Diversity of the bacterial and viral communities in the tropical horse tick, *Dermacentor nitens*, in Colombia

by

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Abstract

Ticks are obligatory hematophagous ectoparasites that can transmit pathogens among various vertebrates, including humans. The presence or absence of pathogenic microorganisms is highly variable in ticks, as is the diversity in the composition of their microbial communities, but the factors driving this diversity have not been explored. The tropical horse tick, *Dermacentor nitens*, is distributed throughout the Americas, and it is recognized as a natural vector of *Babesia caballi* and *Theileria equi*, the causal agents of equine piroplasmosis. We characterized the bacterial and viral communities associated with partially fed *D. nitens* females collected by a passive survey on horses from field sites representing three distinct geographical areas in Colombia, Bolivar, Antioquia, and Cordoba. RNA-seq and sequencing of the V3 and V4 hypervariable regions of the 16S rRNA gene were performed using the Illumina-Miseq platform. A total of 356 operational taxonomic units (OTUs) were identified, in which the presumed endosymbiotic *Francisellaceae/Francisella* spp. dominated. Differences in the relative abundance of the microbial composition among different regions were found independent of the presence of *Francisella*-Like Endosymbiont (FLE). The most prevalent bacteria in different regions were; *Corynebacterium* in Bolivar, *Staphylococcus* in Antioquia, and *Pseudomonas* in Cordoba. *Rickettsia*-like endosymbionts, the main etiological agents of rickettsioses in Colombia, were detected in the Cordoba samples. Metatransctiptomics revealed 13 abundant contigs presenting FLE genes with regional differences. In addition, nine contigs corresponding to six different viruses, in three viral families were also identified, including Chuviridae, Rhabdoviridae, and Flaviviridae. These findings enhance our understanding of the diversity of microbial communities in ticks and may be used to make regional distinctions among the ticks by their bacterial and viral compositions.

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Chapter 1 - Literature review and Thesis structure

Abstract

Ticks transmit diverse human and non-human animal pathogens, contributing to a rising number of new public health concerns around the world. Ticks directly damage the host by skin damage, trauma, and blood loss, and indirectly by the transmission of pathogens that can develop into acute and chronic illnesses, resulting in both short-term and long-term harm. Ticks also carry a diverse group of commensal and symbiotic microorganisms. Endosymbiotic communities can offer positive, neutral, or negative effects to their tick hosts by playing important roles in development, fitness, and immune responses. Nevertheless, much of their biology and potential interactions with transmissible pathogens remains largely unexplored. In addition, pathogenic microorganisms and endosymbiotic bacteria share significant genetic similarities as evidenced by phylogenetic studies of bacterial genera including *Francisella*, *Coxiella*, and *Rickettsia*. Accordingly, the tick holobiome must be studied, where the many different microorganisms and viruses that inhabit ticks can have significant impacts on their biology and vectorial capacity. Here, I briefly review the current status of our knowledge of ticks and the importance of tick endosymbionts in their biology.

Introduction

Tick taxonomic position and life cycle

Ticks belong to the Phylum Arthropoda, Class Arachnida, subclass Acari, and Order Parasitiformes (Anderson & Magnarelli, 2008). There are approximately nine hundred species of ticks described worldwide (Guglielmone et al., 2014). Ticks are further subdivided into the Families Ixodidae (hard-bodied ticks), and Argasidae (soft-bodied ticks), both of which include

species of veterinary and human importance (Anderson & Magnarelli, 2008; Estrada-Peña, 2015; Guglielmone et al., 2014). The Family Ixodidae is subdivided into Prostriata, a monotypic group with the genus (*Ixodidae*), and Metastriata, a group containing 11 genera, mainly represented by *Dermacentor*, *Rhipicephalus*, and *Amblyomma* (Anderson & Magnarelli, 2008).

The life cycle of Ixodidae ticks consists of four developmental stages: one non-feeding stage (egg), and three active parasitic stages (larva, nymph, and adult). All ticks are required to feed on vertebrate hosts to molt into the next stage and complete their life cycle. Thus, a one-, two-, or, more commonly, three-host feeding strategy can be found in their life cycles (Guglielmone et al., 2014). The one-host and the two-host life cycles may have been adopted in response to environmental stress to avoid living without the protection of the microenvironment provided by the host (Estrada-Peña, 2015). In the three-host life cycle, ticks are required to feed through three different attachments to reach the adult stage and complete the life cycle (Anderson & Magnarelli, 2008; Estrada-Peña, 2015) (Figure 1.1). Requiring more than one host throughout their life cycle increases the possibility of acquiring and transmitting a broader diversity of pathogenic microorganisms. The life cycle of the tick, one- or multiple-host cycles, is also influenced by the type of host and abiotic factors, like temperature, which is also one of the main drivers influencing the vectorial capacity of the tick (Cabezas-Cruz et al., 2017).

