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NEXT-GENERATION MASSIVE SEQUENCING AND THE BACTERIAL PROFILE OF TICKS FROM WILD AND DOMESTIC ANIMALS AND BLOOD OF HUMANS IN THE CHIHUAHUAN DESERT

SECUENCIACIÓN MASIVA DE SIGUIENTE GENERACIÓN Y EL PERFIL BACTERIANO DE GARRAPATAS DE ANIMALES SILVESTRES Y DOMÉSTICOS Y SANGRE HUMANA EN EL DESIERTO CHIHUAHUENSE

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Dedicated to

To my family:

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GENERAL ABSTRACT

NEXT-GENERATION MASSIVE SEQUENCING AND THE BACTERIAL PROFILE OF TICKS FROM WILD AND DOMESTIC ANIMALS AND BLOOD OF HUMANS IN THE CHIHUAHUAN DESERT

"TESIS DE DOCTORADO EN RECURSOS NATURALES Y MEDIO AMBIENTE EN ZÓNAS ÁRIDAS, Universidad Autónoma Chapingo"

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RESUMEN

Las garrapatas son un tipo de artrópodo pertenecientes al suborden Ixodoidea, que parasitan a los animales domésticos y silvestres para alimentarse. Durante el proceso de patogénesis, transmiten una gran cantidad de enfermedades bacterianas en los vertebrados parasitados, algunas de éstas son zoonóticas como lo es la fiebre manchada de las montañas rocosas por *Rickettsia rickettsii*, los distintos tipos de Ehrlichiosis, Anaplasmosis granulocítica, fiebre Q, enfermedad de Lyme entre otras. En el contexto de salud pública, tanto a nivel global y en particular en el norte de México, existe una fundada preocupación debido al aumento en las poblaciones de perros callejeros, lo cual incrementa el número de vectores de dichas enfermedades que afectan a los hospederos animales como a la población que residen cerca de estos animales. Asimismo, este tipo de vectores también afectan a diversas especies silvestres importantes en la región bajo análisis, funcionando como reservorios de tick-borne pathogens (TBP) que pueden diseminarse a áreas urbanas, y además pueden afectar la salud de especies amenazadas como en el caso de la tortuga del bolsón de Mapimí (*Gopherus flavomarginatus*). El propósito del siguiente estudio es analizar por medio de next-generation sequencing (NGS) las comunidades bacterianas de la sangre de personas que estuvieron expuestas a picadura de garrapata y compararla con las personas no expuestas a este factor, así como analizar el perfil bacteriano de dos especies de garrapatas de la familia Ixodidae y Agarasidae de importancia en salud pública, ecológica y veterinaria en la región de la Comarca Lagunera y el desierto chihuahuense.

Palabras clave: Garrapatas, zoonosis, Tick-borne diseases, Tick-borne pathogens, picaduras

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ABSTRACT

Ticks are a type of arthropod belonging to the Ixodoidea suborder, which parasitize domestic and wild animals for food. During the pathogenesis process, they transmit many bacterial diseases in parasitized vertebrates, some of which are zoonotic, such as Rocky Mountain spotted fever caused by *Rickettsia rickettsii*, different types of Ehrlichiosis, granulocytic anaplasmosis, Q fever, Lyme disease, among others. In the context of public health, both globally and particularly in northern Mexico, there is well-founded concern due to the increase in the population of stray dogs, which increases the number of vectors of these diseases that affect animal hosts, as well as the population that resides near these animals. Likewise, this type of vector also affects various important wild species in the region under analysis, functioning as reservoirs of tick-borne pathogens (TBP) that can spread to urban areas, and can also affect the health of threatened species as in the case of the Mapimí Bolson tortoise (*Gopherus flavomarginatus*). The purpose of the following study is to analyze by means of next-generation sequencing (NGS) the bacterial communities in the blood of people who were exposed to tick bites and compare it with people not exposed to this factor, as well as to analyze the bacterial profile of two species of ticks of the family Ixodidae and Agarasidae of importance in public, ecological and veterinary health in the Comarca Lagunera region and the Chihuahuan desert.

Keywords: Ticks, zoonoses, Tick-borne diseases, Tick-borne pathogens, bites

1. GENERAL INTRODUCTION

Organisms of the suborder Ixodoidea, commonly known as ticks, are obligate ectoparasites whose most important families are Ixodidae and Agarasidae (Polanco-Echeverry & Ríos-Osorio, 2016). Members of the family Ixodidae are known as hard ticks (they have a chitinous shield that covers the entire back) and those of the family Agrasidae as soft ticks (they lack a chitinous shield). Both families parasitize many animals (reptiles, birds, and mammals) including humans, from which they need to extract blood to survive. However, the properties of tick saliva and the way in which they digest blood are evolutionary characteristics that make them exceptional disease vectors (Márquez-Jiménez et al., 2005). It is estimated that 10% of the approximately 900 known tick species are of both veterinary and public health importance (De la Fuente et al., 2017).

One of the greatest public health concerns are diseases caused by vectors that affect domestic animals with which humans come into contact. Ticks are considered the main vector of pathogens of veterinary interest, and the second most important that affect humans after mosquitoes (Bonnet et al., 2017; de la Fuente et al., 2008). Ticks transmit diseases during the bite either through saliva, feces, coxal secretions or regurgitation (Betancur & Giraldo-Ríos, 2018; Márquez-Jiménez et al., 2005).

Ticks not only carry pathogens, but also constitute a well-defined structure of microbial communities that act as symbionts for them; these microorganisms are not only important for the biology of their hosts, but also constitute an important source of knowledge to understand the dynamics of transmission of pathogenic bacteria (Ahantarig et al., 2013; Bonnet et al., 2017). For example, the genus *Rickettsia* as a symbiont has an important role in the transmission and dynamics of some pathogenic strains of the Rickettsidae family (Ahantarig et al., 2013; Bonnet et al., 2017). Non-pathogenic tick microorganisms influence the abundance and diversity of tick-borne pathogenic bacteria, as well as favor replication and transmission of tick-borne pathogens (TBP) to vertebrate hosts (Bonnet et al., 2017).

Most of the studies to determine the bacterial microbiota have been carried out using conventional culture plate methods. However, most of the bacteria present in both arthropods and vertebrates are intracellular symbionts (endosymbionts) including those that are pathogenic. That is why novel molecular techniques have been developed to know with greater certainty the microorganisms under study. This is the case of next-generation massive sequencing (NGS) of the 16S rRNA gene, which is a technique that allows the identification of a large number of bacteria in a given sample by means of DNA (Couper & Swei, 2018; Frickmann et al., 2015; Simner et al., 2018).

TBP that cause the well-known tick-borne diseases (TBD) have been increasing globally; diseases such as anaplasmosis, ehrlichiosis, Q fever, spotted fever and Lyme disease have gained more global attention by physicians and veterinarians (Dantas-Torres et al., 2012). One of the main diseases transmitted by tick bites to humans is Rocky Mountain Spotted Fever (RMSF), which is currently of great concern due to its high mortality in the Americas. In Mexico, it has been detected in states with an arid and semi-arid climate such as Durango, Sonora, Sinaloa, Baja California, and Coahuila by means of molecular techniques such as PCR and sequencing. During the period 2012-2014, 121 human cases of Spotted Fever were registered in the Comarca Lagunera (Castillo-Martínez et al., 2017), three cases per 100,000 inhabitants reported in Coahuila in 2014, the highest in Mexico in that year (Ortega-Morales et al., 2019). Disease whose main vector is the brown dog tick (*Rhipicephalus sanguineus*). The problem is of great concern since it has been registered not only in stray dogs, but also in dogs with owners (Delgado-de la Mora et al., 2019). In the same sense, it has been observed that in communities with an overpopulation of stray dogs, these are usually spreaders of *Rickettsia rickettsii* (Castillo-Martínez et al., 2015). In addition, the abundance of these arthropods in the Comarca Lagunera (Durango and Coahuila) has been increasing due to climatic, geographic, demographic, and sociopolitical conditions (Castillo-Martínez et al., 2015).

The determination of the microbial community of ticks is of special interest in two different contexts that will be addressed in this thesis; 1) due to the relevance of investigating the microbiota of the tick (*Ornithodoros turicata*), which parasitizes an endemic species of ecological importance in the center of the Chihuahuan desert (Mapimí Biosphere Reserve), such as the Bolson tortoise (*Gopherus flavomarginatus*), that according to the official Mexican norm 059, it is a species that is in danger of extinction. (SEMARNAT, 2010); As well as the brown dog tick (*Rhipicephalus sanguineus*) which is closely related to diseases transmitted to humans by close contact. 2) In another context, analyze and compare the blood microbiota of people who were exposed to tick bites and compare them with people not exposed to this factor to determine if there is a change in its composition. In this regard, it is necessary to mention that blood is not a sterile medium since it harbors microorganisms (Tao *et al*., 2014; Faria *et al*., 2015; Frickmann *et al*., 2015; Païsé *et al*., 2016).

To date, there is no information on the composition of the bacterial microbiota by next-generation massive sequencing of the 16S rRNA ribosomal gene in ticks in the analyzed region; Likewise, no studies have been carried out on the bacterial microbiota in the blood of people who were exposed to tick bites and people who were not exposed, all of them apparently healthy. Therefore, this information will contribute to improve decision-making in the prevention of TBD, which would benefit the actions in epidemiological matters and specifically the health of animals and people living in this region.

2. HYPOTHESIS

There are potentially pathogenic bacteria in the bacterial composition of the ticks *O. turicata* and *R. sanguineus*, as well as significant differences in the blood bacterial profile of people exposed to tick bites regarding those not exposed evaluated througout Next-generation sequencing.

3. OBJECTIVES

3.1 General objetive:

- To analyze the bacterial profile of two species of ticks, as well as from people exposed and not exposed to tick bites in the Chihuahuan desert and the Comarca Lagunera.

3.2 Specific objectives:

- To analyze the bacterial profile of the parasitic soft tick *(Ornithodoros turicata)* in the Chihuahuan desert using Next-Generation sequencing.
- To analyze the bacterial profile of the parasitic hard tick *(Riphicephalus sanguineus)* in the Comarca Lagunera by using Next-Generation sequencing.
- To analyze and compare the blood bacterial profile of people exposed and not exposed to tick bites in the Chihuahuan Desert and the Comarca Lagunera.

4. LITERATURE REVIEW

4.1 Definition of arthropods

The word "arthropod" comes from the Greek arthron, "joint" and podos, "foot" or "leg", which together mean "jointed leg". Arthropods are invertebrates of the Arthropoda phylum that are bilaterally symmetrical, characterized in turn by having segmented bodies, external skeletons (exoskeleton), composed of chitin and other elements, as well as articulated appendages. Arthropods mainly comprise ectoparasites from a parasitological point of view (Duvallet et al., 2018; Mehlhorn, 2016).

The Arthropoda phylum approximately five and two million described species, representing more than 80% of all animal species on the planet. However, estimates indicate that there are between 5 and 10 million species of arthropods (ØDegaard, 2000).

Blood-sucking arthropods comprise two main groups: 1) ticks (Ixodida) and 2) insects, which include bugs (Hemiptera), flies (Diptera), lice (Phthiraptera), and fleas (Siphonaptera) (Verwoerd, 2015). More than 16,000 blood-sucking species are known, of which about 500 are associated with the transmission of pathogens (Duarte, 2013).

4.2 The Acari

Mites are part of the arachnids, they contain mites and ticks, structurally they can be differentiated from insects because they do not have wings or antennae, when they are larvae, they have three pairs of legs, and in the nymph and adult stage they have 4 pairs of legs. They are made up of the head, thorax and abdomen and these are fused into a single part called the idiosome (Fig. 1B). They have a hypostome and two chelicerae to cross the integument of the hosts, as well as two pedipalps that do not go through the skin but do fuse with the cement to adhere strongly to the host, in addition to being sensory structures and allow it to detect the animal they will parasitize. Ticks feed on a wide variety of vertebrate

animals and are mainly divided into two families: Agarasidae or soft ticks and Ixodidae or hard ticks (Duvallet et al., 2018).

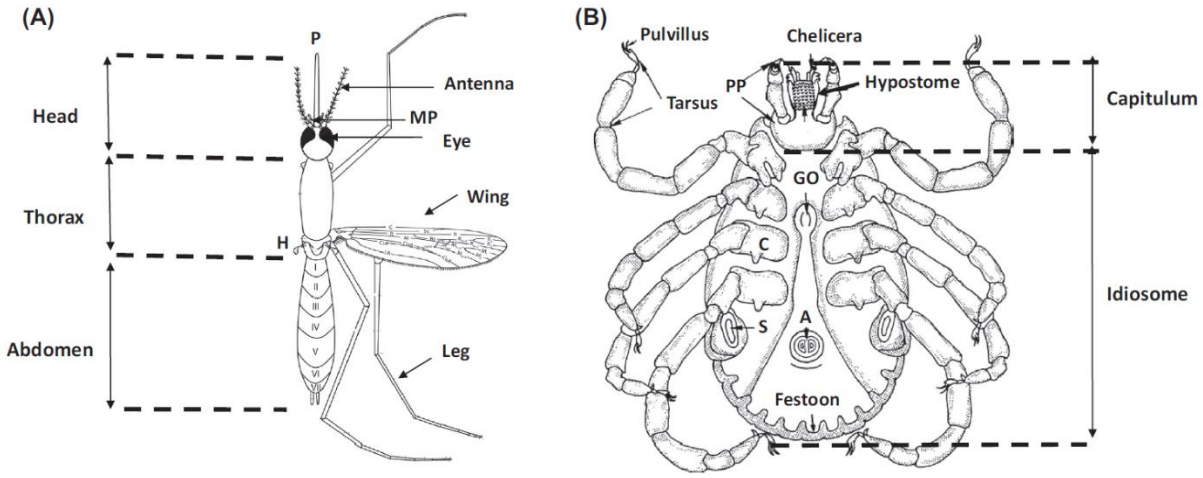

Figure 1. Structural differences between insecta and acari. (A) Mosquito of culicinae female on a dorsal view. H, halter; MP, maxillary palp; P, proboscis. (B) Hard tick (Dermacentor sp.) from a ventral view. A, anus; C, coxa; GO, genital opening; PP, pedipalps; S, stigma. Adapted from Duvallet et al. (2018).

4.3 Vector definition

The term "vector" has been applied in different epidemiological scenarios, which is difficult to define. It can be defined as living organisms that can transmit infectious diseases from human to human or from animals to humans and vice versa. In many cases, these are hematophagous arthropods that ingest the microorganisms during the blood meal of an infected host and later transmit them to a susceptible being during the following blood meal. Among these are mosquitoes, ticks, flies, fleas, freshwater snails and sandflies (WHO, 2014). To adequately define vectors, ecological, evolutionary, and public health perspectives must be considered. Although the term vector is generally and universally applied to hematophagous arthropods as described above, it has also been attributed to animals of other taxonomic classifications (Klimpel & Mehlhorn, 2016; Leonard, 2014; Mühldorfer & health, 2013; Pinaud et al., 2016). Therefore, another broader definition of a vector that can also be used would be any

vertebrate or invertebrate organism that is capable of serving as a carrier and transmitter of pathogens between organisms of different species (Kuno & Chang, 2005), including those that only transport the agent mechanically. Even other authors expand the definition including inanimate objects or fomites as vectors of infectious agents and transfer it to hosts such as syringes (Gibson et al., 2001). In this context the definition of a vector will depend on the epidemiological, ecological, biological and evolutionary perspective, the use of a single definition can lead to the risk of excessive simplification since there may be different appropriate definitions (Wilson et al., 2017).

4.4 Ectoparasites

Ectoparasites are distributed in all ecosystems on the planet, often cause harm to humans and animals, and are generally difficult to eradicate completely; therefore it is a question of controlling them with insecticides and endectocides (Wall & Shearer, 2008). They are organisms that inhabit the skin of humans, domestic and wild animals, as well as laboratory animals, fish, birds and insects (Hopla et al., 1994). These arthropods constitute a very diverse and highly specialized group with the ability to survive on their host (Aldemir, 2007).

Ectoparasites present a wide variety of associations with their hosts: some are obligate or facultative, permanent or intermittent, and they can also occur superficially or subcutaneously. In addition, they can parasitize many domestic and wild animals. Among the parasitic arthropods are mites, ticks, lice and fleas, which, depending on the degree of infestation and the immune status of the animal, can cause dermatological problems (González et al., 2004). Damage associated with the feeding process of ticks ranges from primary and significant loss of blood fluid, inflammation, and direct damage to the skin and other subcutaneous tissues. Presenting signs of excoriation, pruritus, erythema, papules, desquamation and autotrauma associated with the above (Wall & Shearer, 2008).

In addition to all the previous lesions, ticks are transmitters of pathogenic organisms such as bacteria, viruses, and protozoa mainly. These infectious agents, in addition to the associated disease, can also indirectly cause disturbances in their hosts; increased stress, especially due to the itching they cause, decreased reproductive activity, decreased food consumption, among other secondary problems (González et al., 2004).

4.5 Ticks

Obenchain and Galun (2013) cited an Egyptian papyrus from 1550 BC. where tick-borne fever is mentioned. Sometime later, Hoogstraal (1972), who is probably the most prolific researcher of the 20th century on the subject of ticks, cited Homer writing about ticks and their diseases in the year 800 BC. Later, Cato, Aristotle and Pliny referred to ticks as undesirable parasites (Anderson, 2002). However, it was not until 1893 that the ability of ticks to induce disease was published. His research determined that ticks of the species *Boophilus annulatus* were capable of transmitting *Babesia bigemina* and causing Texas cattle fever, was a landmark scientific discovery that demonstrated how parasitic arthropods could transmit and transport infectious organisms (Smith & Kilborne, 1893).

Ticks are a highly specialized group of ectoparasitic arthropods, obligate nonpermanent bloodsuckers that feed on large numbers of vertebrate hosts in most regions of the earth (Sonenshine & Roe, 2013). Regarding human health, mosquitoes are the arthropods that transmit the most pathogens to humans, followed by ticks. However, in veterinary medicine, the most important ectoparasites are ticks because they are the ones that most transmit pathogens to domestic and wild animals (Sonenshine & Roe, 2013). Ticks are classified into two main families Ixodidae (hard ticks) and Agarasidae (soft ticks). In addition to a third family, Nuttaliellidae, exclusive to Africa and only contains a single species. There is a worldwide record of 840 - 860 species (Table 1). The Ixodidae, with a sclerotized dorsal shield, is considered the most important and largest family, comprising 670 species. The Ixodidae family is divided into Prostriata, represented by a genus Ixodes and 244 species, and Meta striata, represented

by 459 species in 14 genera (Guglielmone et al., 2010; Nicholson et al., 2019). The Agarasidae family, has a soft and flexible exoskeleton, and has around 167 known species with 4 genera. All species of both families have four stages in their life cycle, egg, larva, nymph and adult (Anderson, 2002).

Table 1. Known genera of the three families of ticks and number of described species

Family	Genus	Known species
Ixodidae	*Ixodes*	245
	Amblyomma	102
	Aponomma	24
	Haemaphysalis	155
	Hyalomma	30
	Dermacentor	30
	Cosmiomma	1
	Nosomma	1
	Rhipicephalus	70
	Anomalohimalaya	3
	Rhipicentor	2
	Boophilus	5
	Margaropus	3
Agarasidae	*Argas*	56
	Ornithodoros	100
	Otobius	2
	Antricola	8
	Nothoaspis	1
Nutalliellidae	*Nuttalliella*	*1*

In Mexico there are only 100 described species, of which 68 correspond to the Ixodidae family and 32 to the Argasidae family (Guzmán-Cornejo & Robbins, 2010). In the state of Durango, in the political region called Comarca Lagunera, the genera *Dermacentor* (2 species), *Rhipicephalus* (2) and *Haemaphysalis* (1) corresponding to the Ixodidae family were reported; also the genera *Ornithodoros* (3 species), *Otobius* (1) and *Argas* (1) of the family Argasidae (Castillo-Martínez et al., 2016; Hoffmann, 1962).

Ticks are considered interesting organisms not only because of the pathogens they transmit, many of which are widely known and some of which are extremely

rare (Mutz, 2009). They can have direct effects on the productive capacity and reproductive efficiency of some companion animals, particularly when infestations are significant, a situation that worsens in tropical regions (González, 2018).

Ticks, being the most efficient vectors towards animals, can transmit different types of organisms (protozoa, rickettsiae, spirochaetes and viruses); many of these microparasites are widespread and of considerable medical and veterinary interest. It is estimated that about 10% of the 867 known tick species acts as vectors of pathogens in domestic and wild animals and humans. Furthermore, ticks are capable of producing severe toxic conditions such as paralysis and toxicosis, as well as hypersensitivity and irritation, some of these deadly (Jongejan & Uilenberg, 2004). Tick-borne infectious agents have coevolved with different wild hosts around the world in a state of equilibrium. Due to this, the export and import of domestic animals to different endemic areas of TBD have contributed to the spread of diseases in regions where they did not exist before (Jongejan & Uilenberg, 2004). In addition to the economic implications for livestock, the impact they generate on public health is important, especially in the northern hemisphere, mainly due to Lyme borreliosis (LB) and other tick-borne zoonotic diseases such as those of viral origin characterized by encephalitis and hemorrhagic fevers such as RMSF, causing the greatest morbidity and mortality in humans (Jongejan & Uilenberg, 2004). Thus, understanding the links between host biodiversity and pathogen transmission are therefore essential for predicting tick population dynamics and the epidemiology of tick-borne diseases (McCoy et al., 2013).

4.7 Biology of ticks and feeding

Ticks are non-permanent blood-sucking arthropods that feed on mammals, reptiles, birds, and amphibians throughout the world (Keirans & Durden, 2005). Ticks are distributed in practically all latitudes but are much more common in the tropics and subtropics (Balashov, 1972). Regarding the moult and its reproduction, they are determined by the ingestion of blood, by the temperature and the length of the day; females are capable of laying thousands of eggs

(Anderson & Magnarelli, 2008). Morphologically, on the outside they are composed of the capitulum, the body and the legs, and an essential organ to adhere to the skin called the hypostome and it is also vital for feeding (Balashov, 1972).

Blood-based feeding is essential for the development and reproduction of the ectoparasite. Eating involves a series of behavioral events beginning with hunger and ending with satiety (Waladde & Rice, 1982). Through this mechanism, the tick can become coinfected with a series of pathogens and subsequently inoculate them into another susceptible host (Anderson & Magnarelli, 2008). The essential steps for successful feeding are as follows (Anderson, 2002; Anderson & Magnarelli, 2008):

1. Appetite (hunt or seek a host)

2. Attachment (adherence to the skin or fur of the host)

3. Exploration (search on the skin for a suitable insertion site)

4. Penetration (insertion of the mouthparts into the epidermis and dermis of the host)

5. Attachment (established feeding place)

6. Ingestion (absorption of blood and other fluids)

7. Engorgement (partial or complete meals of drawn blood)

8. Detachment (removal of mouthparts)

9. Disengagement (the tick falls off the host)

Hard ticks generally search for their hosts and with the help of their tarsal claws they attach themselves. Once in the preferred site of the species, they cut the epidermis and dermis by means of the chelicerae, and this allows the hypostome to be inserted. The hypostome adheres strongly to the cement secreted in the

saliva or to the tissues of the host. Blood, hemolymph, or tissue fluids pass through the alimentary canal, which is made up of the ventral part of the chelicerae and the dorsal part of the hyopostome, when sucking blood, they also inject saliva at intervals. Saliva flows from the dorsally located salivarium and host materials are swallowed through the opening into the ventrally located pharynx. This is the reason why various pathogens are inoculated into the host system (Anderson, 2002).

The feeding process of the tick favors the dispersal of the organisms to different regions. An example is the case of *Ixodes scapularis* that generally feeds on birds and can migrate several kilometers while feeding. Some can even be transported from one continent to another by the migration of birds. Tick appetite-generating stimuli include odor, shadow, vibration, and visual appearance. In some species, the eyes are located on the flanks or submarginal areas of the shield. However, many species lack eyes, so they resort to other sensory stimuli such as touch, radiant temperatures, and the aforementioned stimuli. They also have hair-like projections (setae) on the palps or other parts of the body that facilitate tactile sensation, these favor the selection of the feeding site on the host's skin, which are generally protected places and difficult to access for the host (Anderson & Magnarelli, 2008).

4.8 Vectorial competence of the ticks

There are two terms widely used in the epidemiology of zoonotic diseases transmitted by arthropods that, although they can be used interchangeably to refer to the ability of an arthropod to act as a transmitter of diseases, are terms that respond to two different conditions; “vectorial capacity” generally it is a way of referring to the capacity of an arthropod as a vector of infectious agents. However, from an environmental and behavioral point of view they affect some variables such as longevity and density. On the other hand, "vectorial competence" this refers to the ability of a vector to transmit pathogens related to genetic factors (Beerntsen et al., 2000, **Box 1**). These genetic factors determine aspects such as the preference of the tick for a specific host, the duration of the blood meal, the

interactions tick-host-pathogen and the microbiome with the pathogen, as well as the susceptibility of the tick to infection by pathogens (Narasimhan et al., 2014; Rynkiewicz et al., 2015; Vayssier-Taussat et al., 2015).

4.9 Soft ticks

Agarasid ticks also known as soft ticks differ from ixodid or hard ticks in several characteristics involving biology, structure or morphology, and ecological issues. The most relevant difference is that they lack a chitinous dorsal shield both in their nymphal and adult stages and in terms of a relevant ecological aspect that differentiates them is their nidicolous to endophilous lifestyle (Pospelova-Shtrom, 1969).

These ticks generally remain outside the focus of study and research, and their vectorial capacity and their importance in human and animal health are often unknown. This is mainly because they are highly specialized to certain microhabitats and generally live close to their host. And on the other hand is the repeated intake of blood meals during each life cycle and these occur quickly so it is difficult to find it in the hosts (Hoogstraal, 1985). Despite this, it is known that it can cause irritation, allergies, anemia, paralysis, toxicosis and different diseases due to the inoculation of pathogenic agents to its hosts during feeding (Jongejan & Uilenberg, 2004; Vial, 2009).

There are 193 described species within the genera *Argas, Antricola, Carios, Nothoaspis, Ornithodoros* and *Otobius,* and they are abundant in arid and semi-arid habitats (Brites-Neto et al., 2015). The main genera of soft ticks with public and veterinary health importance are *Argas, Otobius* and *Ornithodoros*. The nymphs are always parasitic, while the larvae and adults are generally parasitic. However, they return to their shelter after feeding for a short period of time. The genus *Argas* is an exception since it can feed for days on the birds it parasitizes, as well as the larvae and nymphs of *Otobius* which feed for long periods of time in the external auditory canals of their hosts (Jongejan & Uilenberg, 2004).

Soft ticks have several nymphal stages. Adults usually mate on the host; the female feeds several times and oviposits a small number of eggs after each meal in the environment (Jongejan & Uilenberg, 2004). The fundamental result of their biological adaptations is their unusual feeding abilities and their life cycle, which occurs for example with the subfamilies Argasinae and Ornithodorinae, and at the same time with the Otobinae, Antricolinae and Nothoaspinae in structural terms. All with the result of a uniform and fundamental adaptation within the species of the agarasids (Hoogstraal, 1985).

4.10 Hard ticks

This family comprises the ticks known as Ixodidae; the name being attributed to the anterodorsal plate better known as the chitinous shield. There is marked sexual dimorphism, and the trailing edge of the opisthosoma can be divided into sclerotic structures called festoons. They feed on blood and can parasitize various hosts. They present free-living stages in the environment. Its size varies from 2-20 mm (Brites-Neto et al., 2015). The life cycle of the Ixodidae family depends on the number of hosts that need to parasitize and can be from one to three host species. Feeding until engorged from its immature stages and moving on to its later stages in the life cycle. Ticks on a single host molt twice on the same animal from larva to nymph and from nymph to adult, In the case of those with two hosts, they moult only once on a host from larva to nymph, later it is engorged and detaches from the host, moults in the external environment and the resulting adult looks for a new host and this may or may not be from the same species that previously parasitized and finally the ticks of three hosts, these do not moult on the host, the engorged larva detaches and molts in the external environment, looks for a second animal to parasitize and engorges and detaches again until it moults to its adult stage and finds a third host (Jongejan & Uilenberg, 2004). The adult ixodidae mate on the host, the female feeds, engorges and detaches, once in the external environment it oviposits a large number of eggs and finally dies, on the contrary, the male can last attached to the host for months feeding. Ticks

with three hosts lay the most eggs on average, however they are the most at risk since they have to find three hosts (Jongejan & Uilenberg, 2004).

