028	0.056

Median values not sharing a common superscript differ significantly ($p < 0.05$, Dunn’s multiple comparison post-test).

*: P-values based on the Friedman test

**: Q-values adjusted based on the Benjamini & Hochberg False discovery rate

<https://doi.org/10.1371/journal.pone.0223859.t002>

Even though the current study appears analogous to previous investigations, the comparison of microbiome studies utilizing NGS should be performed with discretion as a multitude of variations exist among methodologies for DNA extraction, sequencing, and analysis.

The most relatively abundant bacterial families sequenced in 96–100% of feline eyes sampled were Corynebacteriaceae (7.8%), Helicobacteraceae (7.5%), Moraxellaceae (6.1%), Comamonadaceae (5.6%), Pseudomonadaceae (5.4%), Staphylococcaceae (5.3%), and Streptococcaceae (4.4%) (Table 1). The majority of the most relatively abundant microorganisms isolated were gram-negative (24.6%; Helicobacteraceae, Moraxellaceae, Comamonadaceae, Pseudomonadaceae) compared to gram-positive (17.5%; Corynebacteriaceae, Staphylococcaceae, Streptococcaceae). This finding challenges the previous notion reported in the culture-based literature that the feline ocular surface is dominated by gram-positive bacteria [1–3, 18–22].

By utilizing NGS, this study identified several taxa previously unassociated with the feline ocular surface from culture-based reports, likely due to their lack of cultivability. This includes families from the phyla Proteobacteria (Helicobacteraceae, Comamonadaceae, Halomonadaceae, Xanthomonadaceae, Rhodobacteraceae, Oxalobacteraceae, Sphingomonadaceae), Firmicutes (Aerococcaceae, Ruminococcaceae, Tissierellaceae, Lactobacillaceae, Exiguobacteraceae, Planococcaceae, Gemellaceae, Lachnospiraceae, Erysipelotrichaceae, Clostridiaceae), Actinobacteria (Bifidobacteriaceae), and Bacteroidetes (Weeksellaceae, Bacteroidaceae, Porphyromonadaceae) (Table 1). Of the aforementioned families, Aerococcaceae, Ruminococcaceae, Planococcaceae, and Lachnospiraceae were sequenced from the conjunctiva of cats with and without feline immunodeficiency virus (FIV) in a preliminary study using NGS [41]. A complete understanding of the impact of these recently identified organisms on the health and disease status of the feline ocular surface remains to be elucidated.

There was no difference in beta diversity among control eyes when sampled at three separate time points: day 0, day 7, and day 35. Likewise, a significant difference was not detected in the following alpha diversity matrices in control eyes over time: observed OTUs and Chao1. This finding suggests the ocular surface microbiome maintains temporal stability with regard

Table 3. Linear discriminant analysis of bacterial taxa with LDA scores > 3.0 in control eyes and their associations with each time point.

Taxa	LDA	Time point
Phylum		
Family		
-Genus		
Bacteroidetes	4.87	Day 0
Firmicutes	4.96	Day 0
Proteobacteria	5.15	Day 35
Gemellaceae	3.89	Day 0
Bacteroidaceae	3.90	Day 0
Bifidobacteriaceae	4.01	Day 0
Staphylococcaceae	4.15	Day 0
Corynebacteriaceae	4.38	Day 0
Dietziaceae	3.75	Day 7
Succinivibrionaceae	3.84	Day 7
Cardiobacteriaceae	3.91	Day 7
Planococcaceae	3.93	Day 7
Enterobacteriaceae	3.94	Day 7
Micrococcaceae	4.00	Day 7
Campylobacteraceae	4.03	Day 7
Flavobacteriaceae	4.24	Day 7
Porphyromonadaceae	3.93	Day 35
Burkholderiaceae	5.17	Day 35
-Unclassified Bradyrhizobiaceae	3.61	Day 0
-Bifidobacterium	3.63	Day 0
-Unclassified Gemellaceae	3.68	Day 0
-Bacteroides	3.73	Day 0
-Corynebacterium	4.40	Day 0
-Dietzia	3.30	Day 0
-Arthrobacter	3.31	Day 7
-Capnocytophaga	3.33	Day 7
-Roseburia	3.43	Day 7
-Dorea	3.45	Day 7
-Anaerococcus	3.47	Day 7
-Campylobacter	3.48	Day 7
-Sphingomonas	3.60	Day 7
-Dialister	3.75	Day 7
-Unclassified Nocardoidaceae	3.75	Day 7
-Unclassified Enterobacteriaceae	4.01	Day 7
-Acinetobacter	4.50	Day 7
-Enterococcus	3.60	Day 35
-Porphyromonas	3.77	Day 35
-Burkholderia	5.21	Day 35

<https://doi.org/10.1371/journal.pone.0223859.t003>

to species richness and community structure. Shannon diversity was significantly lower in control eyes on day 35 compared to day 7 (Fig 5). A decrease in Shannon diversity indicates a decrease in the abundance and evenness of species. This finding likely corresponds to a change in the relative abundance of some bacterial taxa over time. Univariate and linear discriminant