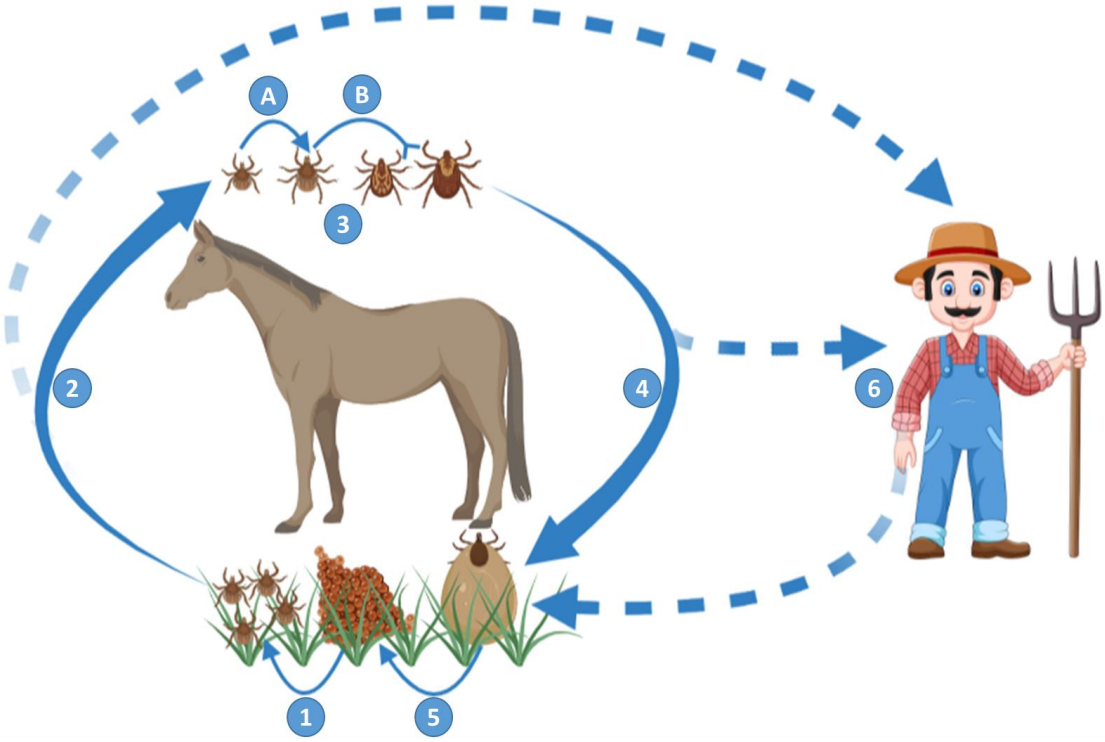

Figure 1.1 The one-host life cycle of *Dermacentor nitens*

This schematic diagram represents the one-host life cycle of *D. nitens*. In the first stage, ticks remain on the same host for the larval, nymphal, and adult stages, only detaching from the host to lay eggs. Eggs hatch into six-legged larvae (1), and larvae seek out and attach to the main host (2). Then molting driven by two episodes (A, B) of blood-feeding occurs to reach the adult stage (3), blood-fed females detach from the host (4), and gravid females lay the eggs in the environment and the cycle repeats. The dotted line represents an alternative way of completing the lifecycle by attaching to an unexpected host (6).

Ticks as vectors of pathogens

Ticks are hematophagous ectoparasites that are competent vectors of a broad range of pathogens of vertebrates (Park et al., 2021). Among arthropod vectors, ticks transmit the most diverse array of human and non-human animal pathogens, leading to an increasing number of new threats worldwide (Cabezas-Cruz et al., 2018). These pathogens are horizontally transmitted to the host during the tick blood-feeding process (Šimo et al., 2017; Swei & Kwan, 2017; Tully & Huntley, 2020). Major tick pathogens are transmitted by Ixodidae ticks, including *Rickettsia*

spp., *Babesia* spp., *Theileria* spp., *Borrelia burgdorferi sensu lato* (*sl*), Crimean-Congo hemorrhagic fever virus (CCHFV), tick-borne encephalitis virus (TBEV), and *Anaplasma phagocytophilum*. These pathogens are responsible for the most prevalent tick-borne illnesses, including spotted fever (*Rickettsia* spp.), human granulocytic anaplasmosis (*A. phagocytophilum*), Lyme disease (*B. burgdorferi s.l.*), Crimean-Congo hemorrhagic fever (CCHFV), tick-borne encephalitis (TBEV), and babesiosis (*Babesia* spp.). Ticks can also occasionally spread other serious human pathogens including *Francisella tularensis sl* and *Coxiella burnetii*. Importantly, cattle and other animals participate in the spread of tick-borne pathogens creating a risk to both animal and human health (Ahantarig et al., 2013; Bonnet & Pollet, 2021; Cabezas-Cruz et al., 2017; Park et al., 2021).

Ticks have remarkable plasticity and have colonized a wide range of ecological niches from the tropics to polar areas (Estrada-Peña, 2015). Studies on pathogens associated with hard ticks have gained global attention as factors such as climate change and urbanization have driven an increase in the number of cases of tick-borne pathogen infection. The colonization of ticks into new areas has been possible mainly due to increasing urbanization of landscapes and climate change. This combined with the modern methods for pathogen detection have sparked increased interest in tick species that were neglected or underestimated in the past, particularly those impacting people related to the livestock industry most likely to be exposed to emergent zoonoses (Raghavan et al., 2016).

The tropical horse tick, *Dermacentor nitens* Neumann.

The tropical horse tick, *Dermacentor nitens*, is distributed throughout the Americas and is recognized as a natural vector of *Babesia caballi* and *Theileria equi*, the causal agents of equine piroplasmosis (Rodrigues et al., 2017; Schwint et al., 2008)(Figure 1.2). *Dermacentor nitens* is

considered a sporadic ectoparasite of humans, with tick infestations likely the result of humans entering infested livestock environments (Guglielmone et al., 2014). The vectorial capacity of *D. nitens* for pathogens related to public health remains unknown; however, agents pathogenic to humans have been previously detected (Cotes-Perdomo et al., 2020; Santodomingo et al., 2019).

D. nitens is a one-host tick, with three to four generations per year (Labruna et al., 2002). The infestations can cause severe lesions, especially in the ears, and predispose the host to secondary infections (Borges et al., 2000). Although equines are the primary host, natural infestations have been reported in other domestic and companion animals, as well as in wild animals (Borges & Silva, 1994; Martins et al., 2015; Nelson et al., 2017). Accidental infestations by *D. nitens* in humans related to agricultural activities may represent a potential danger to human health. In Brazil, *D. nitens* individuals collected from equines used for animal traction work were found positive by PCR as a carrier of *B. burgdorferi s.l.*, the complex known as the causal agent of Lyme borreliosis or Lyme disease in the Americas (Gonçalves et al., 2013).

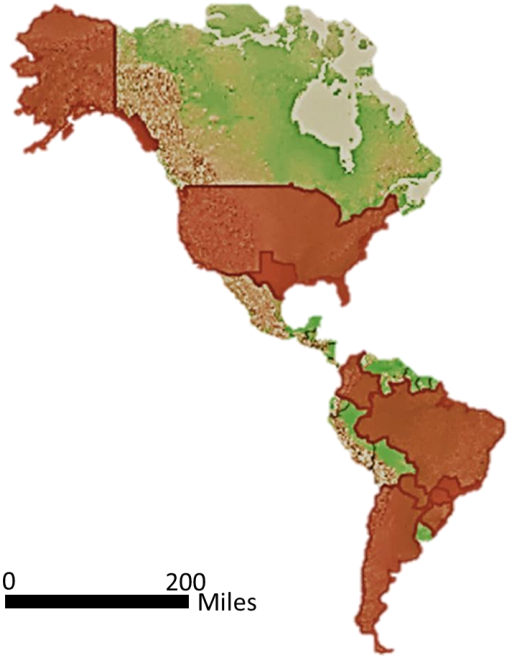

Figure 1.2 Current geographical distribution of *Dermacentor nitens*

Distribution of *D. nitens* in the Americas. The tropical horse tick is distributed currently only in the American continent, primarily in South American countries as represented in red. For North American countries, the state of Texas is also remarked because is the only state where *D. nitens* is currently founded in the US. The map is modified from the CABI Digital Library (https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.85688).

Tick microbial communities

Natural history of ticks and microbial communities

The phylum Arthropoda arose during the Cambrian explosion approximately 539 million years ago; then this phylum diversified into numerous groups, many of which are still extant (Johnson, 2017). One of these groups, the order Acari, includes species that are obligatory blood-feeders, such as ticks (hematophagous), mites are also included in this order and can parasitize humans and non-human animals, while other mites can parasitize plants or be predators of other arthropods (Anderson & Magnarelli, 2008; Estrada-Peña, 2015).

