

## Molecular characterization of bacterial communities of two neotropical tick species (*Amblyomma aureolatum* and *Ornithodoros brasiliensis*) using rDNA 16S sequencing

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### ABSTRACT

Ticks are one of the main vectors of pathogens for humans and animals worldwide. However, they harbor non-pathogenic microorganisms that are important for their survival, facilitating both their nutrition and immunity. We investigated the bacterial communities associated with two neotropical tick species of human and veterinary potential health importance from Brazil: *Amblyomma aureolatum* and *Ornithodoros brasiliensis*. In *A. aureolatum* (adult ticks collected from wild canids from Southern Brazil), the predominant bacterial phyla were Proteobacteria (98.68%), Tenericutes (0.70%), Bacteroidetes (0.14%), Actinobacteria (0.13%), and Acidobacteria (0.05%). The predominant genera were *Francisella* (97.01%), *Spiroplasma* (0.70%), *Wolbachia* (0.51%), *Candidatus* Midichloria (0.25%), and *Alkanindiges* (0.13%). The predominant phyla in *O. brasiliensis* (adults, fed and unfed nymphs collected at the environment from Southern Brazil) were Proteobacteria (90.27%), Actinobacteria (7.38%), Firmicutes (0.77%), Bacteroidetes (0.44%), and Planctomycetes (0.22%). The predominant bacterial genera were *Coxiella* (87.71%), *Nocardioideis* (1.73%), *Saccharopolyspora* (0.54%), *Marmoricola* (0.42%), and *Staphylococcus* (0.40%). Considering the genera with potential importance for human and animal health which can be transmitted by ticks, *Coxiella* sp. was found in all stages of *O. brasiliensis*, *Francisella* sp. in all stages of *A. aureolatum* and in unfed nymphs of *O. brasiliensis*, and *Rickettsia* sp. in females of *A. aureolatum* from Banhado dos Pachecos (BP) in Viamão municipality, Brazil, and in females and unfed nymphs of *O. brasiliensis*. These results deepen our understanding of the tick-microbiota relationship in Ixodidae and Argasidae, driving new studies with the focus on the manipulation of tick microbiota to prevent outbreaks of tick-borne diseases in South America.

### 1. INTRODUCTION

The microbiome is a major factor in mammalian physiology (Rosshart et al., 2017). The microbiota of mammals is associated with obesity (Mulders et al., 2018), diabetes (Inoue et al., 2017), cancer (Kroemer and Zitvogel, 2018; Rosshart et al., 2017), viral infections (Oh et al., 2014; Rosshart et al., 2017), multiple sclerosis (Dopkins et al., 2018), hypertension, chronic kidney disease (Sircana et al., 2019), and other metabolic, nervous, cardiovascular, and immune diseases. These associations have been strengthened by several lines of evidence that link the

effect of the microbiota to the physiology of mammals (mainly humans). However, all metazoans have partnered with a small or large consortium of microbes to enhance their health and survival, which is also valid for ticks (Narasimhan and Fikrig, 2015).

Endosymbioses are interactions in which a smaller partner (the endosymbiont) lives inside the cell of a larger individual (the host). Examples of this relationship include the origin of mitochondria in eukaryotic cells (Dietel et al., 2018), and the *Coxiella* mutualist symbiont, which is essential to the development of *Rhipicephalus microplus* (Guizzo et al., 2017). Ticks are among the main vectors of diseases for

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humans and animals, but little is known about the interactions of these arthropods with their microbiota (Bonnet et al., 2017). It is well known that the microbiota plays a vital role in the physiology of its host, either by preventing infections by pathogens or by the synthesis of compounds necessary for the survival of the host organism (Bonnet et al., 2017; Gerhart et al., 2016). In addition to endosymbionts, pathogens of medical and veterinary importance, such as species of the genera *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, and *Rickettsia*, are present in ticks (Greay et al., 2018). Furthermore, arthropod microbiota could influence the presence and transmission of mammalian pathogens. For instance, commensal and symbiont bacteria can inhibit infection by *Plasmodium* spp., *Trypanosoma* spp. and the Dengue virus in *Anopheles* spp., *Glossina* spp., and *Aedes aegypti*, respectively (Weiss and Aksoy, 2011). In this sense, recently, several studies have addressed the microbiome of ticks of medical importance, such as *Ixodes ricinus* (Hernández-Jarguín et al., 2018), *Ixodes scapularis* (Clow et al., 2018; Narasimhan et al., 2014), *Ixodes pacificus* (Kwan et al., 2017), *Dermacentor andersoni* (Clayton et al., 2015; Gall et al., 2016), *Amblyomma americanum* (Ponnuamy et al., 2014; Trout Fryxell and DeBruyn, 2016), *Amblyomma maculatum* (Budachetri et al., 2014), and *Haemaphysalis longicornis* (Nakao et al., 2013; Zhang et al., 2019; Zhuang et al., 2018).

