

UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS JABOTICABAL

**Analysis of the prokaryotic microbiome of females and cell
culture of *Rhipicephalus sanguineus* sensu lato and sensu stricto
from Brazil**

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Veterinarian

2020

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from Brazil**

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Thesis presented to the Faculty of Agricultural and
Veterinary Sciences – Unesp, Campus of Jaboticabal,
as part of the requirements for obtaining the title of
Master in Veterinary Medicine, Animal Pathology.

2020

L979a Luzzi, Mayara de Cassia
 Analysis of the prokaryotic microbiome of females and
 cell culture of *Rhipicephalus sanguineus sensu lato* and
 sensu stricto from Brazil / Mayara de Cassia Luzzi. -
 Jaboticabal, 2020 45 p.

 Dissertação (mestrado) - Universidade Estadual Paulista
 (Unesp), Faculdade de Ciências Agrárias e Veterinárias,
 Jaboticabal
 Orientadora: Darci Moraes Barros-Battesti
 Coorientador: Luciano Takeshi Kishi

 1. Brown dog tick. 2. Tick embryonic cell culture. 3.
 Endosymbionts. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da
Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos
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CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: ANALYSIS OF THE PROKARYOTIC MICROBIOME OF FEMALES AND CELL CULTURA OF *Rhipicephalus sanguineus* SENSU LATO AND SENSU STRICTO FROM BRAZIL.


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Jaboticabal, 09 de setembro de 2020

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*Força no caminho, amor verdadeiro,
prosperidade justa.*

Dedication

I dedicate this work to my husband Rafael A. de Andrade and to my son Matteo Luzzi de Andrade.

ACKNOWLEDGEMENTS

I would like to give thanks to all financial support I had for executing this research:

- FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo: n. 2007/57749-2, 2015/26209-9, 2020/03190-9 and 2009/53984-2;
- CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico: n. 302420/2017-7, 440639/2015-8, and 131532/2018-9;

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

I would like to thank Dr. Darci Moraes Barros-Battesti, for the confidence. I would like to thank all the collaborators that have helped in the conclusion of this work, especially Lucas Amoroso Lopes de Carvalho.

I would like to thank all the spiritual help I received during this work.

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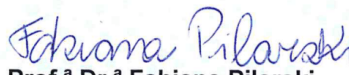
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CERTIFICADO

Certificamos que o projeto de pesquisa intitulado “**Metataxonomia do microbioma de fêmeas, ovos e células embrionárias de *Rhipicephalus sanguineus sensu lato (Acari: Ixodidae)***”, protocolo nº 06761/19, sob a responsabilidade da Prof^a Dr^a Darci Moraes Barros-Battesti, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 13 de junho de 2019.

Vigência do Projeto	01/07/2019 a 11/08/2021
Espécie / Linhagem	<i>Canis familiaris</i>
Nº de animais	6 cães
Peso / Idade	5 kg a 7 kg
Sexo	3 fêmeas e 3 machos
Origem	FCAV – UNESP Jaboticabal

Jaboticabal, 13 de junho de 2019.


Prof.^a Dr.^a Fabiana Pilarski
Coordenadora – CEUA

ANALYSIS OF THE PROKARYOTIC MICROBIOME OF FEMALES AND CELL CULTURE OF *Rhipicephalus sanguineus* SENSU LATO AND SENSU STRICTO FROM BRAZIL

Abstract - Two lineages (temperate and tropical) of *Rhipicephalus sanguineus* are known in Brazil and in part of Latin America, corresponding, respectively, to the southern and northern populations. Here, we investigated the prokaryotic microbiomes of both lineages, using the 16S rRNA (V4-V5 region) gene-based metataxonomic approach, through NGS sequencing on the MiSeq Illumina platform. For this purpose, we selected specimens of females from the environment and samples of primary cultures of embryonic cells, from both lineages in Brazil. This was the first study to investigate the prokaryotic microbiome of tick cell cultures. The results showed that many bacterial taxa detected in the samples studied were typical members of the normal skin, buccal mucosa or respiratory tract. We found that there was significant diversity of microorganisms in *R. sanguineus* females and in embryonic cell cultures from both the temperate and the tropical lineage, 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 these lineages, thus driving different patterns of interactions between ticks and the pathogens that they can harbor or transmit to vertebrate hosts.

Key words: brown dog tick, tropical lineage, temperate lineage, tick embryonic cell culture, *Coxiella*, endosymbionts

ANÁLISE DO MICROBIOMA PROCARIÓTICO DE FÊMEAS E CULTURA CELULAR DE *Rhipicephalus sanguineus* SENSU LATO E SENSU STRICTO DO BRASIL

Resumo - Duas linhagens (temperada e tropical) de *Rhipicephalus sanguineus* são conhecidas no Brasil e em parte da América Latina, correspondendo, respectivamente, às populações do sul e do norte. Aqui investigamos o microbioma procariótico de ambas as linhagens usando uma abordagem metataxonômica baseada no gene 16S rRNA (região V4-V5), por meio do sequenciamento de nova geração na plataforma MiSeq Illumina. Para este propósito, foram selecionadas amostras de fêmeas do ambiente e cultivo primário de células embrionárias, considerando 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 da microbiota da pele, mucosa bucal ou trato respiratório. Verificamos que existe uma diversidade significativa de microorganismos em fêmeas e cultura de células embrionárias nas duas linhagens de *R. sanguineus*, 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

1. INTRODUCTION

Broadly speaking, ticks are hematophagous ectoparasites widely distributed in the world, and have a close relationship with great financial losses in agribusiness, declination of animal performance and transmission of myriad diseases, acting as vectors for zoonosis (Guglielmone et al., 2003, 2006; Dantas-Torres, 2007; Grisi et al., 2014).

