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# Determination of microbial diversity in the ocular conjunctiva of healthy dogs by total DNA sequencing

## Determinação da diversidade microbiana na conjuntiva ocular de cães saudáveis pelo sequenciamento completo do DNA

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**Abstract:** The conjunctiva plays an essential role in eye health and immunity and acts as a barrier to the entry of microorganisms. Conjunctival infections are common in dogs and result from both the invasion of pathogenic microorganisms and the uncontrolled growth of the existing microbiota. Most of the existing data come from studies based on traditional culture methods. These reports indicate the predominance of gram-positive bacteria, especially *Staphylococcus* spp. In the present study, we analyzed the microbiota present on the conjunctival surface from a heterogeneous dog population without ophthalmological disorders using DNA sequencing. After a thorough ophthalmological examination, conjunctival swabs were collected from both eyes of 30 dogs. After processing and nucleic acid extraction, the sample pool was subjected to shotgun DNA sequencing through the Illumina platform and analyzed via the Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) server. A predominance of the phylum Proteobacteria and the genera *Ralstonia* and *Burkholderia* were identified along with a minority of fungi, whereas viruses were not found. Microbial DNA sequencing has provided new data on this subject, revealing the presence of noncultivable organisms that were previously unknown as part of the ocular microbiome.

**Keywords:** bacteria; ophthalmology; eye; microbiome; metagenomics.

**Resumo:** A conjuntiva desempenha papel fundamental na saúde e imunidade ocular e atua como uma barreira à entrada de microrganismos. As infecções conjuntivais são comuns em cães e resultam tanto da invasão de microrganismos patogênicos quanto do crescimento descontrolado da microbiota existente. A maior parte dos dados existentes provém de estudos baseados em métodos de cultura tradicionais. Esses relatos apontam para o predomínio de bactérias gram-positivas, principalmente *Staphylococcus* spp. O presente estudo analisou a comunidade microbiana presente na conjuntiva ocular de uma população heterogênea de cães sem distúrbios oftalmológicos por sequenciamento de DNA. Após exame oftálmico minucioso, foram coletados suabe conjuntivais de ambos os olhos de 30 cães. Após processamento e extração de ácidos nucleicos, o *pool* de amostras foi submetido ao sequenciamento *shotgun* de DNA por meio da plataforma Illumina e analisado no servidor *Metagenomic*

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*Rapid Annotations using Subsystems Technology* (MG-RAST). Foi identificada uma predominância do filo Proteobacteria e dos gêneros *Ralstonia* e *Burkholderia* juntamente com uma minoria de fungos, enquanto vírus não foram encontrados. O sequenciamento do DNA microbiano trouxe novos dados sobre o assunto, revelando a presença de organismos não cultiváveis até então desconhecidos como parte do microbioma ocular.

**Palavras-chave:** bactéria; oftalmologia; olho; microbioma; metagenômica.

## 1. Introduction

The conjunctiva plays an essential role in lacrimal function, immune protection of the eye, eye mobility and corneal regeneration, and acts as a barrier to the entry of pathogenic microorganisms. [1,2] Conjunctival inflammation is one of the most commonly diagnosed eye disorders in veterinary practice; it occurs more rarely in isolation and is more commonly secondary to other inflammatory ocular diseases, such as ulcerative keratitis, tear production deficiencies and glaucoma. In some cases, etiology determination is challenging. [3] Infections can result from both the invasion of a pathogenic microorganism and the uncontrolled growth of the existing microbiota due to poor immunity. [4]

Infectious conjunctivitis in dogs may have bacterial, fungal, viral or parasitic origins. [3] The bacteria that have been described in clinical cases are *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Enterobacter* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Proteus* spp.; [5,6,7] moreover, the fungi that have been described includ *Blastomyces dermatitidis* and *Curvularia* spp. [8, 9] Among the viruses that had been previously reported, canine herpesvirus (CHV-1), canine adenovirus (CAV-1 and CAV-2) and canine distemper virus (CDV) are the main species identified in dogs. [10,11,12]

Regarding the conjunctival microbiota of healthy dogs, no viral species have been described in the studies available in the literature thus far. In contrast, the bacterial microbiota seems to be predominantly composed of *Staphylococcus* spp. according to traditional cultivation methods. [1,7,14,15,16] Furthermore, the fungi *Alternaria* spp., *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp. and yeast *Candida* species have been detected in the conjunctiva of healthy dogs using mycological culture methods, and are considered to be transitional microbiota. [16,17]

With the advent of next generation sequencing (NGS) techniques, knowledge of the uncultivable microbiota from different sites of healthy animals has provided important and more extensive information from those previously known using the culture-dependent methods. To date, only one study has described the ocular microbiota of dogs through NGS, which was specifically accomplished by using 16S rDNA amplicon sequencing. Although the authors demonstrated Firmicutes to be the most prevalent phylum, at the genus level there was a predominance of *Bifidobacterium* spp., a bacterium that had not been previously described in research. *Bifidobacterium* spp. were detected in 92.8% of the samples and accounted for 9.1% of all reads. [18]

When considering the importance of the conjunctiva in eye health and its frequent involvement in infectious conditions, it is extremely important to update the existing data on the ocular microbiota of healthy dogs through more refined molecular techniques, due

to the fact that they are currently available. The initial objective of the present study was to determine whether viruses inhabited the ocular conjunctiva of healthy dogs; if so, we aimed to identify these viruses. Furthermore, we aimed to analyze the conjunctival microbiota present at this site.