Despite the abundance of tick species and the impact of tick-borne diseases in South America, to date, there have been few research studies on the microbiome of South American native ticks. In recent years, microbiome studies of Neotropical ticks have become available, such as for *Amblyomma cajennense* sensu stricto and *Amblyomma sculptum*. In these studies, the genera *Coxiella*, *Francisella*, and *Rickettsia* are consistently present (Binetruy et al., 2019; Binetruy et al., 2020). It is important to note that the genera *Coxiella*, *Francisella*, and *Rickettsia* contain both endosymbiont and pathogen species. Particularly, the genus *Coxiella* harbors a pathogen species, the Q fever agent *Coxiella burnetii*, and a plethora of closely related *Coxiella* endosymbionts. A similar pattern can be observed in *Francisella* and *Rickettsia* species (Binetruy et al., 2019, 2020).

Brazil has a tick fauna composed of 70 species, of which 47 belong to the Ixodidae and 23 to the Argasidae (Dantas-Torres et al., 2019). Considering the Ixodidae, the *Amblyomma aureolatum* tick has been recognized as a vector of Spotted Fever Group (SFG) rickettsiae (Dall'Agnol et al., 2018; Saraiva et al., 2014) and the protozoan *Rangelia vitalii* (Soares et al., 2018, 2014). On the other hand, among the Argasidae ticks, *Ornithodoros brasiliensis* is associated with a toxicosis syndrome in humans and animals (Reck et al., 2013, 2011), and has been linked to the transmission of a *Borrelia* species (Davis, 1952). Therefore, the aim of this work was to investigate the bacterial communities associated with two neotropical tick species of human and veterinary health importance from Brazil: *A. aureolatum* and *O. brasiliensis*.

## 2. MATERIALS AND METHODS

### 2.1. Ticks

From December 2014 to December 2016, *A. aureolatum* (Acari: Ixodidae) adult ticks were collected from their natural host, free-ranging crab-eating fox (*Cerdocyon thous*). Wild canids were captured using Tomahawk live-traps in two areas of environmental preservation, in the Pampa biome in the Rio Grande do Sul (RS) state, southern Brazil: Banhado dos Pachecos (BP) in Viamão municipality, and APA Ibirapuitã, in Santana do Livramento municipality.

Specimens of *O. brasiliensis* (Acari: Argasidae) (fed adults, fed and unfed nymphs) were collected in July 2016 in their natural environment from the Atlantic Rainforest biome in the Jaquirana municipality, Southern Brazil highlands, RS. Ticks were manually collected by sifting the soil. Vertebrate hosts of *O. brasiliensis* include dogs, armadillos, and possibly skunks, besides humans (Reck et al., 2011; Reck et al., 2013).

After the ticks were collected, they were immediately taken to the laboratory and washed thrice in 70% ethanol, followed by a final wash

using sterile ultrapure water to remove debris and to disinfect the surface. Ticks were identified up to species level by dichotomous keys (Barros-Battesti et al., 2006, 2012). Samples of *A. aureolatum* adults were discriminated by gender (males and females), whereas *O. brasiliensis* specimens were classified as fed adult males, fed adult females, and fed or unfed nymphs.

### 2.2. DNA extraction, library preparation, and sequencing

The DNA extraction procedures were performed in a biosafety cabinet to ensure sample protection from environmental contaminants and the protection of researchers from potential infectious pathogens. Genomic DNA was extracted from each individual whole tick using the PureLink Genomic DNA MiniKit (Invitrogen, Carlsbad, CA, USA), including treatment with 50 µL lysozyme (20 mg/mL) (Sigma-Aldrich, Dorset, UK) at 30 min/37 °C for peptidoglycan disruption. In all DNA extraction procedures, a negative control (ultrapure water) was included. Ticks were grouped by host (for *A. aureolatum*), sex, stage/instar, and locality, as indicated in Table 1.

To synthesize the sequencing libraries, the V4 region of the bacterial 16S rRNA gene was amplified according to Kozich and coworkers (2013) and the 16S metagenomic sequencing library preparation guide (Illumina Inc.) ([https://support.illumina.com/documents/documentation/chemistry\\_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf?\\_ga=2.236812035.389663062.1622237564-255547950.1622237564](https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf?_ga=2.236812035.389663062.1622237564-255547950.1622237564)). In all PCR reactions, a negative control (ultrapure water) was included. Sequencing was performed on a MiSeq (Illumina) platform using a 500-cycle v2 kit generating 250-bp paired-end reads. A drawback of our study is that we are not able to include a mock control for library sequencing due to the high cost for the inclusion of additional samples.

### 2.3. Bioinformatics analysis

Raw FASTQ files were used as input for the Mothur MiSeq SOP (Kozich et al., 2013), and data analysis was performed in the statistical language R. The operational taxonomic units (OTU) generated were compared with the ribosomal RNA database Silva (<https://www.arb-silva.de/>).

## 3. RESULTS

The mean number of raw reads obtained per sample before and after quality control was 313,515.2 (100,548 to 765,590) and 276,052.3 (91,728 to 665,380), respectively (Table 2).

