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Metagenomic Analysis of the Lacrimal Caruncle Region of Canis lupus familiaris Using MiSeq Technology

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Metagenomic analysis of the lacrimal caruncle region of *Canis lupus familiaris* using MiSeq technology

By

Seun A. Ikugbayigbe

THESIS

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ABSTRACT

The identification of ocular microbiota may allow early diagnosis and treatment strategies against eye diseases and disorders with canines. However, clinical microbial identification has been limited to cloning and conventional culture-based studies, which typically underestimate community diversity. In this report, Illumina MiSeq analysis of the 16S rRNA gene was used to examine the microbiome of the lacrimal caruncle region from five healthy dogs. The breeds sampled were a Golden Retriever (Dog A), a Weimaraner (Dog B), a Shih Tzu mix (Dog C), a Yorkie mix (Dog D), and a Dachshund (Dog E). MiSeq analysis revealed a total of 370 operational taxonomic units (OTUs) representing 79 families of bacteria. Generally, Dog A had the most unique bacterial profile in terms of families that were represented, with samples from this dog having contributions from families that were not observed above 2% of total OTUs in the other dogs. For example, the Oxalobacteraceae (*Massilia* spp.), Micrococcaceae (*Arthrobacter* spp.), and Enterobacteriaceae (*Pantoea* spp.) families were uniquely found in Dog A at levels above 2% of the total OTUs. Dogs A and B harbored very high percentages of Pseudomonadaceae (up to 65% in the right eye of Dog A), which was attributed entirely to the genus *Pseudomonas.* These dogs also had relatively high percentages of Moraxellaceae (up to 21% in the left eye of Dog A), which were almost entirely from the *Psychrobacter* and *Acinetobacter* genera. The microbiomes from Dog A and Dog B were similar with respect to the families present and their relative abundance, while the microbiomes of Dogs C, D, and E were more similar to each other. Overall, this

study demonstrated the efficacy of Illumina's MiSeq technology as an inexpensive and facile tool for microbiome analysis of ocular bacteria in canines, and highlighted the potential for this technique to be used by veterinarians for clinical investigations.

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INTRODUCTION

Ocular diseases and disorders severely hamper the health and way of life of canines, and may lead to adverse behavioral changes. Common agents responsible for these diseases and disorders are microbial pathogens. For instance, anterior uveitis, blepharitis and keratoconjunctivitis are ocular manifestations of *Leishmania* infection (Pena et al. 2000). Similarly, endophthalmitis, chorioretinitis, and hyphema have been associated with *Brucella canis* infection (Townsend, 2008). Several other pathogenic bacteria have been linked to ocular distress, such as *Borrelia burgdorferi* (Lyme disease), *Ehrlichia* spp., and *Rickettsia rickettsii* (Rocky Mountain spotted fever), which have led to uveitis, hyphema, retinal hemorrhage, and retinal detachment (Townsend, 2008). Additional pathogenic bacteria of canine eyes are frequently encountered at veterinary clinics that require medical treatment, including *Pseudomonas* spp. (Ledbetter et al., 2009; Santos et al., 2011) and *Staphylococcus* spp. (Saijonmaa-Koulumies et al., 2008).

Despite the existence of numerous infectious ocular diseases in canines, there has been a paucity of research examining the microbial community associated with the eye. These microbial community surveys are typically conducted using high-throughput metagenomics analyses of genes encoding ribosomal RNA (Huang et al., 2009). Presently, sequencing of the 16S ribosomal RNA gene is the unanimously accepted and most widely used approach in bacterial community profiling (Petrosino et al., 2008; Davenport and Tummler, 2013). For example, pyrosequencing of the 16S rRNA gene was used to

investigate the canine gastrointestinal microbiome where differences were observed between phyla depending on the diet of the animal (Swanson et al., 2011).

Similarly, a 16S rRNA pyrosequencing study was conducted to compare the duodenal microbiota of dogs exhibiting chronic idiopathic inflammatory bowel disease (IBD) with that from healthy dogs (Suchodolski et al., 2012). That study determined that the canine duodenum harbors a complex microbial community comprised of several bacterial phyla, including Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteria (Suchodolski et al., 2012). Notably, there was an increase in sequences belonging to Proteobacteria, and a decrease in species within Bacteroidetes, Fusobacteria, and Firmicutes with respect to dogs with idiopathic IBD (Suchodolski et al., 2012). An additional pyrosequencing-based metagenomics study investigated the oral cavity of six healthy dogs (Sturgeon et al., 2013). This analysis revealed the existence of several different phyla, including Bacteroidetes, Proteobacteria, Firmicutes, Fusobacteria, and Spirochaetes. Among the genera identified in significant quantity were *Porphyromonas*, *Fusobacterium*, *Capnocytophaga*, *Derxia*, *Moraxella*, and *Bergeyella*. The taxonomic range of the bacteria identified was attributed to the power of metagenomics analysis when compared to traditional culturing techniques (Sturgeon et al., 2013).

The goal of the present study was to build upon the existing canine microbiome knowledge base by exploring the bacterial diversity in the lacrimal caruncle region of domestic dogs using a metagenomics approach. Specifically,

the V4 region of the 16S gene was sequenced from total genomic DNA using Illumina's MiSeq v3 platform (Caporaso et al., 2012). This approach provided snapshots of the bacterial communities associated with the eyes of five individual dogs of varying breeds, including a Golden Retriever (Dog A), Weimaraner (Dog B), Shih Tzu mix (Dog C), Yorkie mix (Dog D) and Dachshund (Dog E).

METHODS

Sample Collection

In accordance with Eastern Illinois University IACUC protocol 13-001, all samples were obtained from dogs (with owner consent) at a local veterinary clinic (Animal Medical Center, Charleston, IL) with a veterinarian present. The five dogs examined represented five different breeds: Golden Retriever (Dog A), Weimaraner (Dog B), Shih Tzu mix (Dog C), Yorkie mix (Dog D), and Dachshund (Dog E) (Table 1). Fluid and debris was collected from the lacrimal caruncle region (Figure 1) with a sterile Cary-Blair swab (rayon tipped) and transported in sterile agar. Total DNA was extracted from each swab using a FastDNA® Spin Kit (MP Biomedicals; Solon, OH) and quantified using an Epoch Microplate Spectrophotometer (BioTek; Winooski, VT).

Table 1. Identification and characteristics of dogs sampled in the current study.

ID	Breed	Sex/Status	Age (years)	Eye Disease History
A	Golden Retriever	F/S	5.5	none
B	Weimaraner	F/S	5.5	none
C	Shih Tzu mix	F/S	unknown/adult	none
D	Yorkie mix	M/N	3.5	none
E	Dachshund	M/N	unknown/adult	none

F=female, M=male, S=spayed, N=neutered

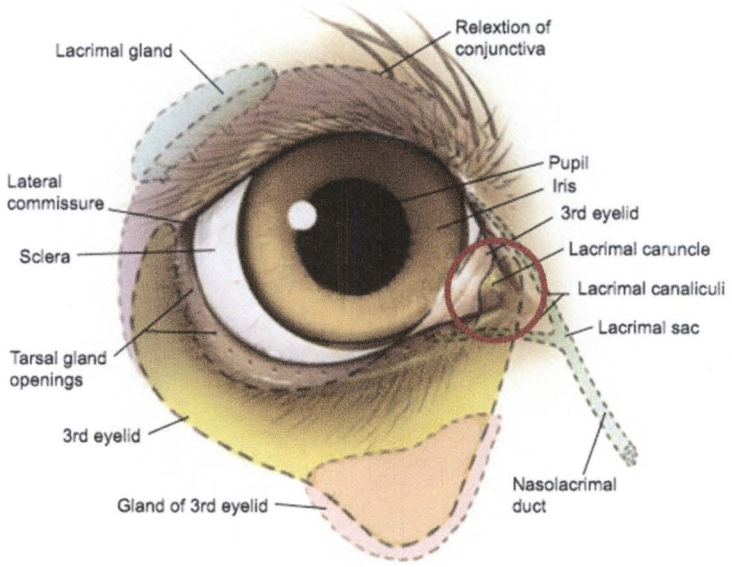

Figure 1. Superficial anatomy of a dog eye highlighting the lacrimal caruncle region that was examined in the present study. Image courtesy of McCracken et al. (2008).

