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VETERINARY AND ANIMAL PRODUCTION - RESEARCH PAPER

# Isolation and evaluation of antagonistic activity against pathogenic bacteria by *Lactobacillus* and *Enterococcus* spp. from the saliva of *Speothos venaticus* and *Chrysocyon brachyurus*

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## Abstract

Saliva plays a crucial role in oral defense across mammals by combining host-derived antimicrobial factors with antagonistic indigenous microbiota. Understanding the composition of the oral lactic microbiota in wild canids may provide valuable insights into microbial ecology and animal health. In this study, *Lactobacillus* and *Enterococcus* spp. isolated from the saliva of South American bush dogs (*Speothos venaticus*) and maned wolves (*Chrysocyon brachyurus*) were enumerated, identified, and evaluated for their antagonistic activity against pathogenic bacteria. For bacterial isolation, MRS agar and BHI agar supplemented with sodium azide (BHI-SA) were used for *Lactobacillus* and *Enterococcus*, respectively. Identification of the bacterial isolates was performed using PCR-ARDRA and multiplex PCR. Antagonistic activity was assessed using a double-layer agar diffusion assay, which detects diffusible inhibitory effects without identifying the compounds involved. A total of 23 bacterial isolates were obtained from BHI-SA and 24 from MRS for bush dogs, while 15 were recovered from BHI-SA and 23 from MRS for maned wolves. Salivary bacterial counts ranged from 4.0 to 5.0 $\log_{10}$ CFU/mL. In bush dogs, *Enterococcus faecalis* and *Enterococcus faecium* were identified, while *E. faecalis* and *Enterococcus hirae* were found in maned wolves. *Limosilactobacillus reuteri*, *Lactiplantibacillus paraplantarum*, *Lactiplantibacillus plantarum* and *Lactobacillus johnsonii* were isolated from bush dogs, while *Ligilactobacillus salivarius* and *Latilactobacillus curvatus* were identified in maned wolves. Antagonistic activity against indicator pathogens was more frequent among *Lactobacillus* isolates. These results suggest that the antibacterial properties observed in the saliva of these wild canids may be partly attributed to indigenous *Lactobacillus* and *Enterococcus* species.

**Keywords** *Lactobacillus* · *Enterococcus* · *Speothos venaticus* · *Chrysocyon brachyurus* · Saliva · Antagonism

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## Introduction

The popular expression "licking their wounds" is used to describe, in a figurative way, the recovery process of animals or humans after physical or psychological injury. It is based on the long-standing observation of the behavior of wounded animals, which usually lick the affected area. This observation also led to a widespread, yet unscientific and inadvisable, belief that suggests humans can let dogs lick their skin wounds due to the supposed protective and healing properties of the saliva of these animals. Actually, saliva is well known to act as a natural barrier against pathogenic microorganisms that may enter the oral cavity, due to its content of immunoglobulins, enzymes (lysozyme, lactoperoxidase), and other protective substances such as lactoferrin, as well as to the presence of indigenous microorganisms with antagonistic properties [3, 4, 7, 17, 22]. Various reports in the literature also described binding or inhibition of pathogenic viruses by components of human saliva [10]. In addition, different antimicrobial protein family members have been identified in dog saliva, including cathelicidin 1, cathelicidin antimicrobial peptide, and CRISP1 [5]. Regarding the indigenous oral microbiota of canids, information is available in the literature about its composition, which shows that, similar to humans, Bacteroidota, Pseudomonadota, Bacillota, and Actinomycetota are the predominant phyla [9, 16, 17, 19]. However, the potential functions of these microorganisms are not yet well understood. Consequently, the role of the indigenous microbiota of canid saliva in protecting against potential wound infection is still only a hypothesis that has been raised in a TV documentary [13].

The Canidae family in Brazil is composed of six wild species, and among them, the South American bush dogs (*Speothos venaticus*, Lund, 1842) (Fig. 1A) and the maned wolf (*Chrysocyon brachyurus*, Illiger, 1815) (Fig. 1B) are the most frequently studied [12]. These animal species have carnivorous or omnivorous-carnivorous diets and form a group of predators with biological functions essential to the balance of the ecosystem, both by controlling their prey populations and as important seed dispersers in the environment [1, 15]. In Brazil, South American bush dogs and maned wolves are considered endangered animals according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [6, 18].