Ticks like most other eukaryotic organisms harbor pathogenic and non-pathogenic microbial communities with both commensal and symbiotic relationships (Swei & Kwan, 2017). These microorganisms can have direct positive or negative effects on the tick. Interactions among the microorganisms in ticks are also an important factor in the transmission of human/animal pathogenic organisms by these vectors (Park et al., 2021).

The composition of the microbiota in ticks is highly variable. Microbial communities vary depending on the tick species, season, geographic region, stage, and feeding status (Travanty et al., 2019). Environmental factors seem to be the main drivers of variation in microbial population structure among field-collected and laboratory-reared ticks (Bonnet et al., 2017; Bonnet & Pollet, 2021; Narasimhan & Fikrig, 2015). Furthermore, microbial communities also vary depending on the presence of pathogens. For example, in *Ixodes scapularis* the endosymbiont population has been shown to impact pathogen infection processes (Bonnet et al., 2017; Bonnet & Pollet, 2021). An unaltered intestinal microbiota favored colonization of *Borrelia burgdorferi s.l.*, whereas inducing microbial dysbiosis blocked the colonization by *Anaplasma phagocytophilum* (Narasimhan et al., 2014). Tick microbial diversity is enhanced by the environmental microorganisms that can also colonize the ticks. For example, the microorganisms present on the host skin may colonize ticks during the blood-feeding process (Clow et al., 2018). A similar situation can occur with the microorganisms present in the soil or vegetation during the detached and questing phases of the tick life cycle (Clow et al., 2018; Van Treuren et al., 2015).

Endosymbionts related to arthropods including ticks can be divided into two main groups, the first group is classified as primary endosymbionts and contains those obligate mutualistic microorganisms that are strictly required to assist the host in essential functions. The

second group is known as secondary endosymbionts, which basically contain those facultative microorganisms that are not required for the survival of the arthropod (Ahantarig et al., 2013; Gurfield et al., 2017). Vertical or transovarial transmission of endosymbionts results in maternal inheritance and uses specific adaptative strategies to colonize, spread, and persist within tick populations (Narasimhan et al., 2021). Tick-endosymbionts are generally tissue-specific with microbial guilds well established in salivary glands, gut, ovaries, etc. (Narasimhan & Fikrig, 2015). Some of these microorganisms, including pathogenic and non-pathogenic bacteria, can be transmitted transovarially to tick offspring (Sumrandee et al., 2014). Among non-pathogenic microbial communities, at least 10 different genera of maternally inherited microorganisms have been identified, but the most common bacterial endosymbionts found in ticks are mainly related to *Rickettsia*, *Coxiella*, and *Francisella* genera (Bonnet et al., 2017; Cabezas-Cruz et al., 2019; Díaz-Sánchez et al., 2019) (TABLE 1.1).

Table 1.1 Vertically-transmitted endosymbiotic bacteria found in ticks.

Vertically-transmitted bacteria	Distribution across ticks	Known effect on ticks
Coxiella-Like Endosymbiont	Very common in ticks, it is considered the most common endosymbiont across tick species.	Required for physiological processes during ontogeny (Ben-Yosef et al., 2020).
Francisella-Like Endosymbiont	Rare in ticks, but main endosymbiont of *Dermacentor* spp.	Required for the synthesis of the vitamin B complex (Duron et al., 2018).
Rickettsia-Like Endosymbiont	Present in most tick species, mainly found in *Ixodes* spp.	Required for folic acid biosynthesis (Bonnet et al., 2017)
Midichloria spp.	Present in most tick species.	Related to tick mitochondria (Budachetri et al., 2018).
Wolbachia spp.	Present in most tick species	Unknown effect in ticks (Duron et al., 2017).
Spiroplasma spp.	Present in ticks	Unknown effect in ticks (Duron et al., 2017).
Rickettsiella spp.	Common in ticks.	Unknown effect in ticks (Duron et al., 2017).
Arsenophonus spp.	Common in ticks, mainly found in *Ixodes Ricinus*.	Unknown effect in ticks (Lejal et al., 2021).
Lariskella spp.	Rarely reported in ticks	Unknown effect in ticks (Duron et al., 2017).

Cardinium spp.	Present in ticks	Unknown effect in ticks (Duron et al., 2017).

Table 1.1 is adapted from (Bonnet et al., 2017; Duron et al., 2017)

Role of non-pathogenic microbial communities in the ticks

The tick microbiome refers to the collection of microorganisms living and interacting inside an arthropod tick vector, either as pathogens, commensals or symbiotic residents. In a broad sense, the tick microbiome includes communities of viruses, bacteria, protozoa, and fungi (Narasimhan et al., 2021; Narasimhan & Fikrig, 2015). Recent experimental approaches to access the bacterial diversity in various species of ticks have been using the power of next-generation sequencing (NGS) of 16S rRNA gene sequence amplicons (16S rRNA) (Gurfield et al., 2017; Klindworth et al., 2013; Xiang et al., 2017). Those studies revealed the diversity of tick bacterial communities, including mammalian pathogens, which are dependent on the host, tick species, and geographic location (Bonnet & Pollet, 2021; Cabezas-Cruz et al., 2018, 2019).

Non-pathogenic microorganisms can also inhibit the replication and transmission of pathogens to vertebrate hosts, but the roles of endosymbionts in promoting or inhibiting the transmission of pathogens have been largely understudied (Duron et al., 2017). The negative association between the prevalence of infection with *Rickettsia rickettsii* (the pathogen) and *Rickettsia peacockii* (the endosymbiont) in *Dermacentor andersoni*, for example, suggests that rickettsial endosymbionts may affect the transmission of other rickettsial diseases (Budachetri et al., 2018). The transmission of *Erlichia chaffeensis* is further hindered by the presence of *Coxiella*-related symbionts in the salivary glands of *Amblyomma americanum* ticks (Clay et al., 2008).

The *Francisella*-Like Endosymbiont (FLE) is widespread, well-conserved, and probably an important core microbiome component among the Ixodidae and Argasidae ticks (Ahantarig et

al., 2013; Gerhart et al., 2016, 2018; Scoles, 2004). The FLE group is well described in species from different continents, including Asia, Europe, and America (Reif et al., 2011; Sperling et al., 2020; Travanty et al., 2019; Y.-K. Zhang et al., 2019). Recent evidence also suggests that the endosymbiotic relationship of the FLE group with ticks may have independently evolved from recently established commensalism (Gerhart et al., 2016). FLE in *Dermacentor* spp. are a source of vitamin B synthesis (Duron et al., 2018), likely an important reason why these FLEs are vertically transmitted in these ticks (Ahantarig et al., 2013; Gall et al., 2016; Gerhart et al., 2016; Scoles, 2004). Additionally, the association between FLE and ticks is considered to be a recent symbiotic relationship based on the low levels of sequence divergence and punctuated phylogenetic distribution in different tick species, but with moderate levels of horizontal transfers among different species and genera (Ahantarig et al., 2013; Buysse et al., 2021; Gerhart et al., 2016, 2018; D. Kumar, Sharma, et al., 2022).