## 2. Materials and methods

The methodology that was applied in this study was approved by the Ethics Committee in Animal Use (CEUA) of the Federal University of Rio Grande do Sul (UFRGS) under protocol 35271. This study was also conducted according to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) on the use of animals in ophthalmic research.

### *2.1 Ophthalmic examination and sample collection*

The first stage of the study was conducted at the Veterinary Clinic Hospital of UFRGS in Porto Alegre, RS, Brazil, and included both eyes of 30 healthy dogs of different sexes, breeds, and ages that were domiciled in different environments. A description of the sampled population is available in Table 1.

All of the animals underwent ophthalmic evaluation performed by a professional ophthalmologist prior to sample collection, including the Schirmer tear test (Schirmer Lacrimal Test, Ophthalmos, São Paulo, Brazil), slit lamp biomicroscopy (SL15, Kowa Company, Nagoya, Japan), a fluorescein test (fluorescein sodium 1%, Ophthalmos, São Paulo, Brazil) and rebound tonometry (Tonovet®, Tiolat, Helsinki, Finland). The exclusion criteria previously used by Ledbetter were used in the current study. [12] The absence of eye diseases was noted. After this evaluation, a drop of anesthetic eye drop (1% tetracaine hydrochloride and 0.1% phenylephrine hydrochloride, Allergan, São Paulo, Brazil) was instilled onto the ocular surface. Under gentle manual restraint, conjunctival samples were collected with a sterile cotton swab (Absorve®, Jiangsu Suyun Medical Materials, Lianyungang, China) that touched the lower and upper palpebral conjunctiva and bulbar conjunctiva without contacting the outer surface of the eyelids.

The swabs were placed in DNase/RNase-free microtubes. No later than two hours after collection, the swabs were taken to the laboratory. Inside of a biosafety cabinet, each sample was diluted in 250 µl of ultrapure water plus 250 µl of DNA stabilization Buffer AS (Qiagen, Germantown, USA) to provide sample stability, after which samples were kept at -80 °C until processing.

**Table 1 Sample composition of the dogs that were used in the study.**

| Dog | Sex | Age (years) | Breed | Home Environment | Reason for being taken to the hospital |
|---|---|---|---|---|---|
| 1 | M | 8 | Lhasa Apso | House with garden | Ophthalmic check-up |
| 2 | F | 6 | Mixed breed | House with garden | Ophthalmic check-up |
| 3 | F | 10 | Yorkshire Terrier | Apartment | Clinical check-up and collection of blood tests |
| 4 | F | 12 | Poodle | Concrete yard house | Ophthalmic check-up |

| | | | | | |
|---|---|---|---|---|---|
| 5 | M | 1 | Pug | Apartment | Pre castration evaluation |
| 6 | F | 5 | Boxer | House with garden | Cardiac evaluation |
| 7 | M | 10 | Mixed breed | Farm | Clinical check-up and collection of blood tests |
| 8 | M | 12 | Mixed breed | House with garden | Ophthalmic check-up |
| 9 | F | 16 | American Cocker Spaniel | Apartment | Clinical check-up and collection of blood tests |
| 10 | F | 11 | Dachshund | Apartment | Cardiac evaluation |
| 11 | F | 6 | Mixed breed | House with garden | Ophthalmic check-up |
| 12 | F | 11 | Dalmatian | House with garden | Ophthalmic check-up |
| 13 | F | 1 | Yorkshire Terrier | House with garden | Pre castration evaluation |
| 14 | M | 2 | Shih Tzu | Apartment | Pre castration evaluation |
| 15 | M | 7 | Golden Retriever | House with garden | Ophthalmic check-up |
| 16 | M | 3 | English Bulldog | Apartment | Ophthalmic check-up |
| 17 | F | 2 | Shih Tzu | Apartment | Pre castration evaluation |
| 18 | M | 12 | Mixed breed | Concrete yard house | Cardiac evaluation |
| 19 | F | 1 | French Bulldog | Apartment | Pre castration evaluation |
| 20 | F | 5 | Shih Tzu | Apartment | Ophthalmic check-up |
| 21 | M | 4 | Lhasa Apso | House with garden | Ophthalmic check-up |
| 22 | M | 13 | Shih Tzu | Apartment | Ophthalmic check-up |
| 23 | M | 6 | Yorkshire Terrier | House with garden | Clinical check-up and collection of blood tests |
| 24 | F | 11 | Mixed breed | Farm | Cardiac evaluation |
| 25 | M | 3 | Miniature Pinscher | Apartment | Neurological evaluation |
| 26 | F | 1 | Shih Tzu | House with garden | Pre castration evaluation |
| 27 | F | 12 | Mixed breed | House with garden | Ophthalmic check-up |
| 28 | M | 10 | Golden Retriever | House with garden | Clinical check-up and collection of blood tests |
| 29 | F | 6 | Yorkshire Terrier | Apartment | Clinical check-up and collection of blood tests |
| 30 | M | 7 | Mixed breed | Apartment | Ophthalmic check-up |