The importance of isolation, identification and characterization of bacteria present in the oral cavity of canids is due to its possible role in maintaining oral and general health of the host, by forming barriers against the installation of pathogenic microorganisms through competition for nutrients or adhesion sites, as well as by production of antagonistic substances (organic acids, bacteriocins, etc.) The presence of lactic acid-producing bacteria, such as *Lactobacillus* and *Enterococcus*, in the oral cavity of canids could confirm part of the antimicrobial activity attributed to their saliva.

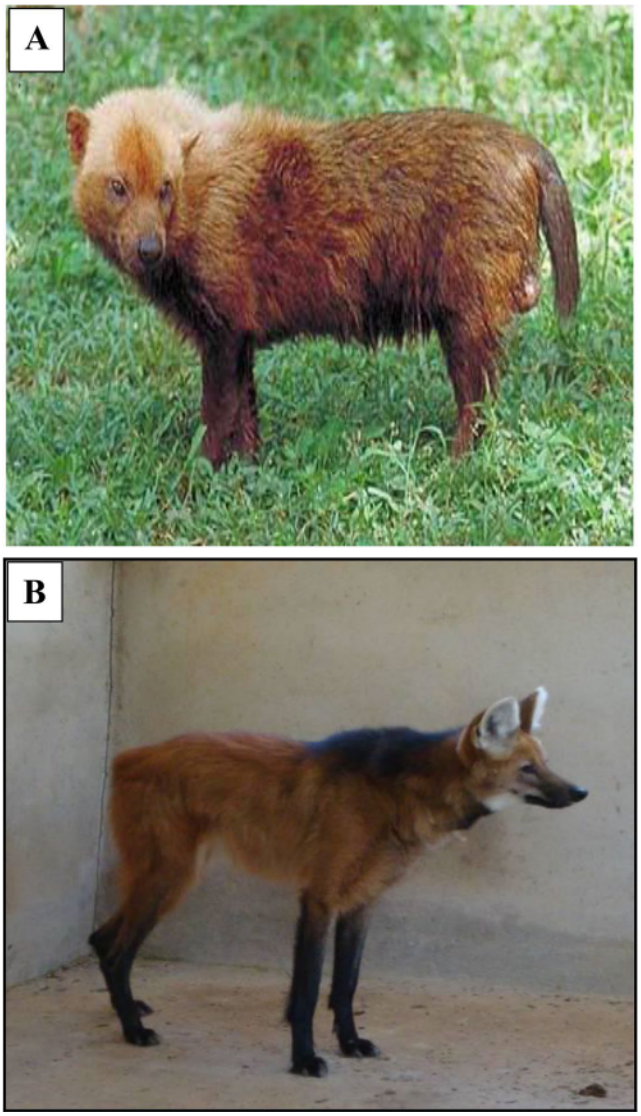


**Fig. 1** Adult specimens of South American bush dog (A - *Speothos venaticus*) and maned wolf (B - *Chrysocyon brachyurus*) at the Zoobotânica Foundation, Belo Horizonte, MG, Brazil

In the present study, *Lactobacillus* and *Enterococcus* spp. isolated from the saliva of South American bush dogs and maned wolves were counted, identified, and their antagonistic abilities against pathogenic bacteria evaluated.

## Materials and methods

### Animals and sampling

Saliva samples were collected at Zoobotânica Foundation, Belo Horizonte, MG, Brazil, where four specimens (one

male and three females) of maned wolf (*C. brachyurus*) and eight specimens (two females and six males) of South American bush dogs (*S. venaticus*) were reared. The number of animals sampled was three maned wolf specimens and five South American bush dogs. For maned wolves, only females aged between eight and 13 years were sampled. The saliva samples from South American bush dogs were obtained from the five male specimens, which were all two years old. A combination of ketamine/xylazine or telazol was used as an anesthetic for immobilizing the animals and sampling, and the recovery of the animals was supervised by a veterinarian from the Zoobotânica Foundation. Saliva was collected using a calibrated sterile loop of 10 µL. After collection, the material was placed into test tubes containing 1 mL of sterile buffered saline solution. The tubes were stored in a container with ice and transported immediately to the laboratory for processing within one hour.