Viral communities and ticks

Viruses are certainly the most frequent and variable biological entities in the world (Koonin, 2010). Arthropods are a prolific source of viruses, and ticks are one category of arthropods that are particularly important for both human and veterinary health. Recently, through metatranscriptomics, it has been discovered that ticks are hosts to a variety of viruses, the majority of which are RNA viruses, and some of which affect both human and animal health (Shi et al., 2018). Excluding the African swine fever virus (family Asfarviridae), most of the tick-borne viruses currently described are RNA viruses (TABLE 1.2). More than thirty-five distinct virus species from six different viral families are harbored by ticks (Mansfield et al., 2017). Tick-borne viruses including Crimean-Congo hemorrhagic fever virus, tick-borne encephalitis virus, Powassan virus, and other recently discovered viruses including Severe Fever

with Thrombocytopenia Syndrome, Heartland, and Bourbon Viruses have become significant public health hazards. The risks of contracting emerging and re-emerging tick-borne viruses are increasing across the globe as a result of anthropogenic changes that increase human interactions with ticks and increased tick populations (Shi et al., 2018).

Table 1.1.2 Common viruses of medical and veterinary importance found in ticks.

Virus name (abbreviation)	Taxonomic classification	Description of genome	Distribution	Primary vector	Reference
Severe fever with Thrombocytopenia syndrome virus (SFTSV)	Bunyaviridae	Negative-stranded RNA	North America, East Asia	*Haemaphysalis longicornis*	(Liu et al., 2014)
Heartland virus (HRT)	Bunyaviridae	Negative-stranded RNA	North America	*Amblyomma americanum*	(McMullan et al., 2012)
African swine fever virus (AFSV)	Asfarviridae	Double-stranded DNA	Africa, Central Europe	*Ornithodoros* spp	(Bernard et al., 2016)
Deer tick virus (DTV)	Flaviviridae	Positive-stranded RNA	North America	*Ixodes scapularis*	(Telford et al., 1997)
Powassan virus (POWV)	Flaviviridae	Positive-stranded RNA	North America, Europe	*Ixodes* spp	(Piantadosi et al., 2016)
Crimean Congo hemorrhagic fever virus (CCHFV)	Bunyaviridae	Negative-stranded RNA	Africa, Asia	*Hyalomma marginatum*	(Ergönül, 2006)
Colorado tick fever virus (CTFV)	Reoviridae	Double-stranded RNA virus	North America	*Dermacentor andersoni*	(Yunker & Cory, 1967)

The study of the microbiome has utilized a variety of approaches from conventional methods to those based on large-scale nucleic acid sequencing which have enabled the study of viral populations found in some tick species (Orozco Orozco et al., 2021; Pettersson et al., 2017; Tokarz et al., 2014). Knowledge of the viral communities present in ticks has been significantly improved by collecting and analyzing these genomic and metagenomic data. More information on the viromes in additional tick species may be a useful approach to continue to expand our understanding of tick-associated viruses, including tick-borne viruses that pose a concern to public health (Pettersson et al., 2017; Shi et al., 2018).

A comprehensive viral survey using metatranscriptomics from different tick species may be an efficient strategy to mitigate potential threats to public health (Madison-Antenucci et al., 2020; Prasad et al., 2015; Shi et al., 2018; Xu et al., 2021). Metatranscriptomic approaches are particularly effective for identifying RNA virus sequences in ticks. Despite considerable insights into bacterial diversity, our understanding of tick-associated viruses is still limited, however, and are largely unexplored when compared with our understanding of bacterial diversity (Tokarz et al., 2014). Virome studies in ticks species collected in Asia (*Haemaphylalis longicornis*), Europe (*Ixodes ricinus*), and North America (*Dermacentor andersoni*, and *Rhipicephalus microplus*) have revealed the emergence of novel pathogenic tick-borne viruses, but the dearth of data on tick viromes suggests a need for viral surveillance and discovery in ticks (Brinkmann et al., 2018; Li et al., 2015; Shi et al., 2018). Advanced sequencing technology providing metagenomics data has provided an approximation of the viral community composition present in a few tick species (Brinkmann et al., 2018; Gómez et al., 2020; Orozco Orozco et al., 2021; Sameroff et al., 2019; Tokarz et al., 2014, 2018; Xu et al., 2021).

Thesis structure and objectives

This thesis dissertation explores the diversity of bacterial and viral communities in partially fed *D. nitens* female ticks from three distinct geographical areas in Colombia. In Chapter 1, I reviewed some aspects about the natural history and life cycle of ticks, mainly the hard ticks, discussing the differences in lifecycles based on the type and number of hosts required to molt until the adult stage. The importance of the microorganisms acquired during the blood-meal process, including both pathogenic and non-pathogenic microorganisms interactions with some specific examples in tick species of public health interest. I also reviewed the current knowledge of viruses discovered in ticks, including the ones that cause diseases in humans and

animals. The main techniques utilized to identify and classify the viral proteins were also pointed out here and correlated with the information reported for the different continents. The information reviewed about the microbial and viral communities here is intended to bring context to the importance of molecular surveillance, including bioinformatic tools to understand the potential variations of tick microbiota in different continents.

In Chapter 2, I have tested the hypothesis that the composition of microorganisms hosted by partially fed *D. nitens* is different based on tick geographical distribution. I have found that the microbial composition overall is very poor, with a low abundance of bacterial groups, but with a clear dominance of the endosymbiont Francisellaceae/*Francisella* OTU. This is not surprising as the genus *Dermacentor* has been closely related to the FLE group, and FLEs have been recognized as the most abundant taxa found in this species (Scoles, 2004). At the same time, I compared the gene expression levels of 13 operons from FLE obtained from metatranscriptomic data and found that the Department of Cordoba is the one with the highest number of Transcripts per Million (TPM). Overall, these observations opens the window for future directions of this work that can be done by surveying different development stages of *D. nitens* including male and female individuals from the regions already studied.

In Chapter 3, I explored the viral diversity of the partially fed *D. nitens* female ticks from the metatranscriptomic data obtained from the RNA extraction of the whole tick body. For this purpose, I manually annotated the viral proteins, and based on a homology search on BLAST, I identified a trend of variation in these viruses when comparing the viral composition per region. The viruses identified here are mainly related to viral families present in different *Dermacentor* species from other continents and this can be related to some of the viruses that can be part of the

core viromes for this species. Overall, I found that the viral diversity in ticks is low, and the viruses identified in our data are apparently independent of the blood-fed status.