In *A. aureolatum*, the predominant bacterial phyla were Proteobacteria (98.68%), Tenericutes (0.70%), Bacteroidetes (0.14%), Actinobacteria (0.13%), and Acidobacteria (0.05%). The predominant genera were *Francisella* (97.01%), *Spiroplasma* (0.70%), *Wolbachia* (0.51%), *Candidatus* Midichloria (0.25%), and *Alkanindiges* (0.13%), as shown in Fig. 1. *Francisella* was the predominant genus in all samples of *A. aureolatum*. In ticks from BP, *Spiroplasma* sp. was found in all samples, whilst in ticks from APA Ibirapuitã, it was found only in one female and in a small proportion. On the other hand, *Wolbachia* sp. was found only in samples from BP, at a larger proportion in males than in females. *Candidatus* Midichloria sp. was also found only in ticks from BP. *Alkanindiges* sp. was found only in males and *Morganella* sp. only in females from BP. *Rickettsiella* sp. was found only in male ticks from BP. A greater amount of unclassified Proteobacteria was found in APA Ibirapuitã than in BP. *Mycobacterium* sp. and *Burkholderia* sp. were also present in a larger amount in BP-collected ticks.

The predominant phyla in *O. brasiliensis* were Proteobacteria (90.27%), Actinobacteria (7.38%), Firmicutes (0.77%), Bacteroidetes (0.44%), and Planctomycetes (0.22%). The predominant bacterial genera were *Coxiella* (87.71%), *Nocardioideis* (1.73%), *Saccharopolyspora* (0.54%), *Marmoricola* (0.42%), and *Staphylococcus* (0.40%), as shown in

**Table 1**

Data about origin of the tick pool used in the DNA metabarcoding analysis.

Sample origin	Biome	latitude	longitude	Tick species	NCBI taxon id of the tick	Library name	Life stage	Sex	Number of ticks
APA Ibirapuitã	Pampa	30°20'32.5"S	55°41'09.7"W	<i>Amblyomma aureolatum</i>	187,763	B	Adult	Male	5
APA Ibirapuitã	Pampa	30°20'32.5"S	55°41'09.7"W	<i>Amblyomma aureolatum</i>	187,763	C	Adult	Female	5
APA Ibirapuitã	Pampa	30°20'32.5"S	55°41'09.7"W	<i>Amblyomma aureolatum</i>	187,763	D	Adult	Female	5
Banhado dos Pachecos	Pampa	30°05'37.9"S	50°51'01.5"W	<i>Amblyomma aureolatum</i>	187,763	E	Adult	Male	5
Banhado dos Pachecos	Pampa	30°05'37.9"S	50°51'01.5"W	<i>Amblyomma aureolatum</i>	187,763	F	Adult	Male	5
Banhado dos Pachecos	Pampa	30°05'37.9"S	50°51'01.5"W	<i>Amblyomma aureolatum</i>	187,763	G	Adult	Female	5
Banhado dos Pachecos	Pampa	30°05'37.9"S	50°51'01.5"W	<i>Amblyomma aureolatum</i>	187,763	H	Adult	Female	5
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	J	Adult	Male	1
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	K	Adult	Female	1
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	L	Adult	Female	1
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	M	Fed nymph	Immature	5
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	N	Fed nymph	Immature	5
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	P	Unfed nymph	Immature	15
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	Q	Unfed nymph	Immature	15
Jaquirana	Atlantic rainforest	29°01'09.8"S	50°25'54.3"W	<i>Ornithodoros brasiliensis</i>	888,526	R	Unfed nymph	Immature	15

**Table 2**

Data on the metagenomic sequencing process, and analysis of bacterial diversity based on the different OTUs.

Sample origin	Tick species	Library name	Life stage	Sex	Number of raw reads	Number of reads after quality control	Shannon index	Simpson index	invSimpson index
APA Ibirapuitã	<i>Amblyomma aureolatum</i>	B	Adult	Male	294,424	277,238	0.097180283	0.019209686	1.019585882
APA Ibirapuitã	<i>Amblyomma aureolatum</i>	C	Adult	Female	346,018	327,598	0.055127549	0.013924067	1.014120674
APA Ibirapuitã	<i>Amblyomma aureolatum</i>	D	Adult	Female	100,548	91,728	0.080087477	0.024767218	1.025396194
RVS Banhado dos Pachecos	<i>Amblyomma aureolatum</i>	E	Adult	Male	318,006	298,018	0.752314061	0.28020152	1.389274852
RVS Banhado dos Pachecos	<i>Amblyomma aureolatum</i>	F	Adult	Male	312,700	285,202	1.597470598	0.561248189	2.279160929
RVS Banhado dos Pachecos	<i>Amblyomma aureolatum</i>	G	Adult	Female	144,174	131,514	0.11195518	0.02694269	1.027688625
RVS Banhado dos Pachecos	<i>Amblyomma aureolatum</i>	H	Adult	Female	239,878	207,742	2.084659654	0.694184435	3.269807541
Jaquirana	<i>Ornithodoros brasiliensis</i>	J	Adult	Male	148,164	119,234	1.116713161	0.267420311	1.365030238
Jaquirana	<i>Ornithodoros brasiliensis</i>	K	Adult	Female	368,288	323,768	1.125283115	0.25330364	1.33922674
Jaquirana	<i>Ornithodoros brasiliensis</i>	L	Adult	Female	429,368	376,904	1.046095937	0.239096409	1.314222253
Jaquirana	<i>Ornithodoros brasiliensis</i>	M	Fed nymph	Immature	381,608	319,306	1.58776802	0.392536779	1.646172909
Jaquirana	<i>Ornithodoros brasiliensis</i>	N	Fed nymph	Immature	234,072	200,520	1.581527038	0.391100196	1.642283497
Jaquirana	<i>Ornithodoros brasiliensis</i>	P	Unfed nymph	Immature	346,760	294,182	0.611494688	0.136234093	1.157719706
Jaquirana	<i>Ornithodoros brasiliensis</i>	Q	Unfed nymph	Immature	765,590	665,380	0.85484268	0.197547578	1.246177156
Jaquirana	<i>Ornithodoros brasiliensis</i>	R	Unfed nymph	Immature	273,130	222,450	0.750464292	0.176728503	1.214663905