## Enumeration and selection of isolates

### Isolation and enumeration of bacteria

The tubes were transferred into an anaerobic chamber (Forma Scientific Co., Marietta, USA) containing an atmosphere of 80% $N_2$, 10% $H_2$, and 10% $CO_2$, and were immediately subjected to serial tenfold dilutions in sterile buffered saline that had been regenerated before use (10 min at 100 °C). Aliquots (0.1 mL) from $10^{-2}$, $10^{-4}$, and $10^{-6}$ dilutions were spread onto Petri dishes containing de Man, Rogosa, and Sharpe agar (MRS, Difco, Sparks, USA) for the isolation of *Lactobacillus*. The dishes were incubated from 48 to 72 h at 37 °C with intermediate readings. The same three dilutions were removed from the chamber, and 0.1 mL was inoculated by spreading onto the surfaces of dishes containing Brain Heart Infusion agar (Difco) supplemented with 0.2% sodium azide (BHI-SA) for the isolation of *Enterococcus*. The plates were incubated at 37 °C for 24 to 48 h before enumeration. Counts were expressed as $\log_{10}$ colony-forming units (CFU)/mL saliva.

### Presumptive identification of bacterial isolates

After incubation and counting, colonies with different morphologies were isolated from each medium and subjected to a process of preliminary identification. The first selection was based on the growth atmosphere test, production of catalase, and morphological appearance after Gram staining (facultative anaerobe or microaerophilic, catalase-negative, Gram-positive cocci or rods). Each isolated strain was stored at −20 °C in BHI broth and MRS broth, both supplemented with 15% glycerol, for presumptive *Enterococcus* and *Lactobacillus*, respectively.

## Antagonistic activity

In vitro tests to verify the production of inhibitory substances were performed by the double agar overlay diffusion method. An aliquot of 5 µL of the culture of each bacterial isolate grown in MRS or BHI broth (Difco) for 24 h at 37 °C under anaerobic or aerobic atmosphere was spotted in the center of a plate containing MRS or BHI agar (Difco). After incubation at 37 °C for 48 h under aerobic or anaerobic conditions, the cells were killed by exposure to chloroform for 20 min. Residual chloroform was allowed to evaporate, and Petri dishes were overlaid with 3.5 mL BHI semi-solid agar (0.7%), which had been inoculated with 0.2 mL of a 24-h culture of the indicator strain to be tested. After incubation at 37 °C for 24–48 h, the plates were evaluated for the presence or absence of inhibition zones around the bacterial spot. The diameter of the halo was measured with a digital caliper (Mitutoyo, Japan). An isolate was considered positive for antagonism when a clear inhibition halo≥2 mm beyond the colony margin was observed in at least one replicate. Inhibition zones were measured in duplicate and results expressed as mean diameter of inhibition zones (mm) and frequency of antagonism (%: number of strains positive for antagonism/total number of strains assessed). The following pathogenic bacteria were used as indicator strains: *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14,028, *Enterococcus faecalis* ATCC 19,433, *Staphylococcus aureus* ATCC 29,213, and *Listeria monocytogenes* ATCC 19,115. The four indicator pathogens were selected for their clinical and zoonotic relevance. They represent both Gram-positive and Gram-negative bacteria frequently associated with foodborne or systemic infections in animals and humans, allowing assessment of a broad antagonistic spectrum.