4.11 *Rhipicephalus sanguineus*

The genus *Rhipicephalus* comprises 84 described species. They are small to medium-sized ticks with eyes and festoons, and short, broad palps. Among arthropod parasites of dogs and humans, the brown dog tick *Rhipicephalus sanguineus* (Latreille, 1806) is an effective vector of a diverse group of pathogens (Dantas-Torres et al., 2012) (Fig. 2). Most *Rhipicephalus* spp. parasitize mammals in Africa. They are usually three-host ticks. Taxonomic identification of ticks of the rhipicephalid group can cause difficulties (Jongejan & Uilenberg, 2004). They are recognized by the hexagonal shape of the base of the capitulum when viewed dorsally. Important species in the genus include the brown dog tick (*R. sanguineus*) and the brown ear tick (*R. appendiculatus*). *Rhipicephalus* ticks are generally found in their adult state parasitizing mammals. *Rhipicephalus sanguineus* has a cosmopolitan distribution, two main lineages are recognized, the tropical and the temperate. Morphological and molecular analyzes revealed the existence of at least four OTUs (i.e., *R. sanguineus* sensu lato*, Rhipicephalus* sp. I, II and III) under the name "*R. sanguineus*" or also known as the *R. sanguineus* group (Dantas-Torres et al., 2013).

The brown dog tick is the tick with the largest geographical distribution in the world and is responsible for transmitting a large number of pathogens to both dogs and humans. The most common host is the dog, both in urban and rural areas. However, it is completely adapted to living in human dwellings and is active throughout the year in tropical, subtropical, temperate and even arid regions (Dantas-Torres, 2010). Different investigations determined that the feeding process of the tick is favored by high temperatures. This suggests that the risk of parasitism in humans may be greater in areas with warm temperatures and long, hot summers, and consequently the risk of contracting TBD.(e.g., *Rickettsia conorii*, *Rickettsia rickettsii, Coxiella Burnetii* and *Ehrlichia canis*) (Dantas-Torres, 2010).

From a behavioral or ethological perspective, it can be said that the *R. sanguineus* tick is a monotropic species or that it feeds on the same species in all its life cycles and on three hosts, since at each stage of its cycle it requires a new host which it parasitizes and is also considered an endophilic tick since it is adapted to living indoors. However, the tick can also live outdoors, especially if there are shelters to do so (tree bark, walls with cracks or limestone). A relevant aspect is that, although it is considered monotropic and will generally parasitize dogs during their life cycles, it can occasionally parasitize humans as accidental hosts since they do not belong to their natural trophic chain (Dantas-Torres, 2010).

Figure 2. *Rhipicephalus sanguineus*. A: larva (mounted in Hoyer's medium; bar = 400 µm). B: nymph (mounted in Hoyer's medium; bar = 0.5 mm). C: female (bar = 1mm). D: male (bar = 1mm). Figure taken from Dantas-Torres (2010).

4.12 *Ornithodoros turicata*

The genus *Ornithodoros* is characterized morphologically mainly by its rounded body shape, the nymphs and adults have a leathery cuticle with innumerable wrinkles and small bumps (mamillas); they lack a lateral suture as in other

species. These differ from the Argas because they are more numerous and shorter (Nicholson et al., 2019). This genus has a worldwide distribution and is favored by the different hosts that it parasitizes and range from birds, reptiles and mammals (Nicholson et al., 2019). The genus *Ornithodoros* are globally distributed and five species have been described in the US: *Ornithodoros coriaceus, O. hermsi, O. parkeri, O. turicata* and *O. talaje*; while *O. turicata* is distributed in the arid regions of the southern United States and in Latin America (northern Mexico) (Barraza-Guerrero et al., 2020; Donaldson et al., 2016; Dworkin et al., 2002). In 1876 it was described for the first time in Guanajuato, Mexico (Dugés, 1876).

O. turicata ticks have relevance for both veterinary and human medicine (Manzano-Román et al., 2012), among the most important infectious agents transmitted by this tick are the spirochetes that cause relapsing fever borreliosis (Anderson et al., 1990). In addition *O. turicata, O. coriaceus* and *O. parkerise* have been experimentally infected with the African swine fever virus (ASFV) (Hess et al., 1987). This virus represents a global alarm for the livestock industry (Costard et al., 2009).

Talking about its ethology and feeding, *O. turicata* is an arthropod with nocturnal and nidicolous habits (Balashov, 1967). They usually fill with blood in about an hour, and it is rare to find them adhered to the hosts (Zheng et al., 2015). Although they can be found in peridomestic sites, their preferred habitat is generally burrows, nests, and caves (Milstrey, 1987). An important aspect of the feeding habits of *O. turicata* is that they are promiscuous in their choice of hosts, and can parasitize mammals such as prairie dogs (*Cynomys* spp.), ground squirrels (*Spermophilus* spp.), cattle, pigs, reptiles such as snakes and tortoises (*Gopherus polyphemus* and *Gopherus flavomarginatus*) (Cooley, 1944; Milstrey, 1987). Additionally, *O. turicata* has been reported feeding on canines and humans (Davis et al., 2002; Rawlings, 1995).

In short, a characteristic of *O. turicata* that allows it to be a vector of different pathogens is its life cycle, this tick can have up to six nymphal stages and survive

unusual periods of time between meals (Beck et al., 1986). The longevity of ticks is quite high, being able to live up to 10 years and even five years between one meal and another (Cooley, 1944; Davis, 1941; Francis, 1938). These long periods of starvation have also been observed in nature (Butler et al., 1984). Ticks feed and reproduce multiple times throughout their lives. Understanding their longevity and resistance allows us to understand ecological factors and how they affect the distribution and vector competition and consequently the control of these potential ectoparasites for humans (Donaldson et al., 2016).

4.13 Tick bites

Ticks have to bite their host to feed, since with the help of some chelicerae located in an extension called hypostome, ticks can go through the skin of the host until they find capillaries or blood vessels or hemolymph to feed; they often do this inadvertently because saliva naturally contains anesthetics and antihistamines and inflammatory lesions are usually not seen (Mutz, 2009). However, tick bites can cause papules, small ulcers or scabs, if they become complicated, although it is rare, a condition called "tick bite granuloma" may occur (Mutz, 2009). Lesions may also have secondary infections. There is also the possibility of developing hypersensitivity reactions derived from the tick's secretions and excretions as they feed on the host (Van den Broek et al., 2003). Ticks must be removed promptly without crushing them, as this could inoculate infectious agents into the circulatory system. If a tick is not detected, it could be a problem after 24 hours for the transmission of agents such as *Borrelia burgdorferi, Rickettsia conorii* or *Babesia*, to name a few examples (Mutz, 2009).

4.14 Ticks as transmitters of diseases

Vector-borne pathogens are of serious public and veterinary health importance (Kuleš et al., 2017). Emerging diseases are alarming worldwide, where the appearance of 30 new pathogens has been detected in the last three decades, of which 60% are of zoonotic origin and it is estimated that a third of these agents come from wildlife (Dikid et al., 2013). The concept of emerging disease refers to the appearance of an infectious agent that has generally been recently identified

and, above all, its ability to cause disease was unknown. Regarding re-emerging diseases, they are caused by infectious agents previously identified and whose pathogenic capacity is known, and this re-enters a human or animal population where it was no longer found, or where its epidemiology or susceptibility to antimicrobial drugs has changed (Heymann, 2009).

TBP is estimated to cause approximately 100,000 cases of human disease worldwide (Bonnet et al., 2017). The tick-pathogen interface is mainly mediated by molecular mechanisms that allow the processes of infection, development and the pathogenesis of PTBs. These mechanisms involve general evolutionary traits and other particular ones of each species, both of the ticks and their agents. The characterization based on molecular technologies has been able to clarify some questions about this tick-pathogen interface, providing new avenues for the development of control strategies and mitigation of parasitosis and TBD. These advances in molecular biology have been able to identify potentially pathogenic organisms never before studied, as well as their functional genes of these and their blood-feeding invertebrate hosts (de la Fuente et al., 2008). Below is a list modified from the table presented by de la Fuente et al. (2008) where only bacterial TBPs are included. It should be noted that it is based on studies carried out by molecular methods for the identification of pathogens.

Table 2. Description of tick-borne pathogens, vectors, geographical distribution, and host species affected. Modified table from de la Fuente et al. (2008).

Pathogen	Disease	Tick vectors	Geographical distribution	Host species affected	References
		Genus *Aegyptianella*			
A. pullorum	Aegyptianellosis	*Argas walkerae, A. persicus, A. reflexus*	Africa, southern Europe, Middle Asia, Indian subcontinent	Domestic poultry	(Hoogstraal, 1985; Jongejan & Uilenberg, 2004)
		Genus *Rickettsia*			
R. rickettsii	Rocky Mountain spotted fever	*Dermacentor andersoni, D. variabilis, Amblyomma cajennense, A.*	Americas	Human, dog	(Merino et al., 2020; Parola et al., 2005b; Parola & Raoult, 2001)

		aureolatum, Rhipicephalus sanguineus			
R. amblyommii	Spotted fever rickettsiae group; no disease name	*Amblyomma americanum, A. neumanni, A. cajennense, A. coelebs*	Americas	Human	(Billeter et al., 2007; Labruna et al., 2007a; Labruna et al., 2007b)
R. conorii conorii	Mediterranean spotted fever	*Rhipicephalus sanguineus*	Europe, Africa, Asia	Human, dog	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. conorii israelensis	Israeli spotted fever	*Rhipicephalus sanguineus*	Israel	Human	(Mutai et al., 2013; Parola et al., 2005a; Parola et al., 2005b)
R. conorii caspia	Astrakhan fever	*Rhipicephalus sanguineus, R. pumilio*	Africa, Asia	Human	(Parola et al., 2005a; Parola et al., 2005b)
R. conorii indica	Indian tick typhus	*Rhipicephalus sanguineus*	India	Human	(Parola & Raoult, 2006)
R. sibirica sibirica	Siberian or North Asian tick typhus	*Dermacentor nuttalli, D. marginatus, D. silvarum, D. sinicus, Haemaphysalis concinna*	Asia	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. sibirica mongolotimonae	Unnamed	*Hyalomma asiaticum, H. truncatum, H. anatolicum excavatum*	Africa, China, France	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. australis	Queensland tick typhus	*Ixodes holocyclus, I. tasmani*	Australia	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. japonica	Oriental or Japanese spotted fever	*Ixodes ovatus, Dermacentor taiwanensis, Haemaphysalis longicornis, H. flava*	Japan	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. africae	African tick-bite fever	*Amblyomma hebraeum, A. variegatum*	Africa, Reunion Island, West Indies	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. honei	Flinders island spotted fever	*Bothriocroton hydrosauri, Amblyomma cajennense, Ixodes granulatus*	Australia, USA, Thailand	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)

R. slovaca	TIBOLA, DEBONEL	*Dermacentor marginatus, D. reticulatus*	Europe, Asia	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. helvetica	Pathogenicitysuspected in humans	*Ixodes ricinus*	Europe	Human	(Fournier et al., 2004)
R. heilongjiangensis	Unnamed	*Dermacentor silvarum*	China	Human	(Parola & Raoult, 2006)
R. aeschlimannii	Unnamed	*Hyalomma marginatum marginatum, H. m. rufipes, Rhipicephalus appendiculatus*	Europe, Africa	Human	(Parola et al., 2005a; Parola et al., 2005b; Parola & Raoult, 2001)
R. parkeri	Unnamed	*Amblyomma. maculatum, A. triste, A. dubitatum*	USA, Mexico, Uruguay, Brazil	Human	(Delgado-de la Mora et al., 2019; Parola et al., 2005b; Parola & Raoult, 2006; Silveira et al., 2007)
R. massiliae	Unnamed	*Rhipicephalus sanguineus, R. turanicus, R. muhsamae, R. lunulatus, R. sulcatus*	Europe, Asia, Argentina, USA	Human	(Cicuttin et al., 2004; Eremeeva Marina et al., 2006; Parola et al., 2005b; Vitale et al., 2006)
R. marmionii	Australian spotted fever	*Haemaphysalis novaeguineae, Ixodes holocyclus*	Australia	Human	(Parola et al., 2005b; Parola & Raoult, 2006)
R. monacensis	Unnamed	*Ixodes ricinus*	Europe, Africa	Human	(Jado et al., 2007; Lauzi et al., 2016; Parola et al., 2005b)
		Genus *Ehrlichia*			
E. chaffeensis	Human monocytic ehrlichiosis	*Amblyomma americanum, Dermacentor variabilis*	USA	Human and various mammals	(Heitman et al., 2016; Parola et al., 2005a; Shaw et al., 2001)
E. ewingii	Canine granulocytic ehrlichiosis, Human ehrlichiosis	*Amblyomma americanum*	USA	Human, dogs	(Heitman et al., 2016; Parola & Raoult, 2001; Shaw et al., 2001)
E. ruminantium	Heartwater, Cowdriosis	*Amblyomma hebraeum, A. astrion, A. cohaerens, A. gemma, A.*	Africa, Caribbean	Mainly cattle	(Jongejan & Uilenberg, 2004; Walker & Olwage, 1987)

		marmoreum, A. lepidum, A. pomposum, A. variegatum, A. americanum			
E. canis	Canine ehrlichiosis	*Rhipicephalus sanguineus*	Southern USA, southern Europe, Mexico, Africa, Middle East, eastern Asia, Australia	Dogs	(Bezerra-Santos et al., 2021; Neave et al., 2022; Ojeda-Chi et al., 2019b; Shaw et al., 2001)
Genera *Anaplasma, Francisella* and *Coxiella*					
Anaplasma phagocytophilum	Human granulocytic anaplasmosis	*Ixodes scapularis, I. pacificus, I. ricinus, I. hexagonus*	USA, Europe	Human and various mammals	(Ebani, 2019; Estrada-Peña & Jongejan, 1999; Parola et al., 2005a)
A. marginale	Bovine Anaplasmosis	Various	Worldwide	Cattle	(Ashford, 2001; Vieira et al., 2019)
A. centrale	Bovine Anaplasmosis	Various	Worldwide	Cattle	(Ashford, 2001; Cabezas-Cruz et al., 2019; Livanova et al., 2018)
A. ovis	Ovine Anaplasmosis	Various	Worldwide	Sheep	(Ashford, 2001; Cabezas-Cruz et al., 2019)
A. platys	Canine ehrlichiosis	*Rhipicephalus sanguineus*	Worldwide	Dog	(Aguirre et al., 2006; Aragón-López et al., 2021; Lara et al., 2020)
F. tularensis	Tularemia	Various	Eurasia, Nearctic	Human and various mammals	(Parola & Raoult, 2001)
C. burnetii	Q. fever	Various	Worldwide	Human and various mammals	(Körner et al., 2021; Parola & Raoult, 2001)
Genus *Borrelia*					
B. burgdorferi	Lyme disease	*Ixodes pacificus, I. persulcatus, I. ricinus, I. scapularis*	USA, Mexico, Canada, Europe, Asia, northern Africa	Human	(Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001; Solís-Hernández et al., 2018)
B. garinii	Lyme disease	*Ixodes persulcatus, I. ricinus*	Europe, Asia, northern Africa	Human	(Estrada-Peña & Jongejan, 1999)

B. afzelii	Lyme disease	*Ixodes persulcatus, I. ricinus*	Europe, Asia, northern Africa	Human	(Estrada-Peña & Jongejan, 1999)
B. valaisiana	Lyme disease	*Ixodes ricinus*	Europe, Asia	Human	(Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. lusitaniae	-	*Ixodes ricinus*	Europe	-	(Estrada-Peña & Jongejan, 1999)
B. spielmani	Lyme disease	*Ixodes ricinus*	Europe	-	(Richter et al., 2004)
B. japonica	Lyme disease	*Ixodes ovatus*	Japan	Human	(Estrada-Peña & Jongejan, 1999)
B. lonestari	-	*Amblyomma americanum*	USA	Human	(Varela Andrea et al., 2004)
B. theileri	Bovine borreliosis	*Boophilus* spp., *Rhipicephulus evertsi*	Africa, Central and South America, Australia	Cattle	(Barbour & Hayes, 1986; Brinkmann et al., 2019)
B. turcica	-	*Hyalomma aegyptium*	Parts of Turkey	-	(Güner et al., 2004)
B. miyamotoi	-	*Ixodes persulcatus*	Asia	-	(FUKUNAGA et al., 1995)
B. hermsii	New World tick borne relapsing fever	*Ornithodoros hermsi*	USA, Canada	-	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. turicatae	New World tick borne relapsing fever	*Ornithodoros turicata*	USA, Mexico	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Krishnavajhala et al., 2018; Parola & Raoult, 2001)
B. parkeri	New World tick borne relapsing fever	*Ornithodoros parkeri*	USA, Mexico	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. mazzottii	New World tick borne relapsing fever	*Ornithodoros talaje*	USA, Mexico	-	(Barbour & Hayes, 1986; Parola et al., 1999)
B. venezuelensis	New World tick borne relapsing fever	*Ornithodoros rudis*	Central and South America	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)

B. duttonii	Old World tick borne relapsing fever	*Ornithodoros moubata*	Africa	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. crocidurae	Old World tick borne relapsing fever	*Ornithodoros erraticus*	Europe, Africa	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. persica	Old World tick borne relapsing fever	*Ornithodoros tholozani*	Asia	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. hispanica	Old World tick borne relapsing fever	*Ornithodoros erraticus*	Spain, Portugal	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. latyschevii	Old World tick borne relapsing fever	*Ornithodoros tartakovskyi*	Iran, Central Asia	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. caucasica	Old World tick borne relapsing fever	*Ornithodoros aspersus*	Asia (Caucasus and Iraq)	Human	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999; Parola & Raoult, 2001)
B. graingeri	-	*Ornithodoros graingeri*	Africa	Human	(Parola & Raoult, 2001)
B. anserina	Avian borreliosis	*Argas* spp.	Worldwide	Birds	(Barbour & Hayes, 1986)
B. tillae	-	*Ornihodoros zumpti*	-	Africa	(Rebaudet & Parola, 2006)
B. coraciae	Bovine epizootic abortion	*Ornithodoros coriaceus*	USA	Cattle	(Barbour & Hayes, 1986; Estrada-Peña & Jongejan, 1999)
B. parkeri	-	*Ornithodoros parkeri*	USA	Human	(Rebaudet & Parola, 2006)
Genus *Dermatophilus*					
D. congolensis	Dermatophilosis	*Amblyomma variegatum*	Africa	Ruminants	(Jongejan & Uilenberg, 2004)

4.15 Vertebrate animals in the study

4.15.1 Species description (*Canis lupus familiars*)

The separation of domestic dogs occurred approximately 100,000 years ago, and the appearance of the different breeds that exist today began around 15,000 years ago (Table 3). This change occurred gradually due mainly to the change from nomadic to sedentary life by human beings (Vilà et al., 1997). The dogs have been domesticated and selected according to their physical, sensory, and behavioral capabilities that give them certain advantages over the rest of the dogs, such as pack dogs (Saint Bernard, Alaskan Malamutes, and huskies), hunters (fox terriers, jack rusell terrier), guarding (chow chow, mastiffs), herding (collies, shepherds), fishermen's helpers (newfoundland, poodles), guarding carriages and riders (dalmatians) and companion dogs (pugs, york shire). The morphology of dogs is very diverse, however all have a common similarity with their wild ancestor the gray wolf (canis lupus) (Database, 2022). Due to its closeness, loyalty and friendship with humans, it is considered the ideal pet species for humans (Otranto et al., 2014). Furthermore, as good companions to humans, dogs share many things with humans, including zoonotic endoparasites and ectoparasites (Dantas-Torres & Otranto, 2014; Otranto et al., 2013).

Table 3. Taxonomic classification of the dog (*Canis lupus familiaris)*.

Taxonomic classification	
Kingdom	Animalia
Phylum	Craniata
Class	Mammalia
Order	Carnívora
Family	Canidae
Genus	*Canis*
Species	*Lupus*
Scientific name: *Canis lupus familiaris* Linnaeus, 1758	

Canis lupus familiaris has been used in different socioeconomic and protection activities, which is why it is highly variable in shape and size (Database, 2022). In general, they are characterized by having a relatively tall body (36 cm to 1.45 m

and 1 to 79 kg), long legs, and a cylindrical, furry tail. It is a sociable animal with a well-established dominance hierarchy. It can reproduce up to twice a year, having a highly variable number of offspring, from 3 to 10 or more. It feeds on all kinds of man's organic waste, but it can be a good hunter of different species of animals (Álvarez-Romero & Medellín, 2005). *Canis lupus familiaris* is strongly associated with human populations, maintaining a commensal-type relationship. Due to the above, the distribution in the country can be seen reflected in the very distribution of the population cores. Feral populations have been identified on islands, such as: Cedros, Guadalupe, María Cleofas, María Magdalena, among others (Álvarez-Romero & Medellín, 2005)

4.15.2 Species description (*Gopherus flavomarginatus*)

In 1959, Legler described a new species of tortoise in the genus *Gopherus* and named it *flavomarginatus*. The word "*Gopherus*" means "excavator" and the name "*flavomarginatus"* is derived from the Latin word flavus, "yellow" and marginatus "fringed", referring to the yellow edges of the carapace scutes (Legler, 1959). This species belongs to the Testudinidae family and is the largest of the five species (*G. agassizii, G. berlandieri, G. polyphemus* and *G. morafkai*) that make up this genus. Its complete and current taxonomic classification is presented in Table 4.

Table 4. Taxonomic classification of the Bolson tortoise (*Gopherus flavomarginatus*)

Taxonomic classification	
Kingdom	Animalia
Phylum	Craniata
Class	Reptilia (Laurenti, 1768)
Order	Testudines (Batsch, 1788)
Family	Testudinidae (Batsch, 1788)
Genus	*Gopherus* (Rafinesque, 1832)
Species	*flavomarginatus*

Scientific name: *Gopherus flavomarginatus* (Legler, 1959)

4.16 Endosymbiont bacteria in ticks

Before 1990, all bacteria present in ticks were considered pathogenic, without really evaluating pathogenicity. However, today we know that there is a well-

structured microbial community in ticks that does not represent a risk to hosts (Cafiso et al., 2019; Garcia Guizzo et al., 2022; Papa et al., 2017; Vila et al., 2019). The capacity of these arthropods to host microorganisms is considerable, it has been shown that the bacterial diversity in these organisms is very high and varies with some intrinsic elements in the biology of the individual, as well as extrinsic ones due to geoclimatic issues (Lalzar et al., 2012). The diversity of these communities can fluctuate depending on various factors, environmental factors are key to the diversification of microbial communities, the structure of the microbiota also depends on the species of ticks found, the season in which they were collected, the geographical region where the study was conducted (Carpi et al., 2011; Lalzar et al., 2012; Williams-Newkirk et al., 2014), the biological stage (Clay et al., 2008; Moreno et al., 2006; Williams-Newkirk et al., 2014; Zolnik et al., 2016) and also the types of food that they carry out (Heise et al., 2010; Menchaca et al., 2013; Zhang et al., 2014), and on the other hand, it has been verified that the dynamics and microbial composition is determined by the pathogenic organisms that the tick harbors (Abraham et al., 2017; Bonnet & Pollet, 2021; Steiner et al., 2008).

Ticks, in addition to potentially pathogenic organisms, harbor a wide variety of endosymbiont microorganisms. For obvious reasons, these organisms have been underestimated in comparison to potentially pathogenic ones, and their biology, functions, and effects on their hosts are not clear (Bonnet et al., 2017). However, these organisms have a very important role in the survival of ticks, some roles they play are beneficial, neutral and in some cases harmful to their hosts. These are from metabolic, nutritional, reproductive, resistance to stressors and external factors and immunity. In addition to the above, they have a key role in the transmission of TBPs, which are of considerable importance in human and veterinary medicine (Bonnet et al., 2017; Bonnet & Pollet, 2021).

In general, there is an intracellular bacteria-arthropod association and these in turn undergo vertical transmission or maternal (transovarial) transmission (Moran et al., 2008; Wernegreen, 2012). In fact, these vertically transmitted obligate

endosymbionts are key to biological processes of the invertebrate host. these crucial characteristics are the biosynthesis or metabolic routes of which arthropods lack, examples of these may be the metabolization of the sap of some plants or the blood of vertebrates that parasitize in the case of hematophagous (Moran et al., 2008; Wernegreen, 2012). However, in most cases they are facultative endosymbionts that facilitate adaptation to environmental changes, protection against potential enemies, nutrition and reproduction (Oliver et al., 2010).

Obligate endosymbionts have coevolved with their hosts, their mutualistic relationships dating back millions of years (Moran et al., 2008). Normally, these obligate endosymbionts are found in a certain host species, as in the case of *Midichloria mitochondrii*, which is very specific to ticks (Smith et al., 2015a; Stefanini & Duron, 2012).

One of the extraordinary symbiotic relationships of arthropods is what occurs with ticks, since the tick, due to its blood-based diet, requires biological symbiotic interactions with its microbiota (Bonnet et al., 2017). At least 10 genera of endosymbionts harboring ticks have been recorded, all of which are maternally inherited obligate endosymbionts to ticks (Duron et al., 2017). Of all these three are exclusive to ticks, *Midichloria*, *Coxiella*-LE and *Francisella*-LE (Bonnet et al., 2017; Duron et al., 2017).

Bacterial composition analysis has been carried out on tick eggs of two different species, verifying that there is >99% of *Coxiella*-LE in the samples analyzed and confirming the efficiency of vertical transmission of this endosymbiont (Duron et al., 2015b). This is mainly due to the tropism that this endosymbiont has for both the ovaries and the Malpighian tubules. On the other hand, studies revealed the metabolic routes developed by this endosymbiont for the synthesis of amino acids and vitamins; it is the main microorganism that encodes genes to synthesize B complex vitamins and their cofactors, which cannot be obtained independently by ticks (Gottlieb et al., 2015; Smith et al., 2015b). It has been shown that in a study where the presence of *Coxiella*-LE was analyzed in 81 different species of ticks

that in the absence of this genus this endosymbiont was replaced with another one also inherited maternally. Among these are *Francisella*-LE and *Rickettsia* (Duron et al., 2017). In birds, for example, the presence of *Francisella*-LE in the ectoparasite *Argas persicus* is vital for the genetic pathways of folic acid, biotin and riboflavin biosynthesis (Gerhart et al., 2016b; Sjödin et al., 2012).

The rickettsiales are other endosymbionts that have the genetic faculty for the synthesis of folic acid, in the species *I. scapularis* and in the *I. pacificus* ticks (Hunter et al., 2015). The survival of different species such as *A. americanum, Dermacentor variabilis* and *I. scapularis* of ticks is closely related to the synthesis routes of essential elements for ticks and this has been observed particularly with rickettsiales in the survival and mortality of larvae (Kagemann & Clay, 2013). In summary, these obligate endosymbionts directly and indirectly affect the ecology and evolution of their invertebrate host and, above all, allow the adaptation of ticks to hematophagy.

Ticks also harbor facultative symbionts belonging to a wide range of genera. A study in 81 tick species demonstrated a considerable list of bacterial facultative symbiont organisms (Duron et al., 2017). Five endosymbionts (*Wolbachia, Cardinium, Arsenophonus, Spiroplasma* and *Rickettsia*) are common in some groups of arthropods and are known to manipulate insect reproduction through induction of parthenogenesis, feminization, male killing, and cytoplasmic incompatibility.

The role of the facultative symbionts has not been fully elucidated. Despite this, it is known, for example, that the genus inhibits the ability to questing for hosts in different genera of ticks, increasing their mortality (Kagemann & Clay, 2013). *Midichloria mitochondrii*, on the other hand, has been linked as an additional source of ATP for tick cells during oogenesis, making it a key player in reproduction (Sassera et al., 2011). In addition, it is known that it can contribute to the molting process of the tick (Zchori-Fein & Bourtzis, 2011). Table 5 below shows the main endosymbiont bacterial taxa associated with ticks.

Table 5. Bacterial genera of maternally inherited bacteria, modified from Duron et al. (2017).