Members of the arachnid class, the Acari subclass, and the Ixodida order, are subdivided into three living families, according to their characteristics: Argasidae, Ixodidae, and Nuttalliellidae (Nava et al., 2017). Supported by a fossil material found in Myanmar, Peñalver et al. (2017) proposed a new family of ticks named Deinocrotonidae, with *Deinocroton draculi* as the unique known representative. Unfortunately, this is an extinct tick whose no genetic data are available.

Archeological records from ticks place their emergence in the Paleozoic Era (Sonenshine & Roe, 2013) and most of them were assembled to amber material. The oldest record of them on any animal species was reported by Otranto et al. (2014), when ticks were retrieved from a dog mummy belonging to Ancient Egypt (1st century – 4th century A.D.). These specimens were identified as members of the *Rhipicephalus sanguineus* s. l. (sensu lato) group.

Brazilian tick fauna is currently composed of 74 species, from which 51 species belong to the Ixodidae family and 23 species to the Argasidae family, distributed all over the country. This number is dynamic since new species are currently being described (Labruna et al., 2017; 2020; Muñoz-Leal et al., 2017, Dantas-Torres et al., 2019; Onofrio et al., 2020).

Rhipicephalus sanguineus s.l. (Latreille, 1806) constitutes a complex of species named “*R. sanguineus* group” formed by 17 species (TABLE 1). Most of these species are siblings and share extremely similar characteristics. Widespread around the world parasitizing mainly domestic dogs (*Canis lupus familiaris*), its popular names include “brown dog tick”, “kennel tick” and “pan-tropical dog tick” (Gray et al., 2015).

Consequently, the taxonomy of this group is a challenge and we observe great problems while identifying each member, moreover regarding *R. sanguineus* ticks, because the type species is lost. But, recently Nava et al. (2018) designed the neotype of *R. sanguineus* s.s. (sensu strictu) based in specimens from France, considering the origin of the taxon (represented by the temperate lineage).

Table 1. *Rhipicephalus sanguineus* including all species of this group (based on Nava et al., 2012, 2018; Dantas-Torres & Otranto, 2015).

Species Name	Description (authorities, year)
<i>Rhipicephalus sanguineus</i> s.s.	Latreille, 1806
<i>Rhipicephalus ziemanni</i>	Neumann, 1904
<i>Rhipicephalus aurantiacus</i>	Neumann, 1907
<i>Rhipicephalus sulcatus</i>	Neumann, 1908
<i>Rhipicephalus rossicus</i>	Yakimov and Kohl-Yakimova, 1911
<i>Rhipicephalus schulzei</i>	Olenev, 1929
<i>Rhipicephalus pumilio</i>	Schulze, 1935
<i>Rhipicephalus pusillus</i>	Gil Collado, 1936
<i>Rhipicephalus turanicus</i>	Pomerantzev, 1940
<i>Rhipicephalus leporis</i>	Pomerantzev, 1946
<i>Rhipicephalus boueti</i>	Morel, 1957
<i>Rhipicephalus guilhoni</i>	Morel and Vassiliades, 1963
<i>Rhipicephalus moucheti</i>	Morel, 1965
<i>Rhipicephalus ramachandrai</i>	Dhanda, 1966
<i>Rhipicephalus camicasi</i>	Morel, Mouchet and Rodhain, 1976
<i>Rhipicephalus bergeoni</i>	Morel and Balis, 1976
<i>Rhipicephalus tetracornus</i>	Kitaoka and Suzuki, 1983

The species *R. sanguineus* s.l. is found in a wide range of ecological habitats, but predominately in urban and suburban areas. With nidicolous behaviour, its evolution is closely related to the domestic dog and displays an outstanding adaptative capacity (Gray et al., 2015). Thus, this tick travelled around the world with multiple routes and was first recorded in Brazil in 1909 by Carlos Jorge Rohr (Rohr, 1909; Caetano et al., 2017). It is a three-host tick that usually feeds on dogs, but it can feed opportunistically on many mammalian hosts or even on humans (Dantas-Torres, 2008; Oskam et al., 2017). Contrastingly to other tick species, they exhibit a negative geotropism. All the evolutive stages need blood meals, for moulting or reproductive purposes (Gray et al., 2015).

Rhipicephalus sanguineus was first described by Latreille (1806) as *Ixodes sanguineus*, and characterized as “blood red, punctate, posteriorly with three impressed lines; no distinct ‘thoracic’ spot anterodorsally”. This data was addressed to the Gallia Region, which included present-day France and borderline territories (Dantas-Torres and Otranto, 2015). Besides this deficient description, there is no *bona fide* specimen documented.

Since *R. sanguineus* (sensu stricto) achieved more importance in the scientific field, many authorities tried to describe this tick deeper. Nava et al. (2018) rediagnosed *R. sanguineus* s.s. according to the International Code of Zoological Nomenclature (ICZN, 1999). They designated a male collected in Montpellier, France, as neotype, and supported the presence of this tick in Europe (France, Italy, Spain, Switzerland, and Portugal) and America [Argentina (southern part), Brazil (southern part), Chile, Uruguay and the U.S.A.], appointing that more studies are needed to clarify the exact geographic range of this taxon. Morphological characteristics of this neotype can be checked in TABLE 2.