Polymerase Chain Reaction (PCR)

Primer sets containing Illumina MiSeq-based barcodes and adaptor sequences (SA507-508 and SA701-709) were used to amplify the 16S rRNA V4 region as previously described (Kozich et al., 2013). Each PCR was performed using 5X Taq Master Mix (New England Biolabs; Ipswich, MA) with 10 pmol of each primer using the following thermocycler program: 95°C for 3 min, followed by 35 cycles of 95°C for 30s, 53°C for 30s, 68°C for 30s, and then 72°C for 5 min. The PCR product was separated using agarose gel electrophoresis, excised and purified using a QIAquick Gel Extraction Kit (Qiagen Laboratories; Hilden, Germany). Samples were quantified as above, each diluted to 1 ng/μL, and combined. The pooled sample was then analyzed using an Illumina MiSeq v3 platform at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

Sequence Analysis

All sequences were processed using the program mothur v.1.33.3 (Schloss et al. 2009) following modified standard operating procedures (Schloss et al. 2011; Kozich et al. 2013). Briefly, individual forward and reverse fastq files were used to generate contigs that were screened whereby all contigs that did not have at least a 50 bp overlap, and/or had ambiguous base calls, were culled. Sequences were aligned against the comprehensive SILVA bacterial alignment (v102; www.arb-silva.de) and pseudo-single-linkage clustered to reduce sequencer origin error (Huse et al. 2010). Potential chimeric reads were identified and removed using the mothur implemented program UCHIME (Edgar et al. 2011) where a total of 4.5% reads were identified as purportedly chimeric and

subsequently culled. Remaining sequences were classified using the naïve Bayesian autoclassifier (Wang et al. 2007) against the mothur implemented bacterial training set (v.10) using a 50% cutoff threshold and all non-bacteria, mitochondrial, and plastid sequences were culled. Operational taxonomic units (OTUs) were binned using an average neighbor algorithm (UPGMA) using 97% similarity threshold for OTU inclusion. This initial clustering resulted in 3,763 OTUs, most of which were exceedingly uncommon. All rare OTUs (defined here as containing 10 or fewer sequences) were eliminated, resulting in 370 OTUs.

Data Analysis

The OTU data were organized into taxonomic levels (order and family) using Microsoft Excel. For visual interpretation, pie charts were created showing those families that contributed 2% or greater of the total OTU reads (1% or greater for Dog A). Summary tables of total OTU counts (including those with less than 2% of total reads) grouped at the family and order levels are appended (Appendix Tables A1-A4). The total OTU counts at the family level for each eye of each dog were assessed holistically using hierarchical clustering analysis with the McQuitty method (Wessa, 2012).

RESULTS AND DISCUSSION

The surface of the canine eye is enriched with nutrients, which supports a host of microorganisms that make up the collective microbiota of the eye (Armstrong, 2000). However, canine eyes have a plethora of defenses to prevent infection, which includes the tear film, the orbit, eyelids, cilia, epithelia of the cornea, as well as that of the conjunctiva. Due to these natural defense mechanisms, canine eyes tend to be sparingly colonized or infected with these microorganisms (Armstrong, 2000). However, they are not immune to pathogenic bacteria, such as those from the genera *Pseudomonas* and *Staphylococcus* (Saijonmaa-Koulumies et al., 2008; Ledbetter et al., 2009; Santos et al., 2011).

Despite linkages of bacteria to eye health, the ocular microbiota of canines and the sum of its genetic parts (the microbiome) has been inadequately investigated. Previous studies have investigated infectious microorganisms involved with ocular disease (*e.g.* uveitis) on an individual basis, such as *Toxoplasma gondii*, *Leishmania donovani*, *Borrelia burgdorferi*, *Dirofilaria immitis*, *Ehrlichia canis*, and *Rickettsia ricketsii* (Massa et al., 2002); however, no single study has explored the bacterial microbiome of the canine periocular region. The ability to efficiently survey the microbiome of the canine eye has the potential to allow veterinarians and researchers to understand the associated microbial community structure, which may help determine a course of treatment to mitigate or prevent disease.

In the present study, the microbiomes associated with the ocular cavity of domestic dogs were investigated. Swab samples from both eyes were taken

from the lacrimal caruncle region at a local veterinary clinic from five dogs of the following breeds: Golden Retriever (spayed female; Dog A), Weimaraner (spayed female; Dog B), Shih Tzu mix (spayed female; Dog C), Yorkie mix (neutered; male Dog D), and Dachshund (neutered male; Dog E). None of the five breeds of dogs had had a history of ocular disease, or had signs and symptoms suggestive of any form of illness or disorder. Total DNA was isolated from each swab, followed by 16S-based sequence analysis using Illumina's MiSeq v3 platform. This generated a total of 370 operational taxonomic units (OTUs) across all five dogs in this study.

To visualize the periocular microbiome of each dog, pie charts were constructed with data at the family level. Any family that contributed to 2% or more of the total number of OTUs from each eye were included, with the exception of Dog A at 1% or higher (Figures 2-6). The lower cutoff point with the Dog A was necessary due to the overwhelming contribution to a single family (Pseudomonadaceae). However, all OTU counts at both the family and order levels can be found in Appendix Tables A1-A4. Using these pie charts to visualize trends, the microbiomes from Dog A and Dog B were similar with respect to the families present and their relative abundance (Figures 2 and 3), while the microbiomes of the small breeds (Dogs C, D and E; Figures 4-6) appear more similar to each other than those of the larger breeds from this study. Hierarchical clustering analysis at the family level confirmed this observation, with the left and right eyes being more similar to each other within the large breed dogs that were examined (Dogs A and B). However, the same analysis

revealed that the microbiomes among eyes of the small breeds do not cluster by individual dog. For example, the left eye microbiomes of Dog E and D are more similar to each other than their respective right eye microbiomes. This was not unexpected when considering the similarity of the microbial profiles among these small breeds (Figures 4-6), especially between Dogs D and E.

Among the more notable observations from the data was that the lacrimal caruncle region of Dogs A and B harbored very high percentages of Pseudomonadaceae (up to 65% in the right eye of Dog A), which were attributed entirely to the genus *Pseudomonas*. This genus of bacteria has been associated with a variety of diseases and disorders in canines, including those affecting the eye (Ledbetter et al., 2009; Santos et al., 2011) and skin (Hillier et al., 2006). *Pseudomonas* has also been isolated from the ear of dogs where it has been implicated with otitis, which is difficult to treat using traditional antimicrobial therapy (Pye et al., 2014). Interestingly, neither Dogs A nor B examined in this study had current or past symptoms of eye or skin distress.

Both Dogs A and B also had relatively high percentages of Moraxellaceae (up to 21% in the left eye of Dog A), which were almost entirely from the *Psychrobacter* and *Acinetobacter* genera. As expected from larger breeds, Dogs A and B in this study routinely spent time outside of the home. This behavior would have exposed these dogs to a broader range of bacteria, which may have led to the increased prevalence of these pathogens. For instance, the smaller and more frequently indoor breeds (Dogs C, D, and E) had less than 3% of total reads per eye from the family Pseudomonadaceae. However, this hypothesis is

extremely speculative, and to explore this further would require additional sampling across a broad range of breeds with varying levels of outdoor activity. Moreover, it should be noted that of the smaller indoor breeds, Dog C also had relatively high levels of Moraxellaceae (19% in the left eye and 5% in the right eye).