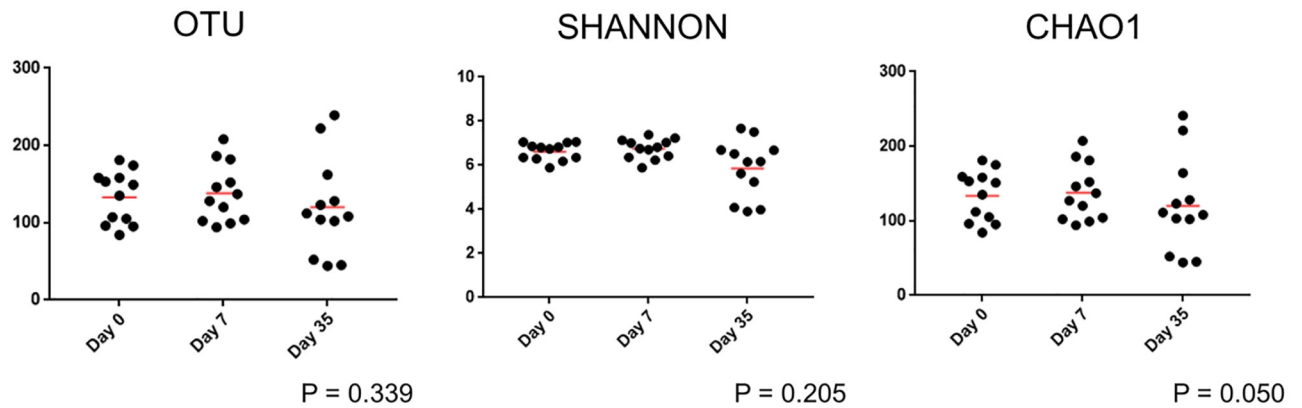


Fig 8. Scatter plots of 16S-rRNA gene sequences obtained from 12 treatment eyes of 12 healthy cats at 3 time points: Baseline (day 0), after one week of topical antibiotic therapy (day 7), four weeks after discontinued topical antibiotic therapy (day 35). There is no difference in alpha diversity in treated eyes over time (Freidman test and Dunn’s post-test).

<https://doi.org/10.1371/journal.pone.0223859.g008>

analyses revealed that Proteobacteria (at the phylum level) and *Burkholderia* spp. (at the genus level) were significantly increased on day 35 compared to day 0 and day 7 (Tables 2 and 3).

The feline ocular surface is likely composed of both a core and transient microbiome. Although there was some temporal variability in community composition detected in control eyes, the vast majority of identified bacterial taxa were present in most eyes at every time point, with no significant change in their relative abundance over time. We speculate many of the bacterial families listed in Figs 7B and 10B belong to the core microbiome; however,

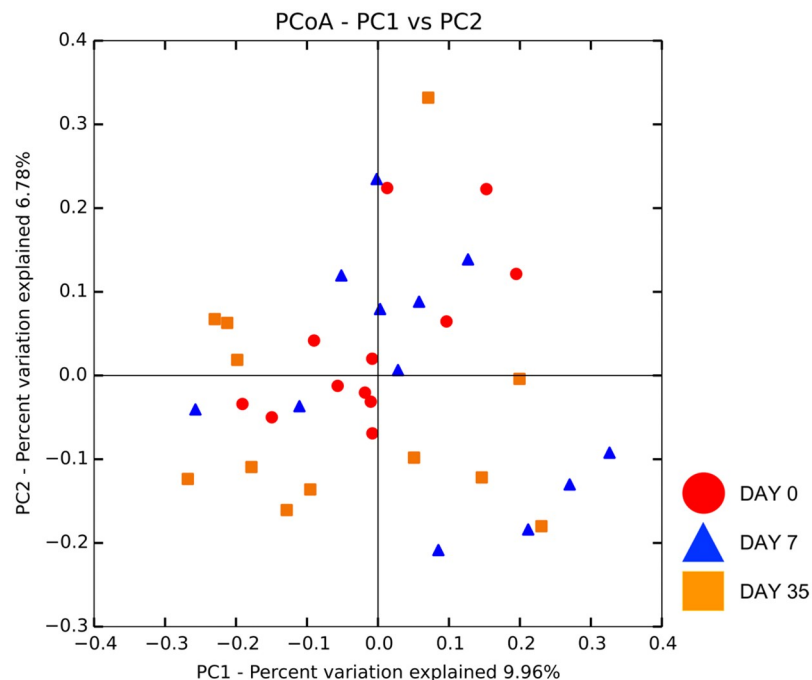


Fig 9. Principle coordinate analysis plot (PCoA) of unweighted UniFrac distance matrices of 12 treatment eyes from 12 healthy cats at three time points: Baseline (day 0), after one week of topical antibiotic therapy (day 7), four weeks after discontinued topical antibiotic therapy (day 35). Clustering was not observed indicating no difference in beta diversity in treatment eyes over time.