**M = male. F = female*

### *2.2 Processing of the samples and sequencing of the nucleic acids*

Nucleic acid extraction procedures were performed with care to reduce cross contamination. All of the samples were thawed at room temperature. After vortex homogenization, a pool with 200 µl of each sample was formed. This pool was filtered through a 0.22 µm sterile syringe filter (Merck Millipore, Darmstadt, Germany) to remove impurities and cellular debris. Afterwards, the pool was ultracentrifuged on a 25% sucrose mattress at 4 °C (27,000 rpm) for two hours. The resulting pellet was treated with DNAse (2 U) and RNAse (5 µl, 20 mg/ml) enzymes [19]. Subsequently, DNA extraction was performed with the phenol/chloroform protocol, and RNA extraction was performed with TRIzol® LS reagent (Life Technologies, Carlsbad, USA) according to the manufacturer›s instructions. The nucleic

acids were subsequently enriched with DNA and RNA amplification kits (Sigma Aldrich, Saint Louis, USA). The nucleic acids were then subjected to purification by using the PureLink® PCR Purification Kit (Thermo Fisher Scientific, Waltham, USA). Its quality and quantity were evaluated by using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and a Qubit fluorometer (Invitrogen, Carlsbad, USA), respectively.

A DNA shotgun metagenomic library was prepared with 50 ng of purified nucleic acids by using a Nextera DNA Preparation Kit (Illumina, San Diego, USA) according to the manufacturer's instructions. Library sequencing was performed with an Illumina® sequencer (Illumina, San Diego, USA) by using the MiSeq v2 300 platform (2x150 cycles).

The sequencing generated a total of 371,504 high-quality reads. These reads were initially trimmed with the FASTQ app and then submitted to a *de novo* assembly tool by using the SPAdes version 3.9 program. The 8,593 generated contigs were compared to the GenBank virus database through Blast2GO version 5.2. Afterward, with the aid of Geneious Prime version 9.1.1 and the BLASTx tool, the results were manually confirmed. During this process, only bacteriophage-like DNA sequences were found, but no other viruses were identified. However, when the reads were analyzed in the MG-RAST 4.0.3 server (https://www.mg-rast.org) a large number of microorganisms were identified, and thereby considered in the analysis. The same contigs that were formed via SPAdes were subjected to this analysis, and the platform was able to recognize all of the sequences, totaling 5,228,105 base pairs (bp), with an average of 608 bp per sequence. The taxonomic distribution was determined based on the lowest common ancestor (LCA) algorithm.

## 3. Results

According to the MG-RAST analysis the majority of the detected sequences belonged to the Bacteria domain, followed by the Eukaryota and Archaea domains. Some sequences were not identified, and a minority corresponded to the virus domain (bacteriophages), as shown in Figure 1.

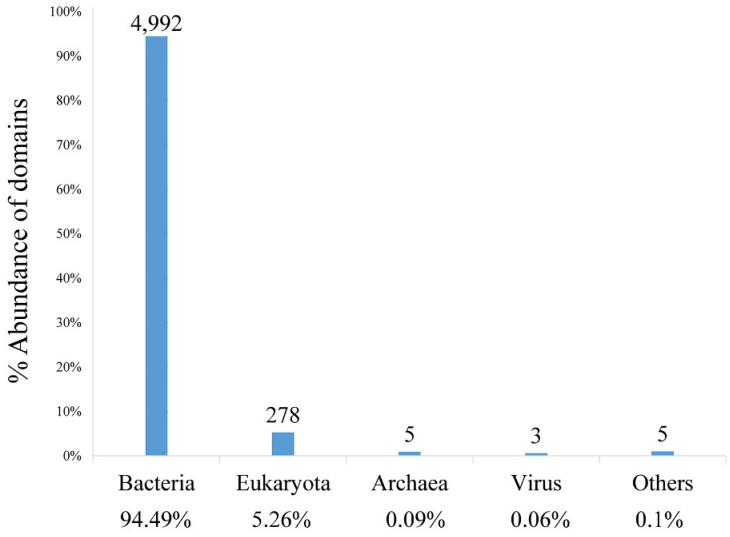


**Figure 1 Taxonomic distribution in the ocular conjunctiva of healthy dogs. The domains were predicted by using the MG-RAST server. The abundance of domains is related to the total number of analyzed contigs. Abundance percentage values are indicated.**

A rarefaction analysis was performed to estimate the microbiological diversity identified in the sequence set. This curve became stable when it reached a value close to 8000 contigs, which indicates that the identification of new organisms is unlikely after this plateau is reached, even if further sequencing is performed (Figure 2). Therefore, the rarefaction curve showed that the depth of sequencing was sufficient to identify the microbial diversity of the sample.