The data also showed a clear similarity between Dogs D and E, and Dog C to a lesser extent (Figures 4-7), with the dominant family members being Verrucomicrobiaceae, Sphingomonadaceae and Flavobacteriaceae. The general microbial community structure of these small dogs was similar to that of Dog B (when excluding the contributions from Moraxellaceae and Pseudomonadaceae; Figure 3). Generally, Dog A had the most unique bacterial profile in terms of families that were represented (Figures 2 and 7), with samples from this dog having contributions from families that were not observed above 2% of total OTUs in the other dogs. For example, the Oxalobacteraceae (*Massilia* spp.), Micrococcaceae (*Arthrobacter* spp.) and Enterobacteriaceae (*Pantoea* spp.) families were uniquely found in Dog A at levels above 2% of the total OTUs.

Conclusion

Metagenomic analysis is becoming increasingly affordable due to advances in sequencing technology, such as Illumina's MiSeq v3 platform that was used in the present study. This has made routine clinical use of metagenomics analysis a possibility. With microbiome data on hand, a veterinarian would be able to assess the bacterial community structure of an area of interest to help prevent, diagnose and/or treat bacterial-influenced diseases or disorders. For example, a

high level of *Pseudomonas* spp. was observed in the lacrimal caruncle region of Dog A examined in the current study. Although this dog had no history of eye disease or disorder, a veterinarian may recommend a course of treatment to prevent future ocular illness from occurring. Furthermore, the routine clinical use of metagenomic techniques would help build a resource base that would allow veterinarians to establish baseline bacterial community structures, which would help to identify potentially harmful pathogens on a case-by-case basis as well as develop correlations to certain diseases and disorders.

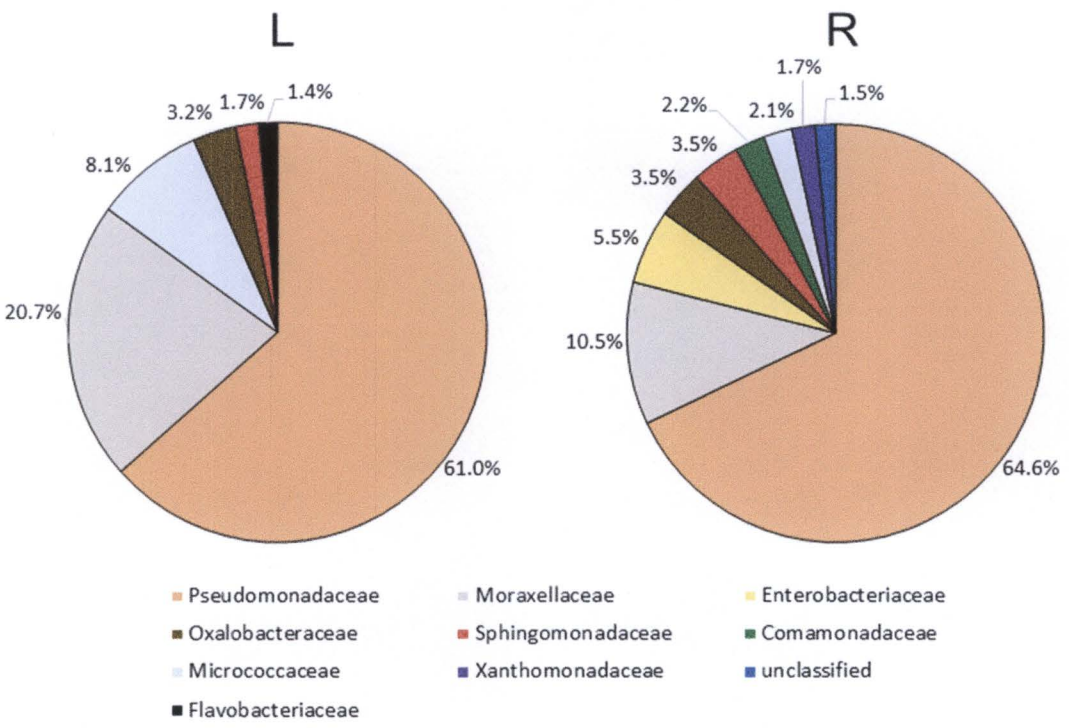

Figure 2. The microbial community profile of the lacrimal caruncle region from the left (L) and right (R) eyes of Dog A. Only those families contributing to 1% or greater of the total community are shown. Data were generated using the Illumina MiSeq v3 platform.

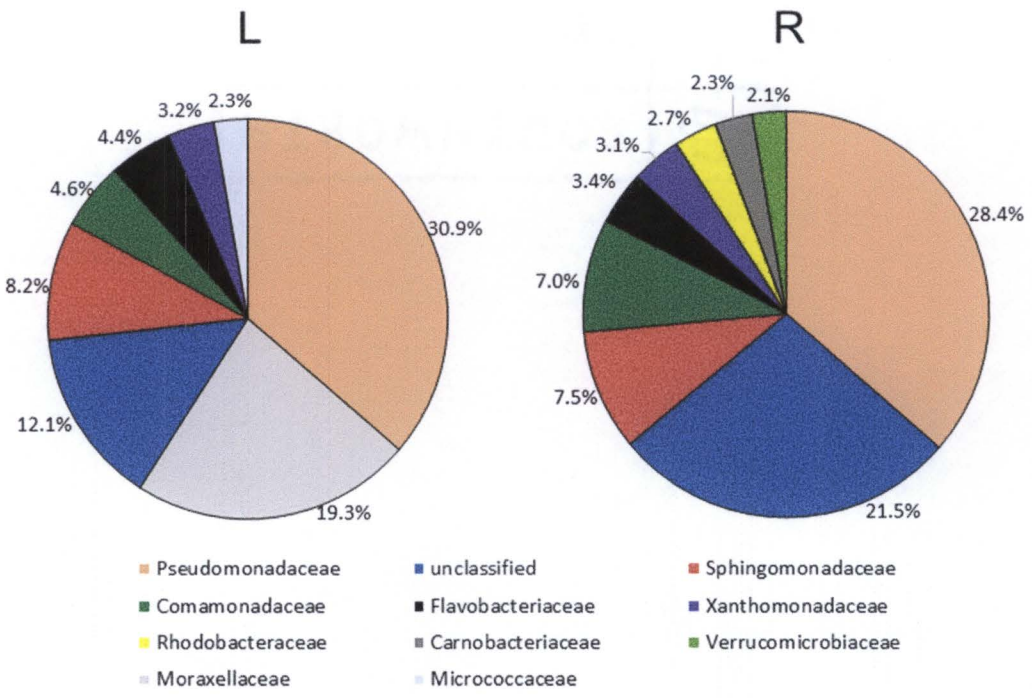

Figure 3. The microbial community profile of the lacrimal caruncle region from the left (L) and right (R) eyes of Dog B. Only those families contributing to 2% or greater of the total community are shown. Data were generated using the Illumina MiSeq v3 platform.

