

# Analysis on the prokaryotic microbiome in females and embryonic cell cultures of *Rhipicephalus sanguineus* tropical and temperate lineages from two specific localities in Brazil

## Análise do microbioma procarionte de fêmeas e cultura celular embrionária de *Rhipicephalus sanguineus* linhagens tropical e temperada de duas localidades no Brasil

Mayara de Cassia Luzzi<sup>1†</sup>; Lucas Amoroso Lopes de Carvalho<sup>2†</sup>; Daniel Guariz Pinheiro<sup>2</sup>; Leidiane Lima-Duarte<sup>4</sup>; Jaqueline Valéria Camargo<sup>1</sup>; Luciano Takeshi Kishi<sup>3</sup>; Camila Cesário Fernandes<sup>3</sup>; Rosangela Zacarias Machado<sup>1</sup>; João Fábio Soares<sup>5</sup>; Marcos Rogério André<sup>1</sup>; Darci Moraes Barros-Battesti<sup>1,4\*</sup> 

<sup>1</sup> Departamento de Patologia, Reprodução e Saúde Única, Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP, Jaboticabal, SP, Brasil

<sup>2</sup> Departamento de Tecnologia, Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP, Jaboticabal, SP, Brasil

<sup>3</sup> Laboratório Multiusuário Centralizado para Sequenciamento de DNA em Larga Escala e Análise de Expressão Gênica – LMSeq, Departamento de Tecnologia, Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP, Jaboticabal, SP, Brasil

<sup>4</sup> Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo – USP, SP, Brasil

<sup>5</sup> Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, RS, Brasil

<sup>†</sup>The authors contributed equally.

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### Abstract

Two lineages of *Rhipicephalus sanguineus* are known in Brazil: the temperate or southern and the tropical or northern populations. The distribution patterns of both lineages of *R. sanguineus* have epidemiological implications that can affect vectorial competence concerning *Ehrlichia canis*, the agent of canine monocytic ehrlichiosis. Intending to identify the microbiomes of both lineages and compare microorganisms in *R. sanguineus*, we used the 16S rRNA (V4-V5 region) gene-based metataxonomic approach, through NGS sequencing on the MiSeq Illumina platform. We selected specimens of females from the environment and samples of primary embryonic cell cultures, from both lineages, and this was the first study to investigate the prokaryotic microbiome in tick cell cultures. The results showed that many bacterial taxa detected in the samples were typical members of the host environment. A significant diversity of microorganisms in *R. sanguineus* females and in embryonic cell cultures from both lineages was found, with emphasis on the presence of *Coxiella* in all samples, albeit in different proportions. The *Coxiella* species present in the two lineages of ticks may be different and may have co-evolved with them, thus driving different patterns of interactions between ticks and the pathogens that they can harbor or transmit to vertebrate hosts.

**Keywords:** Brown dog tick, tropical lineage, temperate lineage, tick embryonic cell culture, *Coxiella*, endosymbionts.

### Resumo

Duas linhagens de *Rhipicephalus sanguineus* são conhecidas no Brasil: populações da linhagem temperada ou do sul, e tropical ou do norte. Os padrões de distribuição de ambas as linhagens de *R. sanguineus* têm implicações epidemiológicas, podendo afetar a competência vetorial de *Ehrlichia canis*, o agente etiológico da erliquiose monocítica canina. Com a intenção de identificar os microbiomas de ambas as linhagens e comparar

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\*Corresponding author: Darci Moraes Barros-Battesti. E-mail: [barros.battesti@gmail.com](mailto:barros.battesti@gmail.com)



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microrganismos de *R. sanguineus*, foi utilizada a metataxonomia, baseada no gene 16S rRNA (região V4-V5), por meio do sequenciamento de nova geração na plataforma MiSeq Illumina. Foram selecionadas amostras de fêmeas do ambiente e cultivo primário de células embrionárias, considerando-se as duas linhagens conhecidas do Brasil. Este é o primeiro estudo que investiga o microbioma procariótico de células de cultura de carrapato. Os resultados mostram que muitos grupos de bactérias detectadas nas amostras são membros típicos do ambiente do hospedeiro. Uma diversidade significativa de microrganismos em fêmeas e cultura de células embrionárias nas duas linhagens de *R. sanguineus* foi encontrada, com ênfase na presença de *Coxiella* em todas as amostras, ainda que em diferentes proporções. Possivelmente, as espécies de *Coxiella* presentes nas duas linhagens de carrapatos são diferentes e co-evoluíram com essas linhagens, conduzindo a diferentes padrões de interação entre carrapatos e patógenos que podem abrigar ou transmitir aos hospedeiros vertebrados.

**Palavras-chave:** Carrapato-marrom-do-cão, linhagem tropical, linhagem temperada, cultura de células embrionárias, *Coxiella*, endossimbiontes.

## Introduction

*Rhipicephalus sanguineus sensu lato* (s.l.) is one of the most important species of ticks in Brazil and is considered to have major medical and veterinary significance (Dantas-Torres et al., 2012). It is widespread around the world and mainly parasitizes domestic dogs (*Canis lupus familiaris*). Its popular names include “brown dog tick”, “kennel tick” and “pan-tropical dog tick” (Gray et al., 2013). The taxon “*R. sanguineus*” forms a group of ticks that includes 17 species (Nava et al., 2012; Dantas-Torres & Otranto, 2015) sharing many morphological characteristics. It is well accepted today that two *R. sanguineus* lineages exist: a temperate population (southern lineage), which corresponds to *R. sanguineus sensu stricto* (s.s.) (Nava et al., 2018); and a tropical population (northern lineage) (Nava et al., 2012; Dantas-Torres & Otranto, 2015). Both of them can be found in Brazil, where the tropical lineage occurs in 15 Brazilian states, and the temperate lineage in at least two Brazilian states (Santa Catarina and Rio Grande do Sul) (Caetano et al., 2017). Besides the region of southern Brazil, the distribution of the temperate lineage in the New World includes populations of *R. sanguineus* in temperate and cold areas of Argentina, Chile, Uruguay and the USA (Moraes-Filho et al., 2011; Nava et al., 2012, 2018). The tropical lineage is distributed in regions of Argentina, Paraguay, Brazil, Colombia, South Africa and Mozambique (Nava et al., 2012).

The distribution patterns of both lineages of *R. sanguineus* also have epidemiological implications, due to differences that can affect vectorial competence concerning *Ehrlichia canis*, the etiological agent of canine monocytic ehrlichiosis (CME) (Nava et al., 2012; Cicuttin et al., 2015; Moraes-Filho et al., 2015).