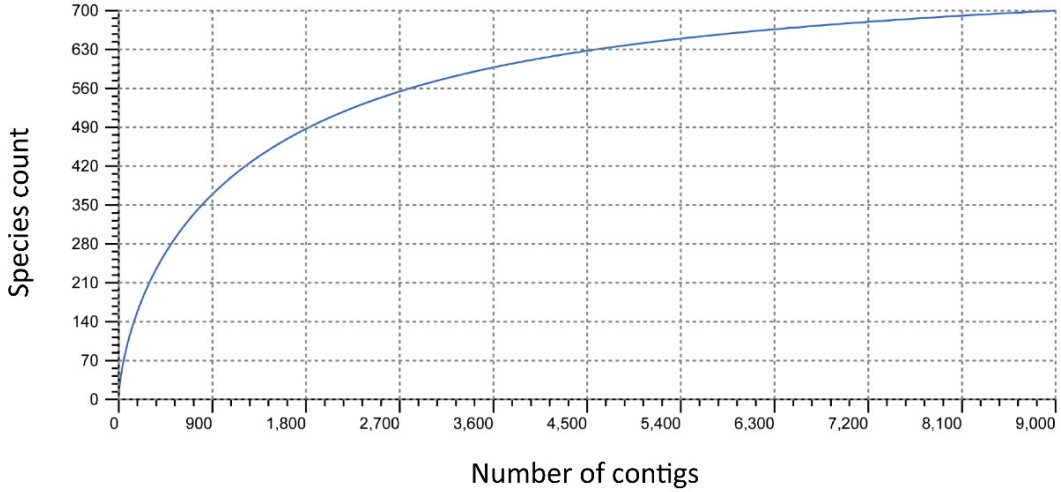


**Figure 2 The rarefaction curve shows the depth of sequencing. The curve shows the relationship between the number of recovered species (x-axis) and the total number of analysed contigs (y-axis).**

Among the bacteriophages, MG-RAST identified only eleven sequences, most of which were from the bacteria present in this analysis, such as *Burkholderia* spp., *Ralstonia* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Bacillus* spp. (Table 2). In the Archaea domain, all of the sequences correspond to the phylum Euryarchaeota, but no further classification has been determined.

**Table 2 Bacteriophages identified through the MG-RAST method.**

| Phage | Identification | Sequence |
|---|---|---|
| *Burkholderia* phage KS9 | NODE_2318_length_665_cov_1.71747_1_665 | RefSeq284c8c671eaf2852cf01728d79c372f7 |
| *Burkholderia* phage BcepF1 | NODE_3356_length_567_cov_1.06591_117_561 | RefSeq82fb224beff6a95296b46f9516b0bcfd |
| *Burkholderia* phage BcepF1 | NODE_439_length_1177_cov_1.43143_1_273 | RefSeq9573319e900ee58963ad81118b706841 |
| *Burkholderia* phage phiE12-2 | NODE_2099_length_692_cov_2.06372_108_692 | RefSeqc4b3ce5a51d01dcc981d710d57bb50e4 |
| *Ralstonia* phage RSM3 | NODE_81_length_1941_cov_2.56229_148_1182 | RefSeq80ea0d09ce3f9846bfd764144c593dd |
| *Staphylococcus* phage 11 | NODE_3771_length_540_cov_2.33898_1_540 | RefSeq2f60c05664cce42f18d2babc8fcea5f5 |
| *Staphylococcus* phage CNPH82 | NODE_2237_length_675_cov_1.79015_1_675 | RefSeqa86769730bc8ced98a9b4f165bd9fd76 |
| *Pseudomonas* phage F116 | NODE_3261_length_575_cov_2.77679_1_575 | RefSeqc2c61a51fe247b405dec5f72d8c66ba0 |
| *Bacillus* phage phi29 | NODE_5659_length_450_cov_18.8947_1_450 | RefSeqce41329b343832940da794a173da94cf |
| *Bacillus* phage phi29 | NODE_8338_length_262_cov_103.704_1_262 | RefSeqce41329b343832940da794a173da94cf |
| *Sinorhizobium* phage PBC5 | NODE_4358_length_505_cov_1.21693_1_505 | RefSeq4e81e8bdb137d9d08e725074393ec2f9 |

In the Eukaryota domain, two fungal phyla (Basidiomycota and Ascomycota) and a Chordata phylum, which includes vertebrates, were found. Figure 3 illustrates this classification.

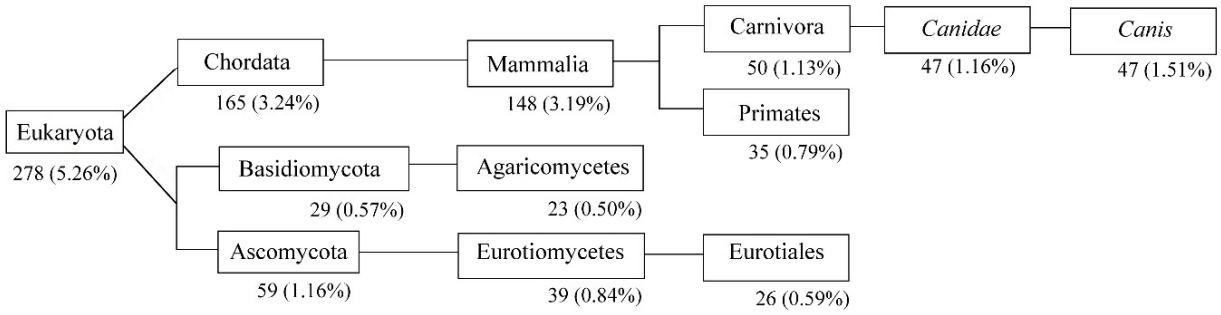


**Figure 3 Composition of the Eukaryota domain detected in the ocular conjunctiva of healthy dogs. Data from the MG-RAST server, including the percent value of each classification were obtained. The percentages are relative to the total number of analysed contigs via the server.**