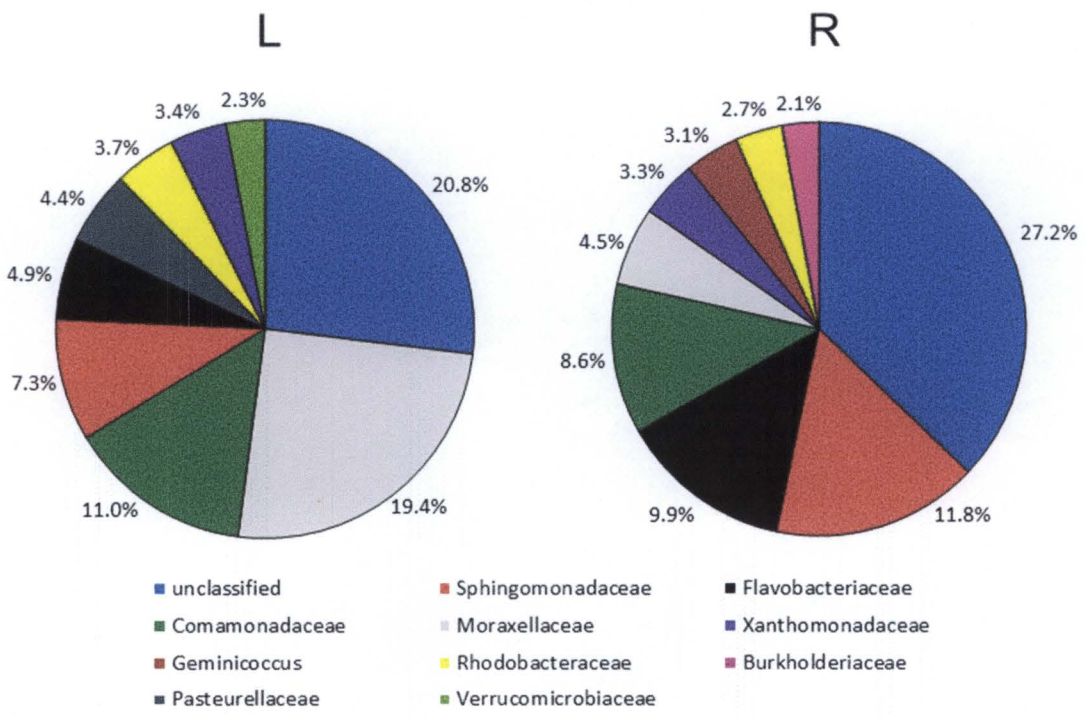

Figure 4. The microbial community profile of the lacrimal caruncle region from the left (L) and right (R) eyes of Dog C. Only those families contributing to 2% or greater of the total community are shown. Data were generated using the Illumina MiSeq v3 platform.

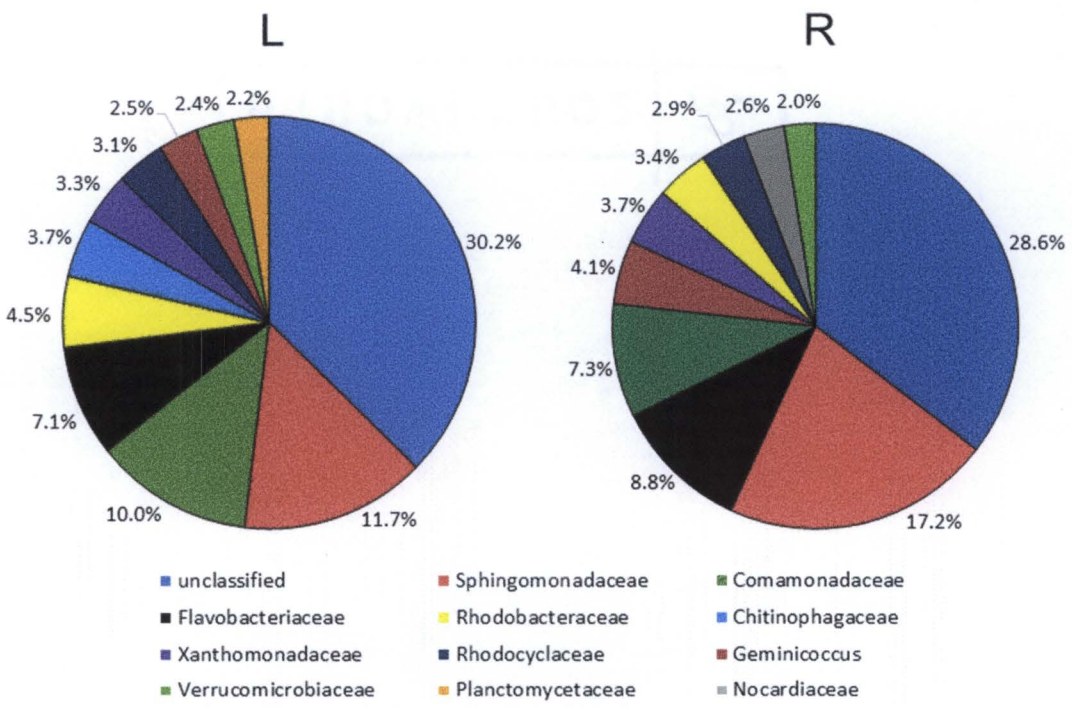

Figure 5. The microbial community profile of the lacrimal caruncle region from the left (L) and right (R) eyes of Dog D. Only those families contributing to 2% or greater of the total community are shown. Data were generated using the Illumina MiSeq v3 platform.

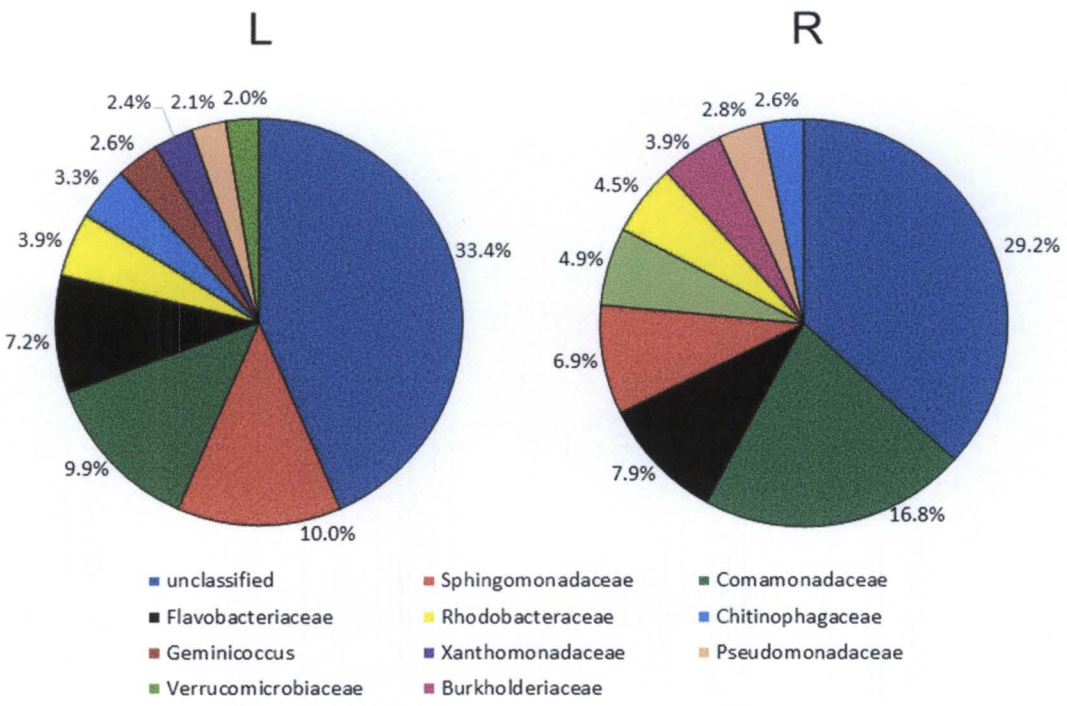

Figure 6. The microbial community profile of the lacrimal caruncle region from the left (L) and right (R) eyes of Dog E. Only those families contributing to 2% or greater of the total community are shown. Data were generated using the Illumina MiSeq v3 platform.