The occurrence of CME in tropical regions of the South America is related to the difference in the vector competence between populations of *R. sanguineus* (s.l.). It was observed that populations of ticks belonging to the tropical lineage are highly competent vectors of *E. canis* while ticks belonging to the temperate lineage (*R. sanguineus* s.s.), distributed in South America (Nava et al., 2012), are incompetent vectors of *E. canis*. This fact coincides with scarcity or absence of CME in areas of distribution of *R. sanguineus* (temperate lineage) (Moraes-Filho et al., 2015). According to Nava et al. (2018), the populations of *R. sanguineus* s.l. from American tropical lineage and those of the *Rhipicephalus* sp. 1 lineage or southeastern European lineage clearly do not belong to *R. sanguineus* s.s.

Sanches et al. (2021) comparatively analyzed the proteomic profiles of both lineages of *R. sanguineus* (tropical and temperate) non-infected and *E. canis*-infected conditions. According to these authors, a higher number of over-represented proteins than under-represented were observed in salivary glands and midgut tissues of both lineages, suggesting that there is a greater translational investment in the tropical lineage when compared with the temperate lineage. Most of these proteins were grouped in the Gene Ontology (GO) terms related with metabolism, suggesting that the tropical lineage is more metabolically active than the temperate lineage. These authors, based on their results added to those known about the differences between these lineages previously observed on morphology, genetics, biology and behavior, concluded that these lineages represent distinct taxonomic units.

Leukocytes infected with *E. canis* were inoculated into cell cultures from several tick species, including *R. sanguineus* s.l., and isolation was successfully achieved mainly in embryonic cell lines from tick species that are not considered vectors (Ferrolo et al., 2016). However, primary embryonic cultures from *R. sanguineus* s.l. (tropical lineage) obtained from Brazilian ticks were found to be susceptible to *E. canis* infection, probably because these cultures maintained most of the cells resembling the vector (Barros-Battesti et al., 2018).

Embryonic tick cells have been cultivated since the 1950s (Weyer, 1952) and more than 40 strains have been established (Bell-Sakyi et al., 2007, 2018). Among the various purposes of cultivating these cells, they can be applied as substrates for the growth and isolation of several microorganisms (Alberdi et al., 2016; Cabezas-Cruz et al., 2016; Mansfield et al., 2017; Barros-Battesti et al., 2018).

Regarding other applicability for tick cell cultures, some are the following: 1) a better understanding of host-pathogen vector relationships, 2) elaborate analyzes for genomics, transcriptomics, and proteomics, 3) obtaining vaccine agents, 4) producing antigens and antibodies, 5) obtaining selective drug screening (Bhat & Yunker, 1979; Varela et al., 2004; Bell-Sakyi et al., 2007). Besides this useful panorama, it is important to characterize the tick cell lines aspects, if researchers want to compare them in *in vivo* relationships. It is important to know the differences and similarities with natural life, making it possible to produce correct questions and prevent probable problems in the research design.

Recently, efforts have been directed towards further elucidation of the tick microbiome (microbiota, commensal and symbiotic microorganisms), to certain factors that could affect ticks' adaptation, development, reproduction, defence and immunity mechanisms (Bonnet et al., 2017; Greay et al., 2018a). The most common bacteria found in general in the microbiome of adult ticks are *Coxiella*, *Francisella*, "*Candidatus Midichloria mitochondrii*", *Anaplasma*, *Borrelia*, *Ehrlichia* and *Rickettsia* (Greay et al., 2018b). With the advent of next-generation sequencing techniques and bioinformatics tools, determining the symbiotic commensal community has been facilitated. These tools can provide the next steps towards identifying the role of this community in ticks.

Tick microbiome can be different depending on tick genotype, biogeographical area, and if the tick is male or female. This is what René-Martellet et al. (2017) concluded analyzing *R. sanguineus* ticks from tropical and temperate lineages originated from Africa, North America, and Europe. The most present endosymbionts detected in DNA pools were *Coxiella*, *Rickettsia*, and *Bacillus*, aiming V5-V6 region of 16S rRNA. Gofton et al. (2015) investigated the bacterial profile of 460 ticks that can bite humans in Australia, using MiSeq Illumina to detect region V1-V2 of 16S rRNA. Many genera of environmental and commensal bacteria were found in the samples, such as Actinomycetales, Bacteroidetes, Firmicutes and Proteobacteria. Similar results were also obtained by other authors (Zhang et al., 2014; Van Treuren et al., 2015; Zolnik et al., 2016) when analyzing 16S rRNA from DNA samples of different tick species by next-generation methods.

Although there are many recent studies in the literature about tick microbiomes, none of them have found to compare tick cell microbiota from different *R. sanguineus* lineages, and neither they tried to figure out if tick cell lines from these ticks are similar or not to adult ticks. Does the female microbiome maintain itself in *in vitro* egg cultivation? Therefore, if there is a microbiome inside tick cell cultures, could it influence crucial parameters, such as growth? At this point, this study aimed to investigate the prokaryotic microbiome of the tropical and temperate lineages of *R. sanguineus* (females and embryonic cell cultures), using the gene 16S rRNA (V4-V5 region). Studying tick embryonic cell microbiome may help to create patterns and provide important information for the establishment of these cultures and their use as a substrate for the growth of microorganisms. They can also allow reducing drastically the number of animals used in host-pathogen experiments. Finally, deciphering the interactions between symbiont microorganisms and ticks provide valuable information on controlling the transmission of pathogens.

## Material and Methods

### Ethics statement

The procedures were performed in accordance with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and were approved by the Ethics Committee for Use of Animals (CEUA; no. 06761/19), FCAV/UNESP, Jaboticabal, SP, Brazil.

### Obtaining females and primary cultures of *R. sanguineus* (tropical and temperate lineages)

Engorged and non-engorged females of *R. sanguineus* were collected directly from dogs, and the ticks they were molecularly identified and the sequences were deposited (GenBank accession numbers MF187515 and KX383849). Females of the tropical lineage were collected from dogs attended at the Governador Laudo Natel Veterinary Hospital of the School of Agrarian and Veterinary Sciences, São Paulo State University (FCAV/UNESP), in Jaboticabal, state of São Paulo, Brazil (21°14'38" S; 48°17'35" W). Females of the temperate lineage were collected from dogs in Porto Alegre, state of Rio Grande do Sul, Brazil (30°01'40" S; 51°13'43" W). These females of the temperate lineage were kindly provided by Prof. João Fabio Soares, of the Federal University of Rio Grande do Sul (UFRGS), in Porto Alegre, state of Rio Grande do Sul. The methodology used to define the number of ticks to be collected followed the recommendations of Tekin et al. (2017).

The female ticks were washed inside a hood with the following solutions: 70% ethanol for 1 minute; 2% sodium hypochlorite (Alphatec, São Paulo, Brazil) for 1 minute; 2% benzalkonium chloride (Polyorganic, São Paulo, SP, Brazil) for 15 minutes; and sterile distilled water containing 5% penicillin/streptomycin and 2.5% µl amphotericin B (Vitrocell Embriolife, Campinas, SP, Brazil) for 5 minutes. The specimens were dried using autoclaved gauze pads (Pudney et al., 1973; Kurtti et al., 1983; Bhat & Yunker, 1977). Five non-engorged *R. sanguineus* females of the tropical and seven of the temperate lineages were simply homogenized using a pistil and the DNA was extracted. The ticks belonging to each lineage were pooled in order to obtain DNA pools from each strain.