Table 2. Morphology of *R. sanguineus* s.s. according to Nava et al., 2018 to neotype designation

Male	<ul style="list-style-type: none"> - Spiracular plate elongated and subtriangular in shape with a dorsal prolongation narrow and usually visible dorsally, with the dorsal prolongation narrower than the width of the adjacent festoon; - Punctations of the scutum moderate in number and unequal in size; - Marginal groove conspicuous, deep and punctate; posteromedian groove distinct and elongated, and posterolateral grooves often sub-circular, shorter than posteromedian groove; - Adanal plates long, wide, and subtriangular in shape, with a clear concavity in its inner margin and posterior margin broadly rounded or truncated; accessory adanal plates with the posterior end pointed, narrower than the width of adjacent festoon.
Female	<ul style="list-style-type: none"> - Combination of broadly U-shaped genital aperture, spiracular plate with a narrow dorsal prolongation visible dorsally, basis capituli hexagonal with broad lateral angles, and scutum barely longer than broad with posterior margin sinuous and punctations moderate in number and unequal in size, larger and more numerous along cervical fields.

Nymph	<ul style="list-style-type: none"> - Basis capituli sub-triangular dorsally with lateral angles slightly curved and presence of ventral processes; - Scutum approximately as long as broad with lateral margins nearly straight, posterior margin broadly rounded; - Cervical grooves short and sigmoid in shape extending posteriorly to the level of the eyes.
Larva	<ul style="list-style-type: none"> - Basis capituli broader than long with lateral angles short and slightly curved and with posterior margin slightly convex; - Cervical grooves short, shallow and subparallel; - Scutum almost twice broader than long.

It is well accepted today the existence of two lineages of *R. sanguineus*: temperate species (southern lineage) and tropical species (northern lineage) (Nava et al., 2012; Dantas-Torres and Otranto, 2015). Both of them can be found in Brazil, but in different regions. The tropical lineage occurs in 19 Brazilian states (including Minas Gerais and São Paulo), and the temperate lineage in at least two Brazilian states (Santa Catarina and Rio Grande do Sul) (Zemtsova et al., 2016; Caetano et al., 2017, respectively).

Zemtsova et al. (2016) collected 136 specimens of *R. sanguineus* s.l. from 23 different countries, and tested for 12S rRNA and 16SrRNA genes by PCR. They have found two main genetic clades, which lead to conclude that there is a clear and solid association between geographical locations of the ticks and temperature. The tropical lineage of *R. sanguineus* was always collected from places with the annual mean temperature $>20^{\circ}\text{C}$ and, in contrast, the temperate clade was present in areas with the annual mean temperature $<20^{\circ}\text{C}$. This data shows not only differences between the lineages, but even the tick adaptability to distinct environmental conditions. Nonetheless, Labruna et al. (2017) showed that the tropical and temperate lineages don't coexist in the same geographical area in Brazil, because of diapause mechanisms. In Portugal, Dantas-Torres et al. (2017) suggested that the temperate lineage of *R. sanguineus* s.l. is the only representative of this tick group in dogs.

Interbreeding experiments between two populations of *R. sanguineus* from Brazil and Argentina were performed by Szabó et al. (2005), and confirmed their reproductive divergence by showing that hybrid females laid mostly unviable eggs. These authors commented that crosses between *R. sanguineus* ticks from São Paulo, Brazil (tropical

lineage) and *R. sanguineus* s.s. ticks from Montpellier (France) (temperate lineage) produced sterile hybrids (Nava et al., 2018). Conversely, the crossbreeding of two lineages of *R. sanguineus* s.l. from Italy and Portugal revealed fertile hybrids descending from the mates, which makes the researchers conclude that the lineages are biologically compatible, but are distinct genetically and phenotypically (Dantas-Torres et al., 2018).

The differences between tropical and temperate lineages are not limited to referring to different ecosystems, but regarding parasites also. Nava et al. (2012) discussed the epidemiological data of Canine Monocytic Ehrlichiosis (CME) in the context of these two populations of *R. sanguineus*. In this study, only the tropical lineage was recognized to be a competent vector for *Ehrlichia canis*. In contrast, ticks belonging to the temperate lineage were identified as incompetent vectors of *E. canis*, coinciding with scarcity or absence of CME in the related geographical area (Moraes-Filho et al., 2011). A few years later, Moraes-Filho et al. (2015) compared the vector competence for *E. canis* in four South American populations of *R. sanguineus*, using in vivo models. After infestation, only the dogs exposed to ticks from southeastern Brazil (tropical lineage) developed CME, attested by clinical illness, seroconversion to *E. canis* and detection of the parasite DNA by PCR.

The brown dog tick is considered a species of major medical and veterinary significance (Dantas-Torres 2012). Vector of several pathogens, such as *Babesia vogeli*, *Hepatozoon canis*, and *Ehrlichia canis*, this tick can cause significant morbidity and mortality to dogs. Not merely the transmission of pathogens, but its massive infestation is an important aspect to consider, for causing exfoliation and reducing animal welfare (Gray et al., 2015).

In human medicine, it has been implicated mainly in the epidemiology of rickettsiosis: *R. conorii* (Demma et al., 2005), *R. rickettsii* (Wikswa et al., 2007; Peniche-Lara et al., 2015), *R. massiliae* (Cicuttin et al., 2015), *R. parkeri* (Dall'agnol et al., 2017) and *R. amblyommatis* (Silva et al., 2018).