**Fig. 2.** In the immature stages (nymphs), *Staphylococcus* sp. and *Escherichia-Shigella* were present in greater numbers compared to adults. Unclassified Gammaproteobacteria were found in greater amounts in unfed nymphs, whilst the genera *Rickettsiella*, *Nitrosospora*, *Rubrobacter*,

*Planctomyces*, and *Acidibacter* were present in greater quantity in adults (males and females).

Bacterial diversity seemed to be higher in the *A. aureolatum* samples collected in BP compared to those from APA Ibirapuitã, and there was no

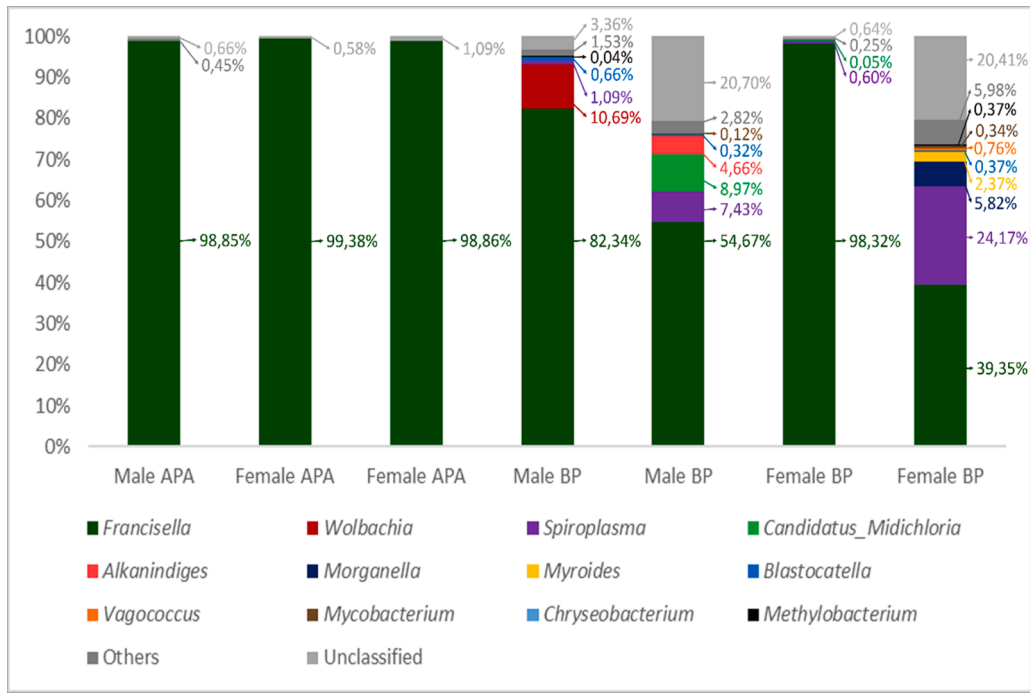


Fig. 1. The relative abundance of bacterial genera in *Amblyomma aureolatum* ticks. Bars represent the proportion of main bacterial genera found in the *A. aureolatum* microbiome, each bar is one different sample (replicate). Different genera are shown as different colors in the bars. Below the bars, the main bacterial genera are shown. Note the comparison among male and female ticks from two different locations (APA, APA do Ibirapuitá; BP, RVS Banhado dos Pachecos).