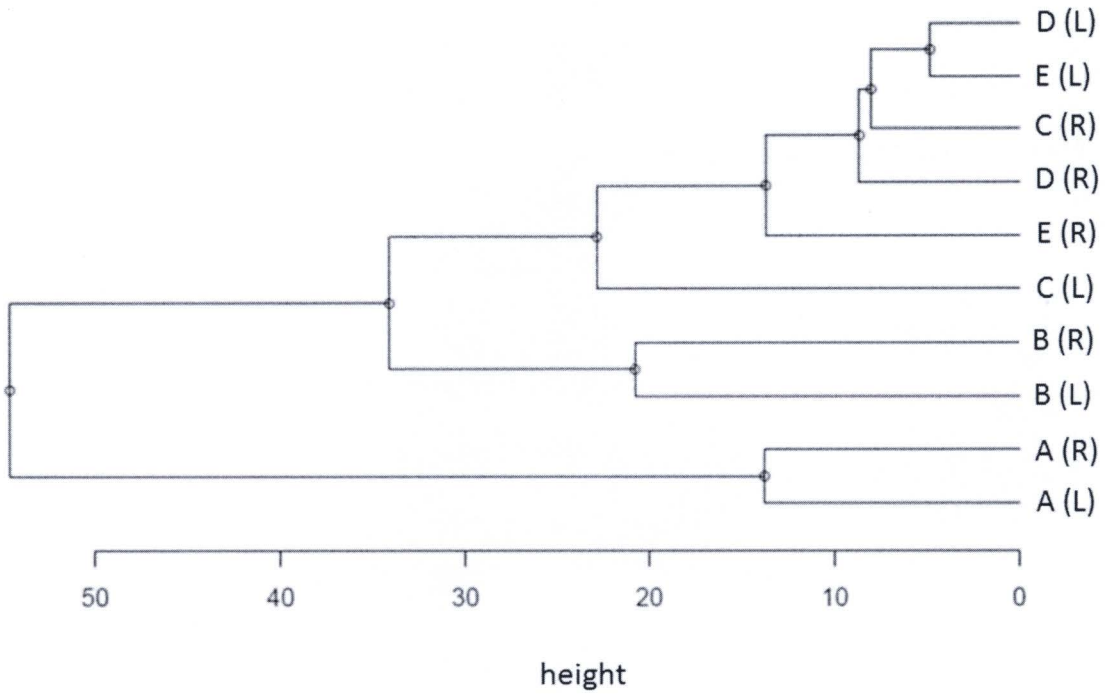

Figure 7. A hierarchical cluster analysis (McQuitty method) at the family level (79 families total) of the lacrimal caruncle microbiomes from the left (L) and right (R) eyes of Dogs A-E.

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APPENDIX

Table A1. The operational taxonomic unit (OTU) counts and percent of total OTUs for each sample at the family level for the left (L) and right (R) eyes of the Golden Retriever (G) and Weimaraner (W).

		GL%	GL	GR%	GR	WL%	WL	WR%	WR
1	unclassified	0.9	17	1.5	12	12.1	215	21.5	260
2	Moraxellaceae	20.7	410	10.5	86	19.3	343	1.5	18
3	Comamonadaceae	0.6	11	2.2	18	4.6	81	7.0	85
4	Sphingomonadaceae	1.7	33	3.5	29	8.2	146	7.5	91
5	Flavobacteriaceae	1.4	27	0.4	3	4.4	79	3.4	41
6	Pasteurellaceae	0.0	0	0.2	2	0.0	0	0.0	0
7	Rhodobacteraceae	0.1	2	0.2	2	1.5	27	2.7	33
8	Xanthomonadaceae	0.5	10	1.7	14	3.2	56	3.1	38
9	Verrucomicrobiaceae	0.1	1	0.5	4	0.8	14	2.1	26
10	Chitinophagaceae	0.2	4	0.1	1	0.4	7	0.9	11
11	Planctomycetaceae	0.0	0	0.0	0	0.3	6	0.9	11
12	Rhodocyclaceae	0.0	0	0.4	3	0.5	9	1.1	13
13	Nocardiaceae	0.	0	0.0	0	0.6	11	0.6	7
14	Bradyrhizobiaceae	0.1	2	0.0	0	0.3	5	0.1	1
15	Alcaligenaceae	0.0	0	0.1	1	0.2	4	0.7	8
16	Corynebacteriaceae	0.0	0	0.0	0	0.0	0	0.2	3
17	Caulobacteraceae	0.1	2	0.6	5	0.1	2	0.3	4
18	Erythrobacteraceae	0.0	0	0.0	0	0.5	8	0.2	3
19	Pseudomonadaceae	61.0	1210	64.6	528	30.9	548	28.4	344
20	Hyphomicrobiaceae	0.0	0	0.0	0	0.2	4	0.7	9
21	Micromonosporaceae	0.0	0	0.0	0	0.0	0	0.1	1
22	Saprospiraceae	0.0	0	0.0	0	0.6	10	0.2	2
23	Acidimicrobiaceae	0.0	0	0.0	0	0.1	1	0.2	3
24	Bacillaceae	0.0	0	0.0	0	0.4	7	0.2	2
25	Micrococcaceae	8.1	160	2.1	17	2.3	40	1.8	22
26	Neisseriaceae	0.0	0	0.0	0	0.3	5	0.6	7
27	Peptostreptococcaceae	0.0	0	0.0	0	0.1	2	0.1	1
28	Acetobacteraceae	0.0	0	0.0	0	0.0	0	0.0	0
29	Anaerolineaceae	0.0	0	0.0	0	0.1	1	0.1	1
30	Gaiellaceae	0.0	0	0.0	0	0.2	4	0.3	4
31	Geminicoccus	0.1	1	0.5	4	1.4	24	1.1	13
32	Methylophilaceae	0.0	0	0.2	2	0.1	1	0.8	10
33	Rhizomicrobium	0.0	0	0.0	0	0.2	3	0.2	3
34	Staphylococcaceae	0.0	0	0.0	0	0.0	0	0.1	1
35	Burkholderiaceae	0.1	1	0.2	2	0.3	5	0.2	3
36	Conexibacteraceae	0.1	2	0.0	0	0.2	3	0.2	3
37	Flammeovirgaceae	0.0	0	0.0	0	0.1	1	0.0	0
38	Hydrogenophilaceae	0.0	0	0.0	0	0.1	1	0.1	1
39	Methylococcaceae	0.0	0	0.0	0	0.3	6	0.5	6
40	Nannocystaceae	0.0	0	0.0	0	0.2	4	0.2	2
41	Rhizobiaceae	0.0	0	0.0	0	0.5	8	0.1	1
42	Sinobacteraceae	0.1	2	0.0	0	0.3	6	0.2	2
43	Acidimicrobineae	0.0	0	0.0	0	0.0	0	0.5	6
44	Burkholderiales	0.0	0	0.0	0	0.1	1	0.1	1
45	Cytophagaceae	0.1	1	0.4	3	0.1	1	0.6	7
46	Gemmatimonadaceae	0.0	0	0.0	0	0.1	1	0.3	4
47	Intrasporangiaceae	0.0	0	0.0	0	0.0	0	0.0	0
48	Microbacteriaceae	0.2	4	0.0	0	0.0	0	0.2	2
49	Nitrospiraceae	0.0	0	0.0	0	0.1	1	0.1	1
50	Oxalobacteraceae	3.2	63	3.5	29	1.1	19	1.2	14
51	Phyllobacteriaceae	0.0	0	0.0	0	0.0	0	0.0	0
52	Rhodobiaceae	0.0	0	0.0	0	0.1	1	0.2	2
53	Thiohalomonas	0.0	0	0.0	0	0.0	0	0.0	0
54	Trueperaceae	0.0	0	0.0	0	0.0	0	0.0	0
55	Aeromonadaceae	0.0	0	0.1	1	0.2	3	0.2	2
56	Alteromonadaceae	0.0	0	0.0	0	0.1	1	0.2	3