Five engorged females (from each lineage), also obtained from both study localities were placed in separate sterile Petri dishes and kept in a biological oxygen demand (BOD) incubator at 80-85% humidity, and 27 °C ± 1 °C for tropical lineage and 21 °C ± 1 °C for temperate lineage, for oviposition to be performed. The eggs were collected every day, and when they reached the age (14-15 days old), they were used for primary embryonic cell cultures, following the protocol described by Barros-Battesti et al. (2018). When the cells reached 90-100% confluence, they were harvested using a cell scraper, washed in 10% PBS solution and subjected to DNA extraction.

DNA was extracted from all the samples using the DNeasy Blood & Tissue kit (Qiagen®) (Ammazzalorso et al., 2015; Cesare et al., 2018; Evans et al., 2018). The concentration of each DNA sample was estimated using a spectrophotometer (Nanodrop, Thermo Scientific®). The samples were then stored at -20 °C until use.

### Amplification of the V4-V5 region of the 16S rRNA gene

Five microlitres of the total DNA of the samples (25ng/µL) was subjected to amplification of the V4-V5 region of the 16S rRNA mitochondrial ribosomal gene, and was sequenced using a 500-cycle Nano Reagent v2 kit using the MiSeq platform (Illumina). This 16S region was selected based on data from Sperling et al. (2017). In total, four samples (females and embryonic cells of *R. sanguineus* of both the tropical and the temperate lineage) were sequenced in triplicate (Table 1). For the amplification process, a two-step PCR protocol was performed, following the manufacturer’s recommendations (Nextera XT® index kit), in which the region of interest (PCR-1) and Illumina adapters and barcodes were attached (PCR-2). The primers used to amplify the V4-V5 region of 16S rRNA were based on data from Caporaso et al. (2011), comprising: 515f: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806r: 5'-GGACTACHVGGGTWTCTAAT-3'. The 16S rRNA gene V4-V5 region was sequenced in a multiplex pool using the 500-cycle Nano Reagent v2 (Illumina®) kit in accordance with the manufacturer's recommendations.

**Table 1.** Samples of *Rhipicephalus sanguineus* (tropical and temperate lineages) collected to investigate metataxonomy.

Sample	Name	Description
1	Trop(F)	DNA pull from non-engorged females of <i>R. sanguineus</i> tropical lineage
2	Temp(F)	DNA pull from non-engorged females of <i>R. sanguineus</i> temperate lineage
3	Trop(C)	DNA of primary cell culture of <i>R. sanguineus</i> tropical lineage
4	Temp(C)	DNA from primary cell culture of <i>R. sanguineus</i> temperate lineage

### Bioinformation analyses

Firstly, quality control was applied to the raw reads, to obtain amplicon sequence variants (ASVs). For each sequenced sample, a report was generated through the FastQC software (FastQC, 2015), which contains information on the average PHRED qualities per sequenced nucleotide, the number of reads produced and the quality distribution per nucleotide position. The function *search\_oligodb*, from the USEARCH software (Edgar, 2010), was used to identify the positions of the reverse/forward sequencing primers in the samples, and these values were applied to perform their removal.

After this, the sequences were subjected to the DADA2 pipeline (Callahan et al., 2016), implemented in the R language (R Core Team, 2019). The values obtained were used as a reference for the *FilterAndTrim* function, through the following parameters: *trimLeft* (removal of adapters), with values of 18 and 19 bases, for forward and reverse, respectively; *maxEE* (low-quality reading removal) of two bases, for both; *truncLen* (read size standardization) of 230 bases, for both; removal of reads from sequencing control (*rm.phix = TRUE*); and exclusion of the corresponding read if the pair failed to pass the quality filtering (*matchIDs = TRUE*).

The eligible reads were then subjected to detection (*learnErrors* function) and correction (*given* function) of possible base-switching errors during sequencing. The paired-end reads (reverse and forward) were merged (*mergePairs* function) and filtered for the presence of chimeric sequences (*removeBimeraDenovo* function). Thus, a table of ASVs was obtained, and these were counted in each sequenced sample (Callahan et al., 2017).

For taxonomic annotation and removal of contaminating sequences, the ASVs were contrasted against taxonomic databases specializing in 16S rRNA sequences, using the *assignTaxonomy* function, also in the DADA2 pipeline. The following taxonomic databases were used: Genome Taxonomy Database (GTDB) (Parks et al., 2018); SILVA (v.138) (Quast et al., 2013); RDP (v. Trainset 16) (Cole et al., 2005); and a version of the RDP supplemented with RefSeq-NCBI (Tatusova et al., 2014). The RDP database classification proved to be the best taxonomy classifier regarding the number of sequences classified according to taxonomic level and, therefore, this database was used in the ensuing analyses. However, the other databases helped to identify contaminating sequences (such as chloroplasts or mitochondria), which were dismissed for the subsequent analysis and annotations.

The tables containing the ASVs counted according to samples, along with their taxonomic classification and sequences in FASTA format, were imported into the QIIME2 pipeline (Bolyen et al., 2019). In this pipeline, using the command *align-to-tree-mafft-fasttree*, the sequences were aligned with the MAFFT algorithm (Kato et al., 2005), and regions that were not phylogenetically informative were masked. Here, it was possible to obtain sequences organized into phylogenetic trees, using the maximum likelihood method of the *FastTree* algorithm (Price et al., 2009).

The MicrobiomeAnalyst platform (Dhariwal et al., 2017) was used to obtain the diversity parameters of the samples. To infer the internal diversity of each sample, the alpha diversity, observed ASV diversity values, Chao1 index and Shannon index were obtained. The Kruskal-Wallis nonparametric statistical test was used to identify whether differences between the conditions studied existed. For pairwise comparison among lineages of the same development stage (cell cultures or adult females), the Mann-Whitney test was used.

To assess the diversity between conditions (beta diversity), UniFrac distances were calculated by using the phylogenetic tree. These were weighted according to the abundance of ASVs in the samples. The results were plotted as PCoA graphs and compared statistically using the PERMANOVA multivariate nonparametric statistical test. Based on these beta diversity indices, a dendrogram was constructed to display the sample groups according to their similarity.

Bar charts of relative abundances were obtained for each taxonomic level, with grouping of low-abundance taxa from the family level onwards, for better visualization.

The differential abundance of microorganism taxa between lineages was ascertained using linear discriminant analysis effect size (*LEfSe*) (Segata et al., 2011) with a p-value cutoff of 0.1 and log linear discriminant analysis (LDA) score of 2.0.