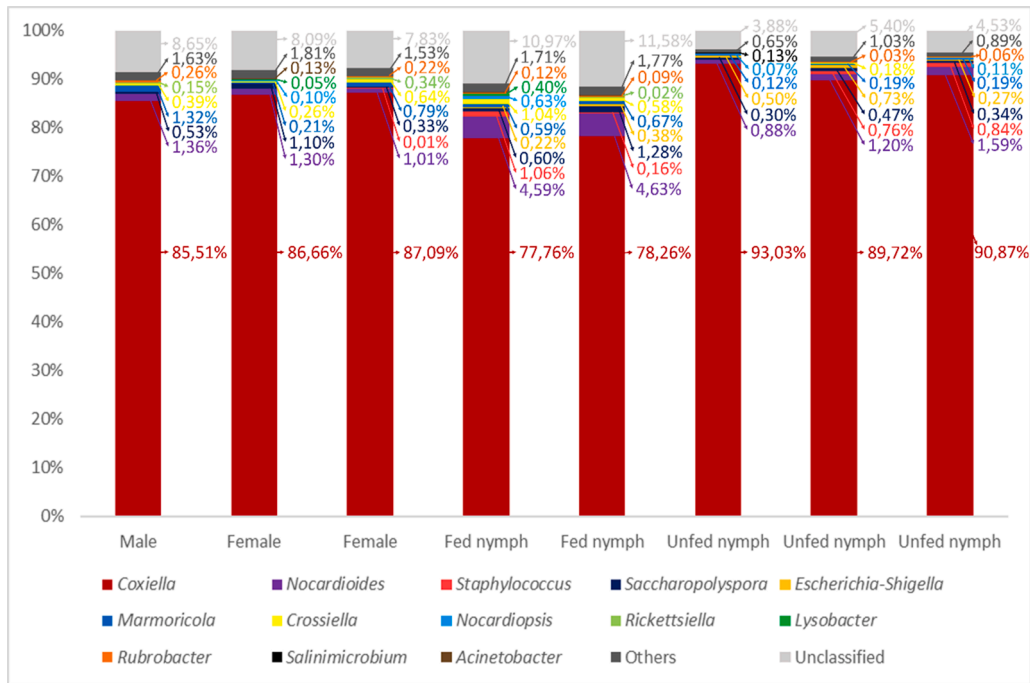
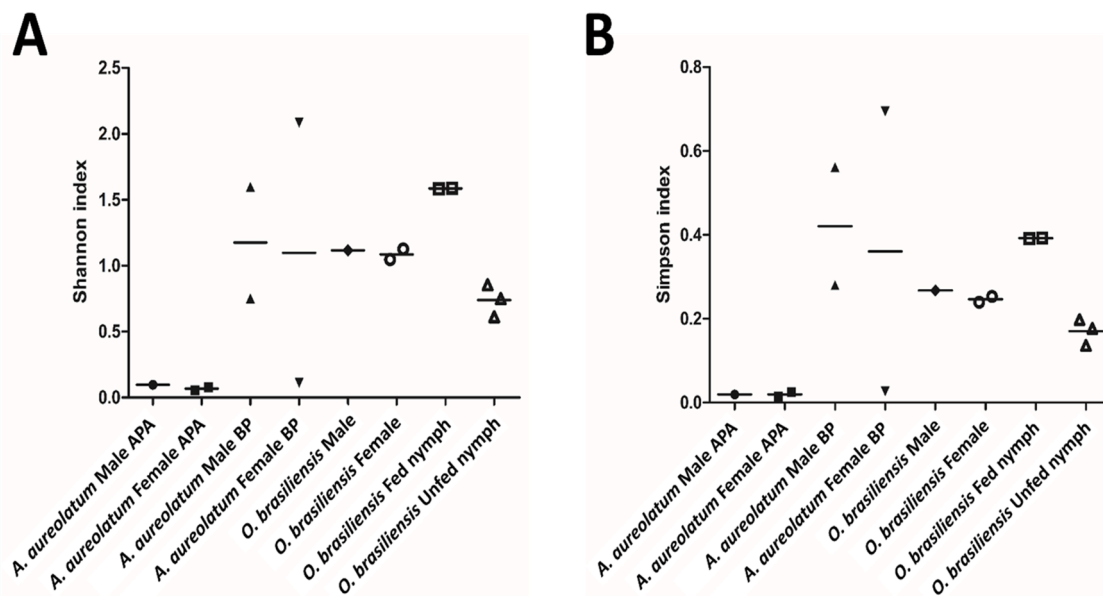


Fig. 2. The relative abundance of bacterial genera in *Ornithodoros brasiliensis* ticks. Bars represent the proportion of main bacterial genera found in the *O. brasiliensis* microbiome, each bar is one different sample (replicate). Different genera are shown as different colors in the bars. Below the bars, the main bacterial genera are shown. Note the comparison among male, female, fed and unfed nymph ticks.

evident difference between males and females (Fig. 3). Since *O. brasiliensis* ticks were collected in only one location, diversity evaluation only allowed a comparison among different stage, with a greater diversity in fed nymphs (Fig. 3).

Considering the genera with potential importance for human and animal health that can be transmitted by ticks, *Coxiella* sp. was found in

all stages of *O. brasiliensis*, *Francisella* sp. in all stages of *A. aureolatum* and unfed nymphs of *O. brasiliensis*, and *Rickettsia* sp. in females of *A. aureolatum* from BP and females and unfed nymphs of *O. brasiliensis*.



**Fig. 3.** The different indexes of alpha diversity of bacterial microbiomes of *Amblyomma aureolatum* and *Ornithodoros brasiliensis*. Panel. (A) Bacterial diversity by Shannon Index. (B) Bacterial diversity by Simpson Index. The horizontal lines show the mean of the replicates, and the symbols (circles, squares, triangles, diamonds) represent each sample analyzed. Abbreviations: APA, APA do Ibirapuitã; BP, Banhado dos Pachecos.