<https://doi.org/10.1371/journal.pone.0223859.g009>

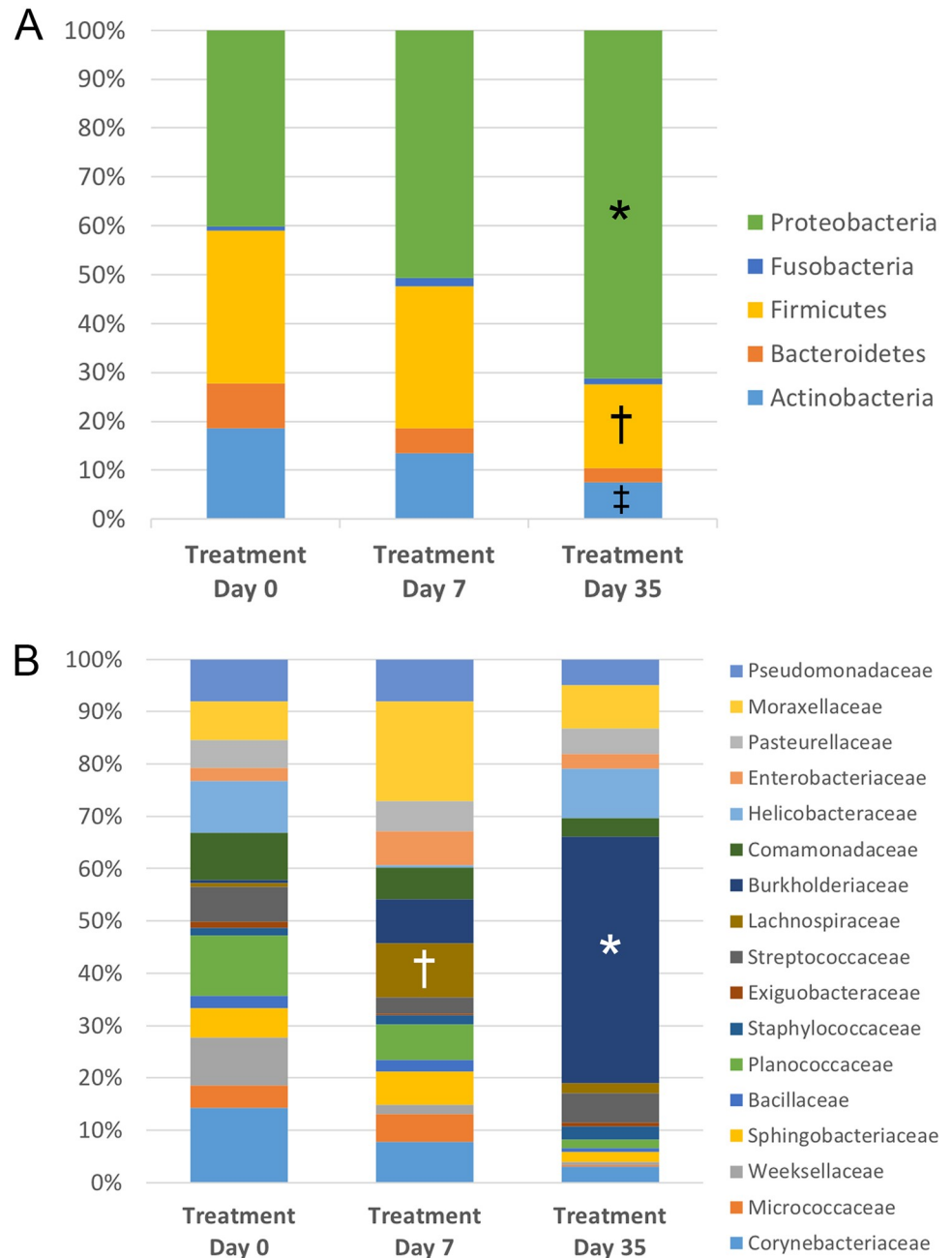


Fig 10. Temporal composition by bacterial phyla (A) and families (B) in treated eyes. Bars represent mean percentage of taxa present at $\geq 3\%$ mean relative abundance. (A) Note the relative abundance of Proteobacteria (*) is increased on day 35, while Firmicutes (†) and Actinobacteria (‡) are decreased on day 35. (B) At the family level, the relative abundance of Burkholderiaceae (*) is increased on day 35 and Lachnospiraceae (†) is increased on day 7.

<https://doi.org/10.1371/journal.pone.0223859.g010>

additional cross-sectional and longitudinal studies are needed to support our findings. Concurrently, there was individual variation in the relative abundance of taxa both between eyes and between cats at baseline. This finding is not unique to the ocular surface, as it is generally recognized that a high degree of interindividual variability exists within human and animal microbiomes, and this is likely attributed to environmental factors and host genetics [53].

Table 4. Temporal variation of bacterial taxa isolated from the ocular surface of treatment eyes of healthy cats at three time points. Median relative percentages and ranges of bacterial groups, annotated to level of phylum, family and genus, based on sequencing of 16S rRNA genes.

Taxa	Day 0		Day 7		Day 35		p-value*	q-value**
	Median %	Range %	Median %	Range %	Median %	Range %		
Phylum								
Family								
-Genus								
Proteobacteria	40.9 ^a	13.2–65.9	46.2 ^{a,b}	31.3–72.2	71.1 ^b	44.7–94.5	0.009	0.037
Burkholderiaceae	0 ^a	0–1.5	1 ^{a,b}	0–43.1	17.4 ^b	0.3–90.5	0.001	0.025
- <i>Burkholderia</i>	0 ^a	0–1.4	0.5 ^a	0–43.1	16.7 ^b	0.3–90.5	<0.001	0.004
Firmicutes	28.4 ^a	20.6–49.4	27.2 ^a	13.7–44.2	11.7 ^b	2.4–48.4	0.018	0.037
Lachnospiraceae	0 ^a	0–2.5	3.2 ^b	0–23.5	0.9 ^{a,b}	0–6.9	<0.001	0.018
Streptococcaceae	4.2 ^a	0.9–8.4	1.4 ^b	0–0.8	0.9 ^b	0–34	0.006	0.071
- <i>Streptococcus</i>	4.2 ^a	0.3–8.4	1.4 ^{a,b}	0–8.8	0.9 ^b	0–34	0.046	0.134
Staphylococcaceae	5.8 ^a	1–24	1.4 ^{a,b}	0–27.5	0.5 ^b	0–9.4	0.028	0.128
- <i>Staphylococcus</i>	5.7 ^a	1–24	1.4 ^{a,b}	0–27.5	0.5 ^b	0–9.4	0.028	0.118
Actinobacteria	13.8 ^a	9.2–43.3	9.7 ^{a,b}	3.5–36.1	5.8 ^b	0.1–20	0.017	0.037
Microbacteriaceae	1.3 ^a	0–5.6	0.4 ^{a,b}	0–5.7	0 ^b	0–0.6	0.002	0.037
Corynebacteriaceae	8.3 ^a	0–29.7	3.1 ^{a,b}	0–16.2	1.0 ^b	0–9.8	0.014	0.102
- <i>Corynebacterium</i>	8.3 ^a	0–29.7	3.1 ^{a,b}	0–16.2	1.0 ^b	0–9.8	0.014	0.080
Bacteroidetes	8.1 ^a	1.6–26.8	4.8 ^{a,b}	0.1–16.3	1.8 ^b	0–7.6	0.050	0.075

Median values not sharing a common superscript differ significantly ($p < 0.05$, Dunn’s multiple comparison post-test).