In chapter 4, I summarize all the findings obtained from the viral and microbial bioinformatic analysis. Overall, we found that the geographical origin shows a unique bacterial composition for the 16s rRNA sequencing analysis, but no statistical differences were found for the regional composition of viruses. Independent of this result, the data suggest a trend of slight differences in the Transcripts per Million comparisons towards the region of Cordoba, a region that is also endemic for other tropical diseases such as malaria or dengue.

Chapter 2 - Bacterial communities in *Dermacentor nitens*

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Abstract

Ticks are obligatory hematophagous ectoparasites that can transmit pathogens among various vertebrates, including humans. The presence or absence of pathogenic microorganisms is highly variable in ticks, as is the diversity in the composition of their microbial communities, but the factors driving this diversity are not well understood. The tropical horse tick, *Dermacentor nitens*, is distributed throughout the Americas, and it is recognized as a natural vector of *Babesia caballi* and *Theileria equi*, the causal agents of equine piroplasmosis. We characterized the bacterial communities associated with partially fed *D. nitens* females collected by a passive survey on horses from field sites representing three distinct geographical areas in Colombia (Bolivar, Antioquia, and Cordoba). Next-generation sequencing of the V3 and V4 hypervariable regions of the 16S rRNA gene was performed using the Illumina-Miseq platform. A total of 356 operational taxonomic units (OTUs) were identified, in which the presumed endosymbiotic Francisellaceae/*Francisella* spp. was predominantly found. Differences in the relative abundance of the microbial composition among the geographical regions were found to be independent of the presence of *Francisella*-Like Endosymbiont (FLE). The most prevalent bacteria found in each region were *Corynebacterium* in Bolivar, *Staphylococcus* in Antioquia, and *Pseudomonas* in Cordoba. *Rickettsia*-like endosymbionts, the main etiological agents of rickettsioses in Colombia, were detected only in the Cordoba samples. These findings enhance our understanding of the diversity of microbial communities in ticks and may be used to make regional distinctions between ticks and their bacterial compositions.

Introduction

Ticks are important vectors of pathogens that cause livestock and human diseases, such as ehrlichiosis, borreliosis, Lyme disease, human and cattle babesiosis, and theileriosis (Bonnet et al., 2017; Madison-Antenucci et al., 2020). The risk of emerging and re-emerging tick-borne diseases remain a continuing threat since prevention and management are hampered by suboptimal diagnostics, lack of treatment options for emerging pathogens, and scarcity of vaccines (Cabezas-Cruz et al., 2018; Prasad et al., 2015). Changes in tick habitats as a result of human activities and globalization have been described as factors directly driving migration and colonization of hosts, ticks, and pathogens (Bouchard et al., 2019). Given the importance of ticks as vectors of many important pathogens, understanding ticks and their symbiont compositions in different ecological systems has arisen as an important area of study (Madison-Antenucci et al., 2020). In addition, global climate change caused by human activities has increased the incidence and diversity of circulating pathogens in new habitats (Dantas-Torres et al., 2012).

Ticks harbor diverse microorganisms, including symbionts and pathogenic organisms, which may have direct positive or negative effects on the tick or other members of the microbial communities (Bonnet et al., 2017; Gall et al., 2016; Narasimhan et al., 2021). Interactions among the microorganisms in the bacterial communities within ticks are considered an important factor in the transmission of human/animal pathogenic organisms. (Park et al., 2021; Wu-Chuang et al., 2022). In *Ixodes scapularis*, the endosymbiont population has been shown to impact pathogen infection processes. An unaltered intestinal microbiota favored colonization of *Borrelia burgdorferi s.l.*, whereas inducing microbial dysbiosis blocked colonization of the *I. scapularis* gut by *Anaplasma phagocytophilum* (Bonnet et al., 2017; Bonnet & Pollet, 2021).

Among non-pathogenic communities, common bacterial endosymbionts found in ticks are mainly related to *Rickettsia*, *Coxiella*, and *Francisella* genera (Bonnet et al., 2017; Cabezas-Cruz et al., 2019; Díaz-Sánchez et al., 2019). These microorganisms act as primary endosymbionts providing essential nutrients involved in survival, development, and tick-fitness, such as biosynthesis of B vitamins and cofactors like riboflavin, folic acid, and biotin (Duron et al., 2018). Tick endosymbionts are generally tissue-specific with microbial guilds well established in salivary glands, gut, and ovaries, among other tissues (Narasimhan & Fikrig, 2015). Some of these microorganisms, including pathogenic and non-pathogenic bacteria, can be transovarially transmitted to tick offspring (Sumrandee et al., 2014).

The Tick microbiome include communities of viruses, bacteria, protozoa, and fungi (Narasimhan et al., 2021; Narasimhan & Fikrig, 2015). Recent experimental approaches to characterize the bacterial diversity in various species of ticks have used next-generation sequencing (NGS) of the 16S rRNA gene sequence amplicons (Gurfield et al., 2017; Klindworth et al., 2013; Xiang et al., 2017). Those studies revealed tick bacterial communities, including mammalian pathogens, are dependent on the tick species, type of host ticks fed on, and geographic location of the tick (Bonnet & Pollet, 2021; Cabezas-Cruz et al., 2018, 2019). Characterizing tick microbial populations may give us a better understanding of the different potential roles of intra- and interspecific microbial interactions and their involvement in vector competence (Cabezas-Cruz et al., 2018; Gall et al., 2016; Wu-Chuang et al., 2021).

Dermacentor nitens, also known as the tropical horse tick is recognized as the natural vector of *Babesia caballi* and *Theileria equi*, the causal agents of equine piroplasmosis (Rodrigues et al., 2017; Schwint et al., 2008). *Dermacentor nitens* is considered a sporadic ectoparasite of humans, where tick infestations are probably a consequence of humans entering

infested livestock environments, resulting in transference of ticks from infested animals to persons (Guglielmone et al., 2014). Accidental infestations by *D. nitens* in humans often related to agricultural activities may represent a potential danger to human health, although the vectorial capacity of *D. nitens* for pathogens related to public health remains unknown despite previous reports of the occurrence of human pathogenic agents in this tick species has been previously reported (Cotes-Perdomo et al., 2020; Santodomingo et al., 2019).

To gain an in-depth understanding of the microbial communities of *D. nitens*, we used 16S rRNA gene sequences combined with metatranscriptomic analysis to identify the main bacterial communities present in ticks collected from different geographical regions. These results provided large numbers of sequences that were annotated as operons of *Francisella*-like endosymbionts (FLE) with notable differences occurring between geographically distinct populations.