Maternally inherited bacteria	Distribution in arthropods	Main properties
	Gamma-proteobacteria	
Coxiella-LE	A symbiont endemic to ticks and very common (Duron et al., 2014; Duron et al., 2015a; Lalzar et al., 2012; Machado-Ferreira et al., 2011)	Obligate symbiont on most ticks (Gottlieb et al., 2015; Zhong et al., 2007). Very close to the pathogen that causes Q fever, *C. burnetii* (Duron et al., 2015a)
Rickettsiella	Widespread distribution in arthropods (Kurtti et al., 2002; Tsuchida et al., 2014), common in ticks (Anstead Clare & Chilton Neil, 2014; Duron et al., 2016; Duron et al., 2015a; Kurtti et al., 2002)	Unknown effects on ticks (Tsuchida et al., 2014; Tsuchida et al., 2010), Some strains are entomopathogenic (Cordaux et al., 2007; Leclerque et al., 2011)
Arsenophonus	Common in arthropods (Duron et al., 2008a; Nováková et al., 2009), present in ticks (Clay et al., 2008; Clayton et al., 2015; Dergousoff & Chilton, 2010)	Facultative symbiont in some arthropods (Jousselin et al., 2013; Nováková et al., 2009)
Francisella-LE	Rare in ticks and not found in other arthropods (Clayton et al., 2015; Niebylski et al., 1997; Scoles, 2004)	Effects unknown, facultative symbiont in at least one tick species (Gerhart et al., 2016a) closely related to the agent of tularaemia *(F. tularensis)* (Sjödin et al., 2012)
	Alpha-proteobacteria	
Wolbachia	Very common in arthropods (Duron et al., 2008a; Hilgenboecker et al., 2008; Zug & Hammerstein, 2012), present in ticks (Andreotti et al., 2011; Carpi et al., 2011)	Unknown effect on ticks (Engelstädter & Hurst, 2009a), At least in the sheep tick, *Ixodes ricinus,* it is related that the presence of *Wolbachia* is due to contamination by a parasite, the hyminoptera (Plantard et al., 2012)
Rickettsia	Common in arthropods (Perlman et al., 2006; Weinert et al., 2009), present in ticks (Clayton et	Reproductive manipulator in many species, unknown effects on ticks (Engelstädter & Hurst, 2009a) closely

	al., 2015; Kurtti et al., 2015)	related to tick-borne pathogens (Kurtti et al., 2015; Perlman et al., 2006; Weinert et al., 2009)
Midichloria	Present in ticks, not found in other arthropods (Dergousoff & Chilton, 2010; Epis et al., 2008; Najm et al., 2012; Qiu et al., 2014; Venzal et al., 2007)	Provides energy to ticks (Epis et al., 2013)
Lariskella	Rare in arthropods (Matsuura et al., 2012; Toju et al., 2013), reported once in ticks (Toju et al., 2013)	Unknown effects
Spiroplasma	Mollicutes Common in arthropods (Duron et al., 2008a; Weinert et al., 2007), presente en garrapatas (Henning et al., 2006; Tully et al., 1995)	Unknown effects on ticks (Engelstädter & Hurst, 2009b)
Cardinium	Bacteroidetes Common in arthropods (Duron et al., 2008a; Duron et al., 2008b), Presente en garrapatas (Benson Micah et al., 2004; Kurtti et al., 1996)	Unknown effects on ticks Reproductive manipulator in some species (Engelstädter & Hurst, 2009b)

4.17 Bacterial characterization of ticks according to the vertebrate host

4.17.1 Dog ticks, their microbiota and main diseases transmitted by their ticks

The hard tick *Riphicephalus sanguineus*, commonly known as the brown tick, is a common tick on dogs, followed by those of the genus *Otobius*. Infestations of this vector have been reported in the southern United States and Mexico at unprecedented rates, perhaps due to the accelerated growth rate of dogs in these countries (Ojeda-Chi et al., 2019; Ortega-Morales et al., 2019; Tinoco-Gracia et al., 2018), the brown dog tick is the one most associated with the transmission of

(RMSF) and in the United States the *Dermacentor* spp. Because the brown tick is cosmopolitan and prefers to feed on dogs, it remains in peri-urban areas. The presence of the pathogens *R. rickettsii, R. rhipicephali*, and *R. rhipicephali* have been detected within this vector (Castillo-Martínez et al., 2015).

One of the most lethal diseases in the Americas is Rocky Mountain spotted fever, caused by *Rickettsia rickettsii.* Recent studies in Mexico have detected *R. rickettsii* in different states of Mexico, especially in states with an arid and semi-arid climate, such as Mexicali, which in 2015 the Mexican Ministry of Health declared a state of epidemiological emergency in different states from northern Mexico (Alvarez-Hernandez et al., 2017), and as of 2018 the (RMSF) has already affected more than 4000 people, and not only in Mexico, in the United States the incidence of this disease has increased four times (Tinoco-Gracia et al., 2018), in 2017, 6,247 cases were reported in the US (Prevention., 2019). Another particular study was carried out in Coahuila in the municipalities of Torreón, San Pedro, Viesca, Fco. I. Madero and Matamoros. They found that, in the municipality of Torreón, Viesca and Francisco I. Madero, the ticks were infected with *R. rickettsii,* the cause of RMSF. They also found another species of the genus *Rickettsia*; *R. rhipicephali*, which had never been found in animals co-infected with *R. rickettsii*. It should be noted that it had not been previously reported in Mexico (Castillo-Martínez et al., 2017). This confirms the prevalence of the pathogen in southern Coahuila and confirms the role of the dog in the spread of the pathogen. Other studies also within the la Comarca Lagunera region, in the cities of Torréon, Gómez Palacio and Lerdo, have found, by molecular methods, the pathogens of dogs, *Anaplasma platys* and *Erlichia canis* (Almazán et al., 2016), which causes the disease Granulocytic Anaplasmosis and Monocytic Ehrlichiosis of the dog and which is also zoonotic and affects humans (Breitschwerdt et al., 2014; Maggi et al., 2013).

4.17.2 Reptile ticks and their microbiota

Some bacteria like *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *B. bissettii, B. turcica, Ehrlichia canis, Coxiella burnetii, Rickettsia* spp., *Pasteurella* spp. and *Francisella tularensis* have been reported in the blood of reptiles, being vectorized by Acari (*Ixodes* spp., *Amblyomma* spp., *Hyalomma* spp. and *Ornithodoros* spp.) are the ticks causing these transmissions (Andoh et al., 2015; Barraza-Guerrero et al., 2020; Jacobson, 2007; Jacobson et al., 2012; Jimenez Castro et al., 2019; Kalmár et al., 2015; Omondi et al., 2017; Široký et al., 2014; Zemtsova et al., 2012).

4.18 Studies of bacteria in blood and the human blood microbiome

The human and animal body possesses a great abundance of bacterial, fungal and viral microorganisms. Regardless of the relationship it establishes with its host (commensal, symbiotic or pathogen). Most of the higher living organisms and their homeostasis are related to the microbial composition they host. In fact, 2% of the weight of the human body (1.5 kg) represents the weight of bacterial cells, approximately the weight of the brain or liver, and this despite the fact that bacteria are 1000 times smaller than the somatic cells of the human body (Molina et al., 2012). Due to our long history of coevolution with microorganisms (Moeller et al., 2016), It is somewhat logical that bacterial genes outnumber us by a factor of 150, 3,300,000 compared to 22,000 genes from our own cells (Qin et al., 2010).

Thanks to the introduction of cutting-edge molecular technologies such as "NGS" as well as whole-genome shotgun sequencing, we are able to study so-called "microbial selves" and identify alterations to the nascent known healthy human microbiome (Segata et al., 2013). One of the goals of many scientists is to establish a microbiota taxonomic base of microorganisms in different compartments and tissues in humans. However, it is even more important to establish the functionality of bacterial taxa (functional microbiota) based on metabolic pathways and thus identify dysbiosis in the human microbiota in different parts of the body (Païssé et al., 2016; Turnbaugh et al., 2009).

The concept of healthy HBM has received criticism because the idea that blood is a sterile medium free of any foreign cells (i.e., bacteria) is well established (Païssé et al., 2016). Nevertheless, there are studies that demonstrate the existence of a blood microbiome in animals (Mandal et al., 2016). In the context of the human being, more and more studies analyze the presence of "foreign" microorganisms in human blood and this does not necessarily equate to an infection or a disease state (Castillo et al., 2019).

Since 1960 there has been controversy about the presence of foreign cells in normal blood. Tedeschi et al. (1969) detected an increase in the absorption of nucleosides and amino acids in the blood of apparently healthy people, which led them to the hypothesis that metabolically active bacteria similar to mycoplasmas were found in the blood. Domingue et al. (1977) tested blood from a healthy human population and showed that 7% contained bacterial growth even after osmotic lysis and filtration.

Nikkari et al. (2001) They detected blood bacterial microbiota in apparently healthy people by means of qPCR and the amplification of the 16S rRNA gene from different phylogenetic groups. Moriyama et al. (2008) In the same way, he also found the presence of bacterial DNA in blood samples from healthy people.

4.19 NGS for the study of microbiota in ticks

The microbiota or microbiome can be defined as a group of organisms that interact with their host as commensals, symbiotics, or pathogens (Ursell et al., 2012). The study of microbial communities was practically based on microscopy and cultures. Microbiology, despite being an inexpensive, fast, and relatively simple technique, lacks sensitivity and objectivity (Houpikian & Raoult, 2002). On the other hand, bacterial cultures are widely used for microbial identification and antibiotic susceptibility studies (Kotsilkov et al., 2015), Possessing low sensitivity, it has been estimated that only 2% of environmental microorganisms can be cultured in the laboratory (Wade, 2002).

Modern molecular techniques have been able to characterize the vast microbial community that ticks harbor (viruses, bacteria and eukaryotes) (Greay et al., 2018). The growth of molecular technologies such as next-generation sequencing (NGS) has been able to study these microbial communities in a more efficient and cost-effective manner. NGS undoubtedly has many advantages over traditional non-molecular techniques that have limited sensitivity and even over conventional molecular techniques such as PCR or Sanger sequencing that have limited scalability and sensitivity (Greay et al., 2018). Studies of the microbial composition of ticks through NGS have increased significantly in recent years, mainly due to the ability to identify several species simultaneously in a single analysis and even to identify rare or difficult taxa (Simner et al., 2018). The study of the microbiota in vectors has been significantly developed by the use of the highly conserved 16S rRNA gene and has allowed the detection of bacterial species under different environmental, biological, ecological and experimental conditions (Couper & Swei, 2018). The technique is based on amplifying this barcode from a sample through PCR, loading the samples into a flow cell for sequencing and finally the analysis of bioinformatics data to match the data obtained with the phylogenetic information (Couper & Swei, 2018). The availability of a conserved marker gene and databases has allowed very precise bacterial profiles to be obtained, especially at the genus level and sometimes at the species level. This methodology allows the detection of microorganisms of a great variety of phylogenies and taxa regardless of the type of microorganism (viruses, bacteria, fungi and parasites) and even the discovery of new organisms, which allows replacing the use of many specific tests with a single test based in NGS assay (Simner et al., 2018).

4.20 References

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5. GENERAL MATERIALS AND METHODS

5.1 Study areas

The Lagunera region is located in the North Central part of the United Mexican States, within the biogeographic region known as the Chihuahuan Desert, at the confluence of the states of Coahuila and Durango at the coordinates (103° 13' 42" west, 25° 31 ' 41" north, at an altitude of 1,100 m). It is made up of 15 municipalities, 10 of them from the state of Durango and 5 from the state of Coahuila (Figure 3). The climate is arid, the average temperature is 19 °C with an annual average rainfall of 250 mm, which occurs mainly from May to July (Escareño Sánchez et al., 2011).

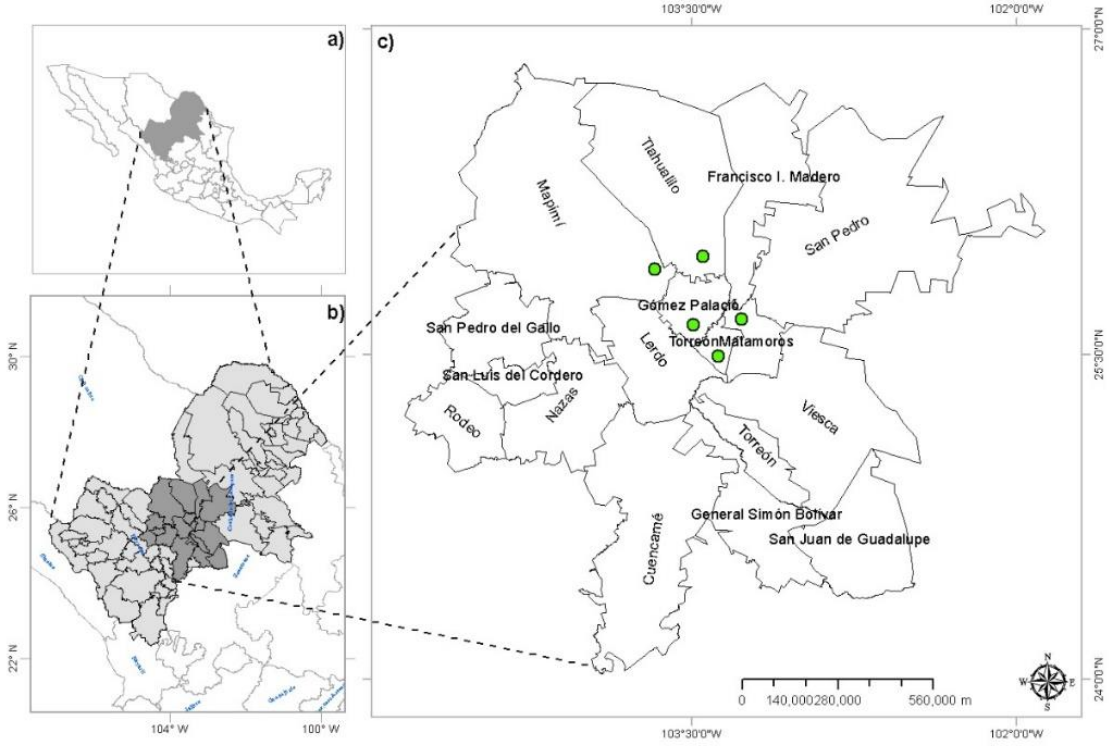

Figure 3. Location map of the study area, within the metropolitan area of the Comarca Lagunera, the green dots indicate the municipalities where the sampling was carried out.

The Mapimí Biosphere Reserve has an approximate area of 342,388 ha and is located within the region known as Bolsón de Mapimí (Figure 4). It comprises part of the municipalities of Tlahualilo and Mapimí in the State of Durango, Jiménez in

Chihuahua and Sierra Mojada in Coahuila (26° 00' and 26° 10' N and 104° 10' and 103° 20' W; Table 4) at an average altitude above sea level of between 1,000 and 1,200 m in the lower parts and 2,000 m on the tops of the highest hills (CONANP, 2006). The average annual temperature is 25.5°C (García & Martínez, 2004). According to the Köppen classification, adapted for Mexico by García (2004), the climate of the area corresponds to the BWhw(e) type, very arid, semi-warm, with summer rains and extreme thermal amplitude, with an average historical precipitation of 145.9 mm. (1993-2003). The predominant vegetation is rosetophilous and microphyllous scrub, as well as halophytic and gypsophilous vegetation (Rzedowski, 2006). The predominant soils are of the yermosol, regosol, xerosol, litosol, solonchack and fluvisol type (García & Martínez, 2004).

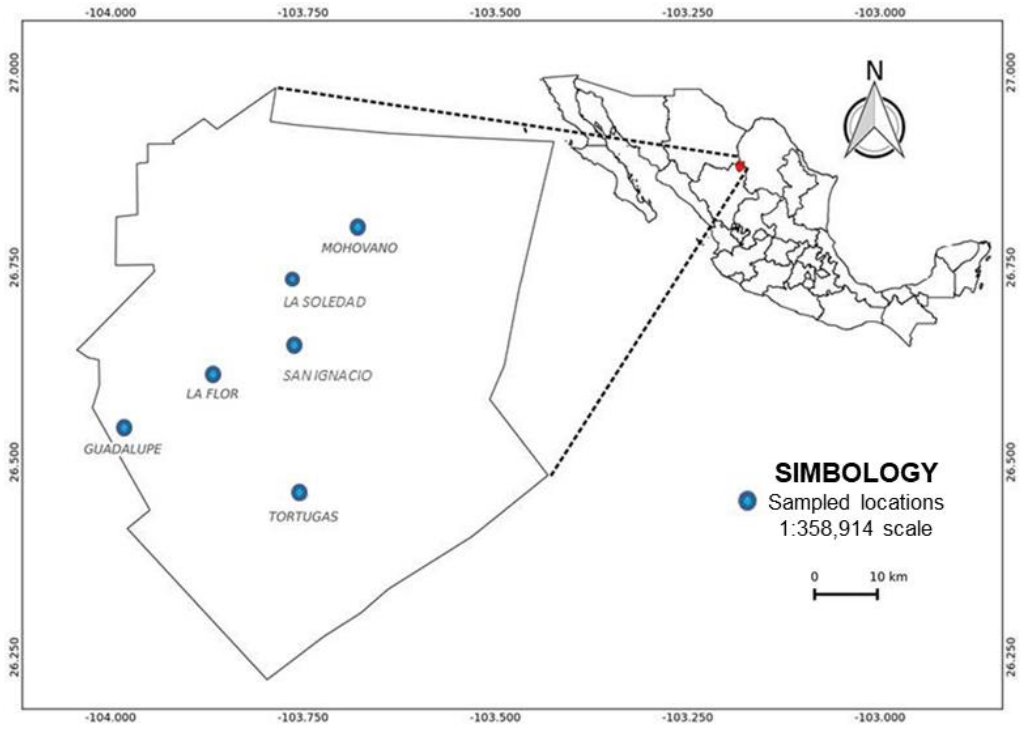

Figure 4. Location of the study area (Mapimí Biosphere Reserve)

5.2 Dog sampling sites

the study was carried out with individuals who live in the urbanized areas of the Comarca Lagunera (Torreón, Matamoros, Gómez Palacio; as well as areas surrounding the Biosphere Reserve (Bermejillo and Tlahualilo). These are areas specially selected due to the prevalence of TBD and the high abundance of stray dogs, the dogs with owners and without owners were included. The type of sampling was random.

5.3 Tortoise sampling sites

The tortoises that were part of this study were captured in the towns of La Flor, Guadalupe, San Ignacio and Mohovano that are located within the Mapimi Biosphere Reserve. Transects of 1 km in length were carried out from 0900 to 1300 h and from 1700 to 2100 h to capture individuals.

5.4 People Sampling Site

Blood samples from people who were exposed to the vectors, with previous symptoms, both in the Comarca Lagunera and in the Mapimí Biosphere Reserve. Samples were taken with their prior consent. In the case of people not exposed to tick bites, people volunteered to donate blood samples for the purposes of the study in the same communities described.

5.5 Tick collection

The samplings were carried out in the summer seasons; for ticks (*O. turicata*) it was carried out from May to July 2017, with respect to ticks (*R. sanguineus*) they were collected from domestic and peridomestic dogs during September 2019. A total of 17 ticks (O. turicata) were collected from 11 tortoises. Regarding the dogs, around 500 adult female and male ticks were collected from 50 domestic and peridomestic dogs. All the samples were deposited in 70% ethanol and transferred to the Laboratorio de Medicina de la Conservación of the Facultad de

Ciencias Biológicas, Universidad Juárez del Estado de Durango, where the taxonomic identification of each individual was carried out morphologically by means of standard taxonomic keys (Morafka, 1977; Walker et al., 2005).

5.6 Creation of tick pools

5.6.1 Soft Tick Pools

Pools of each type of tick species collected in this study were made for the extraction of bacterial DNA. A total of 17 adult ticks were collected and taxonomic keys were used to determine the species, and all the specimens were identified as *O. turicata* (Morafka, 1977). Each tick was placed in an individual 1.5 mL tube containing 500 µL ethanol (70%), 500 µL hydrogen peroxide (H_2O_2), and 200 µL of ultrapurified H_2O; each tube was vortexed for 15 s to remove tick surface contaminants (Couper & Swei, 2018). Later, three pools were formed (five ticks each) and were deposited in BashingBead™. Zymo Research cell lysis tubes containing 750 µL of lysing/stabilizing solution. The tubes were processed in a cellular disruptor (TerraLyzer.) for 20 s. The two extra ticks were deposited in the Entomological collection of the Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, México, for reference purposes.

5.6.2 Hard Tick Pools

Around 500 adult female and male ticks were collected from 50 domestic and peridomestic dogs and preserved in 70% ethanol. All individuals were identified as *R. sanguineus* and were separated by sex (Walker et al., 2005). Ticks were dissected with sterilized microdissection scissors (BioQuip® No. 4715), making a cut in the posterior part of the abdomen; they were held with entomological forceps (BioQuip® No. 4522) from the base of the palps and with the help of a curved forceps (BioQuip® No. 4527) they were slid by lightly pressing the dorsal and ventral part of the specimens to the back of the specimen idiosoma. Five pools were made with the internal content of five female ticks and another five pools for males. Pools were deposited in BashingBeadTM lysis tubes with 750 µL

of Xpedition™ Zymo Reesearch™ lysing/stabilizer buffer. Each tube was placed in a cell disruptor (TerraLyzerTM) for 30 seconds for DNA preservation.

5.7 Collection of blood samples

The blood of 12 apparently healthy volunteers was taken in the months of August - October 2019. The sample was taken from people by venipuncture in the arm vein using the technique of (García, 2009). First of proceeded the antisepsis of the skin with 96% alcohol and deposited in Vacutainer K3EDTA tubes (Vacutainer K3E, BD, USA); three ml per person were extracted and ten drops of blood (50 mg in wet weight) of each collected sample were deposited in a BashingBead™ lysis tube of the DNA Microprep kit, from the Zymo Research™ brand; 750 µL of lysing/stabilizing solution (Xpedition™) were added. Each tube was processed in a cell disruptor (TerraLyzer ™) for 30 s (Barraza-Guerrero et al., 2021).

5.8 Extraction of DNA from ticks and blood

DNA from each pool of ticks and from blood samples were extracted with the Xpedition™ Biomics DNA MiniPrep Kit (Zymo Research™). These processes were carried out in a UV laminar flow hood with all the sterility protocols to avoid contamination of the samples.

The DNA extraction products was run on 1.2% agarose gels at 80V for 45 minutes in a Bio-Rad electrophoresis chamber to visualize the presence of high molecular weight DNA. The visualization was carried out in a GelMaxTM photo documenter (UVP®).

The amount of DNA obtained from the samples were measured in a Qubit® 3.0 brand fluorometer.

5.9 Massive next-generation sequencing of the 16s rRNA gene

The amplification of the V3 and V4 regions of the 16S rRNA gene of each pool of ticks and blood samples will be carried out. The primers suggested by Klindworth et al. (2013). These sequences are 5´-CCTACGGGNGGCWGCAG-3´ and 5´-

GACTACHVGGGTATCTAATCC-3´, which produces an amplicon of ~460 bp. By joining these sequences to the "overhang" adapters of the 16S protocol (Metagenomic Sequencing Library Preparation of Illumina (2021a)) they are as follows:

5´TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3´ y 5´GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3´ (~550 bp amplicon).

The 16S Metagenomic Sequencing Library Preparation (Illumina, 2021a) PCR protocol will be used using 12.5 µl of MyTaqTM Ready Mix 1X (Bioline®), 1 µl of each primer (10 nM), 5 µl of DNA (25 ng total) and 5.5 µl of H_2O Molecular Grade; the following cycle will be used: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes in a Labnet MultigeneTM Gradient PCR thermal cycler. 1 µl of some PCR products will be placed on an Agilent Bioanalyzer 2100 DNALabChip to verify amplicon size (~550 bp). Amplicons will be purified with 0.8% Agentcourt® AMPure® XP beads. Subsequently, the amplicons will be labeled using the Nextera XT Index KitTM for the creation of the libraries, following the Nextera® XT DNA Library Preparation Reference Guide protocol (Illumina, 2021b), using 25 µl of MyTaqTM Ready Mix 1X (Bioline®), 5 µl of each primer (N7xx and S5xx), 5 µl of DNA and 10 µl of molecular grade H2O; the following cycle will be used: 95°C for 3 minutes; 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for five minutes. Libraries will be purified with 1.2% Agencourt® AMPure® XP beads. 1 µl of the final library of a few randomly selected PCR products will be plated onto an Agilent Bioanalyzer 2100 DNALabChip to verify amplicon size expecting a size of ~630 bp. Finally, the final quantification will be carried out with QUBIT, the normalization (equimolarity) of the libraries will be done at 4nM, the grouping of the libraries taking 1.5 µl of each one and the next generation massive sequencing (MiSeq Illumina® of 2x250 paired final readings). following the protocol for 16S metagenomics (Illumina, 2021a).

5.10 Bioinformatic analysis to determine bacterial metagenomic profiles

Sequencing results were stored in the Illumina BaseSpace digital application in FASTQ format. Sequence analysis was performed in an Oracle VM VirtualBox 5.1.14 virtual machine on the MGLinux platform using Quantitative Insights Into Microbial Ecology (QIIME) v.1.9.0 bioinformatics software (Caporaso et al., 2010). The process began by assembling the forward and reverse sequences of the samples using the PEAR program (Zhang et al., 2014) with an overlap of 50 bp, a minimum length per read of 430 bp and a maximum of 470 bp, a Q20 quality criterion and a value of $P<0.001$. The files were then converted to FASTA format and chimeric sequences were eliminated (incomplete PCR products that can hybridize as oligonucleotides (primers) to heterologous sequences; (Acinas, 2007)) of samples with VSEARCH (Edgar, 2010). The selection of operational taxonomic units (OTUs) was carried out with the UCLUS method (Edgar, 2010), at 97% similarity; A representative sequence was obtained for each OTU and the taxonomy was assigned taking the EzBioCloud database as a reference (Yoon et al., 2017). The OTUs tables were built in biom format (Biological observation matrix; (McDonald et al., 2012) and the domains were separated.

The relative abundance of the taxonomic levels of phylum, class and order of each population was obtained and plotted in Excel or in R. The families and genera whose relative abundance was greater than 0.1% were represented in a heat map with the Morpheus software (https://software.broadinstitute.org/GENE-E/).

Tables of absolute abundance of OTUs at the genus level were obtained for each sample of tick pools and blood from people and the number of sequences by the number of OTUs for each of them were plotted in PAST see 3.15 (Hammer et al., 2001) to observe if an adequate coverage depth was achieved (asymptote trend curves). In case of the samples that did not reach a good coverage, a simple rarefaction process was carried out (Weiss et al., 2017) taking a minimum value of sequences from which the subsamples were generated. In this way, a standardized biom file was obtained for all samples. From the standardized biom file, alpha diversity was calculated with the non-phylogenetic indices of Simpson

and Shannon. Monte Carlo parametric tests with 999 permutations were applied to test for a significant difference in alpha diversity between male and female ticks and between people exposed and not exposed to tick bites. Likewise, beta diversity matrices were obtained using the Bray Curtis non-phylogenetic similarity and the UniFrac phylogenetic distance methods Unweighted (qualitative) and weighted (quantitative) (Lozupone & Knight, 2005).

Subsequently, PERMANOVA tests ($p < 0.05$) were applied to prove a significant difference in beta diversity. Samples were then visualized using Principal Coordinate Analysis (PCoA) in Emperor (Vazquez-Baeza et al., 2013). To establish the bacterial taxa at the phylum, family and genus level that contributed to the greatest extent to the differentiation of the microbiota, those groups that presented significant differences in alpha or beta diversity were selected, and a similarity analysis was carried out (SIMPER) with the Bray-Curtis beta matrix (Clarke y Warwick, 1994) in PAST. For those taxa whose contribution was greater than 2%, a Mann-Whitney U test ($p<0.05$) was applied to prove a significant difference between populations; these tests were performed in PAST. Finally, a LEfSe (i.e., linear discriminant analysis effect size) analysis was performed to statistically and biologically determine the key biomarkers which contribute the most to the differences between populations. The clades selected were those less than 0.05 in the alpha value of the Kruskal–Wallis factorial test > 4.0 in the logarithmic LDA score (Segata et al., 2011). This analysis was made on the website http://huttenhower.sph.harvard.edu/lefse/.

5.11 Potentially pathogenic and/or zoonotic bacterial genera recorded in ticks and blood of people

An exhaustive analysis of the current literature was carried out to identify potentially pathogenic and/or zoonotic bacterial genera recorded in ticks and human blood.

6. ARTICLES

6.1 General Microbiota of the Soft Tick *Ornithodoros turicata* Parasitizing the Bolson Tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico

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General Microbiota of the Soft Tick *Ornithodoros turicata* Parasitizing the Bolson Tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico

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Abstract: The general bacterial microbiota of the soft tick *Ornithodoros turicata* found on Bolson tortoises (*Gopherus flavomarginatus*) were analyzed using next generation sequencing. The main aims of the study were to establish the relative abundance of bacterial taxa in the tick, and to document the presence of potentially pathogenic species for this tortoise, other animals, and humans. The study was carried-out in the Mapimi Biosphere Reserve in the northern-arid part of Mexico. Bolson tortoises (n = 45) were inspected for the presence of soft ticks, from which 11 tortoises (24.4%) had ticks in low loads (1–3 ticks per individual). Tick pools (five adult ticks each) were analyzed through 16S rRNA V3–V4 region amplification in a MiSeq Illumina, using EzBioCloud as a taxonomical reference. The operational taxonomic units (OTUs) revealed 28 phyla, 84 classes, 165 orders, 342 families, 1013 genera, and 1326 species. The high number of taxa registered for *O. turicata* may be the result of the variety of hosts that this tick parasitizes as they live inside *G. flavomarginatus* burrows. While the most abundant phyla were Proteobacteria, Actinobacteria, and Firmicutes, the most abundant species were two endosymbionts of ticks (*Midichloria*-like and *Coxiella*-like). Two bacteria documented as pathogenic to *Gopherus* spp. were registered (*Mycoplasma* spp. and *Pasteurella testudinis*). The bovine and ovine tick-borne pathogens *A. marginale* and *A. ovis*, respectively, were recorded, as well as the zoonotic bacteria *A. phagocytophilum*, *Coxiella burnetii*, and *Neoehrlichia* sp. Tortoises parasitized with *O. turicata* did not show evident signs of disease, which could indicate a possible ecological role as a reservoir that has yet to be demonstrated. In fact, the defense mechanisms of this tortoise against the microorganisms transmitted by ticks during their feeding process are still unknown. Future studies on soft ticks should expand our knowledge about what components of the microbiota are notable across multiple host–microbe dynamics. Likewise, studies are required to better understand the host

competence of this tortoise, considered the largest terrestrial reptile in North America distributed throughout the Chihuahuan Desert since the late Pleistocene.