Rhipicephalus sanguineus s.l. is not anthropophilic and some of the reports are believed in fact to be related to other similar ticks, such as *R. turanicus* (Gray et al., 2015). But Szabó et al. (2013) claim our attention to the fact that this tick can infest domestic dogs that move into forest areas, and could be infested by parasites transmitted by other tick species. One example would be *R. sanguineus* tick feeding on a dog during rickettsemia caused by *R. rickettsii*, the pathogen of spotted fever in Brazil transmitted by *Amblyomma* spp.

The One Health approach of *R. sanguineus* needs to be extensively discussed. Numerous tick-borne diseases have reemerged and their mechanisms' description is still unexplored (Brinkman et al., 2019). Ticks are the most significant arthropod vectors after mosquitoes, and they could have a large social and economic impact (Michelet et al., 2016).

Since humanity is facing climate changes and other ecological processes, divergence in temperatures may implicate in increase, reduce or change drastically *R. sanguineus* activity and the pattern of distribution of each lineage. Ecological changes affect trophic relationships and host-pathogen interactions, leading to the establishment of new species into previously free areas and the emergency of diseases (Dantas-Torres et al., 2017).

Tick microbiome

Efforts have been directed towards further elucidation of the tick microbiome, also known as microbiota, formed by commensal and symbiotic microorganisms, non-pathogenic microorganisms, and pathogenic microorganisms too. They can be viruses, bacteria or eukaryotes. Their role is still widely unexplored, but endosymbionts were found to impact the tick's adaptation, development, reproduction, defence and immunity mechanisms, for the good or the bad (Greay et al., 2018; Bonnet et al., 2017; Vila et al., 2019).

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 (Ahantarig et al., 2013).

Gall et al. (2016) evaluated two different populations of *Dermacentor andersoni* Stiles, 1908, 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.

The most common endosymbiont bacteria found in the tick microbiome are *Coxiella*, *Francisella*, *Rickettsia* and "*Candidatus* Midichloria mitochondrii". Among pathogenic bacteria, *Anaplasma*, *Borrelia*, *Ehrlichia*, *Francisella* and *Rickettsia* are most frequently observed (Ahantarig et al., 2013; Greay et al., 2018).

Rickettsia, *Coxiella*, *Francisella*, *Borrelia* and protozoans of the genera *Babesia*, *Theileria* and *Hemolivia* were detected in DNA pools of 280 ticks from 10 different species collected in Turkey, using next-generation methods to analyze 16S and 18S genes (Brinkman et al., 2019). This study is one of many which show the importance of documenting the presence of potential tick-borne pathogens in the tick microbiome.

Tick microbiome can be different depending on tick genotype, biogeographical area, and if the tick is male or female. This is what Renè-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.

To summarize, a major highlight has been attributed to the genus *Coxiella*, which is an essential endosymbiont for tick physiology, which resides in the Malpighian tubules and gonads of the tick (Lalzar et al., 2014). These bacteria play a decisive role in the maturation of *Rhipicephalus microplus* (Canestrini, 1888), and this mutual relationship can be an alternative vector control method if disrupted (Guizzo et al., 2017).

Coxiella sp. is inherited by transovarial via and doesn't have pathogenic characteristics (Duron et al., 2015). But *Coxiella burnetti*, the pathogen of Q-fever, is maintained in the natural cycle of ticks, and its transmission is considered vital. 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. Although this was a significant result, we always have to 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 (Angelakis et al., 2016).

According to Andreotti et al. (2011) and reinforced by Narasinhham & Fikrig (2015), the environmental microorganisms can be found in the tick microbiome either, such as those present in the soil, plants, or even in the skin of animals with *R. microplus*.

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.

With the advent of sequencing tools and bioinformatics, determining the symbiotic commensal community has been easier and can provide the next steps towards identifying its role in the tick. Between the most used techniques to analyze tick microbiome, it is important to highlight the sequencing of one (or more) of the 9 hypervariable regions of the ribosomal gene 16S (Greay et al., 2018).

Tick cell cultures

Efforts have been directed to the study of tick cell lines, that can be used in many fields of research, including tick-borne disease. These cell lines are mostly originated from embryo cells (eggs) but can come from larva or moulting nymphs either (The Tick Cell Biobank - <https://www.liverpool.ac.uk/infection-and-global-health/research/tick-cell-biobank/-14/11/2019>). Even that they are not easier to establishment, tick embryonic cells have been cultivated since the 1950s (Weyner, 1952), and some 90 are nowadays documented at the site Cellosaurus (<https://web.expasy.org/cellosaurus/>). This is an online resource that congregates cell lines of different arthropods. However, none of them were obtained from ticks that occur in the Neotropical region, except for *R. microplus* from the state of Rio de Janeiro (in the establishment) (Bell-Sakyi et al., 2018).

Among the many proposes of cultivating the 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) have been successful at preparing primary cultures from embryonic eggs of *R. sanguineus* (tropical lineage), and these cells showed to be an alternative for the growth of *E. canis in vitro*. Another advantage of studying tick cell lines is that they have a lower cost compared to *in vivo* studies, and the results are achieved faster.

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 (Yunker et al., 1981; Bhat et al., 1979; Blouin et al., 2002; 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.

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?

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.

2. OBJECTIVES

2.1.1 General

Identify the different prokaryotic symbiont microorganisms taxa present in females, eggs and embryonic cell cultures of *R. sanguineus* from two distinct lineages (temperate and tropical), using metataxonomic analysis of 16S rRNA gene.