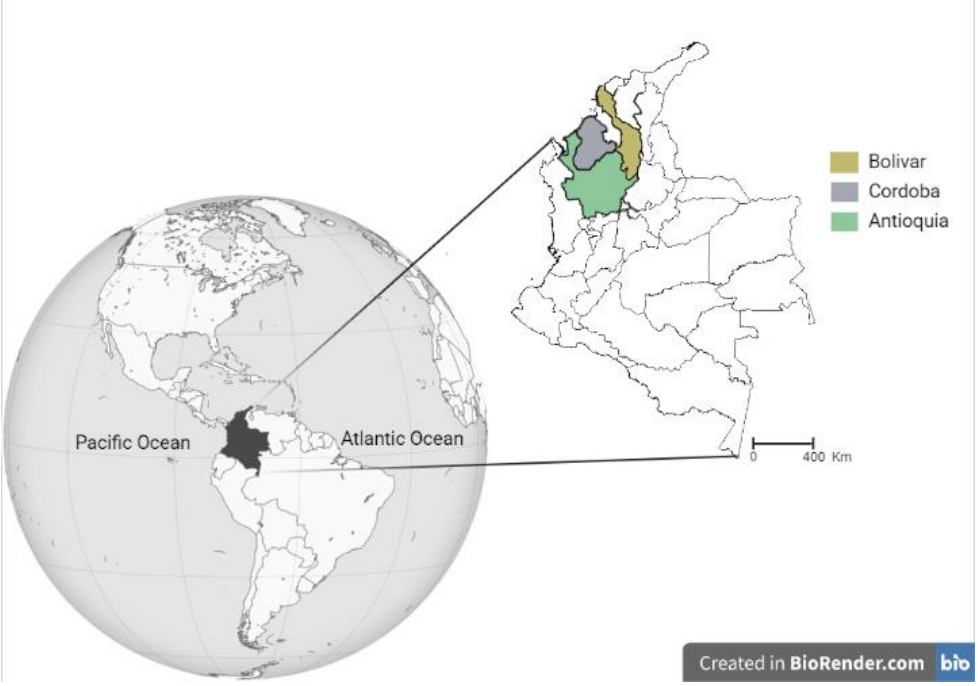
Materials and Methods

Sample collection and nucleic acid extraction

The tick collection was conducted by passive survey at “La Rinconada” slaughterhouse (06°11'26.0"N; 75°22'43.4" W) located in the Rionegro Municipality in the Antioquia Department of Colombia. Samples were collected in July and September of 2019. A total of 45 partially fed *D. nitens* adults were obtained from horses native to three Departments (regions) in Colombia, namely Bolivar, Antioquia, and Cordoba (Figure 2.1). The three Departments are in the northwest of Colombia and share borders mostly with the department of Antioquia. Live ticks were transported to the Universidad de Antioquia facilities for taxonomical identification according to established morphological keys (Barros-Battesti et al., 2006). Ticks were stored at -20 or -80°C until shipment to Kansas State University (Manhattan, Kansas, United States of

America). Female *D. nitens* ticks were pooled using a random selection algorithm to pick five ticks per pool, with three replicated pools per region, generating nine pools with a total of 45 ticks for analysis. Genomic DNA and RNA were extracted independently following manufacturer instructions using Zymo™ DNA and RNA extraction kits (Irvine, California, US).

Figure 2.1 Locations of origin of horses from which ticks were sampled in this study.



Colored zones represent the three different departments. Information was obtained from Instituto Geográfico Agustin Codazzi.

NGS library preparation and data processing

Genomic DNA from tick pools sequenced by the Genome Sequencing Core at the University of Kansas. Amplicon libraries were prepared by Illumina Miseq targeting the V3-V4 region with the primers 16S-F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 16S-R (5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') of the 16S rRNA, with an expected length of ~465 base-pair (bp) for the DNA analysis (Klindworth et al., 2013).

Phylogenetic analyses of bacterial protein sequences and OTU contigs

Phylogenetic analyses compared Bayesian inference, Maximum-Likelihood, Minimum-Evolution, and Neighbor-Joining methods which were performed as an initial assessment. Bacterial protein sequences and the OTUs detected in this study were compared to the reference sequences retrieved from NCBI GenBank database through homology-based BLASTn search. Bacterial protein sequences and partial 16s rRNA nucleotide sequences of FLE were retrieved from GenBank as indicated with the GenBank accession numbers at the end of each branch for the figures 2.3 and 2.4 containing phylogenetic trees. Sequences were aligned by using Muscle in MEGA-X software (S. Kumar et al., 2018). Bayesian inference analysis was done using BEAST v1.10.4 software (Suchard et al., 2018). Phylogenetic trees of the 16s rRNA nucleotide sequences were constructed based on the Neighbor-Joining method with a pairwise deletion. The trees for the V3-V4 regions sequenced in this study were constructed with 500 bootstrap replicates (Felsenstein, 1985; Saitou & Nei, 1987; Tamura et al., 2004) unless otherwise specified. For metatranscriptomic analyses of the FLE sequence, cladograms were constructed using annotated and concatenated genes for each contig by using the Maximum Likelihood method with the Tamura-Nei model and 500 bootstrap replicates (Tamura & Nei, 1993).

Ethical approval

This study was approved by the Bioethics Committee of the Universidad de Antioquia (Approval record No. 15-32-436 of June 2015). It was also granted an environmental license issued by the Colombian government through the National Environmental Licensing Authority

(Autoridad Nacional de Licencias Ambientales-ANLA, Resolution ANLA 00908 of May 27, 2017).

Results

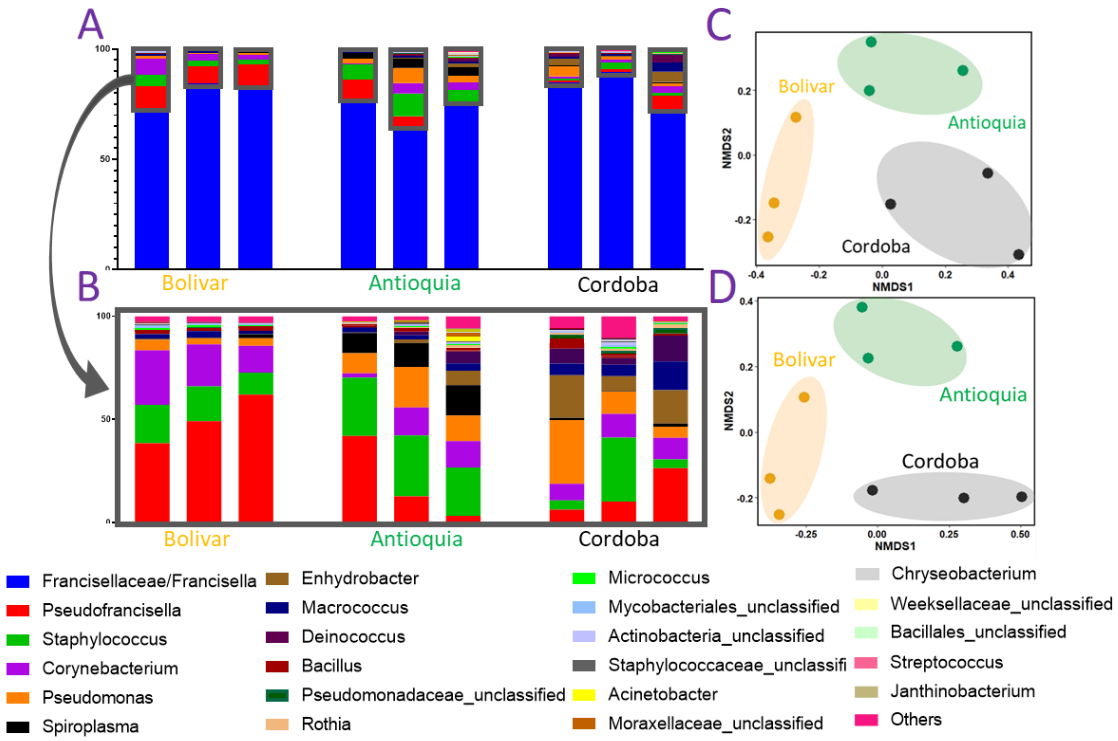
Bacterial diversity investigated using V3-V4 regions of the 16S rRNA sequences.