Keywords: microbiota; soft ticks; Bolson tortoises; bacterial profile; tick-borne pathogens

1. Introduction

Ticks are widely distributed in the world as obligate hematophagous ectoparasites of reptiles, birds, and mammals [1]. In recent years, traditional culture media practices have been replaced by high-throughput techniques such as the next generation sequencing, NGS, used to explore the complete bacterial microbiota of ticks [2]. It is known that tick microbiota is mainly composed of endosymbionts and tick-borne pathogens [3]. Endosymbionts play several beneficial roles to the host (fitness, development, reproduction, immunity, nutritional adaptation, and defense against environmental stress) [4], and tick-borne pathogens are of great concern to animal and human health around the world [3,5]. These two groups of microorganisms form a complex and dynamic ecosystem within ticks, coexisting and interacting with them in a commensal, mutualistic, or pathogenic way [3,6]. From a biological point of view, it is important to know the bacteria that compose of the tick microbiota, but in an epidemiological sense it is fundamental to develop information about the presence and abundance of tick-borne pathogens especially in countries where there is very scarce data.

Tick-borne bacteria have been largely studied in hard ticks (family Ixodidae) *Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp., *Hyalomma* spp., *Ixodes* spp., and *Rhipicephalus spp.*, where zoonotic bacteria *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Ehrlichia chaffensis*, *Francisella tularensis*, and *Rickettsia rickettsi* have been reported [7–10]. In soft ticks (family Argasidae), most studies have focused on the genus *Ornithodoros*, which comprises of more than 60% of the family [1,11]. These ticks usually inhabit sheltered sites (i.e., burrows, caves, cracks, and nests), commonly parasitizing small mammals, birds, reptiles, or bats [12]. Particularly, *O. turicata* is distributed through the arid regions of the Southern United States and Latin America [13]. It has been reported frequently as a promiscuous feeder parasitizing wild animals such as ground squirrels, prairie dogs, snakes, and desert tortoises [14]. In Mexico, this tick has been reported as an ectoparasite of the Bolson tortoise, *Gopherus flavomarginatus* [15], an endemic and endangered reptile species restricted to the Chihuahuan desert [16,17]. *Gopherus* tortoises are considered keystone species because they dig burrows where they spend the most part of its life, providing additional shelter for other animals such as rodents, lagomorphs, birds, and reptiles [18]. However, it is in their burrows where they most likely to acquire soft ticks. Due to the ecological relevance that *G. flavomarginatus* represents for the Chihuahuan desert ecosystem, it is important to generate information on the possible pathogens with which it may have contact, in this case those transmitted by soft ticks. Since there is no previous information regarding the bacterial microbiota of *O. turicata* in Mexico, we established the relative abundance of bacterial taxa in this tick. We hypothesized that the *O. turicata* microbiota will include potentially pathogenic bacteria for the Bolson tortoises, other animals, and even humans.

2. Materials and Methods

2.1. General

All the methods and activities of this study were in strict accordance with accepted guidelines for ethical use, care, and welfare of animals in research at the international level [19]. The federal approval reference number is SEMARNAT-SGPA/DGVS/08406/16. This research has been approved by the Facultad de Ciencias Biológicas UJED ethic committee on 5 February 2017 (ethic code: 0023). The files used in this study (Supplementary files: P1.fasta, P2.fasta and P3.fasta) were deposited into the NCBI Sequence Read Archive (SRA) database (SRA Accession Number: PRJNA649587).

2.2. Location and Environmental Conditions

The study was carried-out in the Mapimi Biosphere Reserve in Mexico, which includes part of the states of Chihuahua, Coahuila, and Durango (26°00′ and 26°10′ N, 104°10′ and 103°20′ W; Figure 1). The Bolson de Mapimi has been defined as an endoreic basin, which includes diverse small sub-basins intermixed along valleys with a mean altitude of 1150 m. This area has very arid climate [20], with an average annual temperature of 25.5 °C, and an average annual precipitation of 264 mm [21]. The predominant vegetation in the reserve is rosette and microphile scrub, as well as halophyte, and gypsophila plants [22]. Interestingly, the microregion concentrates the richest herpetofauna across the whole Chihuahuan desert, having diverse endemic species, including the Bolson tortoise [23].

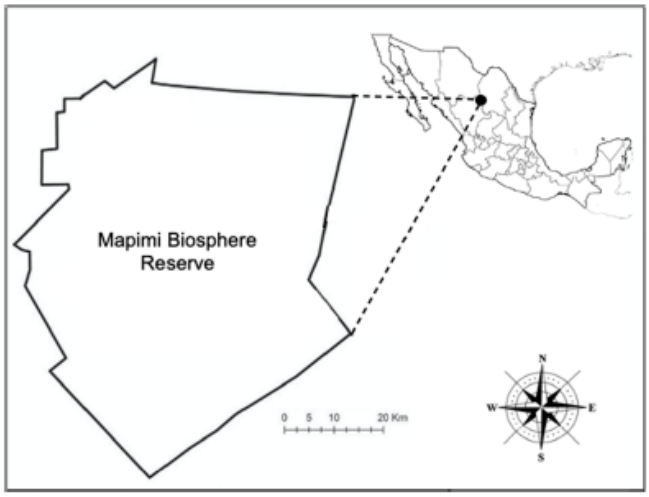

Figure 1. Geographic location of the Mapimi Biosphere Reserve in Northern Mexico.

2.3. Field Work

From May to July 2017, Bolson tortoises were captured by hand from 900 to 1300 h and from 1700 to 2100 h. The search for ticks was carefully carried out on the carapace, neck and skin folds of all four limbs. A total of 45 individuals of *G. flavomarginatus* were captured and revised, but only 11 tortoises (24.4%) carried soft ticks on the carapace or over the skin (Figures 2 and 3). Tortoises carried from one to three ticks per individual. A total of 17 adult ticks were collected and taxonomic keys were used to determine the species [24]. Each tick was placed in an individual 1.5 mL tube containing 500 uL ethanol (70%), 500 uL hydrogen peroxide (H_2O_2), and 200 uL of ultrapurified H_2O; each tube was vortexed for 15 s to remove tick surface contaminants [25]. Later, three pools were formed (five ticks each) and were deposited in BashingBead™ Zymo Research™ cell lysis tubes, containing 750 µL of lysing/stabilizing solution. The tubes were processed in a cellular disruptor (TerraLyzer™) for 20 s. The two extra ticks were deposited in the Entomological collection of the Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, México, for reference purposes.

Figure 2. *Ornithodoros turicata* soft ticks parasitizing the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico.

Figure 3. *Ornithodoros turicata* soft tick (adult) parasitizing the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico. Left is the dorsal view and right is the ventral view.

2.4. DNA Extraction, Visualization, and Quantification

DNA was extracted from the pools using the Xpedition™ Tissue/insect DNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA) in a laminar UV flow hood in sterile conditions. Then, the DNA was placed on a 1.2% agarose gel at 80 V for 45 min in a BIORAD electrophoresis chamber (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The DNA visualization was carried out in a GelMax™ photo documenter (UVP LLC, Upland, CA, USA). The amount of DNA obtained was measured in a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, USA). Then, the V3-V4 region of the 16S rRNA gene was amplified using the following primers [26]: S-D-Bact-0341-b-S-17, 5′-CCTACGGGNGGCWGCAG-3′ and S-D-Bact-0785-a-A-21, 5′-GACTACHVGGGTATCTAATCC-3′.

The PCR protocol was performed by using 12.5 µL of MyTaq™ Ready Mix 1× (Bioline, London, UK), 1 µL of each primer (10 nM), 5 µL of DNA (50 ng total), and 5.5 µL of molecular grade H_2O. The following cycle was used: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min; Illumina (2020a) [27] in a Labnet Multigene™ Gradient PCR thermal cycler (Labnet International, Edison, NJ, USA). Then, 1 µL of each PCR product was placed in an Agilent Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA) to verify the amplicon size (550 bp). The amplicons were purified with Agentcourt® AMPure® XP 0.8% beads (Beckman Coulter Inc., Brea, CA, USA). Thereafter, the Nextera XT Index Kit™ was used to create the library, following the Illumina (2020b) [28] protocol using 25 µL of MyTaq™ Ready Mix 1× (Bioline®), 5 µL of each primer (N7xx and S5xx), 5 µL of DNA, and 10 µL of molecular grade H_2O. Each pool considered the

following cycle: 95 °C for 3 min; 10 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. The libraries were purified with Agencourt® AMPure® XP 1.2% beads. Then, 1 µL of the final PCR product library was randomly selected and placed on a Bioanalyzer DNA 1000 chip to verify the size of the amplicon expecting a size of 630 bp. Thereafter, the amplicon was quantified, normalized, (equimolarity), and sequenced with next generation massive sequencing (MiSeq; Illumina, San Diego, CA, USA) of 2 × 250 paired final readings following the 16S metagenomic protocol [27].

2.5. Bioinformatics Analyses

The DNA sequences were analyzed on MGLinux, in a VM Oracle VirtualBox using Quantitative Insights into Microbial Ecology bioinformatics software (QIIME, Boulder, CO, USA) [29]. Both forward and reverse sequences were assembled using the PEAR program [30] with an overlap of 50 bp, a minimum reading length of 430 bp, and a maximum of 470 bp, a quality criterion Q30 (one false base for every 1000 bases) with $p < 0.0001$. Then the files were converted to the FASTA format, and chimeric sequences were discarded with USEARCH [31]. Thereafter, the operational taxonomic units (OTUs) were selected with the UCLUST method [31] at 97% similarity; a representative sequence for each OTU was obtained, and the taxonomy was assigned and taken as reference to the EzBioCloud database [32]. Next, the OTUs table was built in the Biom format (biological observation matrix) [33] separating domains. A simple random rarefaction process was performed [34] in order to obtain a standardized Biom file for all pools. The Shannon and Simpson alpha diversity indexes were calculated using the standardized Biom file; the mean ± standard deviation for each index was obtained. The relative abundance for the phylum level was represented as a bar chart using Excel, and family and genus levels were visualized as heatmaps using Morpheus software (Broad Institute, Cambridge, MA, USA) [35]; hierarchical clustering (average linkage method with Euclidean distance) was used to visualize pools dendrograms. Each genus and/or species of bacteria registered for *O. turicata* was consulted in the available literature to indicate its possible pathogenic potential for tortoises or zoonotic potential for humans.

3. Results

The average number of sequences assembled was 196,564. After taxonomic designation an average of 190,769 bacterial sequences was obtained. The average number of OTUs was 44,369 (Table 1). Simple random rarefaction was made at 100,000 sequences, since at this point the number of taxa of the three pools reached asymptotes. From the standardized Biom file, the OTUs resulted in 28 phyla, 84 classes, 165 orders, 342 families, 1013 genera, and 1326 species.

Table 1. 16S rRNA V3-V4 region sequences obtained from soft tick *Ornithodoros turicata* pools as an ectoparasite of the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico.

Pool	Total	Assembled	Discarded	ChS	QS	BS	OTUs
P1	424,827	278,006	146,821	1580	276,426	272,339	56,998
P2	457,353	202,476	254,877	1633	200,843	195,622	41,791
P3	159,929	109,211	50,718	278	108,616	104,347	34,318
Mean	347,370	196,564	150,805	1164	195,295	190,769	44,369

Abbreviations are ChS = chimeric sequences eliminated, QS = quality sequences after chimeras elimination, BS = bacteria sequences after taxonomy designation, and OTUs = operational taxonomic units.

While the Shannon diversity index was 8.12 ± 0.39 (SD), the Simpson diversity index was 0.86 ± 0.05. From the 28 phyla obtained, 27 (96.4%) were taxonomically classified, the rest were classified as "other". The most abundant phyla were Proteobacteria (mean = 70.75%), Actinobacteria (mean = 19.67%), and Firmicutes (mean = 5.35 %; Figure 4).

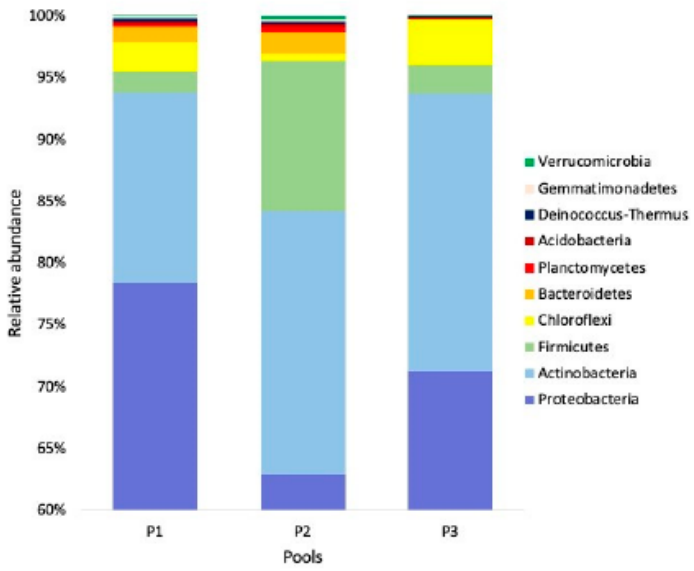

Figure 4. Relative abundance (%) of bacterial taxa at the phylum level from three pools of the soft tick *Ornithodoros turicata* as an ectoparasite of the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico. Only the 10 most abundant phyla are shown.

At the class level, 84 taxa were obtained of which 82 (97.6%) were taxonomically classified, the rest was classified as "other". The most abundant classes were Alphaproteobacteria (mean = 56.91%), Actinobacteria (mean = 18.55%), and Gammaproteobacteria (13.65%; Table S1). From the 165 orders obtained, 158 (95.7%) were taxonomically classified, the rest were classified as "other". The most abundant orders were Rickettsiales (mean = 55.40%), Legionellales (mean = 13.30%), Micrococcales (mean = 5.99%), Propionibacteriales (mean = 5.32%), and Srteptomycetales (mean = 5.11%; Table S1).

At the family level, 342 taxa were obtained of which 325 (95.0%) were taxonomically classified, the rest were classified as "other". The most abundant families were Midichloriaceae (mean = 51.13%), Coxiellaceae (mean = 13.26%), Nocardioidaceae (mean = 5.29%), Streptomycetaceae (mean = 5.10%), and Anaplasmataceae (mean = 4.29%; Figure 5). From the 1013 genera obtained, 897 (88.54%) were taxonomically classified, the rest were classified as "other". The most abundant genera were *Midichloria* (mean = 51.13%), *Coxiella* (mean = 13.25%), *Streptomyces* (5.08%), *Nocardioides* (mean = 5.06%), *Anaplasma* (mean = 4.29%), and *Clostridium* (mean = 2.19%; Figure 6; Table S1). The genus *Mycoplasma*, a potential pathogen of the *Gopherus* spp., was registered in low abundance (mean = 3.33×10^{-6} %). Additionally, 1326 species were obtained, 918 (69.2%) were classified as "other", 268 (20.3%) have a taxonomical key, and 140 (10.5%) have a taxonomical name (Table 2). The most abundant species were *Midichloria*-like bacteria (mean = 50%), *Coxiella*-like bacteria (mean = 13%), *Streptomyces* sp. (mean = 5%), and *Nocardioides* sp. (mean = 5%). However, considering only bacteria with a complete taxonomical name the most abundant was *Midichloria mitochondrii* (mean = 0.72%; Table S1). *Pasteurella testudinis*, a potential pathogen of the *Gopherus* spp., was registered in low abundance (mean = 1.33×10^{-5}%). The bovine and ovine tick-borne bacteria *A. marginale* and *A. ovis*, respectively, were recorded in low abundances; also, the zoonotic bacteria *Coxiella burnetii*, *Anaplasma phagocytophilum*, and *Neoehrlichia* sp. were part of the *O. turicata* microbiota (see relative abundances in Table S1).

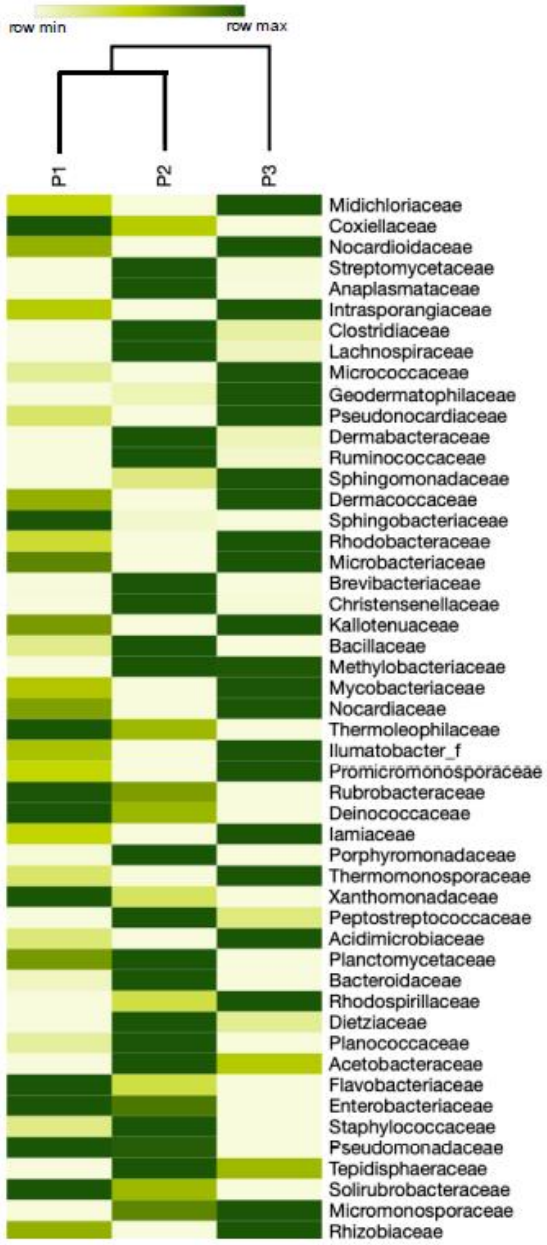

Figure 5. Heatmap of bacterial taxa at the family level from three pools of the soft tick *Ornithodoros turicata* as an ectoparasite of the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico. Only the first 50 more abundant families having a taxonomical name are shown.

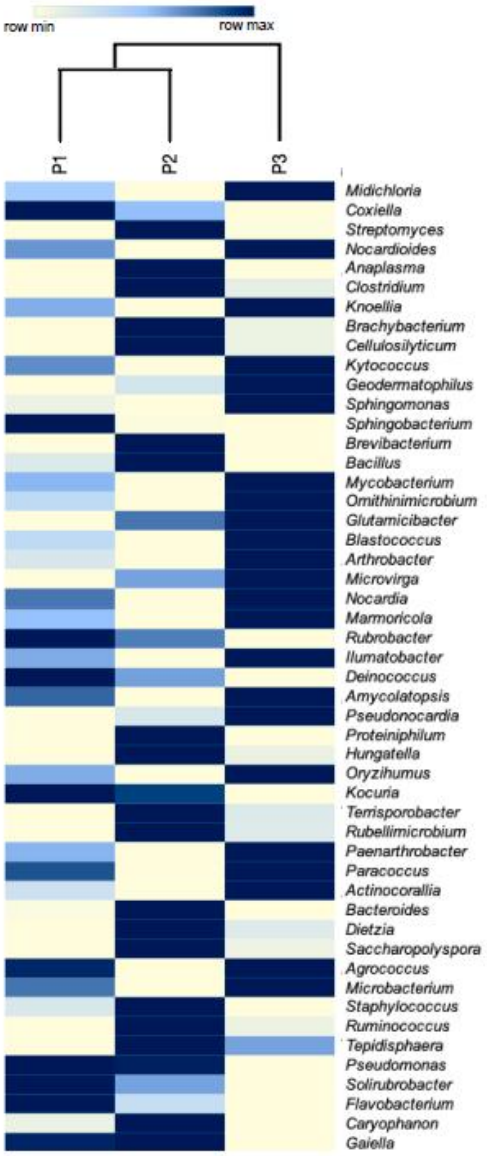

Figure 6. Heatmap of bacterial taxa at the genus level from three pools of the soft tick *Ornithodoros turicata* as an ectoparasite of the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico. Only the first 50 more abundant genera having a taxonomical name are shown.

Table 2. Bacterial species found in the soft tick *Ornithodoros turicata* as an ectoparasite of the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico [1].

Bacterial Species	Bacterial Species	Bacterial Species
Actinocorallia libanotica	*Kribbia dieselivorans*	*Phycicoccus jejuensis*
Actinophytocola timorensis	*Kytococcus aerolatus*	*Phytomonospora endophytica*
Aeromicrobium panaciterrae	*Kytococcus schroeteri*	*Piscicoccus intestinalis*
Agrococcus terreus	*Kytococcus sedentarius*	*Planosporangium thailandense*
Anaerocella delicata	*Lactobacillus sakei*	*Propionibacterium acnés*
Anaplasma marginale	*Lactococcus lactis*	*Rubellimicrobium mesophilum*
Anaplasma ovis	*Lentzea kentuckyensis*	*Saccharopolyspora hirsuta*
Anaplasma phagocytophilum	*Leucobacter celer*	*Salmonella entérica*
Anseongella ginsenosidimutans	*Leucobacter chromiireducens*	*Segetibacter koreensis*
Arthrobacter halodurans	*Leucobacter tardus*	*Seinonella peptonophila*
Bacillus cecembensis	*Leucobacter zeae*	*Serinicoccus profundi*
Bacillus halosaccharovorans	*Lysobacter dokdonensis*	*Sinomonas mesophila*
Bacillus niacini	*Magnospira bakii*	*Skermanella aerolata*
Bauldia litoralis	*Marmoricola bigeumensis*	*Skermanella stibiiresistens*
Blautia wexlerae	*Marmoricola pocheonensis*	*Smaragdicoccus niigatensis*
Brachybacterium ginsengisoli	*Marmoricola terrae*	*Sphingoaurantiacus polygranulatus*
Brachybacterium phenoliresistens	*Massilia brevitalea*	*Sphingobacterium changzhouense*
Brachybacterium squillarum	*Methylobacterium oxalidis*	*Sphingobacterium hotanense*
Brachybacterium zhongshanense	*Micromonospora taraxaci*	*Sphingobacterium mucilaginosum*
Cellulosilyticum lentocellum	*Midichloria mitochondrii*	*Sphingobacterium siyangense*
Citricoccus yambaruensis	*Mobilicoccus pelagius*	*Sphingobacterium suaedae*
Clavibacter michiganensis	*Mycobacterium rutilum*	*Stackebrandtia cavernae*
Clostridium butyricum	*Nocardia brevicatena*	*Staphylococcus cohnii*
Clostridium chartatabidum	*Nocardia puris*	*Staphylococcus hominis*
Clostridium máximum	*Nocardioides aestuarii*	*Staphylococcus saprophyticus*
Coxiella burnetii	*Nocardioides agariphilus*	*Staphylococcus sciuri*
Cronobacter dublinensis	*Nocardioides albertanoniae*	*Staphylococcus succinus*
Dermabacter vaginalis	*Nocardioides daedukensis*	*Stenotrophobacter namibiensis*
Eubacterium eligens	*Nocardioides glacieisoli*	*Streptomyces bacillaris*
Flavobacterium anatoliense	*Nocardioides islandensis*	*Streptomyces baliensis*
Geodermatophilus arenarius	*Nocardioides kongjuensis*	*Streptomyces barkulensis*
Geodermatophilus nigrescens	*Nocardioides lianchengensis*	*Streptomyces cacaoi*
Geodermatophilus obscurus	*Nocardioides luteus*	*Streptomyces drozdowiczii*
Geodermatophilus saharensis	*Nocardioides mesophilus*	*Streptomyces fenghuangensis*
Geodermatophilus soli	*Nocardioides pyridinolyticus*	*Streptomyces glaucosporus*
Glutamicibacter creatinolyticus	*Nocardioides tritolerans*	*Streptomyces mangrovi*
Glutamicibacter nicotianae	*Ornithinimicrobium humiphilum*	*Streptomyces murinus*
Helcobacillus massiliensis	*Ornithinimicrobium kibberense*	*Streptomyces thermoviolaceus*
Janibacter alkaliphilus	*Ornithinimicrobium murale*	*Syntrophomonas curvata*
Janibacter corallicola	*Ornithinimicrobium pekingense*	*Syntrophomonas wolfei*
Kallotenue papyrolyticum	*Ornithinimicrobium tianjinense*	*Taibaiella chishuiensis*
Kibdelosporangium aridum	*Paenarthrobacter nitroguajacolicus*	*Terracoccus luteus*
Kineococcus radiotolerans	*Paraburkholderia caledonica*	*Tetrasphaera elongata*
Kineosphaera limosa	*Paracoccus saliphilus*	*Tetrasphaera remsis*
Knoellia aerolata	*Paracoccus sphaerophysae*	*Tetrasphaera vanveenii*
Knoellia sinensis	*Pasteurella testudinis*	*Thermobaculum terrenum*
Kocuria flava	*Phycicoccus endophyticus*	

[1] Relative abundance is presented in Table S1

4. Discussion

Our working hypothesis stated that the microbiota of *O. turicata* would contain potentially pathogenic bacteria for the Bolson tortoise *Gopherus flavomarginatus*, other animals, and humans.

Therefore, and based on the obtained results, this study demonstrated the presence of some potentially pathogenic and zoonotic bacteria in the microbiota of *O. turicata*.

The most abundant phyla were Proteobacteria, Actinobacteria, and Firmicutes. This is similar to that reported for *Ambylomma tuberculatum* ticks infesting the Gopher tortoise (*G. polyphemus*) in Mississippi, USA, with 89%, 9%, and 1% of the relative abundance, respectively [36]. Currently, Proteobacteria is the broadest phylum in the Bacteria domain [37]. Some of the tick species that have shown dominance of this phylum in their microbiota are *Argas japonicus* in China (42–97%) [38], *Hyalomma dromedarii* in Saudi Arabia (98%) [39], *Dermacentor marginatus* and *D. reticulatus* in Slovakia (60%) [40], *D. marginatus* (97%), *Haemaphysalis punctata* (98%), *Ixodes ricinus* (88%), and *Rhipicephalus sanguineus sensu lato* (99%) in Spain [41], and *I. scapularis* in Massachusetts and Texas, USA (73–100%; [8]).

Alphaproteobacteria and Gammaproteobacteria classes were the most abundant in *O. turicata* microbiota, as has been observed in other tick species [41]. These classes are relevant in the microbiota of all tick species because they group the main intracellular endosymbionts (*Arsenophonus*-like, *Coxiella*-like, *Fransicella*-like, *Midichloria*-like, *Rickettsia*-like, and *Wolbachia*-like) that inhabit the organs of these arthropods [6,42]. These endosymbionts have generally been reported to predominate in the arthropod microbiota and may interfere with the transmission dynamics of pathogenic bacteria [43,44]. In the present study, *O. turicata* showed only two genera of intracellular endosymbionts, *Midichloria* and *Coxiella*. An unidentified species *Midichloria*-like endosymbiont was the dominant taxon in the *O. turicata* microbiota, although *Candidatus Midichloria mitochondrii* (CMM) species was also abundant in this tick. CMM has been reported in a large number of tick genera (*Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp., *Hyalomma* spp., *Ixodes* spp., and *Rhipicephalus* spp.) in different countries of Europe, Asia, and North America [41,45,46]. It is considered a facultative non-pathogenic mutualistic bacterium that lodges in the reproductive tissues of female ticks, specifically in the ovarian mitochondria, where they sometimes invade and destroy this organ [47,48]. By remaining in this organ, vertical transmission to the next generation is carried out. However, its presence has also been recorded in salivary glands from which it is transmitted to vertebrate hosts during tick feeding [49]. Since all the body of *O. turicata* was analyzed in the present study, it is not possible at this time to determine in which organs occur both unknown *Midichloria*-like and CMM. However, it is feasible that they occur in the reproductive organs since some studies have analyzed the possible evolutionary process of intracellular endosymbionts of arthropods, and apparently transovarian transmission is the common factor for all species [50]. Bacterial diversity studies in each organ of *O. turicata* are required to clarify this question.

The second most abundant bacterial species in *O. turicata* microbiota in our study was a *Coxiella*-like endosymbiont. This type of endosymbiont does not appear to be pathogenic and are relatively common in the microbiota of various tick species around the world [51,52]. These microorganisms are known to infect tick ovaries and then transmission occurs vertically through the egg cytoplasm, but they have also been found in Malpighi tubules where they may provide essential nutrients to their host [7]. It should be noted the presence of *C. burnetii* in the present study. This is a zoonotic bacterium that causes Q-fever in both animals and humans. In particular, for the *Ornithodoros* genus there are reports of *C. burnetii* in *O. tartakovskyi*, *O. papillipes* and *O. alactagalis* in the former Soviet Union [53], *O. moubata* in Japan [54], and *O. sonrai* in Senegal [55]. According to Balashov and Daiter (1973) [53] and Eldin et al. (2017) [56], transmission of *C. burnetii* is transovarian in most ticks, except for *Ixodes holocyclus*, *O. hermsi*, and *O. turicata* in which it is transestadial. Direct transmission of *C. burnetii* from infected ticks to humans is not well documented and may occur only rarely in nature [57]. However, the main transmission route is via inhalation of contaminated fecal material from ticks [58]. Since the personnel that monitor *G. flavomarginatus* populations each year in the Mapimi Biosphere Reserve should manipulate tortoises, it is advisable to take precautions to avoid this airborne infection.