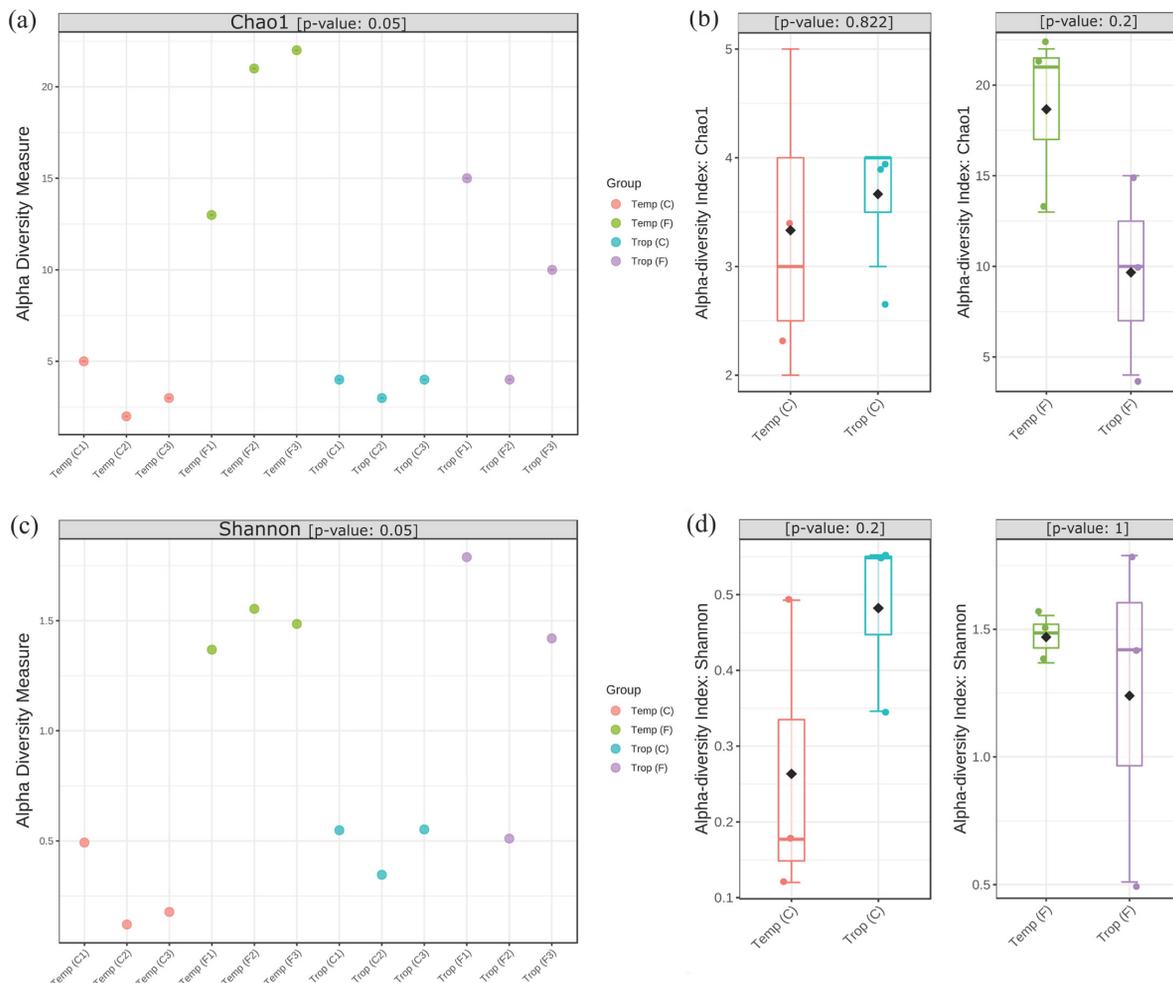
Co-occurrence networks were generated from the normalized abundance matrices of both datasets (female samples and cell lineages) using Pearson's product-moment correlation through the MicrobiomeAnalyst platform. These networks were drawn using Cytoscape v. 3.7.2.

## Results

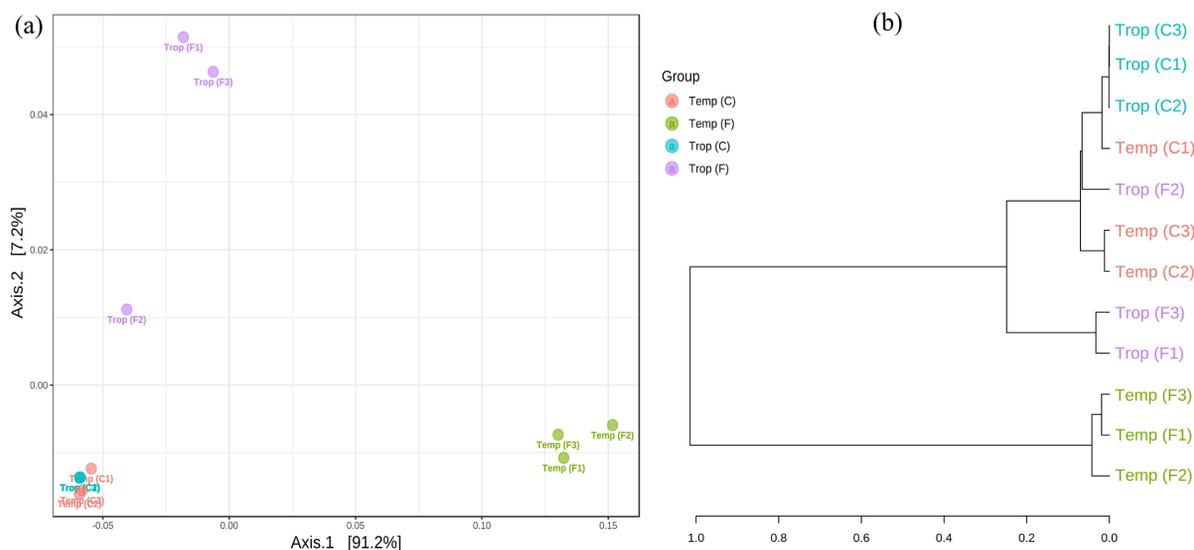
This study generated a total of 15,963 useful reads (after the filtering step) and 67 ASVs, and 31 of these ASVs were counted in two or more samples. We identified 5 phyla, 13 orders, 24 genera and 8 species of bacteria.

Embryonic cell culture samples showed lower alpha diversity than female specimens (Figure 1). However, there was no difference between temperate and tropical lineages in either of the development stages when considering only richness, as shown by the Chao1 index ( $p > 0,05$ ), or when evenness was also taken into account, as seen in the Shannon index. Regarding beta-diversity, the PCoA plot of weighted UniFrac distances (Figure 2) showed that cell cultures were weakly affected by lineage but that this difference was accentuated in adult females (PERMANOVA; F-value: 20.91; R-squared: 0.88; p-value < 0.001). The sample clustering dendrogram based on the same distances showed a closer relationship between tropical females and embryonic cell culture samples, while temperate females exhibited more distinct diversity of composition (Figure 2b).