*: P-values based on the Friedman test

** : Q-values adjusted based on the Benjamini & Hochberg False discovery rate

<https://doi.org/10.1371/journal.pone.0223859.t004>

Currently, there is debate within the physician ophthalmology literature on whether a true core ocular surface microbiome exists, or if it is merely composed of a transient community of microbes from the surrounding environment [10, 13, 37–40, 43, 54]. Compelling arguments can be crafted with supporting evidence that the ocular surface is unfavorable for the establishment of a core microbiome due to its unique innate immune defenses [2, 4, 19 20], thus making a transient population of microorganisms a more likely possibility. Literature has also shown that there is a lower microbial biomass and diversity on the ocular surface compared to other organ systems such as the gastrointestinal tract, oral cavity, nasal cavity, and skin [10, 37, 38, 42, 43]. However, the ocular surface has its own unique and distinctive bacterial microbiome when compared to these other regions of the body, and it appears to be relatively stable over time [38, 43]. With this knowledge, it is conceivable to speculate that the ocular surface microbiome is composed of both a stable commensal core and a transient collection of environmental microbes that encounter the eye.

There were no differences in either alpha or beta diversity among treatment eyes when sampled at baseline (day 0), after one week of topical ophthalmic antibiotic therapy with erythromycin (day 7), and four weeks after discontinuing antibiotic therapy (day 35). As reported with the control eyes, there were statistically significant differences in the relative abundance of some bacterial taxa over time (Tables 4 and 5). Both univariate and linear discriminant analyses revealed *Burkholderia* spp. and their associated family, Burkholderiaceae, and phylum, Proteobacteria, were increased among treatment eyes on day 35 compared to day 0. Additionally, the phyla Firmicutes and Actinobacteria, as well as the family Microbacteriaceae, were most abundant on day 0, while Lachnospiraceae were enriched on day 7 (Tables 4 and 5). A similar trend was noted among control eyes. These findings suggest a short-term course of broad-spectrum topical antibiotics does not alter the feline ocular surface microbiome with

Table 5. Linear discriminant analysis of bacterial taxa with LDA scores > 3.0 in treatment eyes and their associations with each time point.

Taxa	LDA	Time point
Phylum		
Family		
-Genus		
Bacteroidetes	4.45	Day 0
Actinobacteria	4.70	Day 0
Firmicutes	4.85	Day 0
Proteobacteria	5.17	Day 35
Actinomycetaceae	3.66	Day 0
Unclassified_Solibacterales	3.69	Day 0
Neisseriaceae	3.76	Day 0
Microbacteriaceae	4.10	Day 0
Streptococcaceae	4.22	Day 0
Weeksellaceae	4.39	Day 0
Staphylococcaceae	4.45	Day 0
Pseudonocardiaceae	3.66	Day 7
Campylobacteraceae	3.70	Day 7
Succinivibrionaceae	3.99	Day 7
Lachnospiraceae	4.46	Day 7
C111	3.63	Day 35
Burkholderiaceae	5.21	Day 35
-Unclassified_Pirellulaceae	3.48	Day 0
-Jeotgalicoccus	3.54	Day 0
-Haemophilus	3.75	Day 0
-Unclassified_Solibacterales	3.87	Day 0
-Unclassified_Comamonadaceae	3.94	Day 0
-Ralstonia	3.98	Day 0
-Streptococcus	4.18	Day 0
-Cloacibacterium	4.39	Day 0
-Staphylococcus	4.44	Day 0
-Campylobacter	3.62	Day 7
-Paracoccus	3.63	Day 7
-Parabacteroides	3.68	Day 7
-Enhydrobacter	3.72	Day 7
-Faecalibacterium	3.73	Day 7
-Pseudonocardia	3.81	Day 7
-Blautia	4.03	Day 7
-Unclassified_C111	3.73	Day 35
-Unclassified_Clostridiaceae	4.20	Day 35
-Burkholderia	5.24	Day 35

<https://doi.org/10.1371/journal.pone.0223859.t005>

regard to species richness, community structure, and global community composition. Erythromycin is a macrolide antibiotic that is primarily effective against gram-positive bacteria. Minor shifts in abundance of some bacterial taxa over time, such as with *Burkholderia*, a gram-negative organism, were similar in both treatment and control eyes and likely more indicative of transient changes that occur due to external factors in an open system as opposed to the influence of antibiotic therapy. As the bioavailability of topically applied medications is extremely

low (<5%), systemic absorption of erythromycin was unlikely to reach therapeutic dosages to the ocular surface of the contralateral eye [55].

The genus *Burkholderia* consists of rod-shaped bacteria and has approximately 100 validated species with a majority being ubiquitous in the environment as soil inhabitants [56]. Two well-known species of *Burkholderia*, *B. pseudomallei* and *B. mallei*, are associated with zoonotic diseases such as glanders and melioidosis, respectively. Currently, there is no evidence of ocular disease linked to *Burkholderia* species in veterinary medicine. Within the physician ophthalmology literature, *Burkholderia* was suspected to cause endophthalmitis and keratitis in a small number of case reports [57–59]. The temporal increase in the relative abundance of *Burkholderia* spp. in this population of cats remains of unknown consequence and is likely an incidental and transient environmental fluctuation. Another possible consideration for the presence of *Burkholderia* in relatively low biomass samples could be due to contamination from the DNA extraction kit or PCR and amplification reagents [60]. While there is no evidence of *Burkholderia* causing ocular disease in felines, further investigation into the ocular pathogenicity of this genus of bacteria is warranted.