Mycoplasma spp. and *Pasteurella* spp. are bacterial genera that have been reported as part of the microbiota of hard ticks, as in *Ixodes simplex* and *I. ventalloi*, respectively [59,60]. In the present study with *O. turicata*, the presence of *Mycoplasma* spp. and *Pasteurella testudinis* in low abundances was

recorded. An interesting fact is that these bacteria along with some viruses have been reported as some of the possible causes of upper respiratory disease (URTD) in tortoises, causing a decrease in the populations of *G. agassizii* and *G. polyphemus* in the USA [61,62]. It is still unknown if these bacteria can be transmitted directly to the host, and in the case of *G. flavomarginatus* if they could cause disease. For now, these bacteria should remain as potential pathogens for this tortoise, until further studies of vector competence discard this possibility.

Other relevant species recorded in *O. turicata* microbiota were the obligate intracellular bacteria *Anaplasma phagocytophilum*, *A. marginale*, *A. ovis*, and *Neoehrlichia* sp. These species are important for animal and human health because they typically infect hematopoietic or endothelial cells [63]. *Anaplasma phagocytophilum* is a tick-borne pathogen that causes human granulocytic anaplasmosis mainly in Asia, Europe, and the USA; *A. marginale* and *A. ovis* are worldwide tick-borne bacteria causing bovine and ovine anaplasmosis respectively [64,65]. The genus *Neoehrlichia* was discovered in 1999 as an *Ehrlichia*-like bacterium [66], which was later described as *Candidatus Neoehrlichia mikurensis* [67], an emerging tick-borne pathogen detected in ticks and rodents, causing human systemic inflammatory syndrome in Asia and Europe [68,69]. In America, *Candidatus Neoehrlichia lotoris* was registered in a free-living raccoon associated with tick-infested populations [70], which suggests that it is transmitted to mammals by ticks. Undoubtedly, specific molecular studies are needed to determine which *Neoehrlichia* species is the one registered in *O. turicata*, nevertheless, its zoonotic potential is latent.

According to Gofton et al. (2015b) [71], a limitation of 16S bacterial community profiling in ticks is that a high proportion of sequences will belong to bacterial endosymbionts, as CMM as observed in *O. turicata* in the present study. This high abundance may mask the presence of less abundant bacteria with zoonotic potential, as for example the genus *Borrelia*, which was not detected in our study. Species such as *B. mazzottii* recorded in *O. talaje* in Mexico [72], as well as *B. parkeri* in *O. parkeri*, *B. hermsii* in *O. hermsi*, and *B. turicatae* in *O. turicata* in the USA [73], are important indicators of the presence of this bacterial genus in the *Ornithodoros* ticks of America. Perhaps, by carrying out detailed molecular studies in *O. turicata* in the Mapimi Biosphere Reserve, *Borrelia* and other zoonotic bacteria such as *Rickettsia* could be identified. In the same way, since this massive sequencing technique does not reach the species level in many genera documented here for *O. turicata*, it is necessary to carry out specific PCR studies for the most relevant bacteria involved in the biology of this tick and those bacteria important for the health of animals and humans.

It is important to comment that the present study was carried out at a single time of the year, without distinguishing sexes of *O. turicata* to provide an initial overview of the microbiota of this tick species. However, various studies indicate that the microbiota of ticks is dynamic and can change among seasons of the year due to the effect of temperature and humidity, between males and females, between different feeding statuses, among life stages, etc. [3,4]. Therefore, it is guaranteed that detailed studies on these issues will further be carried out.

Finally, the high number of bacterial taxa recorded for *O. turicata* in the present study could be due to the variety of hosts that this tick parasitizes within the burrows of *G. flavomarginatus*, performing a rapid feeding process that usually lasts an hour on average [14,74]. In this way the general feeding habits of *O. turicata* may be keeping pathogenic microbes in circulation, thereby ensuring their survival in the ecosystem. In our study, all *G. flavomarginatus* tortoises found carrying *O. turicata* ticks were apparently healthy, having the possible ecological role of reservoirs of pathogenic bacteria [75,76]. The fact that tortoises have long lives can favor the maintenance of pathogen cycles under normal conditions [77,78]. However, after thousands of years of coevolution, a microbial balance between *O. turicata* and *G. flavomarginatus* must have been reached, although it is not yet known whether this tortoise's defense mechanism is resistance (i.e., capacity to limit pathogen loads) or tolerance (i.e., capacity to survive damage caused by a given pathogen load) [79–81]. To clarify this enquiry, it will be necessary to determine the bacteria that *G. flavomarginatus* carries in the blood and whether they were transmitted by *O. turicata*. Studies of resistance mechanisms and vector competence in this

tortoise will also be relevant from the point of view of immunity and the eco-epidemiology of zoonotic diseases, respectively.

5. Conclusions

Our study established the general relative abundance of bacterial taxa in the soft tick *O. turicata*, documenting the presence of potentially pathogenic species for the Bolson tortoise *G. flavomarginatus*, other animals and humans in the Mapimi Biosphere Reserve, Durango, Mexico. The most abundant phyla were Proteobacteria, Actinobacteria, and Firmicutes. Additionally, Alphaproteobacteria and Gammaproteobacteria classes were the most abundant in *O. turicata* microbiota. The most abundant species were *Midichloria*-like and *Coxiella*-like (endosymbionts of ticks). *Mycoplasma* spp. and *Pasteurella testudinis*, both potentially pathogenic to the Bolson tortoise, were registered. Additionally, the bovine and ovine tick-borne pathogens *A. marginale* and *A. ovis*, respectively, as well as the zoonotic bacteria *A. phagocytophilum*, *Coxiella burnetii*, and *Neoehrlichia* sp. were founded in *O. turicata*. Future studies on soft ticks should expand our knowledge about what components of the microbiota are notable across multiple host–microbe dynamics. Likewise, studies are required to better understand the reservoir competence of this tortoise, considered the largest terrestrial reptile in North America distributed throughout the Chihuahuan Desert since the late Pleistocene.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-7737/9/9/275/s1, Table S1: Relative abundance of bacterial taxa from three pools of the soft tick *Ornithodoros turicata* as an ectoparasite of the Bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico, Supplementary files (P1.fasta, P2.fasta and P3.fasta). The files used in this study were also deposited into the NCBI Sequence Read Archive (SRA) database (SRA Accession Number: PRJNA649587).

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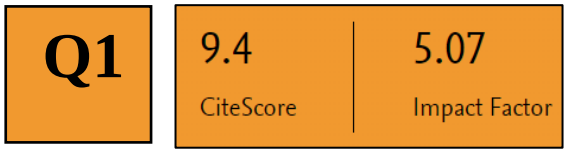

6.2 Bacterial profile of the dog brown tick *Rhipicephalus sanguineus* sensu lato collected from urban dogs in northern Mexico

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Bacterial profile of the dog brown tick *Rhipicephalus sanguineus* sensu lato collected from urban dogs in northern Mexico

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Abstract:

Ticks are obligate ectoparasites that feed on the blood of their hosts to survive, including domestic animals such as dogs. Pathogens of medical and veterinary importance like *Ehrlichia canis, E. weingi, E. chaffensis, Borrelia burgdorferi, Coxiella burnetti, Anaplasma platys, A. phagocytophilum* and *Rickettsia rickettsii* have been previously detected in the brown dog tick (*Rhipicephalus sanguineus*). In September 2019, *R. sanguineus* sensu lato (s.l.) ticks were collected from stray and domestic dogs at the Comarca Lagunera, northern Mexico. The objective was to analyze the bacterial composition and abundance of this tick using next generation sequencing (NGS), emphasizing the prevalence of tick-borne pathogens (TBP). Ten pools, each containing viscera of five adult ticks were prepared; DNA from each pool was extracted, and the V3-V4 region of the 16S rRNA gene was amplified and sequenced using MiSeq Illumina. At genus level, 775 taxa were found. The most abundant taxa were *Coxiella* (mean = 66.16%), *Acinetobacter* (mean = 9.16%), *Ehrlichia* (mean = 3.97%) and *Kocuria* (mean = 1.29%). A total of 93 potential pathogenic bacteria was detected, were seven of them are historically considered TBP (*C. burnetii, E. canis, E. muris, E. chaffeensis, E. minaensis, E. ewingii* and *A. platys*). *Ehrlichia minaensis* is a relatively new potential TBP previously reported in Canada and Brazil and found for the first time in the present study in Mexico in brown dog ticks. We conclude that tick-borne diseases (TBD) are a serious public health problem in this Mexican region; pathogen prevalence studies based on robust molecular technologies should be developed periodically to evaluate the status of these diseases.

Keywords: Next generation sequencing, 16S rRNA, *Ehrlichia, Coxiella, E. minasensis, Anaplasma*

1. INTRODUCTION

Ticks are obligate blood-sucking ectoparasites that parasitize a wide number of animal species in all their life stages, including domestic animals that live closely with humans such as dogs and cats (Saleh et al., 2021). The greatest concern of interest in public health as well as in veterinary medicine are the tick-borne diseases (TBD) that ticks can transmit to their vertebrate hosts; diseases such as Ehrlichiosis, Anaplasmosis, Rickettsiosis, Q fever, Lyme borreliosis, Babesiosis, among other infectious diseases whose pathogens can be transmitted to parasitizing vertebrate hosts, including dogs and humans, which have a close relationship of historical coexistence (Bonnet & Pollet, 2021; Dantas-Torres et al., 2012).

The brown dog tick (*Rhipicephalus sanguineus*) has been found all over the world; it prefers warm climates such as the southern United States and northern Mexico, however, any place where dogs exist, this tick can be found (Saleh et al., 2021). In recent years, the problem involving ticks and their microorganisms has worsened mainly because the tick population and its geographical distribution have increased due to various biotic and abiotic factors; anthropogenic activities that result in climate change, land use change, habitat fragmentation, host availability due to migration of people and human behavior (Dantas-Torres et al., 2012; Geurden et al., 2018; Paddock & Goddard, 2015; Saleh et al., 2021).

Mexico is a country vulnerable to TBD. In the northern part of Mexico, TBDs are largely endemic or are sometimes potentially serious re-emerging diseases, such as Rocky Mountain spotted fever caused by *Rickettsia rickettsii*. The states of Baja California, Coahuila, and Sonora are states mainly affected by this serious disease in the last 10 years (Pieracci et al., 2019). In the case of the Comarca

Laguna, a region located in the west of the Chihuahuan desert, it has the climatic, geographic, demographic, epidemiological and sociocultural conditions for the proliferation of ticks (Castillo-Martínez et al., 2015). TBDs are a recurring problem and some diseases are considered endemic in the region where they have claimed human lives (Castillo-Martínez et al., 2015; Nava-Reyna et al.). Pathogens such as *E. canis, E. chaffensis, A. platys* and *R. rickettsii* have been reported (Almazán et al., 2016; Ortega-Morales et al., 2019). However, TBDs caused by these agents are misdiagnosed with other febrile illnesses Due to the high capacity of ticks to transmit pathogens and the close relationship of dogs to humans, periodic collection of ticks from animals in a region, along with analysis for their potential pathogens can provide important epidemiological information on the distribution and presence of TBPs as a risk factor for both human and veterinary health (Geurden et al., 2018). Therefore, the purpose of this study was to examine the complete bacterial profile of the brown dog tick (*R. sanguineus*) using NGS and to identify the presence of pathogenic organisms in ticks as possible sources of infection for both animals and humans.

2. MATERIALS AND METHOS

2.1. Place, area and period of study

This study was developed from August 2019 to December 2021 in the Conservation Medicine Laboratory of the Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, in collaboration with the Laboratorio de Salud Nacional: laboratorio Nacional en Salud: Diagnóstico Molecular y Efecto Ambiental en Enfermedades Crónico-Degenerativas, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México.

2.2. Tick collection

Ticks were collected in five locations in the Comarca Lagunera: Bermejillo, Tlahualilo, Matamoros, Torreón and Gómez Palacio, located in the states of Coahuila and Durango, Mexico, during

September 2019. Around 500 adult female and male ticks were collected from 50 domestic and peridomestic dogs and preserved in 70% ethanol. All individuals were identified as *R. sanguineus* and were separated by sex (Walker et al., 2005).

2.3. Tick cleaning dissection

Ticks were dissected with sterilized microdissection scissors (BioQuip® No. 4715), making a cut in the posterior part of the abdomen; they were held with entomological forceps (BioQuip® No. 4522) from the base of the palps and with the help of a curved forceps (BioQuip® No. 4527) they were slid by lightly pressing the dorsal and ventral part of the specimens to the back of the specimen idiosoma. Five pools were made with the internal content of five female ticks and another five pools for males. Pools were deposited in BashingBeadTM lysis tubes with 750 µL of Xpedition^{TM} Zymo Reesearch^{TM} lysing/stabilizer buffer. Each tube was placed in a cell disruptor (TerraLyzerTM) for 30 seconds for DNA preservation.

2.4. Extraction and amplification of the 16S rRNA gene

DNA from the samples was extracted using the Zymobiomics MiniPrep (Zymo Research) commercial kit. The extracted DNA was run on 1.2% agarose gels at 80V for 45 minutes in a BIO-RAD electrophoresis chamber to visualize the presence of DNA. Visualization was carried out in a GelMaxTM (UVP®) photodocumenter. The amount of DNA per sample was measured in a Qubit® fluorometer. Amplification of the V3-V4 region of the 16S rRNA gene was made using the primers: S-D-Bact-0341-b-S-17, 5´-CCTACGGGNGGCWGCAG-3´ and S-D-Bact0785-a-A-21, 5´-GACTACHVGGGTATCTAATCC -3´, producing an amplicon of ~460 bp (Klindworth et al., 2013).

2.5. Polymerase Chain Reaction (PCR)

The Illumina PCR protocol was used with 12.5 µL of MyTaq™ Ready Mix 1X (Bioline®), 1 µL of each primer (10 µM), 5 µL of DNA (50 ng total) and 5.5 µL of molecular grade H_2O. The following cycle was used: 95 °C for three minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 30 seconds; 72 °C for five minutes in a Labnet Multigene™ Gradient PCR thermal cycler on a Bioanalyzer DNA 1000 chip to verify amplicon size (approximately 550 bp).

2.6. Purification and creation of libraries

Purification of amplicons was performed with 0.8% Agentcourt® AMPure® XP beads. Subsequently, the amplicons were labeled using the Nextera XT Index Kit™ for the creation of the libraries, following the Illumina protocol, using 25 µL of MyTaq™ Ready Mix 1X (Bioline®), 5 µl of each primer (N7xx and S5xx), 5 µL of DNA and 10 µL of molecular grade H_2O; the following cycle was used: 95 °C for 3 minutes; 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes. Purification of libraries was performed with 1.2% Agencourt® AMPure® XP beads. 1 µL of the final library of a few randomly selected PCR products was placed on a Bioanalyzer DNA 1000 chip to verify amplicon size expecting a size of 630 bp.

2.7. Sequencing by synthesis

Quantitation, normalization (equimolarity), library pooling, and massive next-generation sequencing (MiSeq Illumina® 2 × 250 paired-ended reads) were performed following the Illumina 16S metagenomics protocol.

2.8. Bioinformatic analysis

The sequencing results were compiled into FASTQ files, which were analyzed in the Oracle VM VirtualBox 5.1.14 virtual machine on the MGLinux platform using Quantitative Insights Into Microbial Ecology (QIIME) v.1.9.0 bioinformatics software (Caporaso et al., 2010). Both forward

and reverse sequences were assembled using the PEAR program (Zhang et al., 2013) with an overlap of 50 bp, a minimum reading length of 430 bp, and a maximum of 470 bp, a quality criterion Q30 (one false base for every 1000 bases) with $p < 0.0001$. Then the files were converted to the FASTA format, and chimeric sequences were discarded with USEARCH (Edgar, 2010). Thereafter, the operational taxonomic units (OTUs) were selected with the UCLUST method at 97% similarity (Edgar, 2010). A representative sequence for each OTU was obtained, and the taxonomy was assigned and taken as reference to the EzBioCloud database (Yoon et al., 2017). For abundance at the phylum level, class and order were represented as a bar graph using Excel, and family and genus levels were visualized as heatmaps using Morpheus software (Broad Institute, Cambridge, MA, EE. UU. A random rarefaction process was carried out at a depth of 50,000 sequences. From here, the Chao1 estimated richness index and Shannon, Simpson alpha diversity index were calculated. Non-parametric t-tests (false discovery rate correction) were applied to test differences ($p < 0.05$) between populations for each index. The Bray–Curtis beta diversity (Beals, 1984) was calculated; PERMANOVA was applied to test significant differences ($p < 0.05$) of the internal microbiota between sexes. Each genus and/or species of bacteria recorded for *R. sanguineus* s.l was consulted in the available literature to indicate its possible pathogenic potential for animals and humans.

RESULTS

The average number of assembled sequences was 84,926, with a final average number of sequences after taxonomic assignment of 71,472, and an average number of OTUs (Operational Taxonomic Units) of 48,318 (Table 1). Of these OTU, 18 phyla, 51 classes, 111 orders, 243 families, 775 genera and 1521 species were acquired (Table A). Based on the results obtained in the rarefaction analysis, we obtained an asymptote trend in all sequences that reflects good sequencing coverage against the number of taxa obtained (Fig. A).

Table 1. 16S rRNA V3-V4 region sequences obtained from 10 pools of the internal content of the *R. sanguineus* s.l. colected from domestic and stray dogs.

Sample	Assambled Reads	Discarded	ChS[a]	QS[b]	BD[c]	OTUs[d]	TOTAL
RS1	105,388	24,721	13725	91261	74605	46977	130,109
RS2	77,851	30,967	658	76952	67752	55034	108,818
RS3	95,156	18,971	380	94396	89604	49750	114,127
RS4	72,161	31,795	1570	70341	63771	43084	103,956
RS5	82,236	37,144	11412	70539	53843	46897	119,380
RS6	91,768	35,667	2692	88722	83408	40704	127,435
RS7	75,637	33,167	2,797	72593	59583	50496	108,804
RS8	75,375	23,799	159	74968	69196	47509	99,174
RS9	92,167	37,806	2,827	88990	81613	46682	129,973
RS10	81,517	39,122	836	80402	71343	56046	120,639
Mean	84,926	31,316	3,706	80,916	71,472	48,318	116,242

Abbreviations are [a]Chs= Chimeras found; [b]QS= quality sequences after chimeras elimination; [c]BD = bacteria sequences after taxonomy designation; [d]OTUs = operational taxonomic units.

Of the 18 phyla, 18 were classified with taxonomic name (100%). The most abundant phyla were Proteobacteria with (mean= 88.36%), Actinobacteria (mean= 5.73%), Firmicutes (mean= 4.66%) and Bacteroidetes with (mean= 0.72%; Fig. 1).

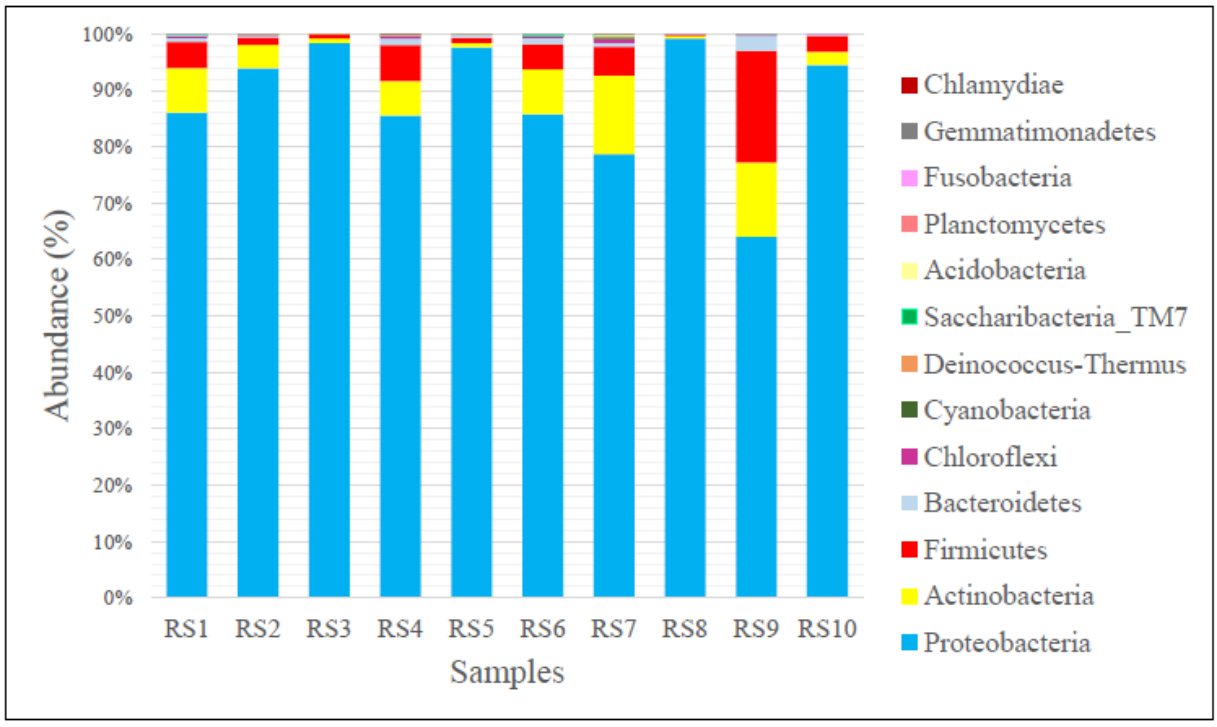

Figure 1. Relative abundance (%) at the phylum level of 10 pools of internal content of *R. sanguineus* s.l. collected from domestic and stray dogs in the Comarca Lagunera, northern Mexico. Only the first 13 taxa are shown.

For the Class level, 50 taxa with taxonomic names (98.03%) were classified, only one of these was classified as "other". The most abundant taxa at this level were Gammaproteobacteria (mean= 79.03%), Alphaproteobacteria (mean= 8.21%), Actinobacteria_c (mean= 5.60%) and Bacilli (mean= 4.24%; Figure 2). At the Order level of the 111 orders, 107 were classified with a taxonomic name (96.39%), the rest were classified as "others". The most abundant taxa were Legionellales (mean= 66.18%), Pseudomonadales (mean= 8.21%), Rickettsiales (mean= 3.97%), Bacillales (mean= 3.73%) and Micrococcales (mean= 3 .37%, Figure 3).

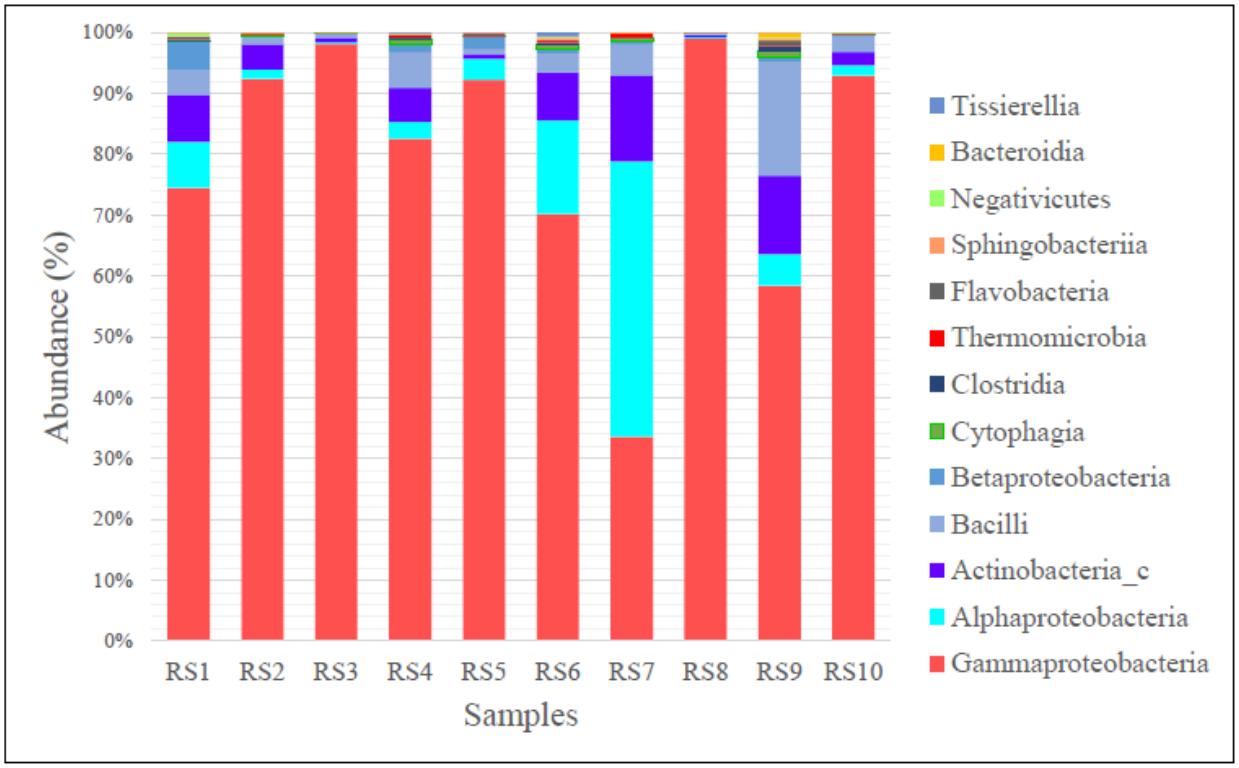

Figure 2. Relative abundance (%) at the class level of 10 pools of the internal content of *R. sanguineus* s.l. collected from domestic and stray dogs in the Comarca Lagunera, northern Mexico. Only the first 13 taxa are shown.

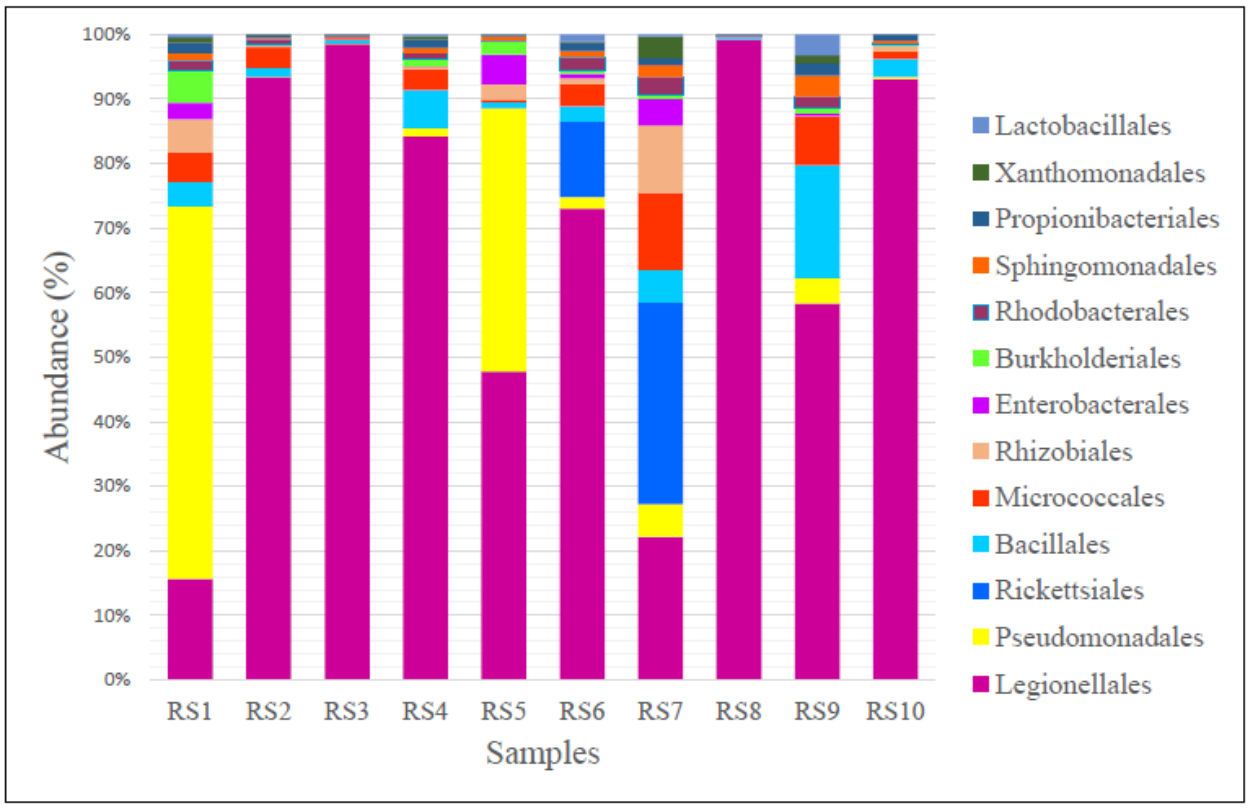

Figure 3. Relative abundance (%) at order level from 10 pools of internal content of *R. sanguineus* s.l. collected from domestic and stray dogs in the Comarca Lagunera, northern Mexico. Only the first 13 taxa are shown.