Within the Bacteria domain, which represented 94.49% of the analyzed sequences, the most prevalent phyla were Proteobacteria and Bacteroidetes, accounting for 97.4% of the total bacteria that were found. The other phyla that were present were Actinobacteria, Firmicutes and Verrucomicrobia, among others. When this domain was divided into classes, Betaproteobacteria (86.73%), Alphaproteobacteria (7.47%), Gammaproteobacteria (2.24%), Sphingobacteria (0.96%), and Actinobacteria (0.87%) accounted for 98.27% of the analyzed sequences. When considering the taxonomic order, 97.89% of the sequences corresponded to Burkholderiales (89.97%), Sphingomonadales (3.38%), Rhizobiales (2.57%), Sphingobacteriales (1.02%), and Pseudomonadales (0.95%). At the family level, 96.98% of the sequences belonged to *Burkholderiaceae* (90.32%), *Sphingomonadaceae* (2.80%), *Bradyrhizobiaceae* (1.67%), *Comamonadaceae* (1.12%), and *Sphingobacteriaceae* (1.07%). Figure 4 demonstrates this classification.

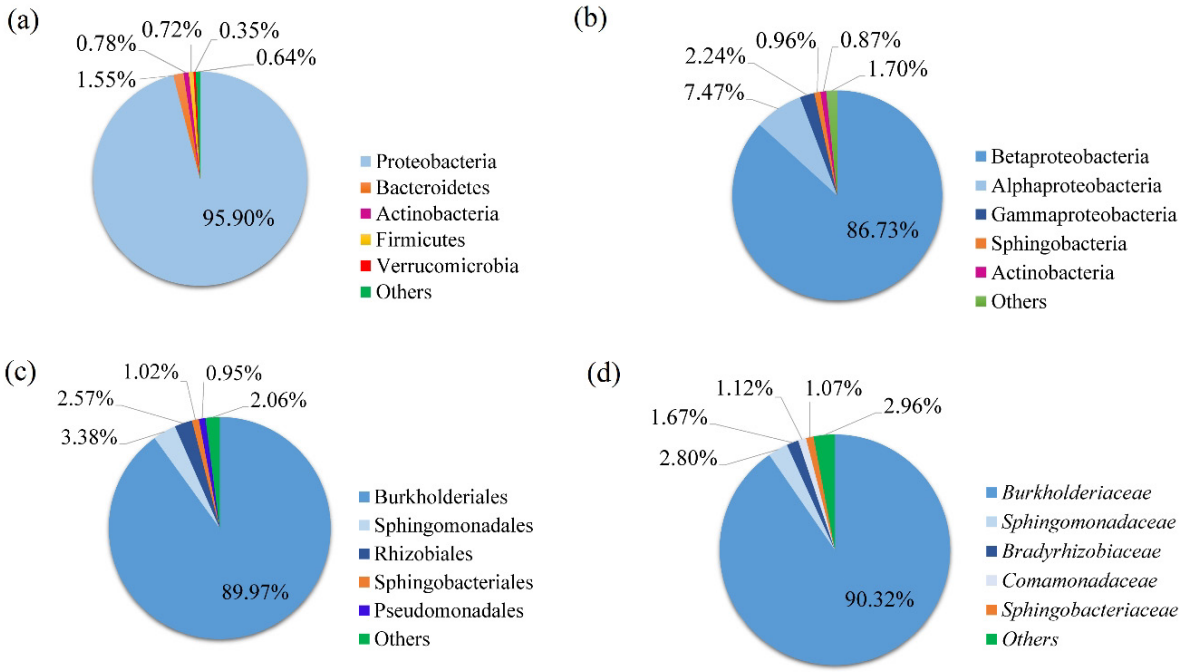


**Figure 4 The bacterial community composition in the ocular conjunctiva of healthy dogs (a) at the phylum level, thus demonstrating the predominance of the Proteobacteria phylum; (b) at the class level, wherein Betaproteobacteria is indicated; (c) at the order level, wherein Burkholderiales predominates, followed by Sphingomonadales; and (d) at the family level, wherein Burkholderiaceae is the most represented family.**

Most of the sequences corresponded to *Ralstonia* (52.35%) and *Burkholderia* (39.58%), representing 91.93% of the total. Figure 5 shows the fifty genera that MG-RAST was able to identify from the analysed sequences. This chart shows only the genera that were represented by more than 10 sequences. Interestingly, the genera *Staphylococcus* and *Bacillus* were identified in only five sequences each, totaling only 0.18% each. The names of all of the bacterial genera that were found in the analysis are available in Figure 6.