Although the ocular surface microbiome was not significantly impacted by a short-course of topical antibiotic therapy in the current study, more frequent and chronic application of ophthalmic antibiotics could have more profound effects and potentially facilitate the emergence of resistant strains [27, 61]. Infectious conjunctivitis occurs frequently in cats and this condition is often treated with topical antibiotics for a prolonged period of three weeks or greater [1, 18, 22]. Additionally, many cats will receive several treatment trials in their lifetime as recurrence is common. Etiologically, feline herpes virus-1, *Chlamydia* spp. and *Mycoplasma* spp. are commonly associated with feline conjunctivitis [1, 18, 22]. Erythromycin accumulates intracellularly allowing for effective treatment against conjunctival *Chlamydia* and *Mycoplasma* infections in cats. In the present study, *Chlamydia* spp. were not isolated from the eyes of 12 healthy cats at any timepoint; however, *Mycoplasma* spp. were identified in 1/24 eyes in the control group at baseline at less than 1% relative abundance (0.002%). Previous culture-based studies have isolated *Mycoplasma* from both diseased and healthy feline eyes [18]. Therefore, its exact role in causing conjunctivitis is unclear. Clinical signs of ocular disease were not present in any cat throughout the study period. Future studies are necessary to evaluate the effects of chronic antimicrobial use in the face of disease on the feline ocular surface microbiome utilizing NGS. Investigating the composition of the ocular surface microbiome and the impact of prolonged antibiotic usage will not only aid clinically with antibiotic stewardship, but also help combat the crisis of antibiotic resistance that plaques physician and veterinary medicine alike.

The homogeneity of the study population provided an ideal circumstance to study the ocular surface microbiome. All 12 cats were adult females, housed in the same building, fed the same diet, and exposed to the same exogenous factors (enrichment toys, caretakers, and research personnel). This provided a highly controlled environment in which to evaluate the ocular surface microbiome. However, there are limitations to studying a homogenous population, as our findings may not represent the general feline population. It is possible that more diverse feline populations with different ages, sex, and environmental factors such as geography, housing, and diet, may demonstrate marked differences in their resident microbial populations. Additionally, cats in this study were housed in groups of 2–4 within the same enclosures, and such close social interactions may affect the composition of the ocular surface microbiome. The feline species is known for its superior ability to groom and allogrooming/allorubbing can be a social activity shared between felids who live in close proximity to reinforce bonds, establish hierarchy, and develop companionships [62]. This may cause them to share a larger percentage of their individual microbiome composition compared to cats in

more solitary living conditions. Measures were not taken to prevent grooming in this study as this is an expected behavior and known environmental factor that occurs in their natural habitat.

Literature on the feline ocular microbiome is scarce at this time with only one other study utilizing NGS to the authors' knowledge. Therefore, interpretation of the significance of the ocular surface microbiome composition, its stability, and response to antibiotic therapy is challenging. Larger scale evaluations from more heterogeneous populations of cats are warranted to limit bias and expound upon the data presented within this study. Further evaluation of the effects of chronic antibiotic use, as well as other exogenous and endogenous factors may allow for a more comprehensive and clinically relevant understanding of the ocular surface microbiome.

Additional limitations of this study are those inherent to NGS. For example, the evaluation of relative abundance does not represent absolute quantities of the microbial populations present [53]. Quantitative PCR of specific organisms is required to determine absolute abundance of a known sequence within a sample. In addition, NGS does not determine the viability of organisms present in a sample. Therefore, organisms that are detected via NGS may represent more than just a living community of organisms on the ocular surface but also a collection of nonviable organisms that have fallen prey to the host's immune defenses [37]. Given the relatively low biomass environment of the ocular surface, contaminating DNA from laboratory reagents may also impact the results obtained [60]. Even with such limitations, NGS provides a plethora of useful knowledge that, along with future investigations, will enhance our understanding of the ocular surface microbiome and its role in health and disease.

Conclusion

This is the first report to investigate the temporal stability of the feline ocular surface microbiome both in untreated eyes and following topical antibiotic therapy. Using molecular-based techniques, a diverse, species-rich bacterial community was shown to inhabit the healthy feline ocular surface. In contrast to culture-based studies, all eyes demonstrated the presence of bacterial microbes, many of which were gram-negative and previously unassociated with the feline eye. A stable bacterial microbiome was identified and discovered to remain consistent with regard to species richness and community structure both over time and following one week of antibiotic therapy. However, significant and similar changes in the abundance of some bacterial taxa over time in both treatment and control eyes indicate the open nature of the ocular surface microbiome is likely influenced by external environmental factors. Further studies are warranted to elucidate if the ocular surface microbiome is altered in the face of disease and chronic topical antibiotic use.

Supporting information

S1 Table. Quantification of nucleic acid (ng/ μ l) extracted from conjunctival swabs of healthy cats.

(DOCX)

S2 Table. Summary of alpha diversity indices at a depth of 15,999 sequences per sample for control and treatment eyes at baseline.

(DOCX)

S3 Table. Summary of alpha diversity indices at a depth of 15,999 sequences per sample for control eyes over time.

(DOCX)

S4 Table. Summary of alpha diversity indices at a depth of 15,999 sequences per sample for treatment eyes over time.

(DOCX)

S5 Table. Summary of alpha diversity indices at a depth of 15,999 sequences per sample comparing control eyes and treatment eyes at day 7 and day 35.

(DOCX)

S1 Fig. Principle coordinate analysis plot (PCoA) of unweighted UniFrac distance matrices of 12 treatment eyes and 12 control eyes from 24 healthy cats at three time points: Baseline (day 0), after one week of topical antibiotic therapy (day 7), four weeks after discontinued topical antibiotic therapy (day 35). No clustering was observed indicating there was no difference in beta diversity in control or treatment eyes over time.