2.2. Specific

- Verifying which endosymbiont bacteria are present in the samples through the amplification of V4-V5 hypervariable region of 16S rRNA gene, using Illumina MiSeq platform;

- Comparing microorganism variations in the samples, between the two *R. sanguineus* lineages and among the different stages of the ticks (females and embryonic cell cultures).

3. MATERIAL AND METHODS

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

Engorged and non-engorged females of *R. sanguineus* s.l. were collected directly from dogs. 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 assess the number of ticks to be collected followed the recommendations of Tekin et al. (2017).

All 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 lineage and seven of the temperate lineage were subjected to homogenization and DNA extraction. The ticks belonging to each lineage were pooled in order to obtain DNA pools from each strain.

Five engorged females (from each lineages) were placed in sterile Petri dishes and kept in a biological oxygen demand (BOD) incubator at 27 °C ± 1 °C and 80-85% humidity, 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 measured using a spectrophotometer (Nanodrop, Thermo Scientific®) and the samples were then stored at -20 °C until use. 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.

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

The total DNA of the samples 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:

- **Trop(F)**: DNA pull from non-engorged females of *R. sanguineus* tropical lineage;
- **Temp(F)**: DNA pull from non-engorged females of *R. sanguineus* temperate lineage;
- **Trop(C)**: DNA of primary cell culture of *R. sanguineus* tropical lineage;
- **Temp(C)**: DNA from primary cell culture of *R. sanguineus* temperate lineage.

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. This procedure was performed at the Centralized Multipurpose Laboratory for Large-Scale DNA Sequencing and Gene Expression Analysis - LMSeq(<http://bit.ly/facility-fcav>), from the Department of Technology of FCAV, UNESP de Jaboticabal (Proc. FAPESP 2009 / 53984-2).

Bioinformatics Analysis

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 (Andrews, 2010), 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., 2012); RDP (v. Trainset 16) (Cole, 2004); 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.

4. 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, 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).

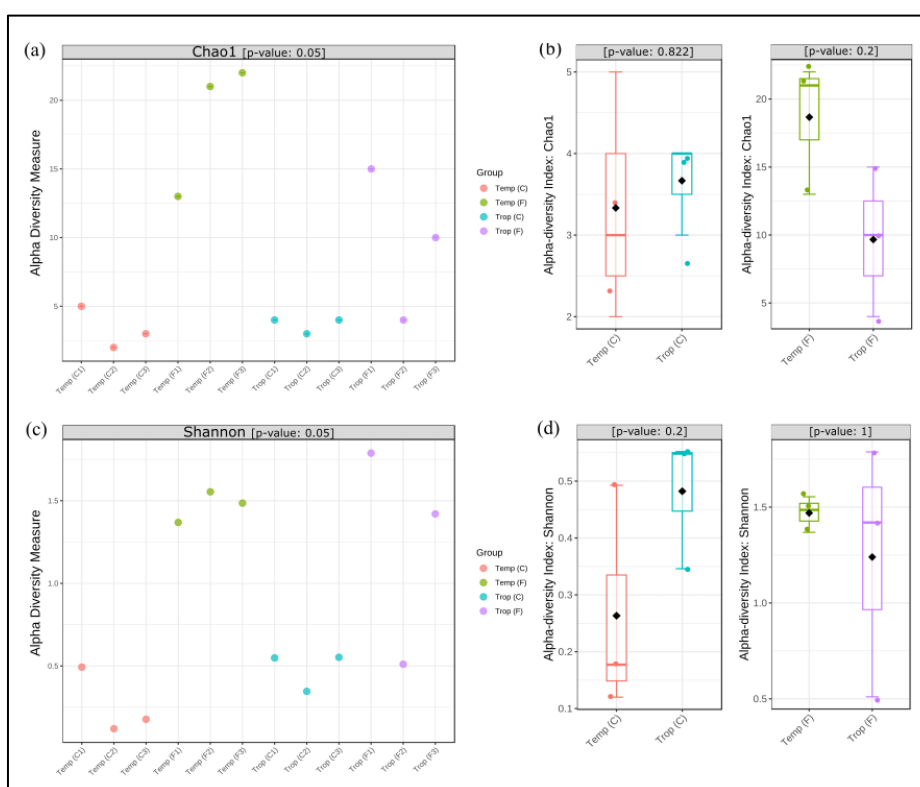


Figure 1. Alpha diversity indices for *Rhipicephalus sanguineus* (s.l. and s.s.) samples. Dot plots represent the alpha diversity of each replicate in the Chao1 (a) and Shannon

(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.