A total of 372,493 sequences after filtering 392,819 raw reads were assembled into 6,686 contigs and assigned to 356 OTUs with a threshold of 97% of sequence identity (Table 2.1). Notably, the sequences consisted of three main OTUs, all identified as FLE (>80%) in all nine pooled tick samples (Figure 2.2A). Among the remaining <20% OTUs, the most prevalent bacteria in different regions were *Corynebacterium* in Bolivar, *Staphylococcus* in Antioquia, and *Pseudomonas* in Cordoba (Figure 2.2B). We also compared the differences in bacterial compositions of the regions through Non-Metric Multidimensional Scaling (NMDS) in the data sets before and after excluding FLE (Figures 2.2C and 2.2D). Our NMDS plots suggested that regional bacterial composition is unique and independent of the presence of FLE and can be useful to differentiate the bacterial compositions from different geographical regions (Figure 2.2).

Library (Paired Reads)	Region	Raw reads	Mapped Reads	Contigs
DNA_Pool_1	Bolivar	48852	46109	706
DNA_Pool_2	Bolivar	41430	39512	508
DNA_Pool_3	Bolivar	37846	36438	503
DNA_Pool_4	Antioquia	45141	42948	842
DNA_Pool_5	Antioquia	39380	37847	1044
DNA_Pool_6	Antioquia	43778	41116	886
DNA_Pool_7	Cordoba	47878	45604	665
DNA_Pool_8	Cordoba	41244	38268	583
DNA_Pool_9	Cordoba	47270	44651	949
Total		392819	372493	6686

Table 2.1 Nine sequencing libraries for the pools for *D. nitens*, targeting V3-V4 regions of the 16 rRNA gene.

Figure 2.2 Bacterial diversity shown by the genera in 16S rDNA sequences from *D. nitens* samples collected from three different regions of Colombia.

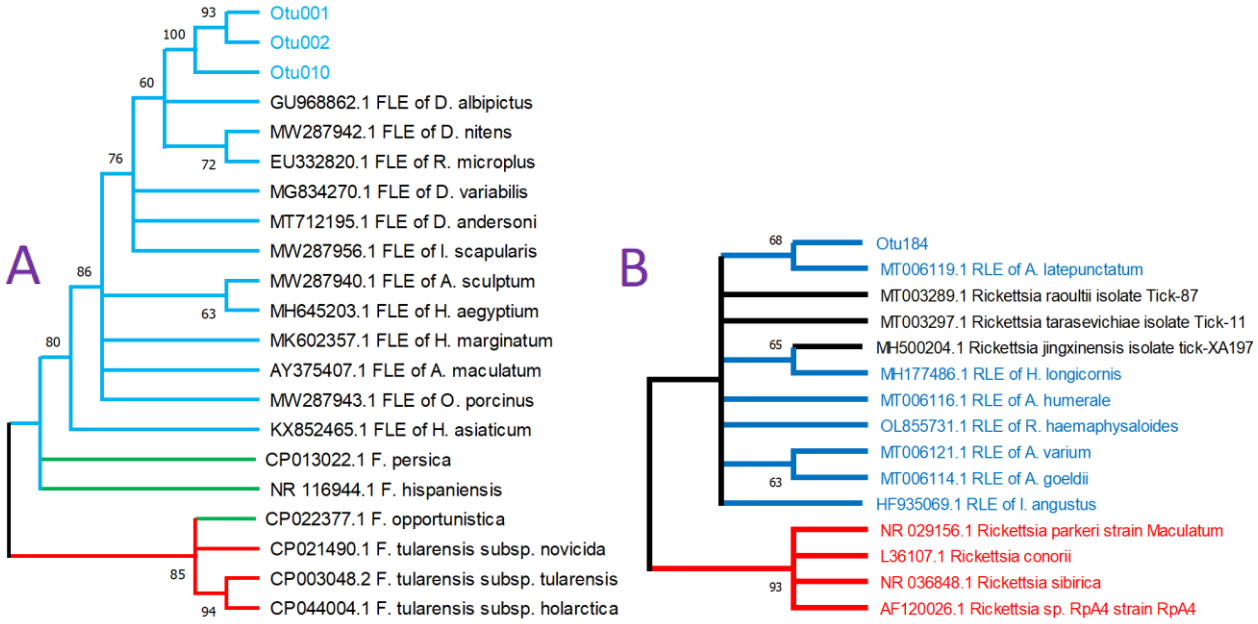


(A) Relative abundance is shown by bacterial genera. **(B)** The relative abundance after excluding the sequences of endosymbionts Francisellaceae/*Francisella* spp. **(C)** Non-metric multidimensional scaling plot (NMDS) plot showing the differences among tick samples from different regions. **(D)** NMDS plot showing the differences among tick samples after excluding the endosymbionts.

The FLEs categorized by a 97% identity threshold were three different OTUs (OTU001, 002, and 010 in Figure 2.3A and Table 2.2). These sequences are significantly different from each other with 20 nucleotide (nt) mismatches between OTU001 and OTU002, 21 nt mismatches between OTU002 and OTU010, and 8 nt mismatches between OTU001 and OTU010. High frequencies of the reads for each FLE OTUs, which are in independent libraries, suggest that the three different FLE OTUs are not sequencing artifacts. The cladogram of the FLE sequences showed these three OTUs clustered in a branch with a bootstrapping value of 100 (Figure 2A). A

single OTU, OTU184, was categorized as *Rickettsia*-like endosymbionts (RLE) in one pool of the Cordoba region. Phylogenetic analysis supports the position of this sequence in the tree clustered with RLE of *Amblyomma latepunctatum* and a clear separation from the pathogenic *Rickettsia* although the bootstrapping value was 68 (Figure 2.3B).