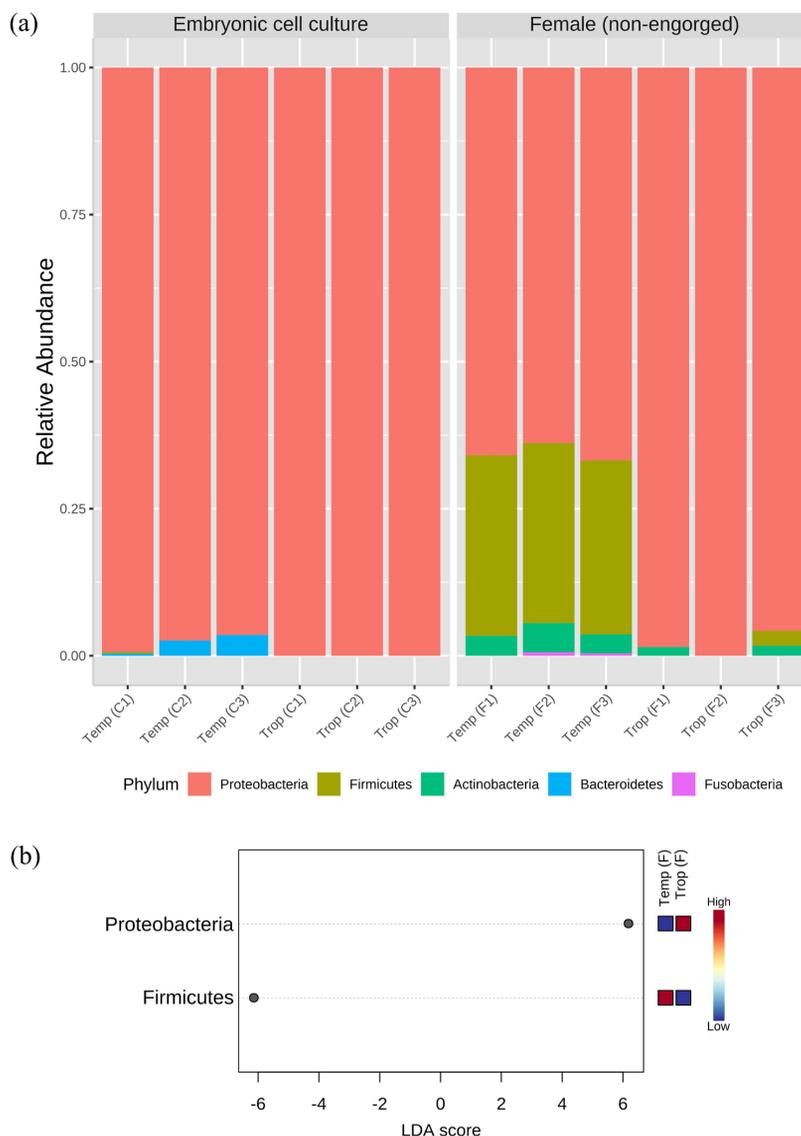
The phylum Proteobacteria predominated in all samples (Figure 3a). While in the embryonic cell culture samples (**C**) of tropical lineage [**Trop (C)**] only this phylum was observed, a relative abundance of 98.8% was observed in the embryonic cell culture samples of temperate lineage [**Temp (C)**], albeit without statistical difference. The female samples (**F**) showed a statistical difference in the proportion of Proteobacteria (Figure 3b), with average relative



**Figure 1.** Alpha diversity indices for *Rhipicephalus sanguineus* (s.l. and s.s.) samples. Dot plots represent the alpha diversity of each replicate in the Chao1 (a) and Shannon (c) indices. The group means were tested using the Kruskal-Wallis statistical test ( $p$ -value < 0.05). Boxplots represent the distribution of indices [Chao1 (b), Shannon (d)] for each pairwise condition. These were compared statistically using a nonparametric two-sample t test and the Mann-Whitney test. There were no statistically significant differences in the diversity indices between lineages.



**Figure 2.** Weighted UniFrac distance analysis for *Rhipicephalus sanguineus* (s.l. and s.s.) samples. Principal coordinate analysis (PCoA) plot (a) of UniFrac distance matrix based on the phylogenetic tree of sequences and weighted according to the actual abundance of the sequences among the samples. Dendrogram diagram (b) showing the clustering of weighted UniFrac distances of the samples using Ward's method.