## Molecular identification

### DNA extraction

Chromosomal DNA was isolated from overnight cultures of all bacterial isolates in 10 mL of MRS or BHI broth. After washing the cells with deionized water, a pellet was obtained by centrifugation at 14,000 *g* for 5 min at 4 °C. This pellet was then suspended in 1 mL of 5 M LiCl and incubated for one hour with constant shaking. After a second washing with 1 mL of deionized water, the pellet was suspended in 1 mL of protoplasting buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mg/mL lysozyme, 100 µg/mL RNAse). After incubation for 1 h at 37 °C and centrifugation at 14,000 *g* for 5 min at 4 °C, the pellet was resuspended in 500 µL protoplasting buffer without lysozyme and supplemented with 100 µL 10% sodium dodecyl sulfate. After lysing, the mixture was extracted once with phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). After

isopropanol precipitation, the DNA was dissolved in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

## Identification of Lactobacillus by PCR amplification of the 16S-23S rDNA intergenic spacer and restriction analysis

DNA from bacteria selected as presumptive lactobacilli was used for identification by amplified ribosomal DNA restriction analysis (ARDRA) as previously described [20]. The 16–23 S intergenic spacer region was amplified using the primer 16–1 A (GAATCGCTAGTAATCG) that anneals a conserved region of the 16 S rRNA genes, and primer 23-1B (GGGTT CCCCCATTCGGA) that anneals a conserved region of the 23 S rRNA genes using a PTC-100® Thermal cycler (MJ Research Inc., Waltham, USA). The reaction mixture (50 µL) contained 10 pM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$, 5 U Taq DNA polymerase (Phoneutria Biotecnologia & Serviços, São Paulo, Brazil), and 5 µL of template DNA solution. The amplification program consisted of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and concluded with a final extension at 72 °C for 5 min. PCR products were electrophoresed on a 1.4% agarose gel and visualized by UV transillumination after staining with an ethidium bromide solution (5 µg/mL).

The amplified 16–23 S rRNA intergenic spacer regions were digested with a set of 12 enzymes chosen after compilation of nucleotide sequences already deposited at GenBank, and in silico restriction digestion using the Webcutter 2.0 tool (Max Heiman 1997; http://rna.lundberg.gu.se/cutter2/). SphI, NcoI, and NheI enzymes hydrolyzed inside the 16 S gene; SspI, Csp45I (an isoschizomer of SfuI), DraI, VspI, HincII, and EcoRI enzymes hydrolyzed inside the intergenic region, and AvrII and HindIII enzymes hydrolyzed inside the 23 S gene. EcoRV enzyme hydrolyses inside the spacer region for the *Lacticaseibacillus casei* group and in the 23 S gene for the *Lactobacillus acidophilus* group. For some *Lactobacillus* species, no spacer nucleotide sequences have been reported, and only fragments of the 16 S and/or 23 S genes were found. All restriction enzymes were purchased from Promega Corporation (Madison, USA), except *Avr*II, which was purchased from New England Biolabs (Ipiswich, USA).

## Identification of enterococcus by multiplex PCR

Identification of presumptive *Enterococcus* was performed using multiplex PCR with species-specific primers for *E. faecium*, *E. faecalis*, *Enterococcus gallinarum*, and *Enterococcus casseliflavus/flavescens*, as previously described [8]. Table 1 presents the primers and amplified genes used for the identification of *Enterococcus* species by multiplex PCR. Bacterial isolates that had not been identified by this method were submitted to an amplification of an approximately 440 bp fragment of the 16 S rRNA gene, located within the second half of the gene, using the universal primers p13B and pjB1F, which included the variable regions V3, V4, and V9, as previously described [14]. PCR products were purified using the Wizard SV gel and PCR clean-up system kit (Promega) and sequenced with BigDye Terminator Cycle Sequencing V3.1 (Life Technologies, Carlsbad, USA) on an ABI 3130 genetic analyzer (Applied Biosystems, São Paulo, Brazil). Sequences were analyzed using Vector NTI Advance v.11 software (Invitrogen, St. Louis, USA) and compared to the Ribosomal Database Project (https://www.glasslab.berkeley.edu/ribosomal-database-project) for higher identity scores.