At the family level of the 243 families, 227 were classified with taxonomic name (93.42%), the rest were classified as "other". The most abundant taxa at this level were Coxiellaceae (mean= 66.17%), Moraxellaceae (mean= 9.54%), Anaplasmataceae (mean= 3.97%), Micrococcaceae (mean= 2.27%) and Bacillaceae (average= 2.00%; Figure 4 A).

Regarding the genus level, of the 775 taxa, 666 (85.93%) were classified with a taxonomic name, the rest were classified as "other". The most abundant taxa were *Coxiella* (mean= 66.16%),

Acinetobacter (mean= 9.16%), *Ehrlichia* (mean= 3.97%), *Kocuria* (mean= 1.29%) and *Pseudomonas* (mean= 1.10%, Figure 4 B).

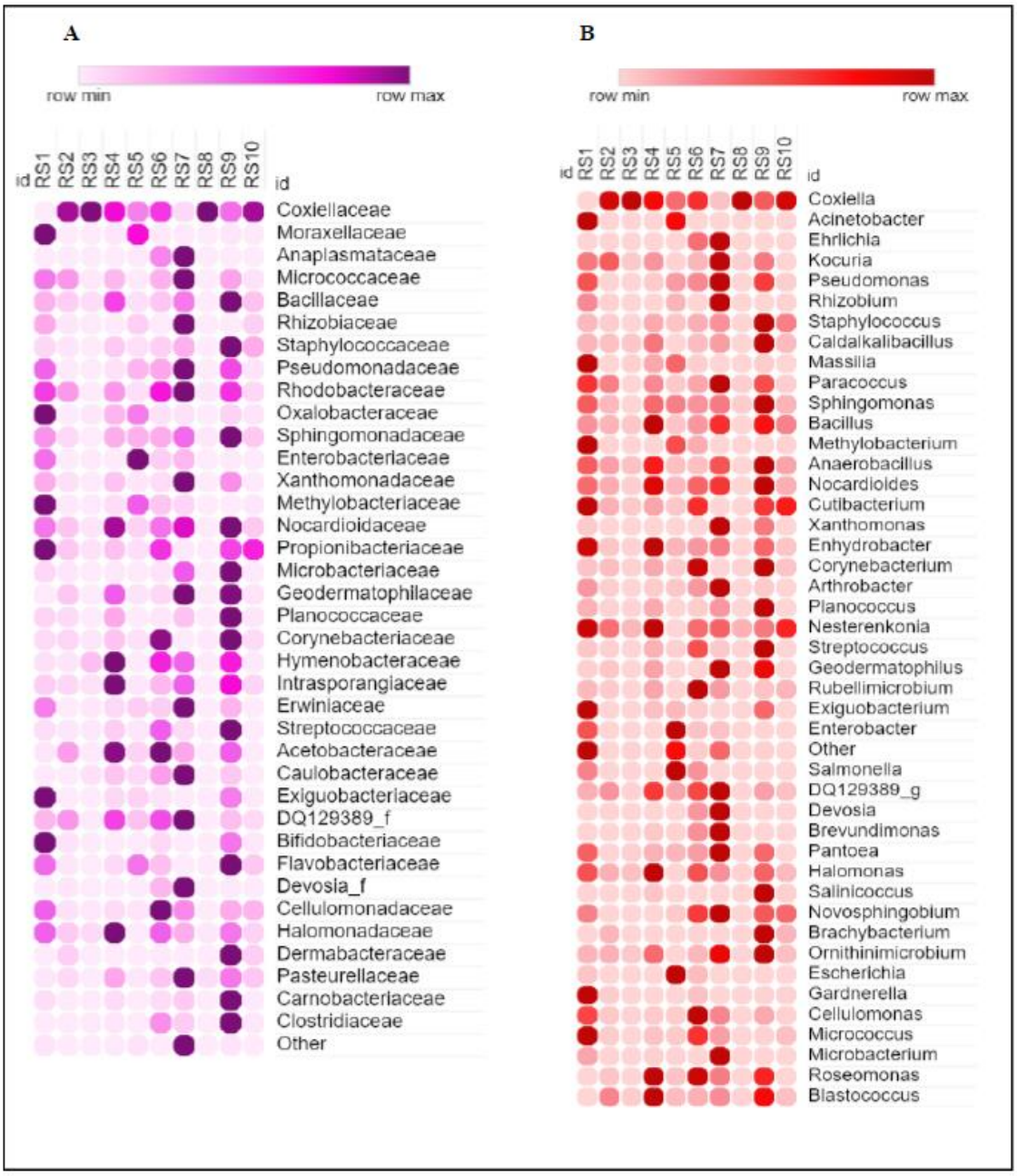

Figure 4. Heatmaps of relative abundance (%) at family level (A) and genus (B) levels from 10 pools of the internal contents of the tick *R. sanguineus* s.l. collected from domestic and stray dogs in the Comarca Lagunera, northern Mexico.

Regarding the species level, 1522 species were found, of which 793 were classified with taxonomic name (52.10%). From this list, an exhaustive bibliographical review was carried out to determine the potentially pathogenic species for both humans and animals, which harbors the brown dog tick in the studied region, 93 potentially pathogenic species were designated (Chentanez et al., 2011; Imai et al., 2019; Knodler & Elfenbein, 2019; Lécuyer et al., 2007; Lee et al., 2019; Moreira et al., 2015; Paczosa et al., 2016; Soldera et al., 2013; Taylor & Unakal, 2021; Van Hoovels et al., 2006) within which seven are historically considered as TBP; *Coxiella burnetii, Ehrlichia canis, Ehrlichia muris, Ehrlichia chaffeensis, Ehrlichia minasensis, Ehrlichia ewingii* and *Anaplasma platys* (Cabezas-Cruz et al., 2016; Fourie et al., 2013; Heitman et al., 2016; Körner et al., 2021; Pritt et al., 2017; Rikihisa, 2015; Snellgrove et al., 2020) in decreasing order of relative abundance (Table 2), the complete list of species is presented in table A.

Table 2. Potentially pathogenic bacteria at the species level found in the brown dog tick *(R. sanguineus* s.l.*)* in north central Mexico, the bacteria are presented in decreasing order of relative abundance (%) from highest to lowest.

Pathogenic bacterial species	Pathogenic bacterial species	Pathogenic bacterial species
Salmonella enterica	*Dolosigranulum pigrum*	*Kocuria rosea*
Staphylococcus cohnii	*Gemella palaticanis*	*Atopobium vaginae*
Leucobacter chromiireducens	*Frederiksenia canicola*	*Leclercia adecarboxylata*
*Coxiella burnetii**	*Cronobacter dublinensis*	*Pantoea eucrina*
Roseomonas gilardii	*Acinetobacter ursingii*	*Streptococcus peroris*
Acinetobacter schindleri	*Haemophilus sputorum*	*Comamonas kerstersii*
*Ehrlichia canis**	*Corynebacterium tuberculostearicum*	*Massilia varians*
Porphyromonas cangingivalis	*Kocuria palustris*	*Cedecea davisae*
*Ehrlichia muris**	*Fusobacterium nucleatum*	*Escherichia hermannii*
Staphylococcus petrasii	*Acinetobacter baumannii*	*Shigella dysenteriae*
Staphylococcus saprophyticus	*Leptotrichia amnionii*	*Klebsiella variicola*
Staphylococcus capitis	*Serratia marcescens*	*Erwinia billingiae*
Tannerella forsythia	*Ehrlichia ewingii**	*Pantoea agglomerans*
Acinetobacter soli	*Enterobacter cloacae*	*Stenotrophomonas maltophilia*
Massilia timonae	*Acinetobacter calcoaceticus*	*Brevundimonas vesicularis*
Haemophilus pittmaniae	*Enterobacter hormaechei*	*Roseomonas mucosa*
Lawsonella clevelandensis	*Klebsiella pneumoniae*	*Paracoccus yeei*

Prevotella amnii	*Acinetobacter nosocomialis*	*Streptococcus minor*
Cutibacterium acnes	*Actinomyces canis*	*Propionibacterium namnetense*
Moraxella osloensis	*Glutamicibacter creatinolyticus*	*Finegoldia magna*
Klebsiella quasipneumoniae	*Neisseria subflava*	*Rhizobium pusense*
Cardiobacterium hominis	*Cellulomonas denverensis*	*Neisseria perflava*
Streptococcus gallolyticus	*Pseudomonas argentinensis*	*Cronobacter sakazakii*
Pseudomonas stutzeri	*Kocuria polaris*	*Corynebacterium pseudotuberculosis*
Lawsonia intracellularis	*Massilia oculi*	*Gordonia polyisoprenivorans*
Phocaeicola abscessus	*Acinetobacter haemolyticus*	*Exiguobacterium acetylicum*
Staphylococcus aureus	*Pseudomonas luteola*	*Staphylococcus delphini*
Actinomyces neuii	*Skermanella aerolata*	*Staphylococcus pseudintermedius*
Staphylococcus succinus	*Fusobacterium canifelinum*	*Roseomonas aerofrigidensis*
*Ehrlichia chaffeensis**	*Citrobacter koseri*	*Pseudomonas otitidis*
*Ehrlichia minasensis**	*Cronobacter malonaticus*	*Anaplasma platys**

The asterisk (*) indicates bacteria historically known as tick-borne diseases

Regarding the analysis of alpha diversity in the group of males, the estimated richness index of Chao1 was 1,176,661.03 ± 701,282.75 in the case of females and 1,018,273.2 ± 404,826.27 in the case of males and there was no significant difference between the groups. ($t = 0.055$, $p = 0.931$). Similar results were shown by the Shannon diversity indices in females 12.74 ± 1.50 and in males 12.53 ± 1.18, with respect to the Simpson index the result was 0.96 ± 0.03 in the case of females and 0.96 ± 0.02 in the group of males and no significant difference was observed with the Monte Carlo test between the two groups respectively ($t = -0.890$, $p = 0.365$; $t = -0.683$, $p= 0.365$). Regarding beta diversity, the Bray-Curtis diversity matrix did not find a significant difference between sexes (PERMANOVA: pseudo-$F = 1.191$; $p = 0.166$).

Discussion

Based on the results obtained, we can say that the proposed hypothesis, which sought to find, through NGS, the V3 - V4 regions of the 16S rRNA gene, potentially pathogenic taxa that cause

TBD in the internal content of *R. sanguineus* s.l. ticks collected from domestic and peridomestic dogs was tested, therefore, we reject the null hypothesis.

The microbiota of *R. sanguineus*

Speaking at the phylum level, the study conducted found the same pattern of bacterial composition as most of the studies where Proteobacteria (mean= 88.36%), Actinobacteria (mean= 5.73%), Firmicutes (mean= 4.66%) and Bacteroidetes (mean = 0.72%) were the most abundant in that order both in this study and in similar studies (Narasimhan et al., 2014; Van Wyk, 2019). Proteobacteria being in most of the studies of bacterial communities in ticks the most abundant phylum (Narasimhan et al., 2014; René-Martellet et al., 2017; Trout Fryxell & DeBruyn, 2016; Zolnik et al., 2018; Zolnik et al., 2016). 316 genera belonging to the phylum Proteobacteria were identified, Proteobacteria being the most abundant and diverse phylum of the bacterial kingdom, hosting more than 450 bacterial genera, therefore they have adapted to a large number of ecosystems and have developed a large number of interactions with members of other bacterial phyla and even with their hosts, including arthropods (Van Wyk, 2019). Agwunobi et al. (2021) designed a study to assess bacterial diversity in ticks in two states of Nigeria and found Proteobacteria to be the second most abundant phylum in *R. sanguineus* parasitizing dogs (43.87%). On the other hand, Wang et al. (2021) they found in engorged ticks collected from dogs in China the phylum Proteobacteria as the most abundant (83.7%). Similarly, Portillo et al. (2019) found that Proteobacteria was the most abundant phylum in 4 genera of anthropophilic ticks, where Gammaproteobacteria followed by Alphaproteobacteria were the most abundant classes in *R. sanguineus*, similar to what was obtained in this study (see Fig. 2), in which genera were detected within of the phylum Proteobacteria with medical, veterinary and ecological importance, including some of them considered as TBP;

Coxiella, Ehrlichia, Pseudomonas, Salmonella, Escherichia, Bordetella, Anaplasma, Campylobacter, Yersinia and *Brucella,* just to mention a few genera detected.

Coxiella was the overrepresented genus within the phylum Proteobacteria with a 66.16% relative abundance in the pools obtained, in addition to having a prevalence of 100%, testing positive in all the pools obtained. Similarly, it has been found to be the most frequent in 315 pools of *R. sanguineus* s.l. collected from ticks parasitizing dogs in Spain (Vila et al., 2019). René-Martellet et al. (2017) analyzed the composition of the bacterial microbiome of *R. sanguineus* in three different regions of the world (Senegal, France, and Arizona) with different *R. sanguineus* genotypes (tropical and temperate) and with different genders (males and females) concluded that regardless of the variables analyzed *Coxiella, Rickettsia* and *Bacillus* were the most dominant taxa. The capacity of obligate and facultative endosymbionts of ticks and their close symbiotic relationship with the genus *Rhipicephalus* is known (Díaz-Sánchez et al., 2019; Duron et al., 2017). *Coxiella*-like endosymbiont (CLE) being a vertically transmitted gram negative obligate endosymbiont common in *R. sanguineus* and responsible for various elementary physiological functions for the survival of the parasitic organism; this endosymbiont typically infects the ovaries (to ensure maternal transmission) and the distal part of the Malpighian tubules, suggesting a possible role in nutrition, osmoregulation, or excretion (Bonnet et al., 2017; Guizzo et al., 2017; Machado-Ferreira et al., 2011). CLE has been shown to have a high maternally transmitted capacity as it has been detected in >99% of tick eggs tested (Duron et al., 2015b). Therefore, it is considered a common bacterium in female hard ticks, where it has been found in greater abundance than in male ticks (René-Martellet et al., 2017). That said, in this investigation no significant difference was found in the bacterial composition between males and females. On the other hand, the genome of the genus *Coxiella* has the capacity to encode metabolic pathways for the synthesis of amino acids and vitamins; like the B complex vitamins;

biotin (B7), folic acid (B9), and riboflavin (B2) and their cofactors, which cannot be obtained in sufficient amounts from blood-based diets (Gottlieb et al., 2015). This is why not only *Coxiella* spp. is recognized as ubiquitous and innocuous for *R. sanguineus*, but supports the hypothesis of being an obligate endosymbiont in some species of ticks. (Guizzo et al., 2017; Smith et al., 2015). In our study, potentially pathogenic species were found, such as *C. burnetti* (see table 2). The presence of *Coxiella*-LE has been shown to be closely linked to *C. burnetti* (Duron et al., 2015a). Previous studies using the same methodology found this relationship in the soft tick *O. turicata* (Barraza-Guerrero et al., 2020). Due to all of the above, subsequent studies on the impact of the genus Coxiella on reproduction, competition, well-being and its relationship with vector competition should be explored in greater depth to try to find possible strategic control points for *R. sanguineus* s.l.

It is important to note that the selected ticks were feeding on dogs at the time of collection, so the blood of the dogs can also interfere with the composition of the tick's microbiota at that time, especially those female ticks that were engorged. So this should be considered as a factor that influences the results obtained. Studies have determined that the type of host ticks feed on influences the bacterial composition of ticks. (Swei & Kwan, 2017; Zhang et al., 2019). Different analyzes of the microbiota in ticks have detected changes in the microbial composition of ticks after feeding. Brinkerhoff et al. (2020) reported that engorged female *I. scapularis* had lower microbial richness compared to unfed males and nymphs, suggesting an impact of blood feeding on tick microbiome diversity. Zolnik et al. (2018) zolnik similarly evaluated the influence of blood feeding on the microbiome of the black-legged tick Ixodes scapularis. They found that unfed nymphs lose bacterial diversity after feeding; They also obtained significant differences between different categories of ticks (fed nymphs, engorged nymphs and adults) unweighted UniFrac ($F = 5.37$, $R2 = 0.32$, $P <$

0.001) and weighted UniFrac (F = 1.57, R2 = 0.12, P < 0.054). However, Zhang et al. (2014) they found no significant difference in diversity with the Shannon index between fed and non-fed ticks (6,472 vs. 6,325). Due to the above, future studies could control this effect on the bacterial composition of ticks and compare fed *R. sanguineus* ticks collected from dogs as in the present study with non-fed ticks and analyze the influence of this factor.

Tick-borne pathogens found

Speaking in particular of those bacteria considered as TBP or those bacteria associated with ticks and transmitted by them, in the study carried out seven bacteria were found (Table 2), among them *C. burnetti* was the most abundant of all the TBPs, something expected. if we consider that *Coxiella* as a genus was the most abundant in the study. Unlike other studies where the prevalence of *C. burnetti* has been around between 5% and 10%, in this study we found *C. burnetti* in 100% of the pools analyzed (Duron et al., 2015b). *C. burnetii* is the bacterium responsible for causing the disease Q fever, which affects both animals and humans (van Roeden et al., 2019). It must be considered that the main reservoirs of Q fever are ruminants and sometimes dogs and infection generally occurs through inhalation of contaminated aerosols, with farmers and veterinarians being the most susceptible (Melenotte et al., 2016). La enfermedad puede cursar como aguda o crónica y puede llegar a ser fatal con una mortalidad de 1% y 13% (Groten et al., 2020; van Roeden et al., 2019). Today the horizontal transmission that occurs due to the bite of different species of ticks is recognized (Duron et al., 2015b; McDade & Marrie, 1990; Philip, 1948; Smith, 1941; Smith & Science, 1941). As previously established, the genus Coxiella is an obligate endosymbiont of some tick species, including R. sanguineus, where they are essential for tick homeostasis in terms of nutrition, development, and reproduction. (Ben-Yosef et al., 2020; Duron et al., 2017; Duron et al.,

2015b; Guizzo et al., 2017; Smith et al., 2015). Of the 67 species of ticks (order Ixodida) registered in Europe and America, between 15 and 17 species of hard ticks have been detected as carriers of both CLE and *C. burnetti*, including genera belonging to *Dermacentor, Ixodes, Haemaphysalis* and *Hyalomma* (Körner et al., 2021). *R.* sangunieus is a frequent carrier of C. burnetti, being detected in different abundances in ticks collected from domestic and wild animals, as well as in humans in various parts of Europe (Bogunović et al., 2018; Chisu et al., 2018; Santos et al., 2018; Satta et al., 2011; Socolovschi et al., 2012; Toledo et al., 2009; Varela-Castro et al., 2018). It has also been identified the American continent (Barraza-Guerrero et al., 2020; Mioni et al., 2020; Noda et al., 2016; Pacheco et al., 2013; Sanders et al., 2008) and in Mexico and in the same study area of this research in the soft tick *Ornithodoros turicata* parasitizing the bolson tortoise *Gopherus flavomarginatus* (Barraza-Guerrero et al., 2020). Despite the fact that the main source of infection in humans is inhalation, it is presumed that among animals the tick is responsible for heterospecific transmission as well as spatial dispersion among vertebrates, thus keeping the pathogen in circulation in ecosystems (Bogunović et al., 2018). It has been determined that there is a positive correlation between *C. burnetti* seropositivity and the presence of ticks in cattle (Asadi et al., 2014; Cantas et al., 2011; Duron et al., 2015b). Regarding the transmission to humans due to tick bites, little has been studied (Beaman & Hung, 1989; Nett et al., 2012; Pascual-Velasco et al., 2007) and in these cases another source of transmission such as inhalation cannot be excluded, so the vectorial capacity of the tick to *C. burnetti* seems limited and they preferably act as an ecological bridge for the transmission of wildlife and domestic hosts (Astobiza et al., 2011; Duron et al., 2015b). There is a constant presence of the pathogen and an important role of ticks as a reservoir of Q fever. Especially with regard to *R. sanguineus* reported with a high prevalence (10.53%) of *C. burnetti* collected from stary dogs (Bogunović et al., 2018). It indicates the importance of dogs in the epidemiology of *C. burnetti* in different regions. It should be noted that to our knowledge in Mexico

there is still no study where the presence of *C. burnetti* in *R. sanguineus* has been reported and even less that a case of Q fever has been attributed to it because of a tick bite, more studies Subsequent tests are necessary to determine the vectorial potential of the Q fever tick.

Other relevant TBPs found in this study were Rickettsiales, with the *Ehrlichia* genus being the third most abundant in ticks; *Ehrlichia canis, Ehrlichia muris, Ehrlichia chaffeensis, Ehrlichia minasensis* and *Ehrlichia ewingi* were the species found, so all recognized potentially pathogenic species of the genus *Ehrlichia* were found only except for *E. ruminantium*. This demonstrates that *R. sanguineus* is a reservoir of a mixture of potentially pathogenic Ehrlichia species and the 16s rRNA gene-based NGS technique is highly effective for analyzing TBD in epidemiological studies. *R. sanguineus* s.l. is the main vector responsible for the worldwide distribution of *E. canis* that causes monocytic Ehrlichiosis (Groves et al., 1975; Khovand et al., 2022). *E. canis* can cause disease in dogs and other canids, which are the main reservoir host for the disease, and can also cause disease in humans, although the incidence is relatively low (Iweriebor et al., 2017). This pathogenic agent has been detected in America (Barrantes-González et al., 2016; Beall et al., 2012; Sosa-Gutierrez et al., 2013; Vinasco et al., 2007), Europe (Bezerra-Santos et al., 2021; Ebani, 2019; Schäfer et al., 2019), Africa (Heylen et al., 2021; Sugimoto et al., 2017), Asia (Kaewmongkol et al., 2017; Khovand et al., 2022; Mengfan et al., 2020) and recently in Oceania (Neave et al., 2022), so it covers all continents.

Mexico is an endemic country in *E. canis,* being *R. sanguineus* an important reservoir of the pathogen in the country. In a study conducted by (Sosa-Gutierrez et al., 2016), using molecular techniques in 22 states of the Mexican Republic, where they analyzed Tick-borne rickettsial diseases (TBRD) in 477 pools with a total of 1,107 ticks collected from both hosts and the environment, they

found that *E. canis* was the second pathogen more frequent and with a distribution mainly in the northern states of Mexico, coinciding with our results, where *E. canis* was found in 4 of the 10 pools analyzed and was the most abundant among the *Ehrlichia* spp. detected. It is important to highlight that *R. sanguineus* was the most frequently tick found in the Sosa study with 52.2%. In the present case *R. sanguineus* had a frequency of 100% of the tick samples collected. which speaks of the relevance of this species as a vector in the Mexican territory (Sosa-Gutierrez et al., 2016). However, in other studies, they have found a high prevalence of *E. canis* in stray dogs and not in the ticks that parasitize them (Aguiar et al., 2007).

It is important to highlight that both the potential pathogens found in ticks, as well as the TBPs, could be being transmitted to the dogs from which they were extracted. Ochoa (2003) reported a 33.1% prevalence of *E. canis* in dogs with febrile states and of these 79% had ticks at the time of the analysis. Ojeda-Chi et al. (2019) reported a 38.46% cumulative incidence in the blood of dogs with 72 positive dogs and a prevalence of 29.26%, similar to that detected by Ochoa (2003), as well as what was detected in the present study, where it was positive in 4 pools, which represents 40% in terms of frequency.

In the Americas, the species with the greatest public health problem is *E. chaffeensis*, also the cause of Monicitic human Ehrlichosis, a disease that causes fever, leukopenia, thrombocytopenia, coagulation disorders, and liver and nervous disorders that, when not treated properly adequate timely the disease can have a mortality of 3 to 5% (Alcántara-Rodríguez et al., 2020; Geier et al., 2016; Gongóra-Biachi et al., 1999). *E. chaffeensis* has been reported in Mexico and with greater frequency in the north-central states of Mexico, the same region of this investigation (Sosa-Gutierrez et al., 2016).

E. minasensis today is considered a widely distributed bacterium, being reported in Malaysia, Ethiopia, China, Pakistan, the Mediterranean island of Corsica, Israel and South Africa. (Cicculli et al., 2019; Hailemariam et al., 2017; Iweriebor et al., 2017; Koh et al., 2018; Li et al., 2019; Rehman et al., 2019; Thomson et al., 2018). It has been detected in livestock and wildlife ticks around the world (Aguiar et al., 2014; Elhachimi et al., 2021; Gajadhar et al., 2010; Lobanov et al., 2012; Qiu et al., 2022). Reported mainly on ticks of the genera *Rhipicephalus*, *R. appendiculatus, R. eversti eversti*, and *R. bursa* (Iweriebor et al., 2017) as well as in the genera *Amblyomma* (Cabezas-Cruz et al., 2012; Carvalho et al., 2016), *Hyalomma* (Cicculli et al., 2019; Rehman et al., 2019) and *Haemaphysalis* (Li et al., 2019). This suggests that *E. minasensis* has more than one vector species and therefore different vertebrate host reservoirs. However, up to the time this document was written, it is the first detection of the species in Mexico (Cabezas-Cruz et al., 2019; Iweriebor et al., 2017). *E. ruminantium* was the only taxon associated with bovine Ehrlichiosis previously (Cabezas-Cruz et al., 2019; Elhachimi et al., 2021). Finding *E. minasensis* at the other end of the phylogeny of *E. ruminantium* and as mentioned before more related to the monocytotropic pathogen *E. canis* (Cabezas-Cruz et al., 2016). However, like several *Ehrlichia* species it can cause clinical disease in animals (Qiu et al., 2022). *E. minasensis* disease has been detected in calves where they were experimentally infected (Aguiar et al., 2014). Moura de Aguiar et al. (2019) detected for the first time the presence of *E. minasensis* by natural infection in calves parasitized with *R. microplus*, developing all the usual clinical signs of Ehrlichiosis (fever, depression, lethargy and thrombocytopenia). *E. minasensis* is not an exclusive pathogen of ruminants and wild animals, it has also been detected in canines. In fact, it is considered that it evolved from *E. canis* in dogs to *E. minasensis* in order to infect cattle (Cabezas-Cruz et al., 2014) in different parts of the world (Melo et al., 2021; Thomson et al., 2018). Melo et al. (2021) detected the presence of antibodies against *E.*

canis and *E. minasensis* in the serum of dogs in Brazil by means of indirect immunofluorescence assay (IFA) and confirmed by ELISA, this work concludes that it is the first report of the presence of *E. minasensis* in dogs in Brazil and highlights the importance of considering other *Ehrlichia* agents infecting dogs in the country and broadening the diagnostic panorama of different Ehrlichias, particularly in areas with high abundance of ticks. Due to all of the above, the presence of this potentially pathogenic bacterium in *R. sanguineus* in the Comarca Lagunera region could be a potential pathogen for both canines, which are the main hosts of *R. sanguineus* (Thomson et al., 2018), as well as with other animals such as dairy cattle that is so important in the region and cause consequences in the livestock industry. Therefore, veterinarians, physicians and epidemiologists should be attentive to the presence and distribution of this new pathogen found in the region.

E. ewingii is another TBP of veterinary and medical relevance found in ticks parasitizing dogs (Thomas et al., 2009), and we also detected it in this study. Beall et al. (2012) conducted a large-scale seroprevalence study in dogs of *Ehrlichia* spp. in the US, finding the highest prevalence in *E. ewingii* (5.1%), demonstrating greater exposure to this agent in a very large and relatively close region, as well as bioclimatic conditions similar to those of the region analyzed in this study. *E. ewingii* is mainly transmitted by *Amblyomma americanum* (Campos-Calderón et al., 2016). *E. chaffeensis together with E. ewingii* are the main etiological agents causing human Ehrlichiosis in the US (Pritt et al., 2011). During 2008–2012, 55 cases of *E. ewingii* infection were reported through the Nationally Notifiable Diseases Surveillance System (NNDSS) in the US. The national incidence range was 0.04 cases per million people per year. The hospitalization rate was 77% and no deaths were reported. Mainly affecting immunosuppressed people and children under 5 years of age where the lethality range increases, however, immunocompetent people and adults can also contract the disease (Heitman et al., 2016).

E. muris in ticks is a TBP that is generally accompanied by other rickettsial species such as E. *canis, E. chaffensis* or *E. ruminantium* in different studies and in different regions (Iweriebor et al., 2017; Livanova et al., 2018). It should be noted that *E. muris* is also zoonotic. Two studies, one carried out from 2007 to 2013 and another in 2009, were detected in several US states, especially in Minnesota and Wisconsin, causing a clinical disease, being a bacterium that is mostly related to rodents, as its main reservoir (Johnson et al., 2015; Pritt et al., 2011; Telford Iii & Goethert, 2008; Xu et al., 2018). *E. muris* can cause infection in humans with a picture similar to other Rickettsial diseases (headache, nausea, vomiting and fatigue) (Telford Iii & Goethert, 2008). Xu et al. (2018) analyzed 8,760 ticks from 45 US states, where the general prevalence was very low, only in five ticks was the agent detected (0.057%) in the species *I. scapularis* and *I. cookei.* In the case of this study, the presence of *E. muris* was detected in two of the pools, where the content of 5 ticks was analyzed, which suggests that the presence of the potential pathogen may have a significant prevalence in ticks from urban dogs of the region studied and with the risk of enzootic cycle towards dogs and humans. However, further studies on individual ticks will be necessary to estimate the prevalence of *E. muris* in the region. On the other hand, despite the fact that *E. muris* is known to potentially cause disease in humans in countries such as Venezuela, Russia, and the United States (Johnson et al., 2015; Nefedova et al., 2008; Perez et al., 1996; Pritt et al., 2011). In Mexico, no information has been documented and there are no reports attributed to *E. muris* in humans and to our knowledge this is the first report of *E. muris* in the brown dog tick in the country, which represents a warning of the possibility of an emerging disease outbreak in the region and speaks of the need for active epidemiological surveillance for this and other possible TBPs circulating in the environment.