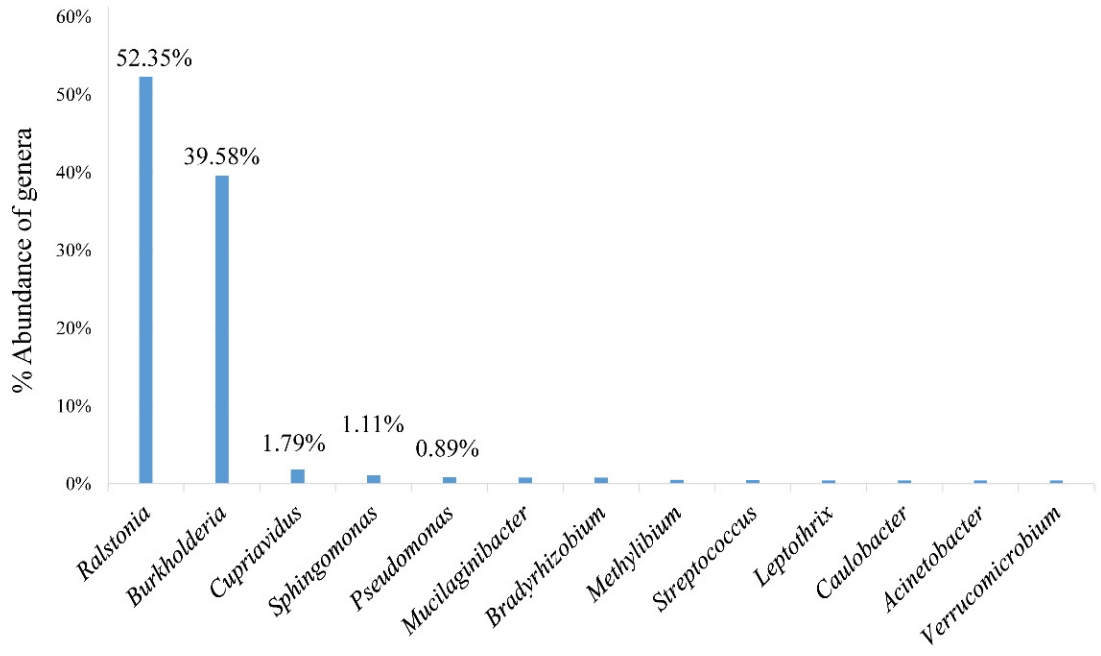


**Figure 5 The most abundant bacterial genera that were identified in the ocular conjunctiva of healthy dogs. The x-axis shows the most prevalent genera matched in the samples. The y-axis shows the percentage of contigs for each identified genus.**

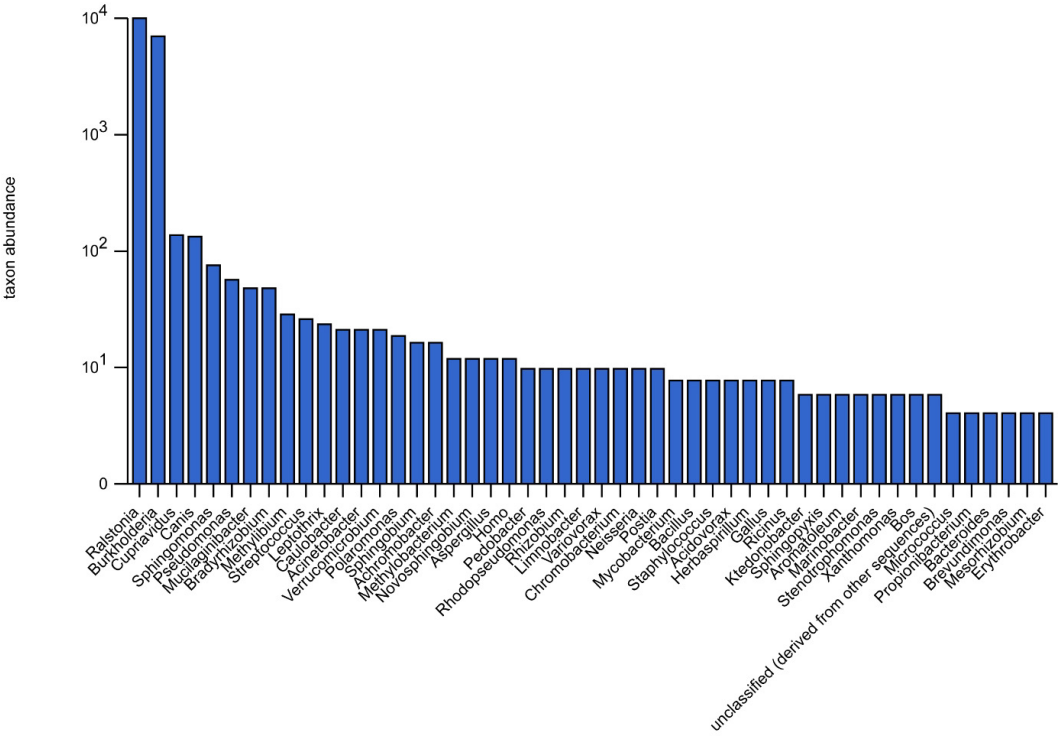


**Figure 6 Bacterial genera composition from the ocular conjunctiva of healthy dogs. The analysis was performed with the MG-RAST server, which identified the 50 most prevalent bacterial genera in the analyzed samples.**

## 4. Discussion

In the present study, we investigated the conjunctival microbial diversity of dogs without ophthalmological disorders through metagenomic DNA sequencing (a shotgun approach) and observed a predominance of the phylum Proteobacteria and the genera *Ralstonia* and *Burkholderia*. A previous study in which amplicons of the 16S rRNA V3-V4 hypervariable region gene were sequenced showed a predominance of the Firmicutes phylum, with Proteobacteria being among the most prevalent in the ocular surfaces of dogs. [18] Similarly, Proteobacteria is also the most commonly found phylum in the conjunctiva of healthy humans. [20,21,22] These microorganisms likely interact with each other and with the host's immune system, allowing constant vigilance of the ocular surface microenvironment.