**Table 1** Primers and amplified genes used for identification by Multiplex PCR of four *Enterococcus* spp

| Enterococcus species | Amplified gene | Primers (5'- 3') | Amplicon (bp) |
|---|---|---|---|
| ***E. gallinarum*** | *van C-1* | +GGTATCAAGGAAACCTC<br>- CTTCCGCCATCATAGCT | 822 |
| ***E. casseliflavus*** | *van C-2*, *van C-3* | +CTCCTACGATTCTCTTG<br>- CGAGCAAGACCTTTA AG | 439 |
| ***E. faecalis*** | *Ddl* | +ATCAAGTACAGTTAG TCT<br>- ACGATTCAAAGCTAA CTG | 941 |
| ***E. faecium*** | *Ddl* | +TAGAGACATTGAATAT GCC<br>- TCGAATGTGCTACAATC | 550 |

## Statistical considerations

This study was designed as an exploratory screening to isolate, identify, and preliminarily characterize lactic acid bacteria with antagonistic potential from canid saliva. Therefore, no inferential statistical analysis was performed. Each antagonism assay was carried out in duplicate to confirm the reproducibility of the observed inhibitory effects, and the data presented in Tables 2, 3, 4 and 5 provide descriptive summaries of these results. Given the limited number of animals sampled, statistical comparisons between groups (e.g., rods vs. cocci or species differences) were not feasible. Consequently, results are expressed descriptively as mean values or frequency counts, providing an overview of the observed microbial and antagonistic profiles rather than a quantitative comparison.

**Table 2** Total population levels (log10 CFU/mL) and number of different morphotypes of catalase-negative Gram-positive cocci (from BHI-SA) and rods (from MRS) in saliva of *Speothos venaticus* and *Chrysocyon brachyurus* in two independent samplings

| Animal and sampling | | Cocci | | Rods | |
|---|---|---|---|---|---|
| | | Level | Nº | Level | Nº |
| ***Speothos venaticus*** | **1** | 5.16 | 8 | 4.82 | 15 |
| | **2** | 3.88 | 15 | 4.16 | 9 |
| **Total** | | | **23** | | **24** |
| ***Chrysocyon brachyurus*** | **1** | 4.17 | 12 | 4.73 | 15 |
| | **2** | 4.53 | 3 | 5.09 | 8 |
| **Total** | | | **15** | | **23** |

Values represent descriptive means from duplicate assays. No statistical comparison was performed due to the exploratory nature of the study and the absence of complete raw data

## Results and discussion

After incubation of the saliva samples and considering the two collections carried out in each animal species, 74 morphotypes were identified on BHI-SA agar and 111 morphotypes on MRS agar. These different morphotypes were preliminarily submitted to catalase and growth atmosphere tests, as well as a Gram stain, to select catalase-negative facultative anaerobes and microaerophilic Gram-positive cocci and rods, presumptively identifying *Enterococcus* and *Lactobacillus*, respectively. Many morphotypes were catalase-positive, and some did not survive storage. From this preliminary selection, a total of 23 bacterial isolates was obtained from BHI-SA agar and 24 from MRS agar for South American bush dogs, and 15 bacterial isolates were recovered from BHI-SA agar and 23 from MRS agar for maned wolves (Table 2). The population levels of the two bacterial genera of interest varied within a range of 4.0 to 5.0 $\log_{10}$ CFU/mL in saliva. These levels are relatively low, likely due to saliva flow, which prevents further expansion of microbial populations by washing them away. As with any other oral ecosystem, concentrations of these bacteria were certainly higher in dental plaque and on the mucosal surface, as described in dogs [16, 19]. This assumption was supported by the observation that the population levels of other Gram-positive (catalase-positive) and Gram-negative bacteria recovered from the two samples were similar to those of the genera of interest in the present study.