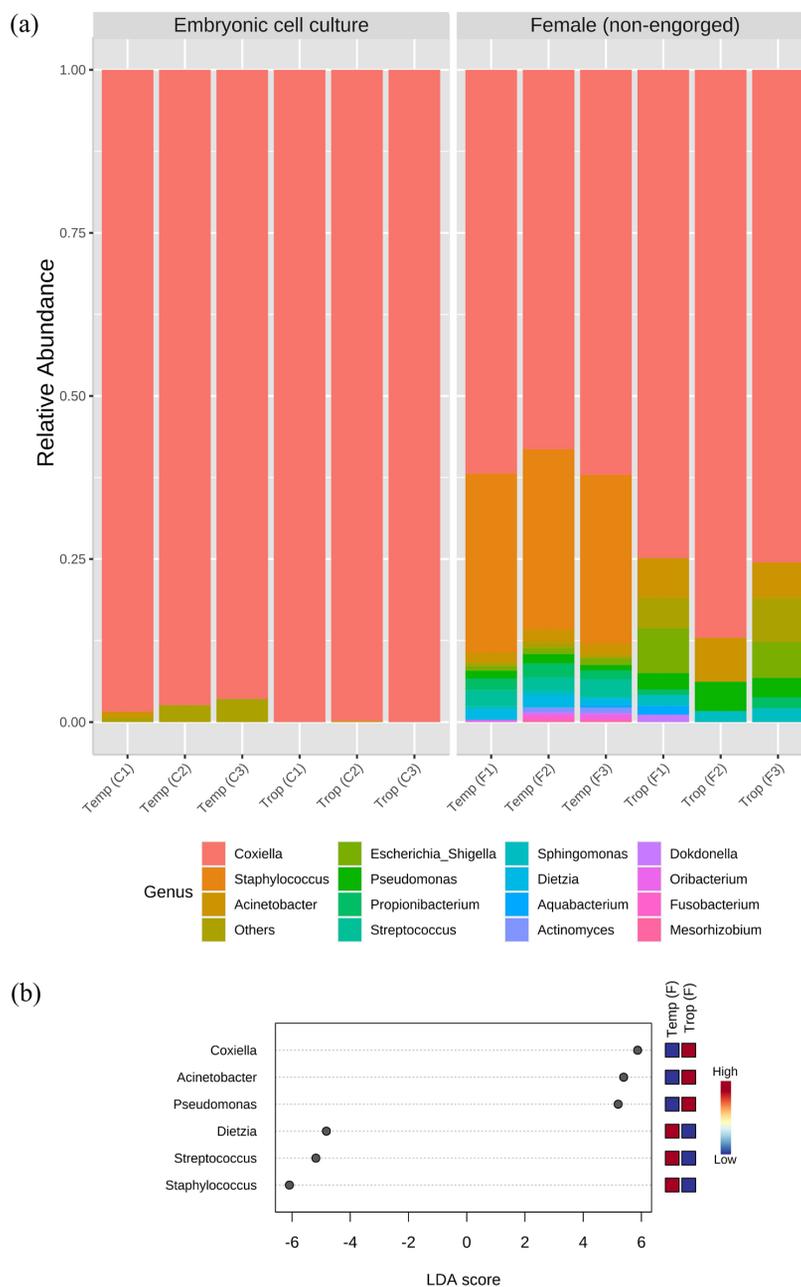


**Figure 3.** Bar charts of relative abundance at phylum taxonomic level (a) and LefSe results regarding differently abundant taxa in *Rhipicephalus sanguineus* (s.l. and s.s.) samples. The differences were significant ( $p < 0.01$ ) only among female samples (b). The threshold of the logarithmic linear discriminant analysis (LDA) score was 2.0.

abundances of 65.26% in female of temperate lineage [**Temp (F)**] and 98.19% in female of tropical lineage [**Trop (F)**]. Other phyla were found in smaller proportions and comprised Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria. While the Bacteroidetes group was only detected in **Temp (C)**, Fusobacteria was only observed in **Temp (F)**. Moreover, **Temp (F)** exhibited differential abundance of the phylum Firmicutes (Figure 3b), which was the second most-present phylum (30.23%). On the other hand, Firmicutes accounted for only 0.49% of **Trop (F)**. The phylum Actinobacteria was found only in adult female samples, in both the tropical and the temperate lineage. Lastly, Bacteroidetes and Fusobacteria were not detected in any sample of tropical lineage.

At the genus level, *Coxiella* showed high relative abundance under all conditions (Figure 4a), corresponding to 99% of the taxa in cell culture samples, albeit without statistical difference between tropical and temperate lineages. On the other hand, the proportions differed in the female samples, comprising 76.8% for **Trop (F)** and 60.2% for **Temp (F)** (Figure 4b).

It was possible to classify some sequences at the species level (Figure 5a). The tropical lineage showed *Coxiella* endosymbiont as the most abundant taxon in embryonic cell cultures (85.45%), while this proportion was considerably smaller in the females of this lineage (only 12.5% in one of three replicates). A second *Coxiella* sequence was detected in both lineages, classified only at the genus level, as *Coxiella* sp. The second *Coxiella* sequence (*Coxiella* sp.) seemed to present a pattern that was the inverse of that of *Coxiella* endosymbiont, since its percentages were 14.50% in **Trop (C)** and 68.56% in **Trop (F)**. Both the cell cultures and the females of the temperate lineage exhibited

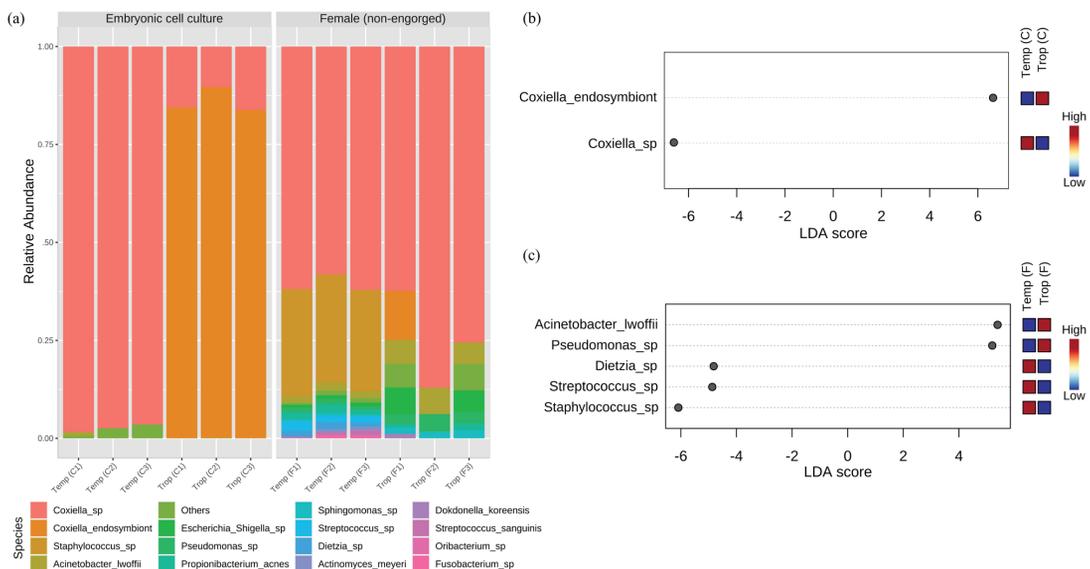


**Figure 4.** Bar charts of relative abundance at genus taxonomic level (a) and LefSe results regarding differently abundant taxa in *Rhipicephalus sanguineus* (s.l. and s.s.) samples. The differences were significant ( $p < 0.01$ ) only among female samples (b). The threshold of the logarithmic linear discriminant analysis (LDA) score was 2.0.