Previous studies have been conducted to analyse the ocular microbiota of dogs using microbiological cultivation techniques. [1,7,14,15,16] Unlike our findings, these authors found a predominance of *Staphylococcus* spp. However, data from such different methodologies cannot be compared because NGS analyses are highly sensitive and can identify microorganisms that are unidentified in conventional culture analyses. [23]

There is considerable variability in the species and amount of bacteria that make up the microbiota of a given site, which is influenced by geographic location, nutrition, and climate. [24] With a goal of achieving wide variability, our study used 30 dogs from different dwellings that were fed in different ways.

The initial objective of the present study was to determine whether there were viruses inhabiting the ocular conjunctiva of healthy dogs; if so, we aimed to determine which viruses were present. The methodology was based on other studies that also sought to identify the virome of various sites from clinical samples, such as feces, [25] serum, [26] organs, [27] and secretions. [28] The virion particle size of the viruses of veterinary interest is extremely variable, with an average variation between 17 and 300 nm in diameter. [29] The 0.22 µm filter is capable of filtering cell debris and bacteria and allows for virus passage, which is a suggested step in processing samples for virus detection. [26] Although the processing method was based on the literature, only 11 sequences were identified as bacteriophages from *Ralstonia* spp., *Burkholderia* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Bacillus* spp., and *Sinorhizobium* spp., but no other viruses were found. This result indicates that viruses may not inhabit the eye conjunctiva of dogs without ophthalmological disorders. Therefore, when other veterinarians or researchers identify viruses in samples from diseased eyes, they should consider them as potential pathogenic agents of the clinical condition in question.

Even with a potential loss of bacteria through a 0.22 µm filter, a wide range of bacteria were detected, and the rarefaction curve was stable. We highlight that this scenario can introduce bias in the recovered sequences. However, as the identified metagenomes were similar to those of previous ocular metagenome analyses, we can explore the data. In addition, an important number of eukaryotic organisms of different sizes have been found; we believe that this is due to plasma membrane lysis generated by manipulation, which allows nucleic acids to pass through the filter. Due to the fact that the utilized technique detects genetic

material and not viable cells, it was possible to recover a wide range of bacteria, as well as some fungal sequences. Therefore, the most abundant microorganism sequences (mainly consisting of bacteria) were carefully analyzed.

Among the eukaryotic organisms, we identified sequences of fungi of the phyla Basidiomycota and Ascomycota. In a previous study, Suchodolski et al. [30] identified fungal DNA in small bowel biopsies of 64 healthy dogs and 71 dogs with enteropathies by using PCR. All 51 identified phylotypes belonged to the Ascomycota (32 phylotypes) or Basidiomycota (19 phylotypes) phyla. Several years later, Foster et al. [31] determined the stool microbiome of 12 healthy dogs and 7 dogs with acute diarrhea through NGS, and the same phyla (Ascomycota and Basidiomycota) were observed to be the most abundant, in addition to being found in more than 50% of the dogs in both groups. Due to orofecal contact and the habit of smelling and even ingesting fecal material, the presence of enteric fungi in the ocular microbiota of dogs is justifiable and expected.

The ocular microbiota of dogs has been the subject of several studies using traditional microbiological culture techniques in recent decades. [1,14,32] These reports indicate the predominance of gram-positive bacteria, especially *Staphylococcus* spp., belonging to the Firmicutes phylum. [1,7,15,32] *Staphylococcus* spp. have also been isolated from the skin and hair of healthy and dermatopathic dogs. [33,34] The eyelids are ocular structures that are internally delimited by the eyelid conjunctiva and externally delimited by skin and fine hairs. [1] Thus, *Staphylococcus* spp. may be present on the outside of the eyelids of dogs with or without conjunctivitis and blepharitis. In addition, the bacterium is also present on healthy human skin, including the region of the hands. [35] However, in the present study, this bacterium was identified in a very small number of sequences (0.18%), as has been observed in other NGS studies, [18] which can be explained by the extreme care to prevent touching of the eyelids and the eyelid margins of the dogs during collection, as well as by the previous washing of hands, the use of gloves during the collection procedure and the subsequent manipulation of the samples in the laboratory. Furthermore, the high detection of *Staphylococcus* spp. in previous studies with traditional culture methods may be related to its possible inhibitory effect on other bacteria in culture. [36]

The only study that has ever been performed using NGS sequencing to determine the ocular microbiome of healthy dogs, but with a different method than ours, demonstrated a predominance of the Firmicutes phylum. However, the most prevalent genus was *Bifidobacterium* spp., which belongs to the Actinobacteria phylum. [18] In our study, the phylum Actinobacteria was identified; however, the phylum Proteobacteria was the most prevalent phylum, which is in agreement with recent data on the ocular microbiome of other species, such as felines, [37] equines, [9] and human. [20,21,22]