**Table 4** Species of *Lactobacillus* isolated from the saliva of *Chrysocyon brachyurus* and *Speothos venaticus*, and in vitro antagonism against four pathogenic indicators (diameter of Inhibition zones: mm)

| Species | S.a. | E.f. | S.T. | L.m. |
|---|---|---|---|---|
| Chrysocyon brachyurus | | | | |
| ***Latilactobacillus curvatus*** | 33.3 | 23.8 | 36.1 | 39.1 |
| ***Ligilactobacillus salivarius*** | 0 | 0 | 31.8 | 0 |
| ***Ligilactobacillus salivarius*** | 37.9 | 30.7 | 49.1 | 52.2 |
| ***Speothos venaticus*** | | | | |
| ***Lactiplantibacillus plantarum*** | 43.4 | 37.3 | 61.9 | 43.9 |
| ***Ligilactobacillus animalis*** | 43.1 | 29.6 | 49.9 | 36.9 |
| ***Ligilactobacillus animalis*** | 28.4 | 22.9 | 31.4 | 36.3 |
| ***Ligilactobacillus animalis*** | 26.9 | 20.7 | 40.5 | 35.2 |
| ***Ligilactobacillus animalis*** | 16.9 | 17.2 | 18.2 | 0 |
| ***Limosilactobacillus reuteri*** | 39.5 | 17.7 | 48.0 | 36.4 |
| ***Lactiplantibacillus paraplantarum*** | 39.8 | 27.1 | 47.3 | 39.5 |
| ***Lactobacillus johnsonii*** | 0 | 0 | 32.1 | 26.8 |
| ***Lactobacillus johnsonii*** | 0 | 25.0 | 43.0 | 33.5 |

*S.a.*: *Staphylococcus aureus*; *E.f.*: *Enterococcus faecalis*; *S.*T.: *Salmonella* Typhimurium; *L.m.*: *Listeria monocytogenes*

Table 3 shows the results of in vitro antagonism tests against four pathogenic indicators performed with the Gram-positive cocci and rods isolated from the saliva of *S. venaticus* and *C. brachyurus* in two independent samplings. The average frequency of antagonism against all the indicator strains assessed was higher for rods than for cocci, and this was seen for both canids. For *S. venaticus*, the mean frequencies were 20.7% and 48.5% for cocci and rods, respectively, and for *C. brachyurus*, these frequencies were 17.3% and 38.1%, respectively. In terms of sensitivity to the

**Table 3** In vitro antagonism against four indicator pathogens by catalase-negative Gram-positive Cocci (BHI-SA) and rods (MRS) isolated from the saliva of *Speothos venaticus* and *Chrysocyon brachyurus*, as mean diameter of Inhibition zones (mm) and frequency of antagonism (%)

| | | | *E.f.* | | *L.m.* | | *S.*T. | | *S.a.* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **mm** | **%** | **mm** | **%** | **mm** | **%** | **mm** | **%** |
| ***Speothos venaticus*** | **Cocci** | **1** | 30.31 | 12.5 | 27.46 | 25.0 | 32.04 | 37.5 | 36.7 | 25.0 |
| | | **2** | 19.3 | 13.3 | 33.6 | 60.0 | 27.7 | 26.7 | 19.2 | 20.0 |
| | **Rods** | **1** | 25.9 | 40.0 | 35.5 | 53.3 | 41.6 | 53.3 | 36.8 | 40.0 |
| | | **2** | 22.4 | 55.5 | 35.1 | 55.5 | 32.8 | 66.7 | 20.1 | 22.2 |
| ***Chrysocyon brachyurus*** | **Cocci** | **1** | 22.5 | 25.0 | 31.7 | 25.0 | 30.3 | 41.7 | 25.8 | 13.3 |
| | | **2** | 0 | 0 | 0 | 0 | 26.1 | 33.3 | 0 | 0 |
| | **Rods** | **1** | 20.2 | 40.0 | 32.5 | 53.5 | 37.1 | 53.3 | 25.4 | 33.3 |
| | | **2** | 25.9 | 25.0 | 31.9 | 50.0 | 40.6 | 37.5 | 37.9 | 12.5 |

*E.f.*: *Enterococcus faecalis*; *L.m.*: *Listeria monocytogenes*; *S.*T.: *Salmonella* Typhimurium; *S.a.*: *Staphylococcus aureus*

%: number of strains positive for antagonism/total number of strains assessed

Data are presented as descriptive means or frequencies based on duplicate determinations. Statistical analyses were not applied due to the exploratory nature of the study