Anaplasma platys was a TBP found in *R. sanguineus* tick pools, although it was only found in one of the pools and in low relative abundance, the presence of this pathogen in the region under study is equally important. *Anaplasma platys* is an obligate intracellular tick-borne pathogen of dogs, which causes canine infectious cyclic thrombocytopenia (CICT) (Ramos et al., 2014). *R. sanguineus* has been linked to the transmission of this bacterium to its vertebrate hosts, mainly due to the recovery of *A. platys* DNA in ticks (Ramos et al., 2014; Sanogo et al., 2003; Snellgrove et al., 2020), as well as its recurrent coinfection with *E. canis,* of which the brown dog tick is the reservoir (Cicuttin et al., 2015; Lara et al., 2020; Lauzi et al., 2016; Pinyoowong et al., 2008; Piratae et al., 2019; Yabsley et al., 2008) and because A. platys infections in dogs is closely related to severe parasitism in dogs (Almazán et al., 2016). However, the competence of this tick as a vector of A. platys has not been fully elucidated. Despite this, different studies have provided evidence to strengthen this hypothesis. Ramos et al. (2014) reported a high prevalence both in dogs (52.9%) as well as in nymphal and adult ticks (48.3%) in an endemic region of the tick and the agent, and reaffirms the competence of *R. sanguineus* as a vector of *E. platys* due to the high occurrence of the bacteria in the dogs analyzed as well as in the ticks that parasitize them. In another more recent study, the competence of *R. sanguineus* to transmit the pathogenic agent to pathogen-naïve New Zealand white rabbits, which have not been identified as susceptible to infection, was also verified, demonstrating that *R. sanguineus* has a very efficient ability to transmit the pathogen to several generations of ticks through the transovarian, transstadial, and horizontal pathways by cofeeding, and demonstrated that rabbits also generated *A. platys* seropositivity, verifying the ability of *R. sanguineus* to *inoculate A. platys* to its hosts (Snellgrove et al., 2020). This strengthens the fact that this TBP is a bacterium of epidemiological relevance. On the other hand, cases have also been reported in humans with non-specific symptoms that include fever, headache, myalgia and lethargy (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014; Maggi et al., 2013). Relatively few studies

have reported A. platys infection, mainly because the studies focus more on the identification of the agent in the hosts, especially dogs. Bezerra da Silva et al. (2016) identified *E. platys* in samples of *R. sanguineus* in Cuba, with a prevalence of 9.80% in ticks in 5 positive pools out of a total of 49, in addition to reporting a prevalence in dogs of 16%, similar to that in the present study where, although it was not evaluated in terms of prevalence, *A, platys* was detected in a pool of ten totals that would correspond to 10%. Few studies have evaluated the occurrence of *Anaplasma spp.* in Mexico. However, *Anaplasma spp.* in both canines and ticks *R. sanguineus* (Escárcega-Ávila et al., 2018; Movilla et al., 2016; Sosa-Gutierrez et al., 2016). There is a very relevant study in terms of the present investigation, Almazán et al. (2016) detected by molecular techniques the presence of *A. platys* and *E. canis* in the same region studied (La Comarca Lagunera). Where they found 3 DNA samples positive for *Anaplasma* and none for *E. canis* in *R. sanguineus.*

Conclusions

The results obtained contribute to previous studies where they recognize the region under study and Mexico in general as an endemic area for TBP, especially regarding tick-borne ricketsial pathogens (TBRP). On the other hand, it demonstrates that the NGS is ideal for infection analysis and TBD monitoring as an epidemiological tool to assess the status of vector-borne zoonotic diseases. In the study, potentially pathogenic species were found in the brown dog tick *(R. sanguineus)*, as well as TBP never reported in the region and even in Mexico.

To the best of the author's knowledge, this study presents the first report of *R. minasensis,* which is a recently discovered potential pathogen. It invites researchers to carry out in-depth complementary

studies to detect new organisms never reported in Mexico that could become a potential public health problem at some point.

The results of the study show that in Mexico and specifically the Comarca Lagunera region, potentially deadly agents persist circulating in the ectoparasites of dogs in close contact with humans. The TBPs and their distribution are constantly changing, so epidemiological studies that help determine the importance of the TBPs found in the region could contribute to minimizing diseases and lethal cases caused by tick bites.

Supplementary Materials: Rarefaction graph is available in the archive Fig. A. The complete relative abundance (%) of Phylum up to species of the bacterial taxa collected from the internal contents of *R. sanguineus* ticks are deposited in Table A.

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6.3 Understanding the long-lasting association between humans and tick-borne diseases thru next-generation sequencing

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Understanding the long-lasting association between humans and tick-borne diseases thru next-generation sequencing

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Abstract: While dogs have been humans´ most enduring animal companion, ticks are the most common external parasites in dogs. Increases in domestic dog neglected populations have increased the risk of contact with their ticks, especially in places where tick bite diseases are endemic. We aimed to characterize the bacterial blood profile of people who were either exposed (HE) or non-exposed (HC) to tick bites using next generation sequencing (NGS). An average of 57,253 sequences assembled was obtained for HC in comparison with 110,009 sequences for HE. The average of quality bacterial sequences was 18,270 for HC and 47,135 for HE. Non-significant differences occurred regarding the alpha diversity between experimental groups, yet, differences in beta diversity were certainly significant. In HE, some potentially pathogenic bacterial taxa were found to be the most abundant: *Kokuria* ($\bar{x}$ = 14.59%) *Staphylococcus* ($\bar{x}$ = 3.05%) and *Treponema* ($\bar{x}$ = 2.93%), in addition to *Chlamydia*, *Clostridium*, as well as *Ehrlichia*, considered as a TBD. The study established differences in the bacterial compositions of people who were exposed to a tick bite regarding those who have never been exposed to ticks. Future studies including an augmented number of replicates should give an improved and sound understanding of the actual fragmentary knowledge we have regarding the blood-microbiome profile, especially pathogenic microorganisms related to TBD; the last being a crucial pending assignment.

Keywords: blood; *Ehrlichia*; microbiota; ticks; tick bites; tick-borne diseases

1. Introduction

In the past two decades tick-borne diseases (TBD) caused by bacteria have tripled [1] and the geographic distribution of ticks has spread widely [2]. Ticks are found within the arthropods that feed on human blood (HBFA), considered a serious problem in public health around the world because of the physical damage caused by adhering to the host and the anaphylactic reactions caused by saliva [3]. Moreover, a wide range of microorganisms that inoculate the host and that cause potentially serious diseases for both domestic and wild animals as well as for humans include bacteria, viruses, fungi, protozoa, and helminths [4]. These are diseases-causing organisms considered as neglected or linked to poverty and are currently the main priorities for the World Health Organization (WHO) along with other international organizations [5]. Such is the case of diseases that include viruses, (i.e., West Nile fever, Crimean hemorrhagic fever- Congo, Omsk hemorrhagic fever and Colorado tick fever), bacteria (i.e., human monocytic ehrlichiosis, human granulocytic ehrlichiosis, Q-fever, rocky mountain spotted fever, borreliosis, relapsing fever and tularemia), fungal (i.e., dermatophytosis), and protozoa as well (i.e., theileriosis, babesiosis,) [6].

In recent years new pathogens have appeared, mainly due to genetic changes that helped the spread of ticks and their pathogens. For example, the appearance of six new bacterial TBDs that had not been previously reported, considered emerging in some areas and re-emerging in

others due to the expansion of the urban sprawl and the availability of hosts [1]. The transmission of these agents occurs simultaneously and is considered a polymicrobial infection, which not only makes diagnosis difficult, but also complicates the clinical picture and prognosis, often resulting in lethal outcomes [2]. Today it is known that blood is not a sterile medium and there are many bacterial phylotypes, as well as archaea, viruses, and fungi that live in our organism in different relation to our body: commensal, mutualistic or pathogenic. The concept of Healthy Human Blood Microbiome (HBM) has grown strongly and the configuration of healthy microbiomes as diseased has been elucidated and comparative analyzes of bacterial profiles are increasingly frequent [7].

The Comarca Lagunera, located in northern Mexico, has been considered an endemic and re-emerging area of the Spotted Fever of the Rocky Mountains(i.e., RMEF), a highly virulent disease with high mortality, caused by *Rickettsia rickettsii* [8]. In fact, in this region the incidence in 2014 ranged from 3 cases per 10,000 inhabitants, showing the highest incidence in Mexico [9]; this health problem was associated to the presence of street dogs aligned to the presence of the brown dog tick *Rhipicephalus sanguineus* (sensu lato) [9, 10].

TBD are common in veterinary and medical clinical settings. However, the studies have been mainly directed to the search for *R. rickettsii* not only in the region but in Mexico, even though that RMSF is a

highly important disease. Therefore, it is crucial to define the presence of other bacteria in the blood from people exposed to tick bites, due to the fact that some tick-borne diseases can have symptoms similar to the RMSF, and other kind of TBD. Such scenario may in turn generate a misdiagnose, such as human monocytic ehrlichiosis or human granulocytic ehrlichiosis, among other diseases. Based in such findings, we hypothesized that the bacterial profile and dynamics of people exposed to tick bites are different from people who have never been exposed to this factor. This study compares the blood bacterial profile of people bitten by ticks and people not exposed to ticks in the Comarca Lagunera, Mexico. To the best of our knowledge, this would be the first work applying more precise molecular techniques such as NGS to this topic. This will provide valuable information for the scientific community interested in public health that intends to analyze and control the risks associated with tick bites.

2. Materials and Methods

2.1 Location

The study was conducted in the north central part of Mexico, at the Comarca Lagunera, a region were the municipalities of Torreón, Gómez Palacio, Tlahualilo, Mapimí and Matamoros converge.

2.2 Groups and samples

The blood healthy apparent volunteers (n=12) was taken during August - October 2019; people not exposed to a tick bite was considered the

116 control group (i.e., HC, n=7), while people exposed to a tick bite within
117 the previous year was considered the exposed group (i.e., HE, n=5).
118 Blood was collected by venipuncture, previously sanitized the puncture
119 area with 96% alcohol and deposited in Vacutainer K3EDTA tubes
120 (Vacutainer K3E, BD, USA); three ml per person were extracted and
121 ten drops of blood (i.e., 50 mg in wet weight) of each collected sample
122 were deposited in a BashingBead™ lysis tube of the DNA Microprep
123 kit, from the Zymo Research™ brand; 750 µL of lysing/stabilizing so-
124 lution (Xpedition™) were added. Each tube was processed in a cell dis-
125 ruptor (TerraLyzer ™) for 30 s [11]. It is formally stated that all the
126 collected samples were taken with the consent of the people involved in
127 this study while the authors reserve the confidentiality (i.e., personal
128 information) of the results obtained.

129 *2.3 DNA Extraction and Visualization*

130 The Zymobiomics DNA Miniprep kit (Zymo Research™) was used to
131 extract the blood DNA in a UV laminar flow hood with all sterility pro-
132 tocols. The DNA extraction products were run on 1.2% agarose gels at
133 80 V for 45 min in a Bio-Rad electrophoresis chamber to visualize the
134 presence of DNA. Visualization was carried out on a GelMaxTM pho-

todocumenter (UVP®, Upland, CA, USA). The concentration and quality of DNA obtained from the samples was measured on a Qubit® 3.0. (Invitrogen, Carlsbad, CA, USA) [11].

2.4 16S rRNA Gene Amplicon Sequencing

Amplification of the V3-V4 region of the 16S rRNA gene was made using the following primers [12]: S-D-Bact-0341-b-S-17 5'-CCTACGGGNGGCWGCAG-3' and S-D-Bact-0785-a-A-21 5'GACTACHVGGGTATCTAATCC-3'. Subsequently, the Illumina PCR protocol was implemented using 12.5 µL of MyTaq™ Ready Mix 1X (Bioline®, London, UK), 1 µL of each primer (10 nM), 5 µL of DNA (25 ng total) and 5.5 µL of ultrapure H_2O; the following cycle was used: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min in a Labnet Multigene™ Gradient PCR thermal cycler (Labnet International, Inc. Global, Edison, NJ, USA). Amplicons were purified with 0.8% Agentcourt® AMPure® XP beads (Beckman Coulter Inc., Brea, CA, USA). Then, the amplicons were labeled using Nextera XT Index Kit™ (Illumina, Inc., San Diego, CA, USA) for the creation of the libraries, following the Illumina protocol [13], using 25 µL of MyTaq™ Ready Mix 1X (Bioline®), 5 µL of each primer (N7xx and S5xx), 5 µL of DNA, and 10 µL of ultrapure H_2O;

the following cycle was used: 95 °C for 3 min; 10 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. Finally, quantification, normalization (i.e., equimolarity), library pooling, and next-generation massive sequencing (MiSeq, Illumina, San Diego, CA, USA) of 2 x 250 paired end reads were performed following the Illumina 16S protocol [14].

2.5 Bioinformatic Analysis

Sequence analysis was performed using Quantitative Insights into Microbial Ecology (QIIME) [15]. The assembly was made using PEAR [16] with Q30. Chimeras were removed with USEARCH [17]. Operational taxonomic units (OTUs) were selected using UCLUST [17] at 97% similarity; taxonomy was assigned using EzBioCloud database [18]. Random rarefaction was carried out at a depth of 8,400 sequences. From here, the Chao1 estimated richness index, also Shannon and Simpson alpha diversity indexes were calculated. Non-parametric t-tests (i.e., false discovery rate correction) were applied to test differences ($p < 0.05$) between populations for each index. The Bray–Curtis beta diversity was calculated; PERMANOVA was applied to test significant differences ($p < 0.05$) of the blood microbiota between groups, and it was visualized

using principal coordinate analysis (PCoA) in Emperor [19]. The relative bacterial abundance was obtained at all taxonomic levels. The most abundant phyla were represented in stacked bar graphs using R, and the family and genera were visualized in a heatmap using Morpheus (https://software.broadinstitute.org/GENE-E/; accessed 15 September 2021). To establish the bacterial taxa at phylum, family and genus levels that contributed the most to the differentiation of the blood microbiota between both populations, a percentage similarity analysis SIMPER [20] was developed using the Bray–Curtis matrix in PAST 4.0. Some of the taxa that contributed the most to the difference between groups were selected and a Mann–Whitney U test ($p < 0.05$) was applied to test for significant differences between populations. Finally, a LEfSe (i.e., linear discriminant analysis effect size) analysis was performed to statistically and biologically determine the key biomarkers which contribute the most to the differences observed between populations. The clades selected were those less than 0.05 in the alpha value of the Kruskal–Wallis factorial test > 4.0 in the logarithmic LDA score [21]. This analysis was made on the website http://huttenhower.sph.harvard.edu/lefse/ (accessed 25 September 2021).

3. Results

An average of 57,253 sequences assembled was obtained for HC in comparison with 110,009 sequences for HE. The average of quality bacterial sequences was 18,270 for HC and 47,135 for HE. The mean of OTU's was 1,892 and 2,774.4 for HC and HE, respectively (Table 1). The OTUs obtained in the analysis corresponded to 15 phyla for HC and 23 for HE, 29 classes for HC and 54 for HE, 51 orders for HC and 96 for HE, 90 families for HC and 178 for HE, 175 genera for HC and 397 for HE, and finally 187 species for HC and 420 for HE.

Table 1. 16S rRNA V3-V4 region sequences obtained from blood of volunteers exposed (HE) and not exposed (HC) to tick bites in the Comarca Lagunera, north central Mexico.

Location	Total reads	Assembled	Discarded	QS[1]	QB[2]	OTUs[3]
HC1	175,290	46,752	128,538	43,249	11,405	1327
HC2	217,267	88,206	129,061	75,542	27,138	2816
HC3	143,397	58,033	85,364	54,139	20,690	1973
HC4	152,472	59,719	92,753	52,483	17,880	2355
HC5	99,672	44,804	54,868	43,911	19,649	1506
HC6	117,165	46,005	71,160	43,618	8417	1381
HC7	133,492	57,252	76,240	52,910	22,708	1886
Mean	148,394	57,253	91,141	52,265	18,270	1892
HE1	114,523	69,396	45,127	66,033	10,472	1923
HE2	106,698	66,459	40,239	62,136	14,831	2373
HE3	115,289	73,685	41,604	71,260	40,107	1692
HE4	601,639	272,832	328,807	265,235	145,328	5853
HE5	133,166	67,673	65,493	61,833	24,937	2031
Mean	214,263	110,009	104,254	105,299.4	47,135	2774.4

[1] QS = quality sequences after chimeras' elimination
[2] QB = quality bacterial sequences
[3] OTUs = operational taxonomic units

No significant differences in the estimated richness index Chao1 occurred between the two groups ($t = 0.271$, $p < 0.789$); the mean for HC was 190.92 ± 49.80 and for HE was 204.57 ± 106.23. The Shannon diversity index was similar between groups ($t = 1.67$, $p < 0.128$); the mean for HC was 4.50 ± 0.44 and for HE was 5.13 ± 0.74. The Simpson's diversity index was also similar ($t = 0.629$, $p < 0.565$); mean 0.91 ± 0.03 and 0.93 ± 0.06, respectively. Nevertheless, significant difference was found comparing the beta diversity between the two groups with the Bray-Curtis index (PERMANOVA: pseudo-F = 1.74; p = 0.002). The results indicate that, although both populations are diverse by themselves, they are significantly different, and each community has a different bacterial profile, as it is demonstrated in the specific segregation between groups in the PCoA (Fig. 1 and 2).

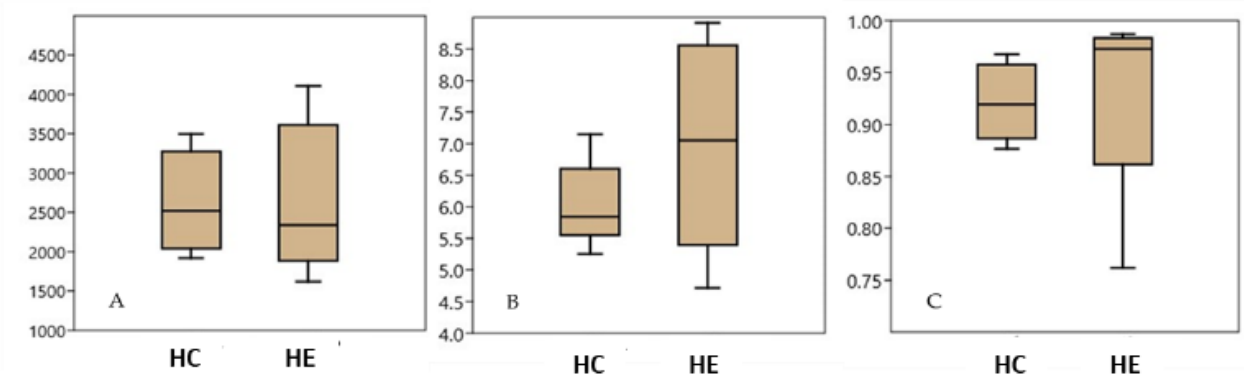

Figure 1. Boxplots of **(A)** Chao1 richness index, **(B)** Shannon alpha diversity index, **(C)** Simpson alpha diversity index for the bacterial microbiota of two groups of volunteers (not exposed = HC and exposed = HE to tick bites) in the Comarca Lagunera, north central Mexico.

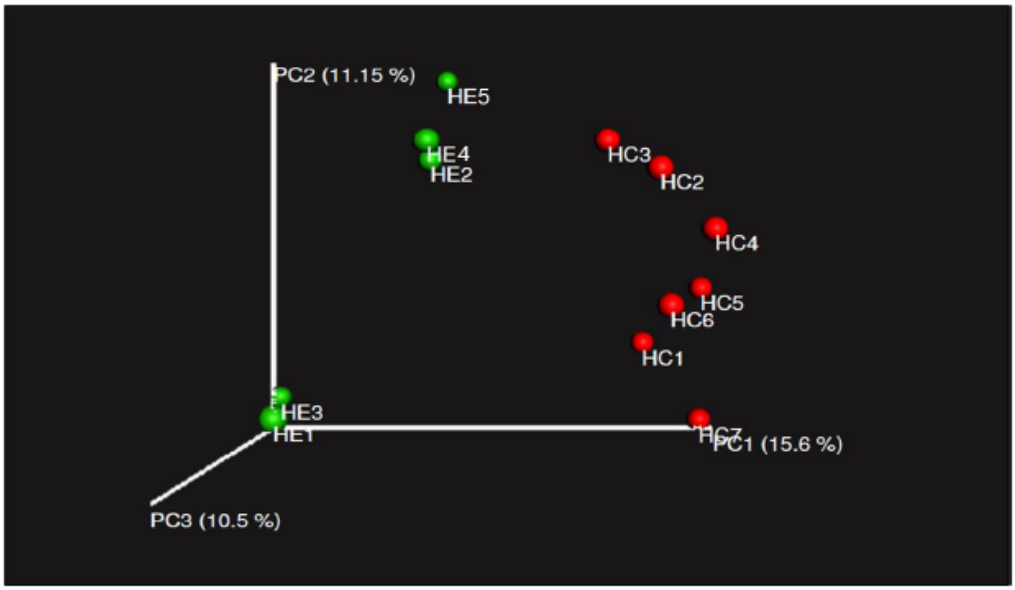

Figure 2. Principal Coordinate Analysis (PCoA) plot based on Bray-Curtis index representing blood samples of volunteers not exposed (HC, red spheres) and exposed (HE, green spheres) to tick bites in the Comarca Lagunera, north central Mexico.

The relative abundance analysis showed that the phylum Firmicutes was the most profuse ($\bar{x}$ = 55.54%) in the HC, followed by Proteobacteria ($\bar{x}$ = 19.92%), Actinobacteria ($\bar{x}$ = 11.69%) and Bacteroidetes ($\bar{x}$ =

239 5.95%). Besides, in the HE group, the most abundant phyla was Actino-
240 bacteria ($\bar{x}$ = 32.02%), followed by Firmicutes ($\bar{x}$ = 26.17%), proteo-
241 bacteria ($\bar{x}$ = 17.04), Bacteroidetes ($\bar{x}$ = 9.11) and Spirochaetes ($\bar{x}$ =
242 3.17) (Fig. 3). At the family level, the most HC abundant taxon was
243 Bacillaceae ($\bar{x}$ = 29.54), followed by Ruminococcaceae ($\bar{x}$ = 10.99),
244 Christensenellaceae ($\bar{x}$ = 8.89), Micrococcaceae ($\bar{x}$ = 4.20), and Halo-
245 monadaceae ($\bar{x}$ = 4.11), while in the HE it was Micrococcaceae ($\bar{x}$ =
246 17.41), Bacillaceae ($\bar{x}$ = 6.01), Peptoniphilaceae ($\bar{x}$ = 4.03), Rumino-
247 coccaceae ($\bar{x}$ = 3.94) and Staphylococcaceae ($\bar{x}$ = 3.41) (Fig. 4). Fi-
248 nally, at the genus level, the relative abundance analysis showed *Anaer-*
249 *obacillus* ($\bar{x}$ = 10.04), a key PAC000748_g ($\bar{x}$ = 9.42), *Bacillus* ($\bar{x}$ =
250 8.96) and *Caldalkalibacillus* ($\bar{x}$ = 7.90) as predominant in the HC,
251 while in the HE group they were *Kokuria* ($\bar{x}$ = 14.59), *Anaerococcus*
252 ($\bar{x}$ = 3.51), *Staphylococcus* ($\bar{x}$ = 3.05) and *Treponema* ($\bar{x}$ = 2.93) (Fig.
253 4). The complete list of the bacteria found in this investigation in the
254 phylum to species levels are deposited in Table S1.

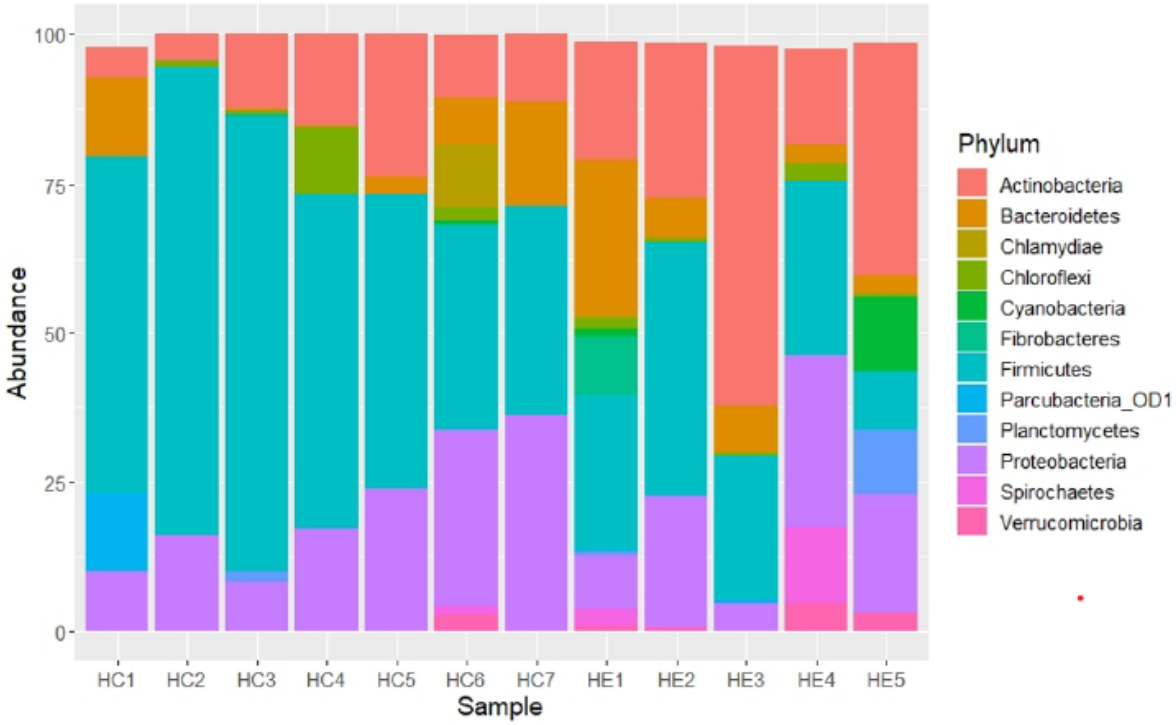

Figure 3. Relative abundance (%) at phylum level of the bacterial microbiota of human blood at the Comarca Lagunera, north central Mexico (HC = not exposed, HE = exposed to tick bites).

The average global dissimilarity according to the SIMPER analysis at the phylum level was 45.15, the contribution of dissimilarity between populations for the taxon firmicutes was 32.96%, the difference being also significant ($p = 0.014$), as well as the difference of Actinobacteria ($p = 0.014$) and Cyanobacteria (0.046). The average global dissimilarity at the family level was 79.07. The taxa that contributed the most to the

dissimilarity were Bacillaceae 14.75% ($p = 0.005$) as well as Halomonadaceae 2.30% ($p = 0.034$) and Spirochaetaceae with a percentage of dissimilarity of 1.88% ($p = 0.046$). An average global dissimilarity of 85.97 at the genus level was obtained; the genus that contributed the most to the difference was *Kokuria* 8.42% ($p = 0.005$), as well as *Anaerobacillus* 5.03% ($p = 0.005$), *Caldalkalibacillus* 3.17% ($p = 0.009$), *Treponema* 1.73% ($p = 0.040$), *Clostridium* 1.19% ($p = 0.009$), among others (Table S2).