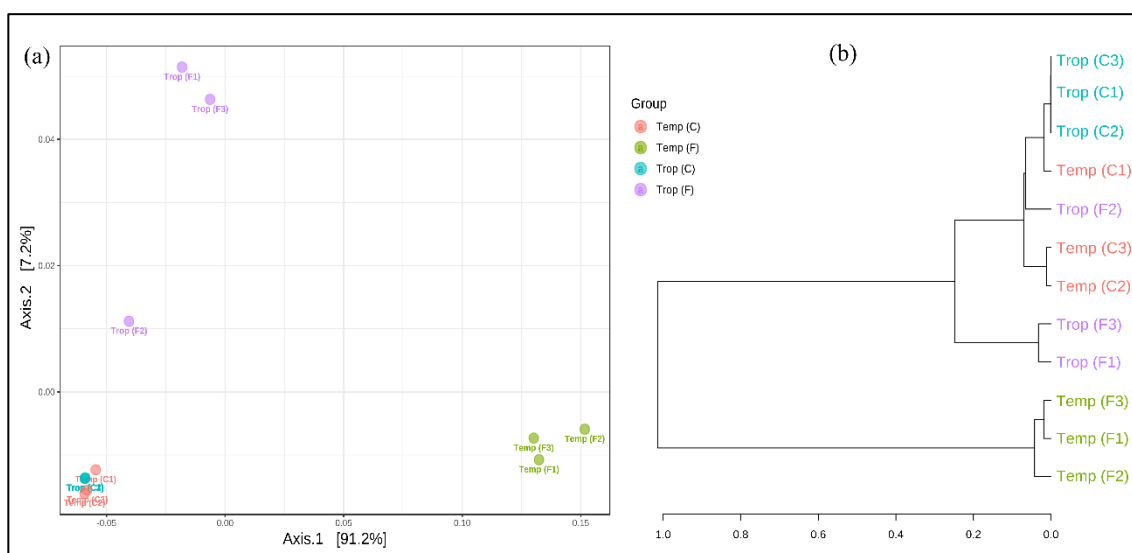


Figure 2. Weighted UniFrac distance analysis for *Rhipicephalus sanguineus* (s.l. and s.s.) samples. Principal coordinate analysis (PCoA) plot (a) of UniFrac distance matrix based on the phylogenetic tree of sequences and weighted according to the actual abundance of the sequences among the samples.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

The phylum Proteobacteria predominated in all samples (Figure 3a). While in the embryonic cell culture samples of tropical lineage (**Trop (C)**) only this phylum was observed, a relative abundance of 98.8% of was observed in **Temp (C)** samples, albeit without statistical difference. The female samples showed a statistical difference in the proportion of Proteobacteria (Figure 3b), with average relative abundances of 65.26% in **Temp (F)** and 98.19% in **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.

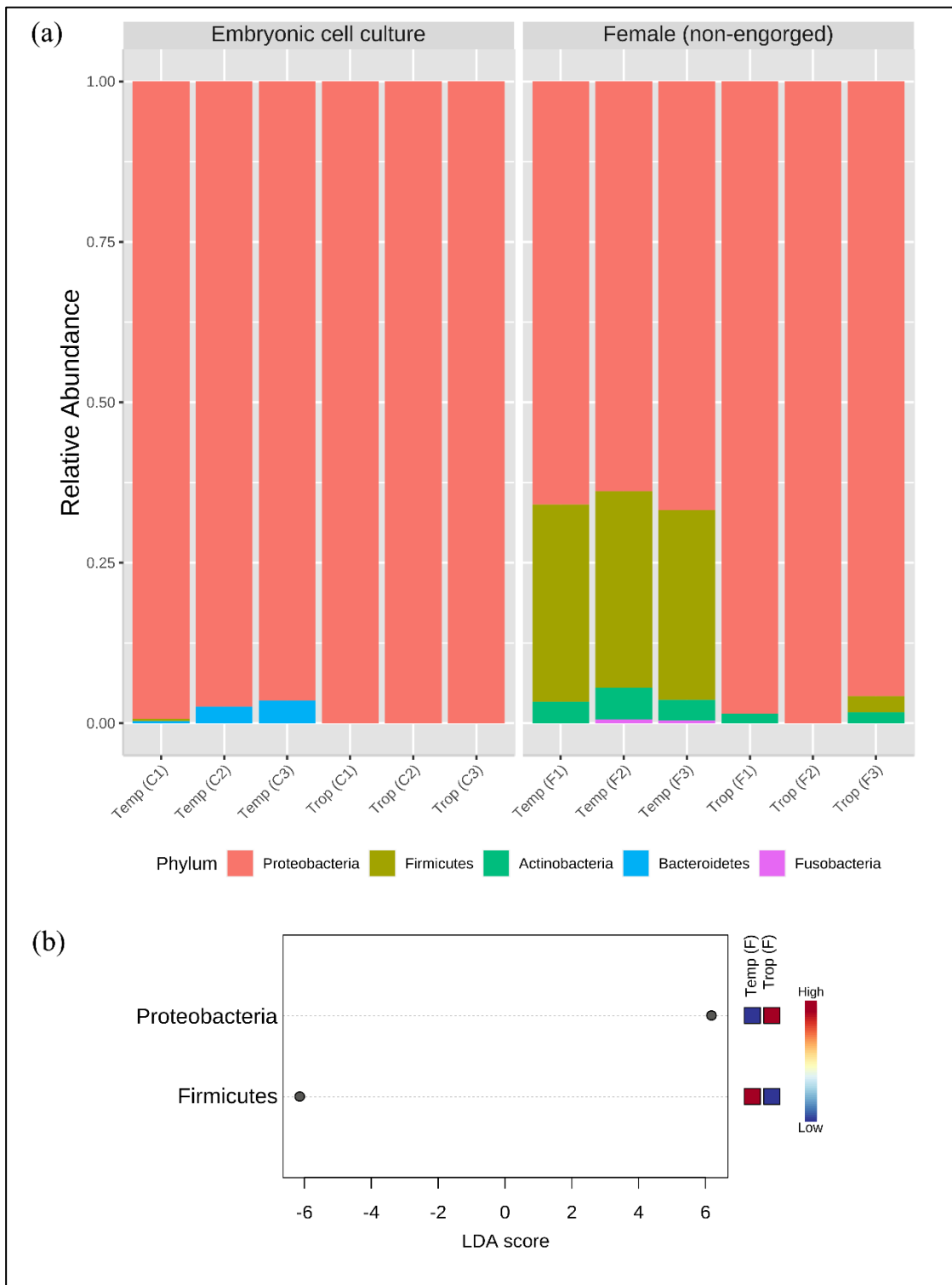


Figure 3. Bar charts of relative abundance at phylum taxonomic level (a) and LefSe results regarding differently abundant taxa in *Rhipicephalus sanguineus* (s.l. and s.s.)

samples. The differences were significant ($p < 0.1$) only among female samples (b). The threshold of the logarithmic linear discriminant analysis (LDA) score was 2.0.