		GL%	GL	GR%	GR	WL%	WL	WR%	WR
57	Beijerinckiaceae	0.0	0	0.0	0	0.0	0	0.2	2
58	Caldilineaceae	0.0	0	0.0	0	0.0	0	0.1	1
59	Carnobacteriaceae	0.4	7	0.0	0	0.3	5	2.3	28
60	Cellulomonadaceae	0.0	0	0.1	1	0.2	3	0.3	4
61	Chromatiaceae	0.0	0	0.0	0	0.2	3	0.1	1
62	Clostridiaceae	0.0	0	0.0	0	0.1	1	0.1	1
63	Cryomorphaceae	0.0	0	0.0	0	0.0	0	0.0	0
64	Cystobacteraceae	0.0	0	0.0	0	0.1	1	0.0	0
65	Enterobacteriaceae	0.3	6	5.5	45	0.5	9	0.0	0
66	Geobacteraceae	0.0	0	0.0	0	0.0	0	0.0	0
67	Kineosporiaceae	0.0	0	0.0	0	0.4	7	0.2	3
68	Leptospiraceae	0.0	0	0.0	0	0.2	3	0.0	0
69	Mycobacteriaceae	0.0	0	0.0	0	0.0	0	0.2	2
70	Nitrosomonadaceae	0.0	0	0.0	0	0.0	0	0.0	0
71	Nocardioidaceae	0.0	0	0.0	0	0.1	1	0.1	1
72	Opitutaceae	0.0	0	0.0	0	0.0	0	0.5	6
73	Polyangiaceae	0.0	0	0.0	0	0.1	1	0.1	1
74	Pseudonocardiaceae	0.0	0	0.0	0	0.1	2	0.2	2
75	Sanguibacteraceae	0.0	0	0.1	1	0.2	4	1.5	18
76	Solirubrobacteraceae	0.0	0	0.0	0	0.1	1	0.0	0
77	Sphaerobacteraceae	0.0	0	0.0	0	0.1	1	0.2	2
78	Sporichthyaceae	0.0	0	0.0	0	0.1	1	0.1	1
79	Xanthobacteraceae	0.0	0	0.1	1	0.2	4	0.0	0
	TOTAL		1976		814		1772		1209

Table A2. The operational taxonomic unit (OTU) counts and percent of total OTUs for each sample at the family level for the left (L) and right (R) eyes of the Shih Tzu mix (S), Yorkie mix (Y), and Dachshund (D).

		SL%	SL	SR%	SR	DL%	DL	DR%	DR	YR%	YR	YL%	YL
1	unclassified	20.8	165	27.2	905	33.4	1922	29.2	189	30.2	317	28.6	176
2	Moraxellaceae	19.4	154	4.5	151	0.9	51	0.6	4	0.5	5	0.3	2
3	Comamonadaceae	11.0	87	8.6	286	9.9	570	16.8	109	10.0	105	7.3	45
4	Sphingomonadaceae	7.3	58	11.8	393	10.0	573	6.9	45	11.7	123	17.2	106
5	Flavobacteriaceae	4.9	39	9.9	330	7.2	412	7.9	51	7.1	75	8.8	54
6	Pasteurellaceae	4.4	35	0.3	9	0.0	0	0.0	0	0.0	0	0.0	0
7	Rhodobacteraceae	3.7	29	2.7	90	3.9	224	4.5	29	4.5	47	3.4	21
8	Xanthomonadaceae	3.4	27	3.3	110	2.4	138	1.1	7	3.3	35	3.7	23
9	Verrucomicrobiaceae	2.3	18	1.8	61	2.0	114	4.9	32	2.4	25	2.0	12
10	Chitinophagaceae	1.9	15	1.9	62	3.3	187	2.6	17	3.7	39	1.8	11
11	Planctomycetaceae	1.8	14	0.9	29	1.3	77	0.2	1	2.2	23	1.1	7
12	Rhodocyclaceae	1.5	12	1.1	35	1.7	99	1.5	10	3.1	32	2.9	18
13	Nocardiaceae	1.3	10	1.7	58	1.3	72	0.6	4	1.0	10	2.6	16
14	Bradyrhizobiaceae	1.1	9	0.6	19	0.6	32	0.3	2	1.2	13	0.8	5
15	Alcaligenaceae	1.0	8	0.6	20	0.7	38	0.5	3	0.3	3	1.0	6
16	Corynebacteriaceae	1.0	8	0.4	13	0.0	0	0.0	0	0.0	0	0.0	0
17	Caulobacteraceae	0.9	7	0.4	14	0.5	30	0.8	5	0.8	8	0.5	3
18	Erythrobacteraceae	0.9	7	0.5	18	0.7	38	0.2	1	0.6	6	0.2	1
19	Pseudomonadaceae	0.9	7	1.6	52	0.2	9	2.8	18	0.9	9	1.5	9
20	Hyphomicrobiaceae	0.6	5	1.1	38	0.6	35	0.3	2	0.1	1	0.5	3
21	Micromonosporaceae	0.6	5	0.1	4	0.3	15	0.0	0	0.3	3	0.0	0
22	Saprospiraceae	0.6	5	0.4	14	0.8	48	0.0	0	0.4	4	0.7	4
23	Acidimicrobiaceae	0.5	4	0.0	0	0.5	27	0.0	0	0.2	2	0.0	0
24	Bacillaceae	0.5	4	0.2	5	0.3	19	0.2	1	0.5	5	0.0	0
25	Micrococcaceae	0.5	4	0.4	13	0.3	16	0.6	4	0.2	2	0.3	2
26	Neisseriaceae	0.5	4	0.6	19	0.5	26	0.5	3	0.1	1	1.0	6
27	Peptostreptococcaceae	0.5	4	0.1	4	0.4	25	0.0	0	0.2	2	0.7	4
28	Acetobacteraceae	0.4	3	0.1	4	0.1	3	0.0	0	0.5	5	0.0	0
29	Anaerolineaceae	0.4	3	0.0	0	0.1	6	0.0	0	0.0	0	0.0	0
30	Gaiellaceae	0.4	3	0.7	22	0.9	50	0.0	0	0.3	3	0.7	4
31	Geminicoccus	0.4	3	3.1	102	2.6	147	1.2	8	2.5	26	4.1	25
32	Methylophilaceae	0.4	3	1.2	39	1.4	78	1.4	9	1.6	17	0.3	2
33	Rhizomicrobium	0.4	3	0.2	6	0.4	24	0.0	0	0.0	0	0.2	1
34	Staphylococcaceae	0.4	3	0.8	25	0.0	0	0.0	0	0.0	0	0.0	0
35	Burkholderiaceae	0.3	2	2.1	71	0.4	25	3.9	25	1.8	19	0.2	1
36	Conexibacteraceae	0.3	2	0.2	8	0.4	22	0.0	0	0.1	1	1.3	8
37	Flammeovirgaceae	0.3	2	0.2	5	0.2	10	0.0	0	0.1	1	0.3	2
38	Hydrogenophilaceae	0.3	2	0.1	3	0.0	0	0.8	5	0.0	0	0.0	0
39	Methylococcaceae	0.3	2	0.3	11	0.6	32	0.3	2	0.5	5	0.0	0
40	Nannocystaceae	0.3	2	0.1	3	0.2	13	0.0	0	0.5	5	0.3	2
41	Rhizobiaceae	0.3	2	0.2	7	0.3	15	0.0	0	0.4	4	0.7	4
42	Sinobacteraceae	0.3	2	0.6	21	0.3	20	0.6	4	0.2	2	0.2	1
43	Acidimicrobineae	0.1	1	0.2	6	0.5	28	0.0	0	0.2	2	0.0	0
44	Burkholderiales	0.1	1	0.2	5	0.4	23	0.6	4	0.2	2	0.2	1
45	Cytophagaceae	0.1	1	0.7	22	0.5	29	0.9	6	1.1	12	0.0	0
46	Gemmatimonadaceae	0.1	1	0.5	15	0.3	19	0.0	0	0.7	7	0.3	2
47	Intrasporangiaceae	0.1	1	0.2	6	0.1	5	0.0	0	0.0	0	0.2	1
48	Microbacteriaceae	0.1	1	0.2	8	0.2	14	0.3	2	0.1	1	0.2	1
49	Nitrospiraceae	0.1	1	0.0	1	0.1	8	0.3	2	0.0	0	0.3	2
50	Oxalobacteraceae	0.1	1	0.4	12	0.6	34	0.5	3	0.	5	0.2	1
51	Phyllobacteriaceae	0.1	1	0.0	0	0.1	7	0.0	0	0.2	2	0.2	1
52	Rhodobiaceae	0.1	1	0.1	3	0.3	18	0.0	0	0.0	0	0.0	0
53	Thiohalomonas	0.1	1	0.2	6	0.1	4	0.0	0	0.0	0	0.0	0
54	Trueperaceae	0.1	1	0.0	0	0.1	4	1.1	7	0.1	1	0.2	1
55	Aeromonadaceae	0.0	0	0.3	11	0.3	18	0.0	0	0.2	2	0.2	1
56	Alteromonadaceae	0.0	0	0.4	14	0.3	20	0.3	2	0.2	2	0.7	4
57	Beijerinckiaceae	0.0	0	0.1	4	0.1	4	0.0	0	0.0	0	0.0	0
58	Caldilineaceae	0.0	0	0.0	0	0.1	7	0.5	3	0.0	0	0.0	0
59	Carnobacteriaceae	0.0	0	0.1	3	0.0	2	0.0	0	0.1	1	0.0	0