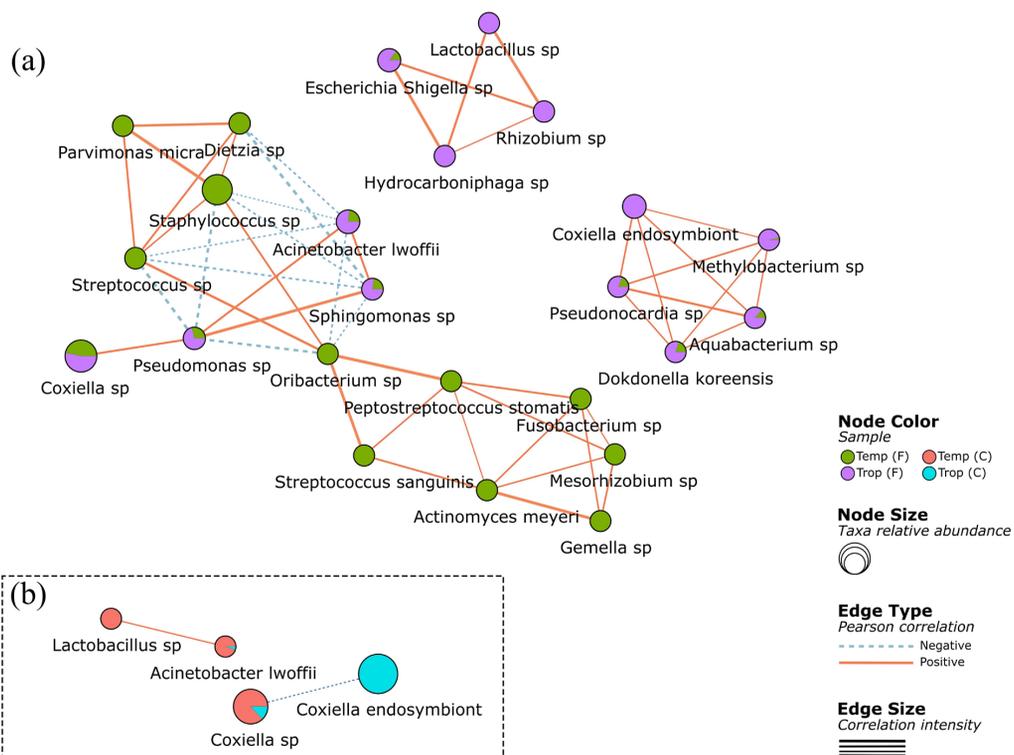
only *Coxiella* sp. sequences, which represented 98.06% and 60.20% of the species diversity, respectively. The LefSe evaluation showed that the cell cultures differed in relation to the two *Coxiella* sequences (Figure 5b), whereas the females differed in relation to several less relatively abundant species (Figure 5c).

Looking more closely into each sequence, they were composed of 374 bp, with only a single base that differed. In a quick and simple comparison of the sequences obtained through BLAST, the alignments were:

- *Coxiella* sp.: 100% with KM079626 and KM079624, deposited in 2014, in which the host was identified as *R. sanguineus*, originally from Algeria;
- *Coxiella* endosymbiont: 100% with 41 sequences obtained from the host *R. sanguineus*, from different countries: Brazil (KR820015), India (MG050151) and Australia and Thailand (MK671684 – MK671708). Also, this sequence aligned with *Coxiella burnetii*, deposited from China (MT498683) and with *Candidatus* *Coxiella mudrowiae*, from Israel (CP024961).



**Figure 5.** Bar charts of relative abundance at species taxonomic level (a) and LfSe results regarding differently abundant taxa in *Rhipicephalus sanguineus* (s.l. and s.s.) samples. There were significant differences ( $p < 0.01$ ) among cell culture (b) and female (c) samples. The threshold of the logarithmic linear discriminant analysis (LDA) score was 2.0.



**Figure 6.** Co-occurrence networks generated from the normalized abundance matrices. Female (a) and cell lineage (b) datasets.

The co-occurrence network of female tick datasets revealed that there were distinct connections, in comparing the **Temp (F)** and **Trop (F)** groups (Figure 6a). Notably, while the prokaryotic microbiome observed in **Temp (F)** showed greater interconnectivity, the microbiome of **Trop (F)** was fragmented into three smaller subnetworks.

Moreover, only a few nodes (*taxa*) were observed co-occurring and with some abundance in both groups. The most abundant ASVs in the female samples, which were assigned as *Coxiella sp.*, did not show any statistically significant difference between the two groups ( $\log$  LDA score  $< 2$ ;  $p$ -value  $> 0.1$ ). However, these taxa only showed a strong correlation with *Pseudomonas sp.* (Pearson coefficient: 0.91), which was differentially abundant in **Trop (F)** samples (Figure 5c) ( $\log$  LDA score: 5.2;  $p$ -value: 0.07). In **Trop (F)**, *Pseudomonas sp.* was correlated with *Sphingomonas*

sp. and *Acinetobacter lwoffii*. Although these three microorganisms were present in both groups of females, only the last of them was statistically more abundant in this group (log LDA score: 5.39; p-value: 0.04).

In another subnetwork, *Pseudonocardia* sp., *Aquabacterium* sp., *Methylobacterium* sp. and *Dokdonella koreensis* also occurred in both groups for some samples, with greater mean abundance in the **Trop (F)** group, despite the lack of statistical difference (LEfSe p-value > 0.1). In addition, all of the taxa in this subnetwork showed positive correlations with *Coxiella* endosymbiont, which was exclusive to **Trop (F)** (Pearson coefficient > 0.95; p-value < 0.01).

The most differentially abundant species with relationships present in the main female network, for the **Temp (F)** group, were *Dietzia* sp., *Streptococcus* sp. and *Staphylococcus* sp., which were all positively correlated (Pearson coefficient > 0.9; p-value < 0.01).

The co-occurrence network for the cell lineage datasets was unquestionably simpler (Figure 6b). It showed a negative abundance correlation (Pearson coefficient: -0.99; p-value < 0.001) between the two *Coxiella* nodes in the **Temp (C)** and **Trop (C)** groups.

## Discussion

The different microbiomes composition found comparatively in the samples can be explained by tick's intrinsic mechanisms and environmental factors, like life stage and the collection site (Kueneman et al., 2021). However, this difference was not greatly expressed comparing the number of species in the samples, and neither an when comparing different ecosystems (females and embryonic cell culture).

Four phyla found in our samples (Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes) (composed by female ticks and tick cell cultures) corroborate with data detected in *Amblyomma maculatum* Koch and *Rhipicephalus microplus* (Canestrini) adult ticks, by different methods (Varela-Stokes et al., 2018; Segura et al., 2020). On the other hand, Fusobacteria, detected only in **Temp (F)**, is a phylum that has not been associated with any tick microbiome yet. Members of this phylum are gram-negatives and commonly compose animal and the human microbiome from the oropharynx and gastrointestinal tract. Jose et al. (2021) found that *Philornis downsi* (Muscidae) shifts their gut microbiome from predominant Proteobacteria members to a substantial constitution of Firmicutes and Bacteroidetes during its life stages, suggesting that this arthropod acquires different bacteria in the microbiome depending on the environment, in the nest or affected by blood diet. So, we infer that the Fusobacteria presence in the DNA of **Temp (F)** could be originated from the tick contact with the animal-host skin.

Endosymbiont can affect the tick depending on their species and depending on the mixture of microorganisms it has inside, but it is known that they co-circulate with pathogenic agents and can interact with them (Ahtarig et al., 2013). Molecular apparatus and the microbiome can have an effect on tick fitness and pathogen infection and transmission; even these systems are not fully characterized and even other mechanisms can be involved, pathogen-specific tick receptors affect vector competence (de la Fuente et al., 2017). Gall et al. (2016) evaluated two different populations of *Dermacentor andersoni* Stiles, previously known with diverse susceptibility to *Anaplasma marginale*. They found that an increase of *Rickettsia belli* on tick microbiome decreased *A. marginale* levels. Moreover, low levels of endosymbiont *Francisella* were related to a reduction of *Francisella novicida*. The authors concluded that the manipulation of tick microbiome can be used for biocontrol in the future, diminishing tick's susceptibility to pathogens. This point should be better investigated regarding *Rhipicephalus sanguineus* microbiome. Also, some authors showed that microbiome composition and immune system can affect vector competence in other arthropods (Dennison et al., 2014; Jupatanakul et al., 2014).