#### 4. DISCUSSION

Ticks are one of the main vectors of pathogens for humans and animals worldwide. However, they harbor non-pathogenic microorganisms that are important for their survival, helping in both their nutrition and immunity. Ticks depend on bacteria, such as *Coxiella*-like and *Francisella*-like endosymbionts, that probably provide nutrients that are lacking in their diet. In addition to endosymbionts, ticks may also be present and transmit pathogens to mammals, including *C. burnetii* and *Francisella tularensis*. However, the evolutionary relationship between endosymbiotic and pathogenic species of the same genus is puzzling (Duron et al., 2015; Gerhart et al., 2016). Analyzing the genome of *Coxiella*-like endosymbionts (CLE) of *Rhipicephalus sanguineus sensu lato* and *Rhipicephalus turanicus*, Tsementzi et al. (2018) observed that both genomes encode numerous pseudogenes, consistent with an ongoing genome reduction process. Similarly, *Francisella*-like endosymbionts (FLEs) contain pseudogenized versions of virulence genes present in *F. tularensis*, indicating that the common ancestor of FLEs and mammalian pathogens were equipped to function as a pathogen (Gerhart et al., 2018). *In silico* flux balance metabolic analysis (FBA) revealed the excess production of L-proline for CLE genomes, indicating a possible proline transport from *Coxiella* to the tick. Additionally, CLE genomes encode multiple copies of the proline/betaine transporter *proP* gene (Tsementzi et al., 2018). Moreover, FLE genomes contain intact pathways for the synthesis of several B vitamins and cofactors lacking in vertebrate blood (Gerhart et al., 2018). This possible symbiotic mechanism of B vitamin provisioning by FLE has formally been demonstrated by Duron et al. (2018) through genomic and microbiome studies.

The active search for new microorganisms harbored in ticks should ideally be coordinated to prevent new cases of diseases, since tick-associated microorganisms of unknown pathogenicity may be identified first, and then an association with human or animal diseases could be found (Varela-Stokes et al., 2017). For instance, *Rickettsia parkeri* was recovered from *Amblyomma maculatum* in Texas approximately 60 years prior to the index case in humans, which was only reported in 2004. In addition, the spirochete *Borrelia miyamotoi*, first identified in *Ixodes persulcatus* in Japan, was considered as a non-pathogenic microorganism until the first human cases were reported only 15 years later (Varela-Stokes et al., 2017).

In *A. aureolatum* ticks, the principal genus found was *Francisella*. This

corroborates the results from Pavanelo and coworkers (2020), who found a dominance of the genus *Francisella* in the *A. aureolatum* midgut microbiota. Budachetri and coworkers (2014) and Varela-Stokes and coworkers (2018) also reported *Francisella* as the main genus in *A. maculatum* (another *Amblyomma* species restricted to the Americas). Additionally, the presence of *Francisella* sp. has been reported in high abundance in *Dermacentor* species, including *Dermacentor variabilis*, *Dermacentor andersoni*, *Dermacentor hunteri*, *Dermacentor nitens*, *Dermacentor occidentalis*, and *Dermacentor albipictus* (Budachetri et al., 2014; Niebylski et al., 1997; Scoles, 2004; Sun et al., 2000). On the other hand, the most abundant genera in *Amblyomma variegatum* (an Afrotropical tick species) were *Clavibacter* (13.4%) in males and *Borrelia* (8.6%) in females (Nakao et al., 2013). In *Amblyomma testudinarium* (an Oriental-Palaearctic tick species) nymphs, the predominant genus was *Pseudoalteromonas* (17.2%) (Nakao et al., 2013). In *Amblyomma americanum* (a Nearctic tick species), *Coxiella* was found in a greater amount in nymphs (26%), whereas *Bradyrhizobium* and *Phenylobacterium* were predominant in adults (28 to 45%) (Menchaca et al., 2013). However, Ponnusamy and coworkers (2014) observed that three of the most common genera found in *A. americanum* were *Rickettsia*, “*Candidatus* Midichloria mitochondrii”, and *Ehrlichia*, all members of the order Rickettsiales, representing 53% (median; interquartile range, 31 to 75%) of the reads. Trout Fryxell and DeBruyn (2016) found that the most dominant OTUs were highly variable among specimens of *A. americanum*, but the most abundant ones (> 1% relative abundance) across all specimens were *Flavobacterium* (24.4 ± 13.3%), an unclassified Gammaproteobacteria (2.22 ± 12.4%), *Rickettsia* (9.1 ± 14.5%), *Sphingomonas* (4.6 ± 3.6%), *Singulisphaera* (1.91 ± 1.81%), *Hymenobacter* (1.95 ± 3.00%), and *Bacillus* (1.86 ± 11.7%). In *Amblyomma tuberculatum* (a Nearctic tick species), the main genera found were *Rickettsia* (55.8%) and *Francisella* (35.2%) (Budachetri et al., 2016). Budachetri and coworkers (2017) determined the microbiomes of *Amblyomma longirostre*, *Amblyomma nodosum*, *Amblyomma maculatum*, and *Haemaphysalis juxtakochi* collected from migratory bird species and found that the most prevalent genera observed, with abundance levels above 1%, were *Lactococcus*, *Raoultella*, *Wolbachia*, *Francisella*, *Propionibacterium*, *Ewingella*, *Elizabethkingia*, *Rickettsia*, *Massilia*, and *Methylobacterium*.

Besides *Francisella*, the main genera found in *A. aureolatum* were *Spiroplasma*, *Wolbachia*, *Candidatus* Midichloria, and *Alkanindiges*.