		SL%	SL	SR%	SR	DL%	DL	DR%	DR	YR%	YR	YL%	YL
60	Cellulomonadaceae	0.0	0	0.1	3	0.2	9	0.0	0	0.1	1	0.0	0
61	Chromatiaceae	0.0	0	0.0	0	0.1	4	0.0	0	0.2	2	0.0	0
62	Clostridiaceae	0.0	0	0.0	1	0.1	8	0.0	0	0.1	1	0.7	4
63	Cryomorphaceae	0.0	0	0.2	6	0.3	19	0.0	0	0.1	1	0.0	0
64	Cystobacteraceae	0.0	0	0.2	7	0.0	1	0.0	0	0.2	2	0.2	1
65	Enterobacteriaceae	0.0	0	0.0	0	0.1	8	0.0	0	0.0	0	0.0	0
66	Geobacteraceae	0.0	0	0.0	1	0.1	6	0.0	0	0.0	0	0.0	0
67	Kineosporiaceae	0.0	0	0.8	26	0.4	24	0.0	0	0.4	4	0.8	5
68	Leptospiraceae	0.0	0	0.1	3	0.1	5	0.0	0	0.2	2	0.0	0
69	Mycobacteriaceae	0.0	0	0.2	8	0.2	14	0.3	2	0.1	1	0.2	1
70	Nitrosomonadaceae	0.0	0	0.2	7	0.3	15	0.0	0	0.2	2	0.0	0
71	Nocardioidaceae	0.0	0	0.0	1	0.1	4	0.3	2	0.2	2	0.0	0
72	Opitutaceae	0.0	0	0.0	0	0.1	4	0.0	0	0.2	2	0.0	0
73	Polyangiaceae	0.0	0	0.0	1	0.1	4	0.6	4	0.0	0	0.0	0
74	Pseudonocardiaceae	0.0	0	0.0	6	0.2	9	0.3	2	0.1	1	0.0	0
75	Sanguibacteraceae	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
76	Solirubrobacteraceae	0.0	0	0.1	3	0.0	2	0.3	2	0.0	0	0.0	0
77	Sphaerobacteraceae	0.0	0	0.2	8	0.0	1	0.0	0	0.1	1	0.2	1
78	Sporichthyaceae	0.0	0	0.1	4	0.1	7	0.0	0	0.3	3	0.2	1
79	Xanthobacteraceae	0.0	0	0.1	4	0.1	6	0.0	0	0.1	1	0.2	1
	TOTAL		793		3284		5636		631		1046		613

Table A3. The operational taxonomic unit (OTU) counts and percent of total OTUs for each sample at the order level for the left (L) and right (R) eyes of the Golden Retriever (G) and Weimaraner (W).

		GL	GL%	GR	GR%	WL	WL%	WR	WR%
1	Burkholderiales	77	3.9	53	6.5	129	7.3	140	11.6
2	Sphingobacteriales	38	1.9	30	6.5	165	9.3	110	9.1
3	unclassified	9	0.5	3	0.4	95	5.3	107	8.8
4	Flavobacteriales	27	1.4	3	0.4	79	4.4	41	3.4
5	Rhodobacterales	2	0.1	2	0.2	27	1.5	33	2.7
6	Rhizobiales	3	0.2	1	0.1	33	1.9	27	2.2
7	Xanthomonadales	12	0.6	14	1.7	62	3.5	40	3.3
8	Actinomycetales	170	8.6	23	2.8	74	3.5	70	5.8
9	Rhodocyclales	0	0.0	3	0.4	9	0.5	13	1.1
10	Gp6	1	0.1	2	0.2	22	1.2	24	2.0
11	Alphaproteobacteria	1	0.1	4	0.5	27	1.5	16	1.3
12	Verrucomicrobiales	1	0.1	4	0.5	14	0.8	26	2.1
13	Planctomycetales	0	0.0	0	0.0	6	0.3	11	0.9
14	Gp16	0	0.0	0	0.0	10	1.2	18	1.5
15	Methylophilales	0	0.0	2	0.2	1	0.1	10	0.8
16	Myxococcales	1	0.1	0	0.0	11	0.6	18	1.5
17	Pseudomonadales	1620	81.7	614	75.2	891	50.2	362	29.9
18	Cytophagales	1	0.1	3	0.4	2	0.1	7	0.6
19	Gp4	1	0.1	0	0.0	12	0.7	11	0.9
20	Caulobacterales	2	0.1	14	1.7	2	0.1	4	0.3
22	Bacillales	0	0.0	2	0.2	9	0.5	5	0.4
23	Gemmatimonadales	0	0.0	0	0.0	1	0.1	4	0.3
24	Gp17	0	0.0	0	0.0	9	0.5	8	0.7
25	Ohtaekwangia	0	0.0	1	0.1	5	0.3	5	0.4
26	Solirubrobacterales	2	0.1	0	0.0	14	0.8	15	1.2
28	Sphingomonadales	0	0.0	0	0.0	8	0.5	3	0.2
29	Acidimicrobiales	1	0.1	0	0.0	6	0.3	15	1.2
30	Methylococcales	0	0.0	0	0.0	6	0.3	6	0.5
31	Rhodospirillales	0	0.0	0	0.0	0	0.0	0	0.0
32	Chromatiales	0	0.0	0	0.0	3	0.2	1	0.1
33	Clostridiales	0	0.0	0	0.0	3	0.2	2	0.2
34	Gaiellales	0	0.0	0	0.0	4	0.2	4	0.3
35	Aeromonadales	0	0.0	1	0.1	3	0.2	2	0.2
36	Alteromonadales	0	0.0	0	0.0	1	0.1	3	0.2
37	Gp10	0	0.0	0	0.0	3	0.2	2	0.2
38	Nitrosomonadales	0	0.0	0	0.0	0	0.0	0	0.0
39	Opitutales	0	0.0	0	0.0	0	0.0	6	0.5
40	Spirochaetales	0	0.0	0	0.0	3	0.2	0	0.0
41	Deinococcales	0	0.0	0	0.0	0	0.0	0	0.0
42	Lactobacillales	7	0.4	0	0.0	5	0.3	28	2.3
43	Neisseriales	0	0.0	0	0.0	5	0.3	7	0.6
44	Sphaerobacterales	0	0.0	0	0.0	1	0.1	2	0.2
45	Anaerolineales	0	0.0	0	0.0	1	0.1	1	0.1
46	Blastocatella	0	0.0	0	0.0	2	0.1	0	0.0
47	Caldilineales	0	0.0	0	0.0	0	0.0	1	0.1
48	Chlamydiales	0	0.0	0	0.0	2	0.1	0	0.0
49	Desulfuromonadales	0	0.0	0	0.0	0	0.0	0	0.0
50	Enterobacteriales	6	0.3	45	5.5	9	0.5	1	0.1
51	Gammaproteobacteria	0	0.0	0	0.0	0	0.0	0	0.0
52	Hydrogenophilales	0	0.0	0	0.0	1	0.1	1	0.1
53	Nitrospirales	0	0.0	0	0.0	1	0.1	1	0.1
54	Pasteurellales	0	0.0	2	0.2	0	0.0	0	0.0
	Total	1982		826		1776		1211	