**Table 5** *Enterococcus* species isolated from the saliva of *Chrysocyon brachyurus* and *Speothos venaticus*, and in vitro antagonism against four pathogenic indicators (diameter of Inhibition zones, mm)

| Species | S.a. | E.f. | S.T. | L.m. |
|---|---|---|---|---|
| *Chrysocyon brachyurus* | | | | |
| ***Enterococcus faecalis*** | 0 | 0 | 23.5 | 0 |
| ***Enterococcus faecalis*** | 38.7 | 21.9 | 40.0 | 0 |
| ***Enterococcus hirae*** | 0 | 32.3 | 29.0 | 28.6 |
| ***Enterococcus hirae*** | 0 | 0 | 26.0 | 0 |
| ***Speothos venaticus*** | | | | |
| ***Enterococcus faecalis*** | 0 | 0 | 32.3 | 0 |
| ***Enterococcus faecalis*** | 25.1 | 0 | 0 | 0 |
| ***Enterococcus faecalis*** | 20.5 | 0 | 0 | 0 |
| ***Enterococcus faecalis*** | 23.3 | 21.7 | 25.9 | 19.2 |
| ***Enterococcus faecalis*** | 0 | 0 | 38.1 | 49.2 |
| ***Enterococcus faecium*** | 14.3 | 0 | 22.3 | 0 |
| ***Enterococcus faecium*** | 0 | 17.8 | 0 | 34.6 |
| ***Enterococcus faecium*** | 0 | 0 | 28.8 | 21.3 |

*S.a.*: *Staphylococcus aureus*; *E.f.*: *Enterococcus faecalis*; *S.*T.: *Salmonella* Typhimurium; *L.m.*: *Listeria monocytogenes*

Values represent descriptive means from duplicate assays. No statistical comparison was performed due to the exploratory nature of the study

antagonism, *L. monocytogenes* and *S.* Typhimurium showed higher mean frequencies (40.3% and 43.7%, respectively) than *E. faecalis* and *S. aureus* (26.4% and 20.8%), considering all the bacterial isolates evaluated.

Antagonistic activity against both Gram-positive and Gram-negative bacteria is frequently observed among *Lactobacillus* species. Various diffusible compounds can manage this phenomenon, including organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like compounds [21]. Although less frequent, the production of inhibitory compounds by *Enterococcus* species has also been described [11]. The in vitro detection method used in this study is commonly applied in studies on diffusible inhibitory substances, and it is justified by its easy application to various microbial species, its practical methodology, and the fact that it results in satisfactory data. Nevertheless, it is important to emphasize that the technique has certain limitations. The antagonistic response may result from more than one substance with quite different chemical structures, and the method cannot specify what substances were involved in the phenomenon. Although many bacterial isolates did not show antagonistic activity in vitro against indicator strains, this does not mean that they cannot show antagonism in vivo or under other growing conditions, and vice versa. The production of these substances may depend on several factors, such as pH, nutrient availability, temperature, and other physical or chemical inducers, which constitute the methodological variables of the in vitro test that aims to simulate conditions found in vivo. Finally, some bacterial isolates exhibited inhibition zones with varying diameters, depending on their specific nutritional and physicochemical needs [2]. For the next step, only bacterial isolates that exhibited antagonistic activity against at least one indicator pathogen were subjected to molecular identification.

ARDRA and multiplex PCR were chosen as rapid and cost-effective methods for species-level discrimination within the target genera. These techniques allow processing of a large number of isolates while maintaining acceptable taxonomic resolution. Preliminary molecular identification by PCR of 16–23 S spacers showed the presence of distinct species of *Lactobacillus* in the oral cavity of South American bush dogs and maned wolves. The technique can distinguish and identify other than lactobacilli, and this distinction is possible due to duplication and insertion of sequences present in the internal transcribed spacer 1 (ITS 1) of the 16–23 S rRNA [20, 23]. *Streptococcus* spp., *Enterococcus* spp., and *Lactobacillus* show one, two, and three spacers, respectively. Among the 19 bacterial isolates selected for identification due to their antagonistic abilities, 12 were identified as species of *Lactobacillus*. Their amplicons were treated with 12 restriction enzymes, allowing for a comparison of the obtained product with a theoretical digestion profile to identify them at the species level [20]. The enzymatic digestion identified a total of seven different *Lactobacillus* species in the two canids: *Ligilactobacillus salivarius* (2) and *Latilactobacillus curvatus* (1) from maned wolves, and *Ligilactobacillus animalis* (4), *Lactobacillus johnsonii* (2), *Limosilactobacillus reuteri* (1), *Lactiplantibacillus paraplantarum* (1), and *Lactiplantibacillus plantarum* (1) from South American bush dogs (Table 4). The species identified as *L. animalis* and *L. reuteri* needed to be identified by multiplex PCR and sequencing. Nine of the 12 lactobacilli showed antagonism against all the indicator assessed strains, and the overall antagonistic frequency in these lactobacilli species was 85.4%.