(TIF)

Acknowledgments

The authors thank Caleb Coursey, So Young Park, and Ben Sarawichitr for technical support.

Author Contributions

Conceptualization: Erin M. Scott, Carolyn Arnold, Jan S. Suchodolski.

Data curation: Erin M. Scott, Carolyn Arnold.

Formal analysis: Erin M. Scott, Carolyn Arnold.

Funding acquisition: Joshua E. Darden, Erin M. Scott, Jan S. Suchodolski.

Investigation: Joshua E. Darden, Erin M. Scott, Elizabeth M. Scallan.

Methodology: Carolyn Arnold, Jan S. Suchodolski.

Project administration: Bradley T. Simon, Jan S. Suchodolski.

Resources: Erin M. Scott, Bradley T. Simon, Jan S. Suchodolski.

Supervision: Bradley T. Simon, Jan S. Suchodolski.

Visualization: Joshua E. Darden, Erin M. Scott, Carolyn Arnold.

Writing – original draft: Joshua E. Darden, Erin M. Scott.

Writing – review & editing: Carolyn Arnold, Elizabeth M. Scallan, Bradley T. Simon, Jan S. Suchodolski.

References

1. Gerding PA, Kakoma I. Microbiology of the canine and feline eye. *Vet Clin North Am Small Anim Pract.* 1990; 20(3): 615–625. [https://doi.org/10.1016/s0195-5616\(90\)50053-4](https://doi.org/10.1016/s0195-5616(90)50053-4) PMID: 2194350
2. Büttner JN, Schneider M, Csokai J, Müller E, Eule JC. Microbiota of the conjunctival sac of 120 healthy cats. *Vet Ophthalmol.* 2018; <https://doi.org/10.1111/vop.12598> PMID: 30095211
3. Espínolaz MB, Lilienbaum W. Prevalence of bacteria in the conjunctival sac and on the eyelid margin of clinically normal cats. *J Small Anim Pract.* 1996; 37(8): 364–366. <https://doi.org/10.1111/j.1748-5827.1996.tb02415.x> PMID: 8872935
4. McDermott AM. Antimicrobial compounds in tears. *Exp Eye Res.* 2013; 117: 53–61. <https://doi.org/10.1016/j.exer.2013.07.014> PMID: 23880529
5. Gilger BC. Immunology of the ocular surface. *Vet Clin North Am Small Anim Pract.* 2008; 38(2): 223–231. <https://doi.org/10.1016/j.cvsm.2007.11.004> PMID: 18299004

6. Cavuoto KM, Mendez R, Miller D, Galor A, Banerjee S. Effect of clinical parameters on the ocular surface microbiome in children and adults. *Clin Ophthalmol*. 2018; 12: 1189–1197. <https://doi.org/10.2147/OPTH.S166547> PMID: 30013312
7. Dorn ES, Tress B, Suchodolski JS, Nisar T, Ravindran P, Weber K, et al. Bacterial microbiome in the nose of healthy cats and in cats with nasal disease. *PLoS One*. 2017; 12(6): <https://doi.org/10.1371/journal.pone.0180299> PMID: 28662139
8. Graham JE, Moore JE, Jiru X, Moore JE, Goodall EA, Dooley JS, et al. Ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. *Invest Ophthalmol Vis Sci*. 2007; 48(12): 5616–5623. <https://doi.org/10.1167/iops.07-0588> PMID: 18055811
9. Isaiah A, Hoffmann AR, Kelley R, Mundell P, Steiner JM, Suchodolski JS. Characterization of the nasal and oral microbiota of detection dogs. *PLoS One*. 2017; 12(9): <https://doi.org/10.1371/journal.pone.0184899> PMID: 28934260
10. Kugadas A, Gadjeva M. Impact of microbiome on ocular health. *Ocul Surf*. 2016; 14(3): 342–349. <https://doi.org/10.1016/j.jtos.2016.04.004> PMID: 27189865
11. Langdon A, Crook N, Dantas G. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. *Genome Med*. 2016; 8(1): <https://doi.org/10.1186/s13073-016-0294-z> PMID: 27074706
12. Lee SH, Oh DH, Jung JY, Kim JC, Jeon CO. Comparative ocular microbial communities in humans with and without blepharitis. *Invest Ophthalmol Vis Sci*. 2012; 53(9): 5585–5593. <https://doi.org/10.1167/iops.12-9922> PMID: 22836761
13. Lu LJ, Liu J. Human microbiota and ophthalmic disease. *Yale J Biol Med*. 2016; 89(3): 325–330. PMID: 27698616
14. Meason-Smith C, Diesel A, Patterson AP, Older CE, Johnson TJ, Mansell JM, et al. Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing. *Vet Dermatol*. 2017; 28(1): 71–e17. <https://doi.org/10.1111/vde.12373> PMID: 27553477
15. Meason-Smith C, Diesel A, Patterson AP, Older CE, Mansell JM, Suchodolski JS, et al. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol Ecol*. 2015; 91(12): <https://doi.org/10.1093/femsec/fiv139> PMID: 26542075
16. Minamoto Y, Hooda S, Swanson KS, Suchodolski JS. Feline gastrointestinal microbiota. *Anim Health Res Rev*. 2012; 13(1): 64–77. <https://doi.org/10.1017/S1466252312000060> PMID: 22853923
17. Older CE, Diesel A, Patterson AP, Meason-Smith C, Johnson TJ, Mansell J, et al. The feline skin microbiota: The bacteria inhabiting the skin of healthy and allergic cats. *PLoS One*. 2017; 12(6): <https://doi.org/10.1371/journal.pone.0178555> PMID: 28575016
18. Whitley RD. Canine and feline primary ocular bacterial infections. *Vet Clin North Am Small Anim Pract*. 2000; 30(5): 1151–1167. [https://doi.org/10.1016/s0195-5616\(00\)05012-9](https://doi.org/10.1016/s0195-5616(00)05012-9) PMID: 11033880
19. Gaskin JM. Microbiology of the canine and feline eye. *Vet Clin North Am Small Anim Pract*. 1980; 10(2): 303–316. [https://doi.org/10.1016/s0195-5616\(80\)50031-8](https://doi.org/10.1016/s0195-5616(80)50031-8) PMID: 6251592
20. Gerding PA, Cormany K, Weisiger R, Kakoma I. Survey and topographic distribution of bacterial and fungal microorganisms in eyes of clinically normal cats. *Feline Pract*. 1993; 21(3): 20–23.
21. Kielbowicz Z, Płoneczka-Janeczko K, Bania J, Bierowiec K, Kielbowicz M. Characteristics of the bacterial flora in the conjunctival sac of cats from Poland. *J Small Anim Pract*. 2015; 56(3): 203–206. <https://doi.org/10.1111/jsap.12304> PMID: 25482666
22. Shewen PE, Povey RC, Wilson MR. A survey of the conjunctival flora of clinically normal cats and cats with conjunctivitis. *Canadian Vet J*. 1980; 21(8): 231–233.
23. Andrew SE, Nguyen A, Jones GL, Brooks DE. Seasonal effects on the aerobic bacterial and fungal conjunctival flora of normal thoroughbred brood mares in Florida. *Vet Ophthalmol*. 2003; 6(1): 45–50. PMID: 12641842
24. Prado MR, Rocha MFG, Brito ÉHS, Girão MD, Monteiro AJ, Teixeira MFS, et al. Survey of bacterial microorganisms in the conjunctival sac of clinically normal dogs and dogs with ulcerative keratitis in Fortaleza, Ceará, Brazil. *Vet Ophthalmol*. 2005; 8(1): 33–37. <https://doi.org/10.1111/j.1463-5224.2005.04061.x> PMID: 15644098
25. Furiani N, Scarpella F, Anna Martino P, Panzini I, Fabbri E, Ordeix L. Evaluation of the bacterial microflora of the conjunctival sac of healthy dogs and dogs with atopic dermatitis. *Vet Dermatol*. 2011; 22(6): 490–496. <https://doi.org/10.1111/j.1365-3164.2011.00979.x> PMID: 21535255
26. McDonald PJ, Watson ADJ. Microbial flora of normal canine conjunctivae. *J Small Anim Pract*. 1976; 17(12): 809–812. <https://doi.org/10.1111/j.1748-5827.1976.tb06947.x> PMID: 1011806