*Spiroplasma* is common in arthropods (for a comprehensive review, see Bonnet et al., 2017 and O. Duron et al., 2017) and, although their effect in ticks is unknown, these bacteria showed a male-killer effect in diverse insect species (Engelstädter and Hurst, 2009). The genus *Wolbachia* is highly common in arthropods and is also present in ticks (Bonnet et al., 2017). The effect of this genus on ticks is largely unknown, but it is responsible for reproductive alterations in many arthropods (Engelstädter and Hurst, 2009). It is a facultative mutualist (defensive symbiosis) in mosquitoes (Brownlie and Johnson, 2009; Hamilton and Perlman, 2013) and an obligate symbiont in bed bugs (Hosokawa et al., 2010; Nikoh et al., 2014). At least in the case of *I. ricinus*, it has been demonstrated that the detection of *Wolbachia* sp. was due to contamination by a hymenopteran parasitoid (Plantard et al., 2012). In several ticks, *Candidatus* Midichloria sp. resides in high numbers in female reproductive tissues. It lives inside tick mitochondria, but its effects on tick physiology and metabolism remain unknown (Buysse and Duron, 2018; Epis et al., 2013). Nevertheless, it has recently been suggested that *Candidatus* Midichloria sp. is an obligate nutritional symbiont providing B vitamins (O. Duron et al., 2017) since its genome encodes pathways for the synthesis of major B vitamins and cofactors (Buysse and Duron, 2018; Sasser et al., 2011). Male *I. holocyclus* ticks also appear to inherit and harbor *Candidatus* Midichloria sp.; however, *I. ricinus* males fail to establish stable bacterial populations. In addition, *Candidatus* Midichloria sp. is found in *I. ricinus* salivary glands, from where it is introduced to vertebrate hosts, including humans, during feeding. However, the consequences of *Midichloria* infection in vertebrate hosts, if any, are unknown (Gofton et al., 2015; Mariconti et al., 2012; Serra et al., 2018). *Alkanindiges* sp. is not commonly found in high proportions in ticks, although *R. microplus* eggs have been described to present a relative abundance of 0.2% (Andreotti et al., 2011). It seems that *Alkanindiges* sp. are found in oilfield soils (Bogan et al., 2003; Chang et al., 2011; Fuentes et al., 2016; Sun et al., 2015; Zheng et al., 2018), patients with parotid abscesses (Woo et al., 2005) and with, bone and joint infections (Fenollar et al., 2006), patients with asthma (Fazlollahi et al., 2018), activated sludge systems (Klein et al., 2007), tonsils of healthy pigs (Lowe et al., 2012), lettuce (Erlicher et al., 2014; Rastogi et al., 2012), and drinking water wells (Karwautz and Lueders, 2014).

To the best of our knowledge, there are only few studies on the microbiome characterization of Argasidae. Ticks of this family could exhibit characteristics remarkably different from those of Ixodidae, for instance, fast feeding (minutes) and a long life (some species may live for several years). They can also induce severe injuries directly associated with the tick bite (tick toxicosis), may have several nymphal stages, and females do not die after oviposition (Ramirez et al., 2016; Reck et al., 2013). In *Ornithodoros tholozani* collected from buffaloes from Pakistan, the main bacterial genera found were *Ralstonia* (40.0%), *Staphylococcus* (22.8%), *Enterococcus* (13.9%), *Saccharomonospora* (4.5%), and *Bacillus* (4.3%) (Karim et al., 2017). The most predominant genus found in *O. brasiliensis* was *Coxiella*. This bacterium was also the main symbiont of *Rhipicephalus* spp. (Bernasconi et al., 2002; Guizzo et al., 2017). Guizzo and coworkers (2017) showed that, in *Rhipicephalus microplus*, 99 and 98.3% of bacteria present in eggs and larvae, respectively, were *Coxiella* sp. The levels of *Coxiella* sp. were affected in fully engorged females injected with tetracycline; the development of larva with reduced levels of the *Coxiella* sp. was arrested at the metonymph stage. In Argasidae, the endosymbiont *Coxiella* sp. was found in *Carios capensis* (Reeves, 2005), *Argas monolakensis* (Reeves, 2008), *Ornithodoros rostratus* (Almeida et al., 2012), *Ornithodoros muesebecki* (Al-Deeb et al., 2016), and in at least 10 other soft tick species, including *O. brasiliensis* (O. Duron et al., 2017).

Besides *Coxiella* sp., the main genera found in *O. brasiliensis* were *Nocardioideis*, *Saccharopolyspora*, *Marmoricola*, and *Staphylococcus*. *Nocardioideis* was a symbiont of ants and was involved in ant-plant mutualisms (Hanshew et al., 2015; Reyes and Cafaro, 2015); it was also isolated from the water flea *Daphnia cucullata* (Crustacea: Cladocera) (Toth et al., 2008). *Saccharopolyspora* was also found associated with

ants (Reyes and Cafaro, 2015) and was isolated from the gut of a termite (*Speculitermes* sp.) (Sinma et al., 2011). Members of the genus *Saccharopolyspora* are a potentially rich source of natural products, but only erythromycin, produced by *Saccharopolyspora erythraea*, and spinosad, an insecticide based on chemical compounds found in the bacterial species *Saccharopolyspora spinosa*, are currently commercially available (Prabhu et al., 2011; Sinma et al., 2011). The genus *Marmoricola* was also found in spiders from Japan (Iwai et al., 2009) and ants from the USA (Ishak et al., 2011). Andreotti and coworkers (2011) showed that the genus *Staphylococcus* was relatively abundant in males (32%) and in the eggs (18%) of *R. microplus* ticks, but not in adult females (0.7%).