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).

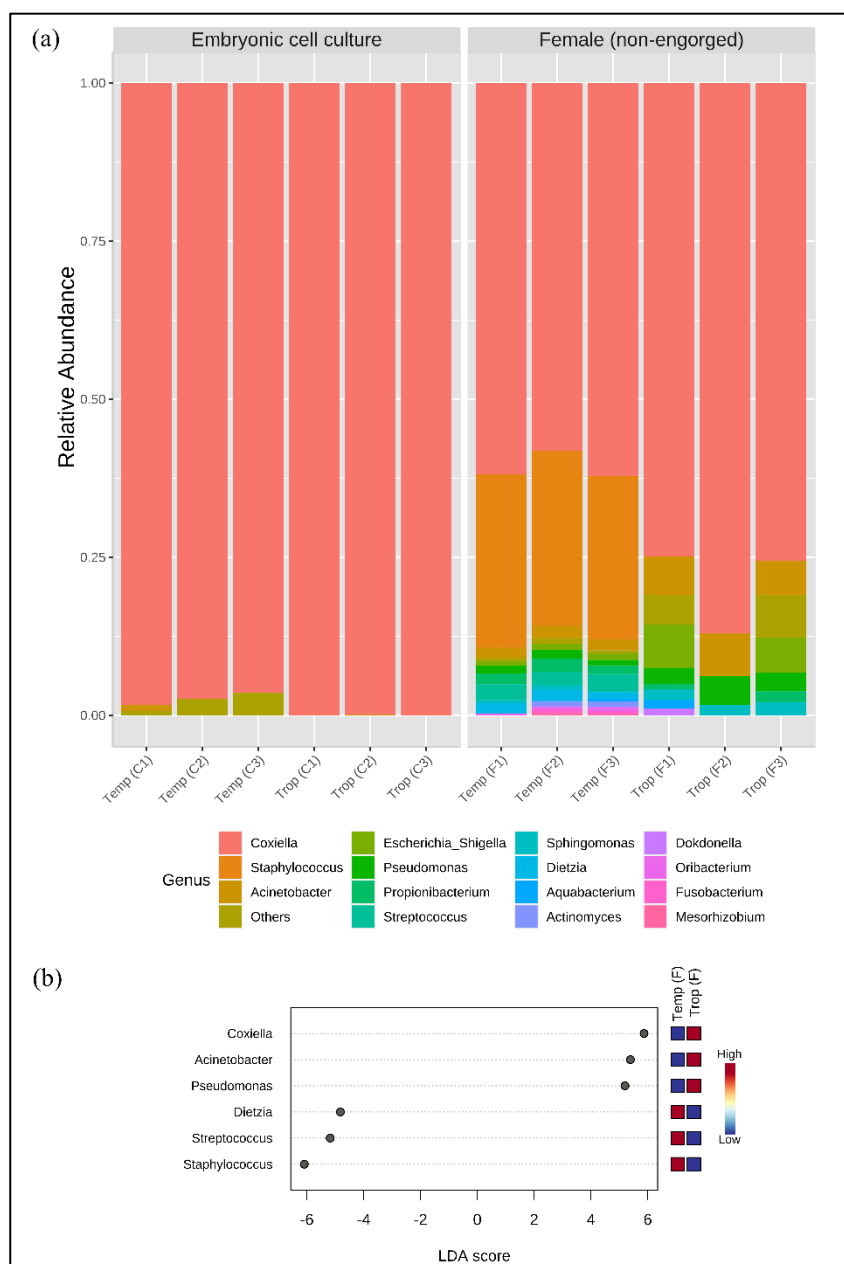


Figure 4. Bar charts of relative abundance at genus taxonomic level (a) and LefSe results regarding differently abundant taxa in *Rhipicephalus sanguineus* (s.l. and s.s.)

samples. The differences were significant ($p < 0.1$) only among female samples (b). The threshold of the logarithmic linear discriminant analysis (LDA) score was 2.0.

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 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).