Table A4. The operational taxonomic unit (OTU) counts and percent of total OTUs for each sample at the order level for the left (L) and right (R) eyes of the Shih Tzu mix (S), Yorkie mix (Y), and Dachshund (D).

		SL	SL%	SR	SR%	DL	DL%	DR	DR %	YR	YR%	YL	YL
1	Burkholderiales	108	13.6	461	13.8	904	15.7	164	25.3	176	16.8	66	10.7
2	Sphingobacteriales	83	10.5	481	14.4	838	14.6	63	9.7	173	16.5	122	19.8
3	unclassified	100	12.6	466	14.0	913	15.9	108	16.7	152	14.5	83	13.5
4	Flavobacteriales	39	4.9	336	10.1	431	7.5	51	7.9	76	7.2	54	8.8
5	Rhodobacterales	29	3.7	90	2.7	224	3.9	29	4.5	47	4.5	21	3.4
6	Rhizobiales	29	3.7	120	3.6	195	3.4	14	2.2	41	3.9	21	3.4
7	Xanthomonadales	29	3.7	131	3.9	158	2.7	11	1.7	37	3.5	24	3.9
8	Actinomycetales	33	4.2	208	6.2	236	4.6	28	4.3	34	3.2	31	5.0
9	Rhodocyclales	12	1.5	35	1.1	99	1.7	10	1.5	32	3.1	18	2.9
10	Gp6	11	1.4	57	1.7	152	2.6	14	2.2	30	2.9	17	2.8
11	Alphaproteobacteria	6	0.8	108	3.2	171	3.0	8	1.2	26	2.5	26	4.2
12	Verrucomicrobiales	18	2.3	61	1.8	114	2.0	32	4.9	25	2.4	12	2.0
13	Planctomycetales	14	1.8	29	0.9	77	1.3	1	0.2	23	2.2	7	1.1
14	Gp16	0	0.0	44	1.3	121	2.1	6	0.9	18	1.7	7	1.1
15	Methylophilales	3	0.4	39	1.2	78	1.4	9	1.4	17	1.6	2	0.3
16	Myxococcales	2	0.3	25	0.8	48	0.8	6	0.9	14	1.3	5	0.8
17	Pseudomonadales	161	20.3	203	6.1	169	2.9	22	3.4	14	1.3	11	1.8
18	Cytophagales	3	0.4	27	0.8	39	0.7	6	0.9	13	1.2	2	0.3
19	Gp4	10	1.3	58	1.7	88	1.6	10	1.5	13	1.2	6	1.0
20	Caulobacterales	7	0.9	14	0.4	30	0.5	5	0.8	8	0.8	3	0.5
22	Bacillales	8	1.0	49	1.5	41	0.7	2	0.3	7	0.7	0	0.0
23	Gemmatimonadales	1	0.1	15	0.5	19	0.3	0	0.0	7	0.7	2	0.3
24	Gp17	0	0.0	9	0.3	41	0.7	0	0.0	7	0.7	1	0.2
25	Ohtaekwangia	3	0.4	22	0.7	57	1.0	2	0.3	6	0.6	8	1.3
26	Solirubrobacterales	9	1.1	55	1.7	83	1.4	7	1.1	6	0.6	25	4.1
28	Sphingomonadales	7	0.9	18	0.5	38	0.7	1	0.2	6	0.6	1	0.2
29	Acidimicrobiales	5	0.6	27	0.8	111	1.9	6	0.9	5	0.5	7	1.1
30	Methylococcales	2	0.3	11	0.3	32	0.6	2	0.3	5	0.5	0	0.0
31	Rhodospirillales	3	0.4	4	0.1	3	0.1	0	0.0	5	0.5	0	0.0
32	Chromatiales	0	0.0	3	0.1	5	0.1	5	0.8	4	0.4	0	0.0
33	Clostridiales	4	0.5	5	0.2	33	0.6	0	0.0	3	0.3	8	1.3
34	Gaiellales	3	0.4	22	0.7	50	0.9	0	0.0	3	0.3	4	0.7
35	Aeromonadales	0	0.0	11	0.3	18	0.3	0	0.0	2	0.2	1	0.2
36	Alteromonadales	0	0.0	14	0.4	20	0.3	2	0.3	2	0.2	4	0.7
37	Gp10	1	0.1	5	0.2	13	0.2	0	0.0	2	0.2	6	1.0
38	Nitrosomonadales	0	0.0	7	0.2	15	0.3	0	0.0	2	0.2	0	0.0
39	Opitutales	0	0.0	0	0.0	4	0.1	0	0.0	2	0.2	0	0.0
40	Spirochaetales	0	0.0	3	0.1	5	0.1	0	0.0	2	0.2	0	0.0
41	Deinococcales	1	0.1	0	0.0	4	0.1	7	1.1	1	0.1	1	0.2
42	Lactobacillales	0	0.0	3	0.1	2	0.0	0	0.0	1	0.1	0	0.0
43	Neisseriales	4	0.5	19	0.6	26	0.5	3	0.5	1	0.1	6	1.0
44	Sphaerobacterales	0	0.0	8	0.2	1	0.0	0	0.0	1	0.1	1	0.2
45	Anaerolineales	3	0.4	0	0.0	6	0.1	0	0.0	0	0.0	0	0.0
46	Blastocatella	3	0.4	4	0.1	5	0.1	0	0.0	0	0.0	0	0.0
47	Caldilineales	0	0.0	0	0.0	7	0.1	3	0.5	0	0.0	0	0.0
48	Chlamydiales	0	0.0	3	0.1	3	0.1	0	0.0	0	0.0	0	0.0
49	Desulfuromonadales	0	0.0	1	0.0	6	0.1	0	0.0	0	0.0	0	0.0
50	Enterobacteriales	0	0.0	0	0.0	8	0.1	4	0.6	0	0.0	0	0.0
51	Gammaproteobacteria	1	0.1	6	0.2	4	0.1	0	0.0	0	0.0	0	0.0
52	Hydrogenophilales	2	0.3	3	0.1	0	0.0	5	0.8	0	0.0	0	0.0
53	Nitrospirales	1	0.1	1	0.0	8	0.1	2	0.3	0	0.0	2	0.3
54	Pasteurellales	35	4.4	9	0.3	0	0.0	0	0.0	0	0.0	0	0.0
	TOTAL	793		3330		5753		648		1049		615	