27. Sandmeyer LS, Bauer BS, Mohaghegh Poor SM, Feng CX, Chirino-Trejo M. Alterations in conjunctival bacteria and antimicrobial susceptibility during topical administration of ofloxacin after cataract surgery in dogs. *Am J Vet Res.* 2017; 78(2): 207–214. <https://doi.org/10.2460/ajvr.78.2.207> PMID: 28140645
28. Samuelson DA, Andresen TL, Gwin RM. Conjunctival fungal flora in horses, cattle, dogs, and cats. *J Am Vet Med Assoc.* 1984; 184(10): 1240–1242. PMID: 6539761
29. Wang L, Pan Q, Zhang L, Xue Q, Cui J, Qi C. Investigation of bacterial microorganisms in the conjunctival sac of clinically normal dogs and dogs with ulcerative keratitis in Beijing, China. *Vet Ophthalmol.* 2008; 11(3): 145–149. <https://doi.org/10.1111/j.1463-5224.2008.00579.x> PMID: 18435654
30. Urban M, Wyman M, Rheins M, Marraro RV. Conjunctival flora of clinically normal dogs. *J Am Vet Med Assoc.* 1972; 161(2): 201–206. PMID: 5036187
31. Cattabiani F, Cabassi E, Allodi C, Gianelli F. Bacterial flora of the conjunctival sac of the horse. *Ann Sclavo.* 1976; 18(1): 91–119. PMID: 788654
32. Gemensky-Metzler AJ, Wilkie DA, Kowalski JJ, Schmall LM, Willis AM, Yamagata M. Changes in bacterial and fungal ocular flora of clinically normal horses following experimental application of topical antimicrobial or antimicrobial-corticosteroid ophthalmic preparations. *Am J Vet Res.* 2005; 66(5): 800–811. <https://doi.org/10.2460/ajvr.2005.66.800> PMID: 15934607
33. Khosravi AR, Nikaein D, Sharifzadeh A, Gharagozlou F. Ocular fungal flora from healthy horses in Iran. *J Mycol Méd.* 2014; 24(1): 29–33. <https://doi.org/10.1016/j.mycmed.2013.10.006> PMID: 24411178
34. Rosa M, Cardozo LM, Da Silva Pereira J, Brooks DE, Martins ALB, Florido PSS, et al. Fungal flora of normal eyes of healthy horses from the State of Rio de Janeiro, Brazil. *Vet Ophthalmol.* 2003; 6(1): 51–55. PMID: 12641843
35. Whitley RD, Moore CP. Microbiology of the equine eye in health and disease. *Vet Clin North Am Large Anim Pract.* 1984; 6(3): 451–466. PMID: 6393541
36. Rappe MS, Giovannoni SJ. The uncultured microbial majority. *Annu Rev Microbiol.* 2003; 57(1): 369–394.
37. Willcox MD. Characterization of the normal microbiota of the ocular surface. *Exp Eye Res.* 2013; 117: 99–105. <https://doi.org/10.1016/j.exer.2013.06.003> PMID: 23797046
38. Doan T, Akileswaran L, Andersen D, Johnson B, Ko N, Shrestha A, et al. Paucibacterial microbiome and resident DNA virome of the healthy conjunctiva. *Invest Ophthalmol Vis Sci.* 2016; 57(13): 5116–5126. <https://doi.org/10.1167/iovs.16-19803> PMID: 27699405
39. Dong Q, Brulc JM, Iovieno A, Bates B, Garoutte A, Miller D, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci.* 2011; 52(8): 5408–5413. <https://doi.org/10.1167/iovs.10-6939> PMID: 21571682
40. Zegans ME, Van Gelder RN. Considerations in understanding the ocular surface microbiome. *Am J Ophthalmol.* 2014; 158(3): 420–422. <https://doi.org/10.1016/j.ajo.2014.06.014> PMID: 25132249
41. Weese SJ, Nichols J, Jalali M, Litster A. The oral and conjunctival microbiotas in cats with and without feline immunodeficiency virus infection. *Vet Res.* 2015; 46:21. <https://doi.org/10.1186/s13567-014-0140-5> PMID: 25879465
42. Ozkan J, Coroneo M, Willcox M, Wemheuer B, Thomas T. Identification and visualization of a distinct microbiome in ocular surface conjunctival tissue. *Invest Ophthalmol Vis Sci.* 2018; 59(10): 4268–4276. <https://doi.org/10.1167/iovs.18-24651> PMID: 30140925
43. Ozkan J, Nielsen S, Diez-Vives C, Coroneo M, Thomas T, Willcox M. Temporal stability and composition of the ocular surface microbiome. *Sci Rep.* 2017; 7(1):9880. <https://doi.org/10.1038/s41598-017-10494-9> PMID: 28852195
44. Scott EM, Arnold C, Dowell S, Suchodolski JS. Evaluation of the bacterial ocular surface microbiome in clinically normal horses before and after treatment with topical neomycin-polymyxin-bacitracin. *PLoS One* 2019; 14(4): <https://doi.org/10.1371/journal.pone.0214877> PMID: 30943258
45. Leis ML, Costa MO. Initial description of the core ocular surface microbiome in dogs: Bacterial community diversity and composition in a defined canine population. *Vet Ophthalmol.* 2018; <https://doi.org/10.1111/vop.12599> PMID: 30095241
46. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, et al. QIIME2: Reproducible, interactive, scalable, extensible microbiome data science. *PeerJ Preprints.* 2018; <https://doi.org/10.7287/peerj.preprints.27295v1>
47. 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. <https://doi.org/10.1038/nmeth.3869> PMID: 27214047
48. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol.* 2006; 72(7): 5069–5072. <https://doi.org/10.1128/AEM.03006-05> PMID: 16820507

49. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved GreenGenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J*. 2012; 6(3): 610–618. <https://doi.org/10.1038/ismej.2011.139> PMID: 22134646
50. Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J Roy Stat Soc B Met*. 1995; 57(1): 289–300.
51. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011; 12(6): <https://doi.org/10.1186/gb-2011-12-6-r60> PMID: 21702898
52. Zakrzewski M, Proietti C, Ellis JJ, Hasan S, Brion M-J, Berger B, et al. Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. *Bioinformatics*. 2017; 33(5): 782–783. <https://doi.org/10.1093/bioinformatics/btw725> PMID: 28025202
53. Tyler AD, Smith MI, Silverberg MS. Analyzing the human microbiome: a "how to" guide for physicians. *Am J Gastroenterol*. 2014; 109: 983–993. <https://doi.org/10.1038/ajg.2014.73> PMID: 24751579
54. Huang Y, Yang B, Li W. Defining the normal core microbiome of conjunctival microbial communities. *Clin Microbiol Infect*. 2016; 22(7): 643.e7–.e12.
55. Dey S, Gunda S, Mitra AK. Pharmacokinetics of erythromycin in rabbit corneas after single-dose infusion: role of p-glycoprotein as a barrier to in vivo ocular drug absorption. *J Pharmacol Exp Ther*. 2004; 311(1): 246–255. <https://doi.org/10.1124/jpet.104.069583> PMID: 15175422
56. Eberl L, Vandamme P. Members of the genus *Burkholderia*: good and bad guys. *F1000Res*. 2016; 5: <https://doi.org/10.12688/f1000research.8221.1> PMID: 27303639
57. Eser I, Altan T, Stahl JE, Aydin MD, Inan N, Kapran Z, et al. Two cases of *Burkholderia cepacia* endophthalmitis. *Br J Ophthalmol*. 2006; 90(9): 1211.
58. Chen KJ, Sun MH, Hou CH, Sun CC, Chen TL. *Burkholderia pseudomallei* endophthalmitis. *J Clin Microbiol*. 2007; 45(12): 4073–4074. <https://doi.org/10.1128/JCM.01467-07> PMID: 17913931
59. Ibrahim M, Yap JY. *Burkholderia cepacia*: a rare cause of bacterial keratitis. *BMJ Case Rep*. 2018; <https://doi.org/10.1136/bcr-2018-224552> PMID: 29695396
60. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*. 2014; 12(87): <http://www.biomedcentral.com/1741-7007/12/87>
61. Ozkan J, Zhu H, Gabriel M, Holden BA, Willcox MDP. Effect of prophylactic antibiotic drops on ocular microbiota and physiology during silicone hydrogel lens wear. *Optom Vis Sci*. 2012; 89(3): 326–335. <https://doi.org/10.1097/OPX.0b013e318243280e> PMID: 22246331
62. van den Bos R. The function of allogrooming in domestic cats (*Felis silvestris catus*); a study in a group of cats living in confinement. *J Ethol*. 1998; 16(1): 1–13.