*Ralstonia* spp. was the most frequently observed genus in our analysis (Figure 4). It consists of Betaproteobacteria that inhabit the environment and are mainly found in water and soil, with some opportunistic pathogenic species. [38] Hoffmann et al. [39] evaluated the skin microbiome of healthy and allergic dogs, including ocular surface collection and other

mucosal sites, via pyrosequencing. The genus *Ralstonia* was the most abundant genus in the healthy dog samples and comprised 35% of the total bacteria found in the eye conjunctiva. The proportion of *Ralstonia* spp. found in allergic dogs was significantly lower than that in healthy dogs. These authors concluded that *Ralstonia* spp. originated from the environment, given the frequent interaction of dogs with the outdoor environment. [39] Our study corroborates this previous consideration, as the main habitats of most of the sampled dogs were yards or garden houses. However, considering that this was the second identification of *Ralstonia* spp. using metagenome sequencing and that this was the predominant bacterium in the eye conjunctiva, it may not only be a transient environmental bacterium, but may also be a permanent part of the eye microbiota of healthy dogs. A recent NGS study in horses also demonstrated that the eye microbial community was dominated by the phylum Proteobacteria and the genus *Ralstonia.* [40]

In the current study, the genus *Burkholderia* was the second most common genus in the sample, accounting for with *Ralstonia* spp. almost 92% of the resulting sequences (Figure 4). This bacterium has been found to be associated with ocular melioidosis in humans and felines, [41,42] as well as with systemic diseases in dogs. [43] Although the present study did not reach the taxonomic level of species, the observation of *Burkholderia* spp. on the ocular conjunctiva of dogs warrants further research to better clarify this phenomenon.

Although it had a much smaller occurrence than the first two genera, *Cupriavidus* spp., which is a bacterium from the *Burkholderiaceae* family, was the third most frequent genus and was found in 1.79% of the sequences (Figure 4). There have been reports of the isolation of *Cupriavidus* spp. in diverse environments, including soil and water, [44,45] as well as in humans with systemic diseases. [46]

In our study, the presence of *Sphingomonas* spp., which is an Alphaproteobacteria from the family *Sphingomonadaceae,* was verified in 1.11% of the sequences. It has already been isolated from air and dust samples, [47] as well as from dog skin microbiota. [48] The human ocular microbiome has been examined through NGS, and *Sphingomonas* spp. were identified among the most prevalent bacteria, representing 1% to 10% of all of the detected genera. [20,22] Recently, it was also identified at an abundance of 7.2% in the ocular microbiome of healthy horses. [49] All of these results led us to believe that the results of the current study indicate actual colonization of the ocular conjunctiva of dogs by *Sphingomonas* spp.

To date, this is the first study to identify *Pseudomonas* spp. on the ocular conjunctiva of healthy dogs by using metagenomic DNA sequencing (Figure 4). This bacterium was identified in the conjunctival sac of healthy dogs and dogs with ulcerative keratitis in China through bacterial culture methods. [15] *Pseudomonas* spp. are essentially opportunistic in cases of eye diseases in dogs, whereby they cause tissue destruction and lead to blepharitis, conjunctivitis, keratitis, scleritis, and endophthalmitis, among other diseases. [6] *Pseudomonas* spp. is also part of the human eye microbiome, which has been confirmed via NGS. [21]

A specific limitation of this study was the lack of differentiation between resident and transient microbiota found in the canine eye conjunctiva. However, it is still difficult to perform

resident/transient microorganism differentiation at permanently exposed mucous sites with direct air contact. Although transient microbiota may exist, their identification is relevant, as transient microorganisms can become pathogenic depending on the conditions that they encounter. Previous studies have also been unable to perform this differentiation. [7,15,16,18] New studies are necessary to better understand the relationship between the conjunctival microbiota and cases of clinical disease. It would be interesting to replicate our methodology using samples from dogs with clinical conjunctivitis.

## 5. Conclusion

In this study, we mainly observed *Ralstonia* spp., *Burkholderia* spp., *Cupriavidus* spp., *Sphingomonas* spp., and *Pseudomonas* spp. inhabiting the eye conjunctiva of dogs without ophthalmological disorders. Although fungi were identified in low percentages, eukaryotic viral agents were not identified; however, some sequences related to bacteriophages were found. These findings suggest that these bacteria may compose the conjunctival microbial diversity, which is relevant for maintaining eye surface health. The shotgun DNA sequencing approach has provided new data on this subject, thus demonstrating the presence of bacteria previously unknown as being part of the ocular environment. It is important to identify the microbiota of the eye to understand the infectious processes that affect it and to rationally direct the appropriate utilized treatments.

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None.

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### Conflict of interest

None declared.

### Authors' contributions

Marcela Torikachvili, Joao Antonio Tadeu Pigatto and Claudio Wageck Canal performed the conceptualization;

Marcela Torikachvili, Fabiana Quoos Mayer, Michelle Becker Petersen, Mariana Soares da Silva, Renata da Fontoura Budazeswski and Matheus Nunes Weber performed the methodology;

Marcela Torikachvili, Mariana Soares da Silva, Renata Fontoura Budazeswski and Franciele Maboni Siqueira did the formal analysis;

Marcela Torikachvili did the writing – original draft, review and editing;

Franciele Maboni Siqueira, João Antonio Tadeu Pigatto e Claudio Wageck Canal did the supervision and project administration

### Ethics approval

This study was approved by the Ethics Committee in Animal Use (CEUA) of the Federal University of Rio Grande do Sul (UFRGS) under protocol 35271.

## Data availability statement

All of the data that are relevant to the study are included in the article or uploaded as supplementary information.

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