Identification of Proteobacteria as the main taxon in this study corroborates the findings relating to the microbiome of *Ixodes* ticks, reported by Zolnik et al. (2016) (90-99.7%) and by Van Treuren et al. (2015) (81.7%). *Coxiella*, a member of this phylum, was the most abundant genus, and this result had been expected (Ahtarig et al., 2013; Bonnet et al., 2017; René-Martellet et al., 2017; Greay et al., 2018a).

The genus *Coxiella* is an essential endosymbiont in tick physiology (Lalzar et al., 2014). In *R. microplus*, *Coxiella* plays a decisive role in the maturation and ecdysis, and this mutual relationship can be an alternative vector control method if disrupted (Guizzo et al., 2017). Also, this bacterium contributes to micro and micronutrients acquisition in *R. sanguineus* (Ben-Yosef et al., 2020). Zhong et al. (2007) concluded that the removal of *Coxiella* endosymbiont reduces the reproductive performance in the species *Amblyomma americanum* (Linnaeus). *Coxiella* sp. is inherited by transovarial via, and does not have pathogenic characteristics (Duron et al., 2015), but *Coxiella burnetti*, the pathogen of Q-fever and member of the same genus, is maintained in the natural cycle of ticks. Oskam et al. (2017) have found *Coxiella* sp. in 100% of *R. sanguineus* samples collected in Australia (n=199), but *C. burnetti* in none of them.

Angelakis et al. (2016) remember that alternation in generations is faster in ticks and pathogens, and mutations can lead to a non-pathogenic endosymbiont becomes an inductor of infections.

Indeed, Duron et al. (2017) commented that all ticks of the genus *Rhipicephalus* have *Coxiella*. Another aspect to point is the fact that even *Coxiella* is a natural member of tick's microbiome, endosymbionts can turn out to be pathogenic and the description and characterization of new members is useful to identify which one can be facultative endosymbiont (Raoult & Roux, 1997; Ivanov et al., 2011). Although the degree of confidence in the classification at the species level after MiSeq sequencing is lower than through other platforms, it was possible to categorize two different taxa of highest abundance: *Coxiella* sp. and *Coxiella* endosymbiont.

It was notable that *Coxiella* at the genus level was relatively more abundant in the embryonic cell cultures and formed almost 100% of the composition of **Temp (C)**. In **Temp (F)** the percentage of *Coxiella* was lower in all reads than in **Trop (F)**. At the species level, we could infer that the sequence identified as *Coxiella* endosymbiont was undetectable in the temperate lineage, such that only a sequence variant classified as *Coxiella* sp. was found. May *Coxiella* sp. influence the vectorial competence of these lineages? It was observed that ticks of the temperate lineage that fed on dogs that were clinically positive for *E. canis* presented a higher proportion of *Coxiella* endosymbiont, whereas the opposite effect was observed in the tropical lineage of *R. sanguineus* ticks under the same conditions (unpublished data). The *Coxiella* species present in the two lineages may be different and may have co-evolved with these two strains, thus driving different patterns of interactions between ticks and the pathogens that they can harbor or transmit to vertebrate hosts.

Most of the other bacterial taxa detected in the samples studied were typical members of the normal skin, buccal mucosa or respiratory tract flora. We infer that these bacteria might have originated from dogs' skin, blood or even saliva because it is known that dogs lick where ticks have bitten. According to Andreotti et al. (2011) and reinforced by Narasimhan & Fikrig (2015), the environmental microorganisms can be found in the tick microbiome and may be difficult to separate from where they belong (O'Neal et al., 2021). Another bacterium found, namely *Dokdonella koreensis*, is a common bacterial species present in the soil. The results found in the present study showed that the cleaning and asepsis methods that were used to extract DNA from tick samples might have not prevented us from finding bacterial taxa from the environment, because these could have formed part of the ticks' ingests. *Coxiella*, *Staphylococcus*, and *Streptococcus* were also found by Andreotti et al. (2011) in *R. microplus* adult female ticks in Texas, USA, through pyrosequencing.

It should be remembered that the tick cell cultures that were used here were maintained under controlled artificial conditions, unlike the female specimens, which were collected directly from dogs. However, we used primary embryonic tick cell cultures, which are closer to natural conditions than established cell cultures (Barros-Battesti et al., 2018); this proximity would probably explain our findings of the phyla Bacteroidetes and Actinobacteria in the **Temp (C)** culture sample, albeit small amounts. Thus, in the present study, we showed that primary cultures of embryonic cells can reveal the existence of a microbiome. This shows the importance of testing cultures before performing other procedures, even regarding established cell lines. The dendrogram showed a more distant relationship between the diversity composition of **Temp (F)** and embryonic cell culture samples. A hypothesis is that the female microbiome of this lineage is not easily conserved when stressing the cells to an *in vitro* situation.

Tekin et al. (2017) concluded that ticks harboring a more diversity-rich bacteriome may be more resistant to bacterial invasion and dissemination of pathogens. Narasimhan & Fikrig (2015) confirmed that symbionts might influence the vectorial competence of ticks because they modulate the immune status of the tick gut. Sanches et al. (2021) compared infected and non-infected tissues of *R. sanguineus* from both lineages and observed that some proteins were only found under infected conditions. According to these authors, four of the most represented proteins present during *E. canis* infection could play important roles in the cement cone structure and function, which would favor infection in the tropical lineage of *R. sanguineus*. On the other hand, proteins relating to detoxification and defensive response modulation were more represented in the temperate lineage of *R. sanguineus* and would have the capacity to reduce the vector competence of this lineage about *E. canis* (Sanches et al., 2021). It can therefore be asked whether this might be the reason why the temperate lineage of *R. sanguineus* is unable to transmit *E. canis*. Hence, it is necessary to make efforts to identify taxa more precisely in future studies using additional methods, regarding the limitation of the technology employed in this study, to shed some light on the factors involved in the differences in vector capacity between the tropical and temperate lineages of *R. sanguineus*, regarding transmission of *E. canis*.

## Conclusion

The metataxonomic analysis showed that significant diversity of bacterial taxa exists in *R. sanguineus* females and embryonic cell cultures from both the temperate and the tropical tick lineage, with emphasis on *Coxiella* in all samples, albeit in different proportions. The *Coxiella* species present in the two lineages may be different and may have co-evolved with these two lineages, thus driving different patterns of interactions between ticks and the pathogens that they can harbor or transmit to vertebrate hosts.

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