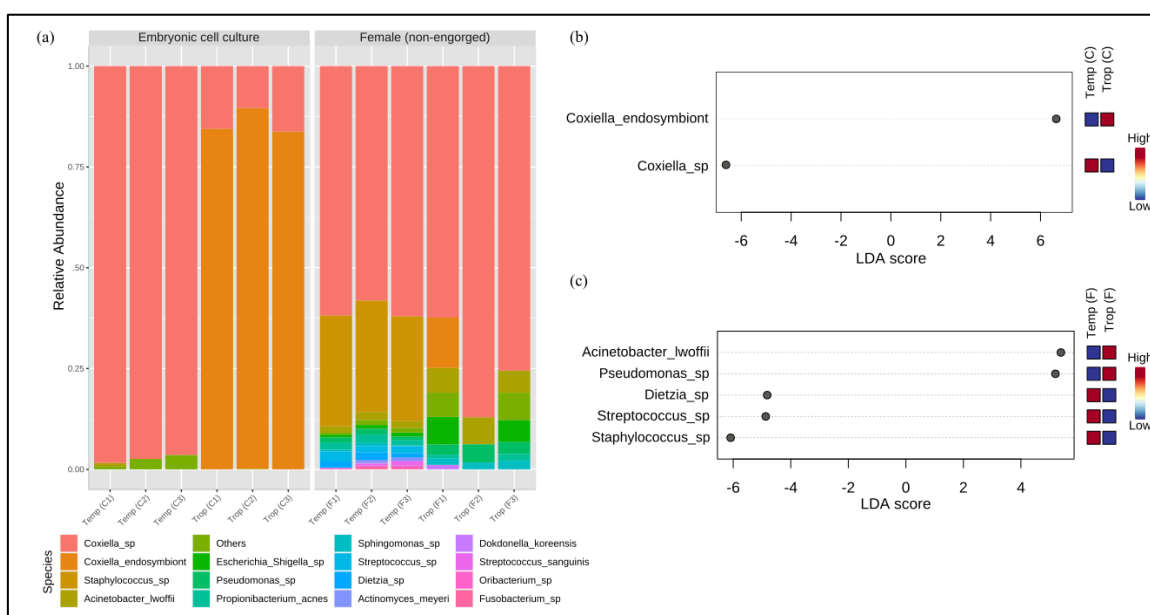


Figure 5. Bar charts of relative abundance at species taxonomic level (a) and LEfSe results regarding differently abundant taxa in *Rhipicephalus sanguineus* (s.l. and s.s.) samples. There were significant differences ($p < 0.1$) among cell culture (b) and female (c) samples. The threshold of the logarithmic linear discriminant analysis (LDA) score was 2.0.

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).

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.

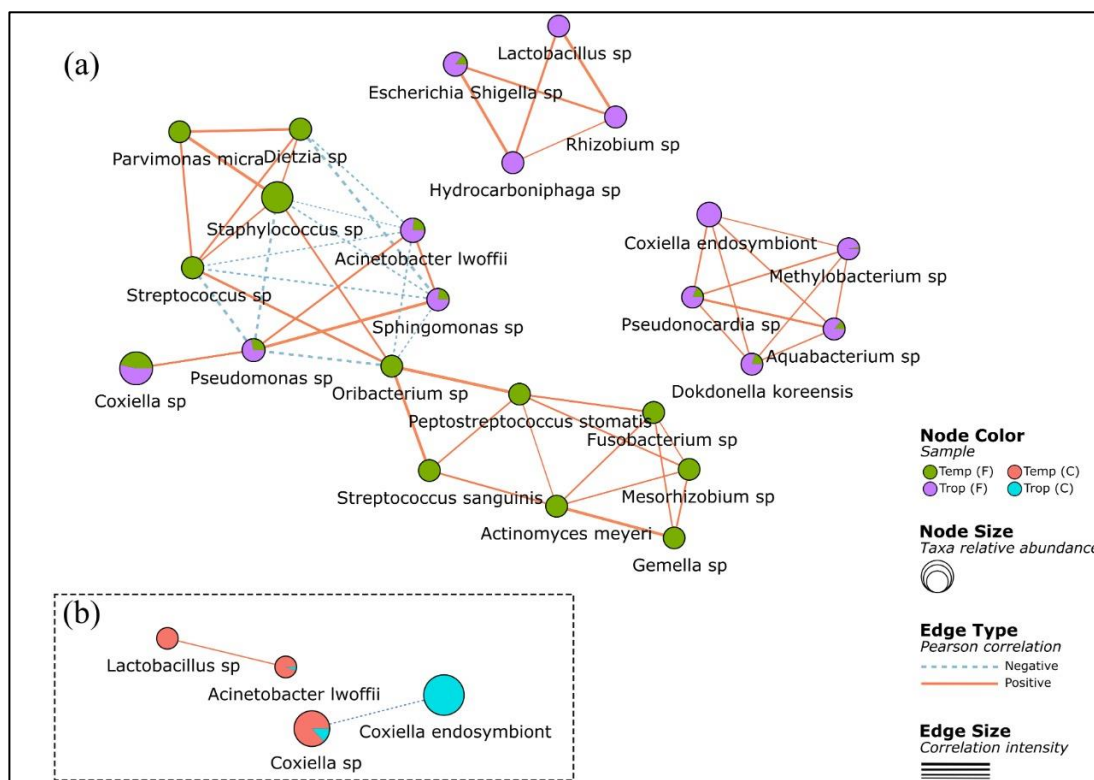


Figure 6. Co-occurrence networks generated from the normalized abundance matrices. Female (a) and cell lineage (b) datasets.

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.

5. DISCUSSION

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 Teuren et al. (2015) (81.7%). *Coxiella*, a member of this phylum, was the most abundant genus, and this result had been expected (Ahantarig et al., 2013; Bonnet et al., 2017; Renè-Martellet et al., 2017; Greay et al., 2018).

Indeed, Duron et al. (2017) commented that all ticks of the genus *Rhipicephalus* have *Coxiella*. 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 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 at the genus level as *Coxiella* sp. was found. 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. These bacteria might have originated from dogs' skin, blood or even saliva, because it is known that dogs lick where ticks have bitten. 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

Rhipicephalus (Boophilus) 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), and this 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 are able to reveal the existence of a microbiome. This shows the importance of testing cultures before performing other procedures, even with regard to established cell lines.

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 and 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 found 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 in relation to *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, in order 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*.

6. 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 strains, thus driving different patterns of interactions between ticks and the pathogens that they can harbor or transmit to vertebrate hosts.

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