In our study, among the genera with potential importance for human and animal health that can be transmitted by ticks, the presence of *Coxiella*, *Francisella*, and *Rickettsia* is noteworthy. We found *Rickettsia* sp. in *A. aureolatum*, which corroborates the studies regarding its importance as a vector of spotted fever in Brazil (Dall'Agnol et al., 2018; Saraiva et al., 2014). We also found *Rickettsia* sp. in *O. brasiliensis*. Bacteria of the genus *Rickettsia* have never been reported in *O. brasiliensis* and have only recently been found in argasid ticks in Brazil (Labruna et al., 2014; Luz et al., 2019; Muñoz-Leal et al., 2019; Peixoto et al., 2021). In recent years, the number of reports of *Rickettsia* spp. in Argasidae has increased, including the description of novel species (Duh et al., 2010; Karim et al., 2017; Lafri et al., 2015; Milhano et al., 2014; Moreira-Soto et al., 2017; Pader et al., 2012; Sánchez-Montes et al., 2016; Socolovschi et al., 2012; Tahir et al., 2016). Therefore, tick-transmitted rickettsioses may be a neglected subject that may have an impact on public health, considering the amount of human cases of parasitism by Argasidae, mainly by *O. brasiliensis* (Martins et al., 2011; Reck et al., 2013). Further studies may clarify the potential roles of *Rickettsia* spp. in the syndrome associated with the *O. brasiliensis* bite.

Finally, it was not possible to detect *Borrelia* sp. in *O. brasiliensis*. Several species of *Ornithodoros* (such as *Ornithodoros hermsi*, *Ornithodoros sonrai*, *Ornithodoros turicata*, *Ornithodoros erraticus*, *Ornithodoros moubata*, and *Ornithodoros rudiis*), especially those presenting a public health impact, have been associated with the transmission of *Borrelia* spp., belonging to the relapsing fever group (Boyle et al., 2014; Lopez et al., 2011; McCoy et al., 2010; Muñoz-Leal et al., 2018; Schwan et al., 2012; Trape et al., 2013). Conversely, Davis (1952) reported the isolation of *Borrelia* sp. in samples of *O. brasiliensis*. This difference corroborates the results found in this study, as we were able to describe significant differences in the bacteriome composition within individuals of a single species and found that the factors sex, developmental stage, mammal host, and environment may influence it. Additionally, these data show the importance of investigating pathogen presence, specifically since bacterial genera present in small proportions in a sample may not appear in the analysis of rRNA 16S gene metabarcoding (Frey et al., 2014). Abundant bacterial endosymbionts limit the effectiveness of next-generation 16S bacterial community profiling in arthropods by masking less abundant bacteria, including pathogens (Gofton et al., 2015). In addition, the samples of *O. brasiliensis* were collected only from one place. The presence of *Borrelia* spp. in samples from different locations and using techniques with a higher sensitivity could help to clarify this issue (Boyle et al., 2014; Muñoz-Leal et al., 2018).

In *A. aureolatum*, bacterial diversity was higher in samples collected in BP compared to those from APA Ibirapuitã. The BP is a wildlife conservation refuge surrounded by small farms, with stray dogs carrying ticks in and out. The APA Ibirapuitã has an extremely low human population and animal density in comparison to the BP. In *O. brasiliensis*, fed nymphs had a greater microbial diversity than unfed nymphs, most likely because the former enters into contact with the host's microbiota during the blood meal. Males and females also had a lower diversity compared to fed nymphs; this difference may be due to the fact that the adult ticks were analyzed individually and the fed nymphs in pools of five individuals. There may also be variation among specimens.

In this study, we determined the bacteriomes of two tick species native to Brazil. Furthermore, we were able to compare the

*A. aureolatum* bacterial diversity from specimens of different ecological characteristics. These results facilitate our understanding of the tick-microbiota relationship in Ixodidae and Argasidae and may drive new studies with a focus on the manipulation of tick microbiota to prevent outbreaks of tick-borne diseases in South America.

### Authors' contributions

BD did most of the experiments, collaborated in the study design, analyzed data, and wrote the manuscript. JR and CASF co-supervised the project, conceived/designed the study, and wrote the paper. PAR, UAS and RLD participated in the field collections and did some experiments. AW and UAS collaborated in some experiments and data analysis. FQM performed the DNA sequencing and contributed to the study design. JAM contributed with data analysis. All authors read and approved the final version of the paper.

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### Ethics approval

The study protocol was approved by the Committee for Animal Care and Experimentation of IPVDF (CEUA/IPVDF 28/2014) and by the Brazilian Ministry of Environment (SISBIO 47,357–3).

### Consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and material

Data available on request from the authors.

### Code availability

Not applicable.

### Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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