Among the 21 bacterial isolates of cocci selected for their antagonistic activities, 12 belonged to the genus *Enterococcus*. The molecular identification by multiplex PCR showed the presence of three distinct species of *Enterococcus* in canids: *E. faecalis* (2) and *E. hirae* (2) in maned wolves, and *E. faecalis* (5) and *E. faecium* (3) in South American bush dogs. In the oral cavity of both canids, all the *Enterococcus* species showed the ability to antagonize at least one of the indicator strains (Table 5). Only one *E. faecalis* strain isolated in the South American bush dog saliva was able to inhibit all the indicator strains assessed. The overall antagonistic frequency in *Enterococcus* species was 47.9%.

In the literature, there is no data on the oral microbiota of the two species of canids studied here, although some information is available on other canids. In a qualitative study on the composition of the oral microbiota of dogs, Elliott and colleagues [9] compared their results with those found

in humans. As in the present study, isolation was based on a culture-dependent method, and identification was performed using a molecular method (PCR). The results showed some similarities between humans and animals in terms of bacterial genera, but major differences at the species level. Unlike the present study, no species of *Lactobacillus* or *Enterococcus* were found in the work mentioned above. Robert and colleagues [17] also conducted a comparative study on the oral microbiota of Beagle dogs and humans using molecular identification methods. However, the material was collected from dental plaque and soft tissue (tonsil, cheek, and tongue). The results again showed a similarity in terms of bacterial genera between humans and dogs. As in the study by Elliott and colleagues [9], neither *Lactobacillus* nor *Enterococcus* was isolated. Equivalent results were obtained in more recent studies in dogs [16, 19].

In conclusion, the results of this study suggest that the antimicrobial activity attributed to canid saliva may be partly due to the presence of indigenous *Lactobacillus* and *Enterococcus* species in *S. venaticus* and *C. brachyurus*.

## Limitations of the study

All samples were collected from individuals maintained in a single wildlife facility (Zoobotânica Foundation, Brazil), under controlled dietary and environmental conditions. Therefore, the results represent the microbiota of managed animals and may not fully reflect wild populations. Moreover, *C. brachyurus* samples were from adult females (8–13 years), while *S. venaticus* samples were from young males (~2 years), and these demographic factors might affect oral microbial composition. Broader studies including multiple locations, age ranges, and both sexes will be necessary to confirm species-level representativeness.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s42770-026-01877-0.

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**Author contributions** The process of conceptualization, data curation, formal analysis, funding acquisition, project administration, and supervision validation were performed by JRN and FSM. Writing original draft, reviewing and editing were conducted by MLOMS, JRN and FSM. Data curation, formal analysis, and investigation methodology were provided by MLOMS, AKSM, SHCS, LBA, ACN, ILBCC, HPT, JRN and FSM. Project administration and resources were provided by ACN, ILBCC, JRN and FSM. All authors read and approved the final version of the manuscript.

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**Data availability** The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics statement** This is exploratory research that was performed after approval by the Ethics Committees for Animal Experiments of the UNA University Centre and the Zoobotânica Foundation in Belo Horizonte, MG, Brazil.

**Generative AI statement** The authors declare that Generative AI was used to improve the grammar of some phrases in this manuscript.

**Conflict of interest** The authors declare there are no conflicts of interest related to this study.



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