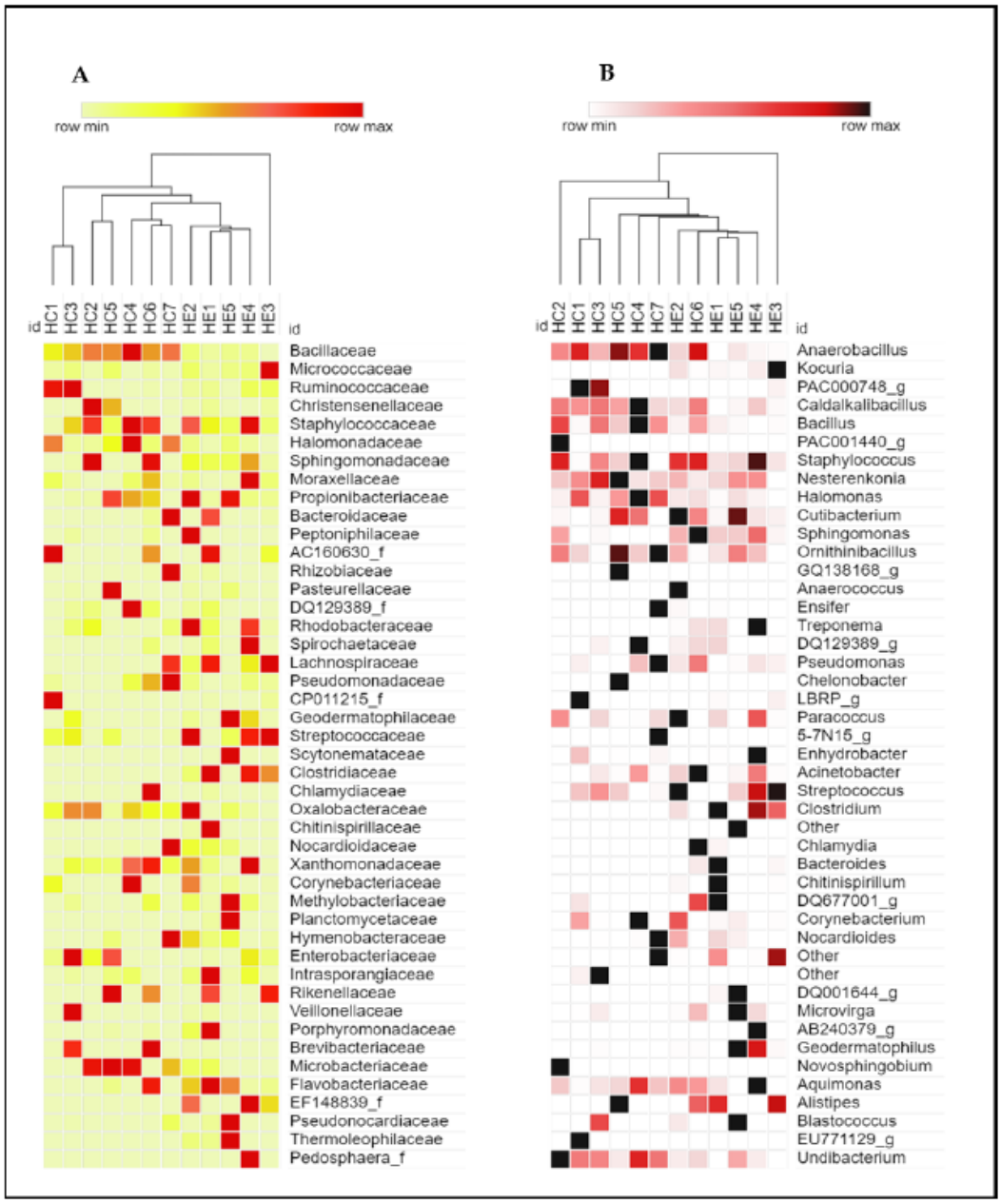

Figure 4. Heatmaps for family (A) and genus (B) levels of the bacterial microbiota of human blood at the Comarca Lagunera, north central Mexico (HC = not exposed, HE = exposed to tick bites).

The biomarkers that most significantly differentiated the groups in the LEfSe LDA score in the HC were Firmicutes phyla, Bacillaceae family, Bacillales order, and Bacilli class, among others. Regarding the HE group, they were Actinobacteria Phyla, Actinobacteria class, *Kocuria* genus, Spirochaetes phyla, Spirochaetia class, Cyanobacteria phyla, Spirochaetales order and *Treponema* genus, among others (Fig. 4).

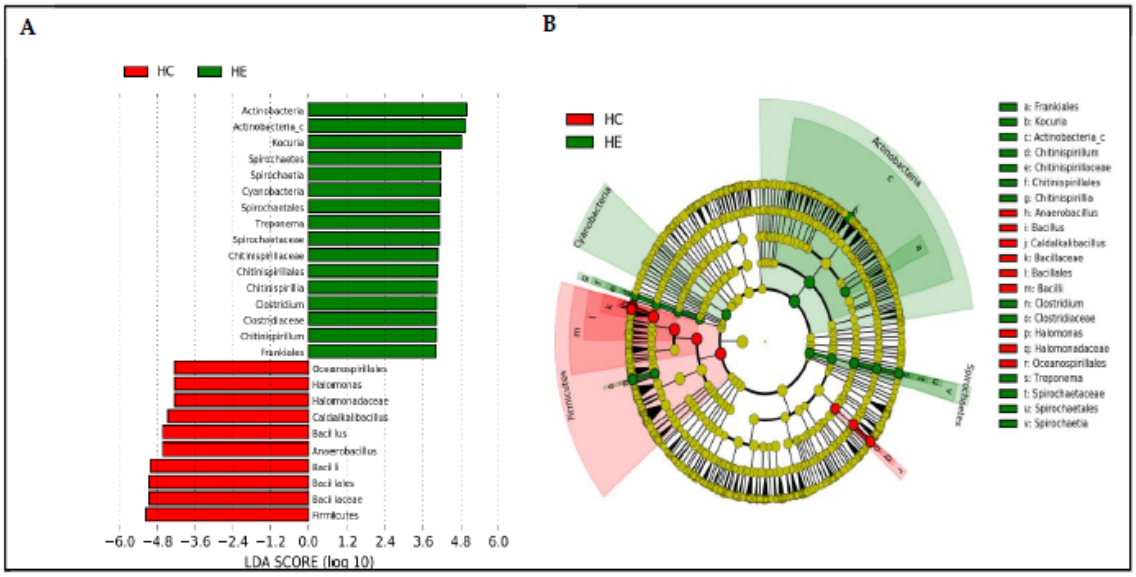

Figure 5. LEfSe analysis of bacterial microbiota of human blood of volunteers not exposed (HC) and exposed (HE) to tick bites in the Comarca Lagunera, north central Mexico. A) Bar graph shows LDA scores which indicates the taxonomic key for differentiation in both groups. B) The cladogram generated by LEfSe

indicates the main biomarkers between both groups. Each successive circle represents one phylogenetic level. Red-colored regions indicate taxa enriched in the HC, while green-colored regions indicate taxa enriched in the HE.

4. Discussion

The blood microbiota has been increasingly studied in order to better understand the meaning and role it has on humans; although a core microbiome has not been found in human blood, some dysbiosis have been highlighted in this environment related to certain pathologies. New molecular technologies such as NGS have certainly allow the identification of bacteria in the blood of apparently healthy people [22]. In this study, we compared the blood of apparently healthy people exposed (HE) to a tick bite in the previous year of the study, regarding people never exposed in their lives to a tick bite (HC). Several studies have demonstrated the presence of a healthy blood microbiota or healthy human blood microbiome (HBM), and due to this it is also possible to evaluate abnormal changes or modifications of the blood microbiota (i.e., dysbiosis) in individuals who present some type of pathology, for example, cardiovascular diseases, inflammatory bowel disease, diabetes, asthma, cirrhosis, diabetes, etc. [7].

Today there is still controversy while difficult to clarify whether dysbiosis is a consequence of some disease or the cause of diseases. However, there are no comparisons of individuals with apparently healthy HBM compared with groups of people exposed to some tick

316 bite. Therefore, our working hypothesis focused on comparing and an-
317 alyzing the differences between bacterial profiles of the blood of hu-
318 mans exposed (HE) or not exposed (HC) to a tick bite. Our research
319 outcomes reveled substantial differences, not only in composition but
320 also denoted that the HE group have greater abundance of the sequences
321 obtained in the analysis with a total of $\bar{x}$= 148,394 sequences from
322 which $\bar{x}$= 57,253 were assembled in the HC group, while in the HE $\bar{x}$=
323 214,263 were obtained, of which $\bar{x}$= 110,009 sequences were assem-
324 bled (Table 1). That is, more than double the sequences even though the
325 HE had fewer analyzed individuals. Besides, the number of OTUs ob-
326 tained in both populations also differed; 2774.4 in the HE group versus
327 1892 in the HC (Table 1).

328 No differences occurred between groups in alpha diversity, how-
329 ever, a considerable high diversity occurred in both cases when analyz-
330 ing through the Chao1, Shannon and Simpson approaches (Fig. 1). Also,
331 difference in beta diversity was detected using the Bray-Curtis index
332 (Fig. 2). This result indicates how the compositions of the HE and the
333 one with the healthy HBM microbiota are different between their con-
334 figurations, being highlighted through the PCoA analysis where the spe-
335 cific segregation between both groups occurred (Fig. 2). Although di-
336 versities were observed between groups, it is still elusive why one bac-
337 teria taxon is found in one group or another. Some studies suggest that
338 the presence of bacteria in the blood is due to translocations of other

parts of the body, such as the intestinal tract, the skin, the mucous membranes, or even suggest that some are vertically inherited by the mother [7, 23].

The most abundant phyla in the HC group were Firmicutes ($\bar{x}$ = 55.54%), Proteobacteria ($\bar{x}$ = 19.92%), Actinobacteria ($\bar{x}$ = 11.69%), and Bacteroidetes ($\bar{x}$ = 5.95%) (Fig. 3). The most common phyla reported in other studies on healthy HBM, included as the most dominant phyla to Proteobacteria followed by Actinobacteria, Firmicutes and Bacteroidetes [23-25]. Panaiotov et al. [26] identified Proteobacteria as the most abundant phylum in blood samples from healthy people with 93% in non-cultured samples and 46% in cultured samples. In the same way, Actinobacteria and Firmicutes with 2% were identified among non-cultivated samples. While among cultured samples Firmicutes was identified with 25%, Actinobacteria 14%, Bacteroidetes 6%, Fusobacteria 3% and Cyanobacteria with 2% [26]. Other study reported an overrepresentation of Proteobacteria phylum with 73.4% in healthy humans or with some type of disorder such as Bipolar disorder, Schizophrenia, Amyotrophic Lateral Sclerosis and a control group [27]. These findings coincide with the microbiota of other parts of the human body, mainly the oral cavity and the intestine, so they are probably bacteria translocated from these areas [27, 28]. In 2016, Païssé et al. [23] analyzed different fractions of human blood and found Proteobacteria and Firmicutes as the most abundant phyla in whole blood.

362 The genus *Bacillus* was detected in both plasma and red blood cells
363 in the blood of donors [29]. In our study, it was also one of the most
364 dominant in the HC group while was a highly significant genus in the
365 observed dissimilarity between the two groups (Table S2) as well as in
366 biomarkers (Fig. 5). However, other phyla were more abundant in the
367 bacterial profiles of healthy people. In 2008, Moriyama et al. [30] iden-
368 tified a set of bacterial taxa in their study of bacteria from the blood of
369 two healthy individuals comprising mainly *Bacillus, Flavobacteria,*
370 *Stenotrophomonas,* and *Serratia*.

371 The genus *Ehrlichia* within the Anaplasmataceae family was found
372 only in 20% of the HE group. This genus is reported in the blood of
373 people with suspected tick-borne disease [31] and the only genus clas-
374 sified as TBD found in the present study. However, several pathogenic
375 bacterial phyllotypes were found. The genus *Kocuria* was the most
376 abundant among the HE regarding the HC, it was also the genus that
377 most differentiated the two populations according to the SIMPER anal-
378 ysis with 8.42%, and the dissimilarity was highly significant ($p = 0.005$)
379 (Table S2). These results are consistent with an investigation in the
380 same region of the study area (i.e., Comarca Lagunera) and with the
381 same methodology used in the present work, where the genus *Kocuria*
382 was found as the most abundant in the blood of dogs (*Canis lupus fa-*
383 *miliaris*) infested with ticks ($\bar{x} = 6\%$) at relative abundance (Mejia-Gar-
384 cía et al. 2022., unpublished data); it was also a biomarker that differ-
385 entiated both groups according to the LEfsE analysis (Fig. 5). *Kocuria*

386 is related to clinical cases of different indoles, in addition, it is related
387 to enzymatic processes because of the production of proteases and cat-
388 alases as well as volatile compounds in food. In addition, the production
389 of biofilm as well as its resistance to antibiotics makes it potentially
390 undesirable [32]. Furthermore, some species of the genus *Kocuria* have
391 been related as causing various diseases, including sepsis, peritonitis,
392 infective endocarditis, meningitis, cholecystitis, urinary tract infections,
393 catheter-linked bacteremia, peritonitis, and abscesses [33-35].

394 The diagnosis of *Kocuria* has been undervalued mainly due to mis-
395 identifications by *Staphylococci*, due to limited biochemical tests and
396 automatic identification systems. Although the virulence of the genus is
397 not well established, today it is considered an important pathogen in
398 human medicine and a recurrent genus in bacteremia [35]. Different
399 studies have found *Kocuria* within the internal microbiota of ticks [36-
400 38]. Li et al. [38], by sequencing the 16 sDNA gene, identified *Kocuria*
401 as the most isolated genus of samples from the midgut of ticks, the in-
402 testines being the structures that have the first contact with pathogens
403 within the vector; from there, they migrate to other parts of the body of
404 the tick, playing a key role in the transmission and prevalence of infec-
405 tious tick diseases. Certain considerations must be considered about the
406 possibility of the genus *Kocuria* as an opportunistic organism that can
407 trigger a clinical picture in immunosuppressed people [38].

408 While *Treponema* was found in 80% of the HE, and only was ob-
409 served in 20% of the HC, it was one of the most abundant genera in our

study. Certainly, *Treponema* ($\bar{x}$ = 2.93), represented graphically on the heatmap relative abundance and SIMPER analysis, had the greatest contribution in dissimilarity between groups (Table S2). This genus has been identified as a bacterium transmitted by transfusions of people exposed to ticks [39]. Also, *Treponema* has been identified in analysis of bacterial profiles in ticks [40] and related to seropositive people to Lyme disease by *Borrelia burgdorferi* [41]. There are very marked similarities in tick-borne spirochetes, both morphological and protein nature of the diseases they cause [42]. Despite said similarities, the most common disease caused by these spirochetes is attributed to the etiological agent *Treponema pallidum*, causing the venereal syphilis disease, although our study did not find this species, we observed the EU463520_s, and the *Treponema* species *T. brennaborense*; this last could possibly be a new species described in the blood of humans, which previously has been associated to bovine digital dermatitis, periodontal diseases, and skin diseases in humans and animals [43].

Kingry et al. [31] found Spirochaetales and Rickettsiales as the most representative in the blood of donors suspected of some tick-borne disease, as in the present study where the LEfSe analysis indicates the same relationship (Fig. 5). It is important to clarify that the presence of spirochetes in the blood is not indicative of disease unless the patient has clinical signs [44]. Nonetheless, the people exposed to tick bites in the study, even though they had no apparent clinical signs at the time of sampling, stated that they had signs and symptoms at the time of being

exposed to the ticks; symptoms such as headache, fever, myalgias were described by the people under analysis.

Methods for detecting pathogens by sequencing have some limitations, especially with regard to samples with a very low biomass, such as blood, where some environmental or laboratory contaminants are very common, especially taxa non-pathogenic like *Bradyrhizobium* [45], detected in a single sample of the present study. Despite this, the superposition of contaminating taxa with pathogens or possibly pathogens do not occur in a common way in bacterial analyzes derived from vectors. It is even proposed directed metagenomics as a recommended analysis in the tick-borne pathogens scenario than in other bacterial infections [46], especially because it is possible to find potentially pathogenic bacteria not yet reported [31].

A disturbing concern regarding TBD is the complexity of reaching an accurate diagnosis, mainly because these diseases have non-specific clinical signs that are very similar to each other. In most cases these diseases are self-limiting. However, in the case of *Ehrlichia chaffeensis, Anaplasma phagocytophilum* and *Rickettsia rickettsii,* they can be potentially fatal, especially in the elderly, immunocompromised, children or people with comorbidities. Children under 10 years of age are the most affected [47]. RMSF is the most common rickettsial disease in Mexico, with lethal outbreaks in different northern states (i.e., Coahuila, Durango, and Sonora) [9]. In our study, unlike other scientific and press publications where a high incidence and mortality of RMSF due to *R.*

458 *rickettsii* have been reported, it was not detected in people exposed to
459 tick bites. It is known that the Comarca Lagunera is endemic for this
460 disease and it has been detected both in studies with people as well as
461 in dogs and their main ectoparasites (*R. sanguineus*) using different
462 methods both in Mexico and in the region under study [9, 10, 48, 49].
463 The lethality of the RMSF in the state of Coahuila was 14.5% in 2012,
464 50% in 2017, 50% in 2020, 60% in 2021, and 76.2% in 2022, the highest
465 in the last 10 years. A total of 21 cases of this disease in Coahuila until
466 September was reported, with 17 deaths registered in different munici-
467 palities of the region [50]. However, the data provided do not rule out
468 the possibility that these people faced bacterial co-infections because of
469 the tick bite. Therefore, more robust studies of bacteria detection by
470 molecular techniques are required to provide more epidemiological in-
471 formation on TBD in the region and not simply PCR as a diagnostic
472 technique, since it is aimed at a particular pathogenic organism and can
473 be misdiagnosed.

474 **5. Conclusions**

475 To our knowledge, this is the first study comparing bacteria in the
476 blood of healthy people exposed to a tick bite regarding unexposed peo-
477 ple, using next-generation massive sequencing. Our study confirms that
478 the blood of healthy people is colonized by a considerable number of
479 bacterial organisms. Additionally, our study detected significant differ-
480 ences both in the diversity and in the microbiological composition in
481 both experimental groups. While *Ehrlichia* was identified as the only

TBD in the HE group, different pathogens such as *Kocuria*, *Treponema*, and *Clostridium* were found in the HE group compared to the HC group. Undoubtedly, we still have a fragmentary knowledge about the main microbiome interactions between humans and the main ectoparasites of the neglected dogs. Future studies must be developed to better understand the possible association of blood dysbiosis and people bitten by ticks.

Supplementary Materials: The following are available online at (link) Table S1: Relative abundance of bacterial taxa from 12 blood samples of people, five exposed to tick bites and 7 not exposed to tick bite in the Comarca Lagunera. Table S2: SIMPER analysis compares the tow population under study. (12 fastaq files available). Sequences used in this study were deposited in the NCBI web Accession Number (BioProject PRJNA908861).

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Informed Consent Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Facultad de Ciencias Biológicas UJED, protocol code 0019, date of approval: June, 15, 2021.

Author contribution statement

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7. GENERAL CONCLUSIONS

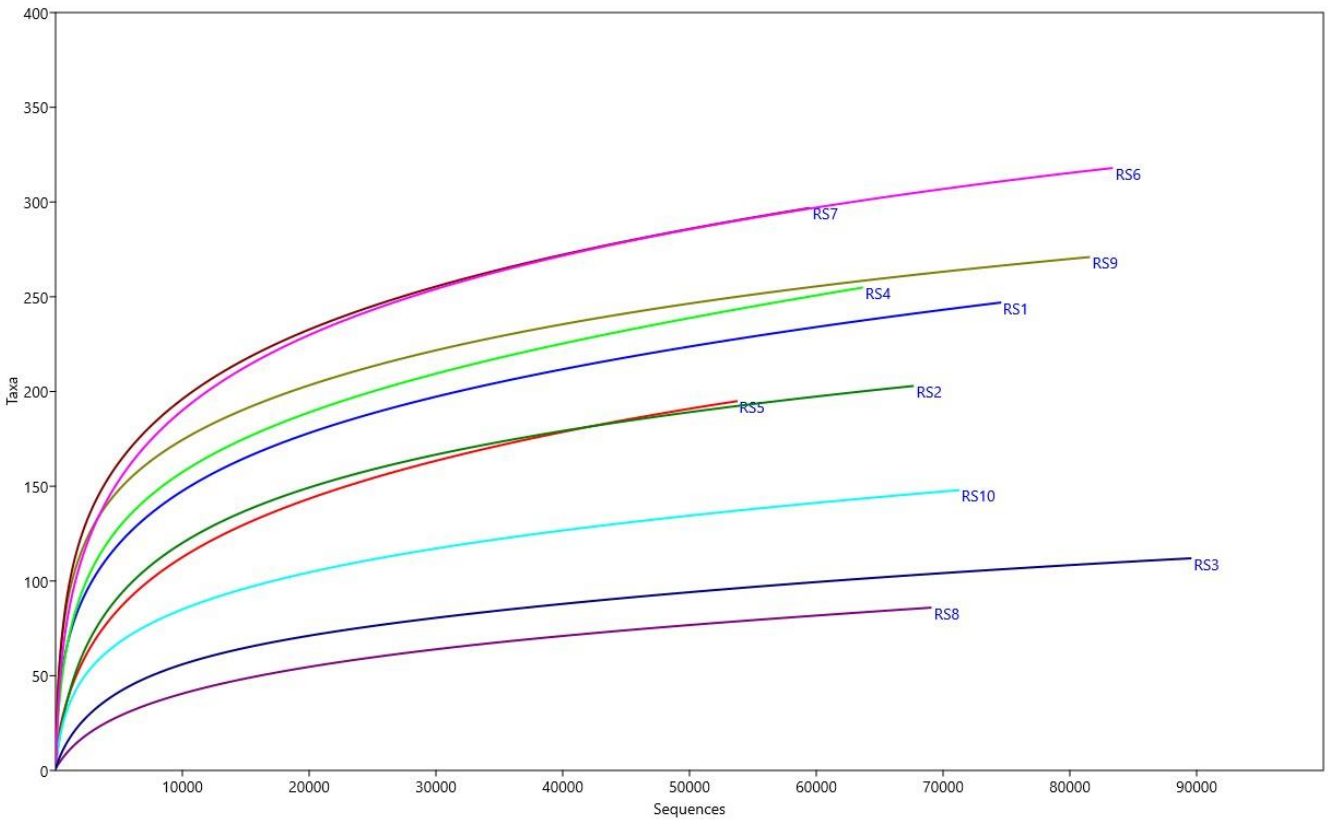
Based on the results obtained in the present doctoral studies, we can say that potential pathogens were detected both in the soft tick (*O. turicata*) parasitizing the bolson tortoise, as well as in the brown dog tick (*R. sanguineus*). The presence of potentially pathogenic bacterial species was detected by means of next generation massive sequencing, where some of them are recognized as TBD. In the case of the study carried out in humans exposed to tick bites vs. people not exposed, the bacterial composition differed between experimental groups. For all the above, the null hypothesis is rejected in that our working hypothesis in this research work was certainly proven.

Something that was particularly interesting was the overrepresentation of the genus *Coxiella* in the ticks analyzed in this study; the pathogenic species *C. burnetti* was found in both tick species, and in the brown dog tick in high abundance with a frequency of 100% (found in all pools analyzed). On the other hand, *A. marginale, A. ovis* and *A. phagocytophilum* were detected in *O. turicata*. Besides, with respect to *R. sanguineus, A. platys* was detected, all these recognized as TBP that affect the animals they infect, having zoonotic potential to can cause serious diseases. Additionally, the genus *Ehrlichia* in *R. sanguineus* was found to be the third most abundant genus in that study. It should be noted that all the species of the genus *Ehrlichia* were found, only except for *E. ruminantium*, including *R. minasensis*, a recently discovered pathogen in Brazil, never reported in Mexico before and whose origin, pathogenesis and zoonotic capacity is largely unknown, which speaks of the importance of this genus in the brown dog tick and the latent risk of emerging diseases in the region under study.

In the case of the study conducted in humans, unlike the reports of high prevalence and incidence in the region with respect to RMSF, the *Rickettsia* genome was neither detected in tick samples nor in the human samples. However, it was possible to detect the *Ehrlichia* genus within people exposed to tick bites. To our knowledge this represents the first study of its kind comparing the blood microbiome of people under the influence of exposure to a tick bite, where a significant difference was found in beta diversity between the two

experimental groups. Additionally, the healthy HBM hypothesis was again verified, finding a wide diversity and abundance in the people analyzed in the study, either the exposed and those unexposed. Future complementary studies increasing the number of samples both from pools of tick bacterial DNA and from people who are presenting symptoms compatible with TBD will be necessary with the purpose of expanding the epidemiological knowledge of these diseases in the region. This could clarify questions regarding the vector-host-pathogen dynamics and promote strategies for control, mitigation, diagnosis, and possibly treatment in the field of human and veterinary medicine.

8. APPENDIX



Appendix 1. Rarefaction curve of the three pools created for the *R. sanguineus* samples, all the sequences tend to plateau at 50,000 sequences.

Appendix 2. Percentage similarity analysis (SIMPER) considering the average of dissimilarity (AVD) of the bacteria in the blood at phyla, family, and genus levels from two groups of volunteers, not exposed (HC) and exposed (HE) to tick bites in the Comarca Lagunera, north central Mexico.

Taxon	AVD	Contrib. %	Cum. %	Mean HC	Mean HE	U	p
Phylum							
Firmicutes	14.88	32.96	32.96	0.552	0.263	2	0.014 *
Actinobacteria	10.47	23.2	56.15	0.119	0.322	2	0.014 *
Proteobacteria	5.469	12.11	68.26	0.201	0.169	14	0.626
Bacteroidetes	4.265	9.447	77.71	0.0596	0.0926	13	0.515
Spirochaetes	1.605	3.554	81.27	0.0023	0.0317	14	0.623
Cyanobacteria	1.394	3.086	84.35	0.00107	0.0277	6	0.046 *
Family							
Bacillaceae	11.66	14.75	14.75	0.294	0.0608	0	0.005 *
Micrococcaceae	7.001	8.854	23.61	0.0426	0.174	7	0.104
Ruminococcaceae	6.2	7.84	31.45	0.109	0.0387	13.5	0.562
Christensenellaceae	4.674	5.911	37.36	0.0887	0.0114	17	1
Peptoniphilaceae	2.091	2.644	40	0.00349	0.0398	13	0.468
Halomonadaceae	1.822	2.305	42.31	0.0414	0.00636	4	0.034 *
Sphingomonadaceae	1.79	2.264	44.57	0.0268	0.023	10	0.252
Moraxellaceae	1.679	2.124	46.69	0.0153	0.0315	16.5	0.935
Bacteroidaceae	1.652	2.089	48.78	0.0198	0.0194	16.5	0.923
Staphylococcaceae	1.523	1.926	50.71	0.0341	0.0348	17	1
Spirochaetaceae	1.49	1.884	52.59	0.00223	0.0295	6	0.046 *
Propionibacteriaceae	1.362	1.723	54.32	0.0154	0.0272	9	0.193
Rhodobacteraceae	1.258	1.591	55.91	0.00546	0.0261	10	0.252
Rhizobiaceae	1.242	1.571	57.48	0.0235	0.00224	17	1
Pasteurellaceae	1.224	1.548	59.03	0.0215	0.00481	10.5	0.259
○ Anaplasmataceae	0.0131	0.01656	99.69	0	0.000262	14	0.310
Genus							

Kocuria	7.244	8.426	8.426	0.000714	0.146	0	0.005 *
PAC000748_g	4.787	5.568	13.99	0.0941	0.00383	15	0.669
Anaerobacillus	4.327	5.033	19.03	0.0996	0.0131	0	0.005 *
Bacillus	4.186	4.869	23.9	0.0899	0.00702	2	0.014 *
PAC001440_g	3.012	3.504	27.4	0.0602	0.000143	14	0.562
Caldalkalibacillus	2.731	3.177	30.58	0.0783	0.024	1	0.009 *
Halomonas	1.822	2.119	32.7	0.0414	0.00636	4	0.034
Anaerococcus	1.735	2.018	34.71	0	0.0347	10.5	0.104
Staphylococcus	1.571	1.827	36.54	0.0341	0.0309	16	0.870
Treponema	1.49	1.733	38.27	0.00223	0.0295	6	0.040 *
GQ138168_g	1.378	1.603	39.88	0.0276	0	15	0.498
Nesterenkonia	1.369	1.592	41.47	0.0382	0.0232	14	0.626
Cutibacterium	1.347	1.566	43.04	0.0154	0.0266	9.5	0.222
Sphingomonas	1.337	1.556	44.59	0.017	0.0215	9	0.185
Ensifer	1.192	1.387	45.98	0.023	0.00117	15.5	0.771
Enhydrobacter	1.123	1.306	47.28	0.00378	0.0203	15	0.728
Clostridium	1.11	1.291	48.58	0	0.0222	3.5	0.009 *
Other	1.11	1.291	49.87	0	0.0222	14	0.31
Chelonobacter	1.043	1.214	51.08	0.0209	0	15	0.498
Chitinispirillum	1.027	1.195	52.27	0	0.0205	7	0.033 *
DQ129389_g	1.016	1.182	53.46	0.0188	0.00502	17	0.862
LBRP_g	1.012	1.177	54.63	0.0187	0.0021	14	0.522
Bacteroides	0.9961	1.159	55.79	0.00114	0.0194	13	0.393
Ornithinibacillus	0.9447	1.099	56.89	0.0227	0.0124	14	0.626
○ *Ehrlichia*	0.0131	0.01523	98.82	0	0.000262	14	0.310

* Indicates significant difference in dissimilarity between groups
○ Indicates the taxa considered as TBP
AVD: Average of dissimilarity
Contrib. %: Contribution of dissimilarity in percentage
Cum. %: Cumulative in percentage
U: U Man-Whitney test
p: p-value