Figure 2.3 Phylogenetic analyses for the *Francisella*-Like endosymbionts (FLE) and *Rickettsia*-like endosymbionts (RLE) identified in this study for *Dermacentor nitens* samples.



(A) Neighbor-joining cladogram rooted to *Francisella tularensis* strains representing the phylogenetic relationship of 16S rDNA sequence OTUs classified as *Francisella* spp. in *D. nitens*. The tree was built using the pairwise deletion method. The blue branches represent the FLE clade, the green branches represent opportunistic pathogenic *Francisella* species, and the red branches represent the pathogenic *Francisella tularensis* strains as an outgroup. **(B)** Neighbor-joining cladogram rooted to pathogenic *Rickettsia* strains to represent the phylogenetic relationship of rickettsial 16S rDNA sequences with the OTU184 classified as *Rickettsia* spp. in the *D. nitens* sample. The red branches represent pathogenic *Rickettsia* spp., blue branches represent the sequences of RLE, and dark branches represent candidate-human pathogenic *Rickettsia*. The OTUs were determined by a 97% identity threshold. Bootstrapping percentages in 500 replications are shown on the nodes with a 60% cut-off. The GenBank accession numbers for each sequence are shown at the beginning of the names of taxa.

OTU Id	Bolivar	Antioquia	Cordoba
Otu001	61%	56%	59%
Otu010	18%	15%	22%
Otu002	10%	5%	3%

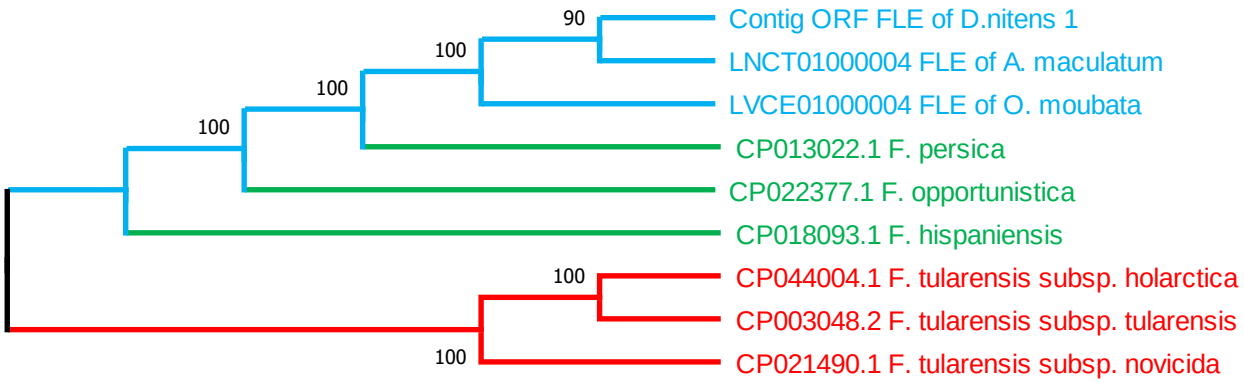
Table 2.2 Relative abundances of *Francisella*-Like Endosymbiont (FLE) captured in 16S sequencing.

The heat map shows the percent abundance for each bacterial OTU identified as *Francisella/*Francisellaceae.

Phylogenetic analyses of *Francisella* spp contigs

Thirteen FLE contigs identified by metatranscriptomics were further analyzed for their phylogenetic positions. All 13 FLE contigs clustered with other FLE identified in tick species when rooted in the pathogenic and opportunistic *Francisella* groups. The sequences had a 100% bootstrapping value for the tick endosymbiont clade represented by *Amblyomma maculatum* and *Ornithodoros moubata* in the figure 2.4 showing the phylogeny of concatenated sequences of 13 contigs (Gerhart et al., 2016). The overall similarity was 90% with the FLE of the Ixodidae family represented by *A. maculatum*. The green branched clade, containing *F. persica*, *F. opportunistica*, and *F. hispaniensis* represents the opportunistic pathogens that have been linked as potential causative agents of illness episodes in humans (Díaz-Sánchez et al., 2019; Gerhart et al., 2016, 2018). The red-branched cluster, shown as the outgroup, are the pathogenic strains of *Francisella tularensis sl*. To show the relationship of the contigs identified with the FLE clade, the sequence named Contig_ORF_FLE_of_*D. nitens*_1 was used as a representative sequence for the phylogenetic analysis, mainly because all 13 contigs grouped with the tick endosymbiont clade. The total coverage found for the 13 contigs classified as FLE was 12,515, with contigs 13 and 1 being the most predominant among all pools of samples (Table 2.3).

Figure 2.4 Phylogenetic relationship of the *Francisella*-Like Endosymbiont in the *D. nitens* samples in this study.



Translated sequences were used for constructing the concatenated open reading frames. The selected contig contains nine genes (Table 2.4) annotated with a total length for the concatenated contig of 3323 amino acids (9969 bp) and 1892 transcripts per million (TPM) in the pooled metatranscriptome. The tree is for a maximum likelihood cladogram built using the complete deletion method. Bootstrapping percentage values are based on 500 replications and are shown at the nodes. The outgroup is for the sequences of pathogenic *F. tularensis* strains. The blue lines correspond to tick FLE, the green lines correspond to opportunistic pathogens, and the red lines correspond to pathogenic strains of *F. tularensis*. The GenBank accession numbers are shown at the beginning of each label.

Sequence name	GenBank accession number	Bolivar	Antioquia	Cordoba
Contig_ORF_FLE_of_D.nitens_13		3.75	5.71	8.03
Contig_ORF_FLE_of_D.nitens_1		2.97	4.78	7.17
Contig_ORF_FLE_of_D.nitens_9		2.70	5.09	6.91
Contig_ORF_FLE_of_D.nitens_7		2.32	4.31	6.41
Contig_ORF_FLE_of_D.nitens_8		2.28	4.22	6.34
Contig_ORF_FLE_of_D.nitens_6		1.85	4.18	6.20
Contig_ORF_FLE_of_D.nitens_3		1.89	4.11	5.98
Contig_ORF_FLE_of_D.nitens_2		1.88	3.94	6.06
Contig_ORF_FLE_of_D.nitens_4		1.78	4.09	5.96
Contig_ORF_FLE_of_D.nitens_5		2.08	3.46	5.89
Contig_ORF_FLE_of_D.nitens_12		1.45	3.37	5.61
Contig_ORF_FLE_of_D.nitens_11		0.56	2.77	5.06
Contig_ORF_FLE_of_D.nitens_10		0.64	2.16	4.62

Table 2.3 The coverages of contigs for *Francisella*-Like Endosymbiont (FLE) genes.

The coverages are shown by the $\log_{10}$(transcript per million) in the metatranscriptome analysis. In the heat map, white is for low and red is for high frequency.

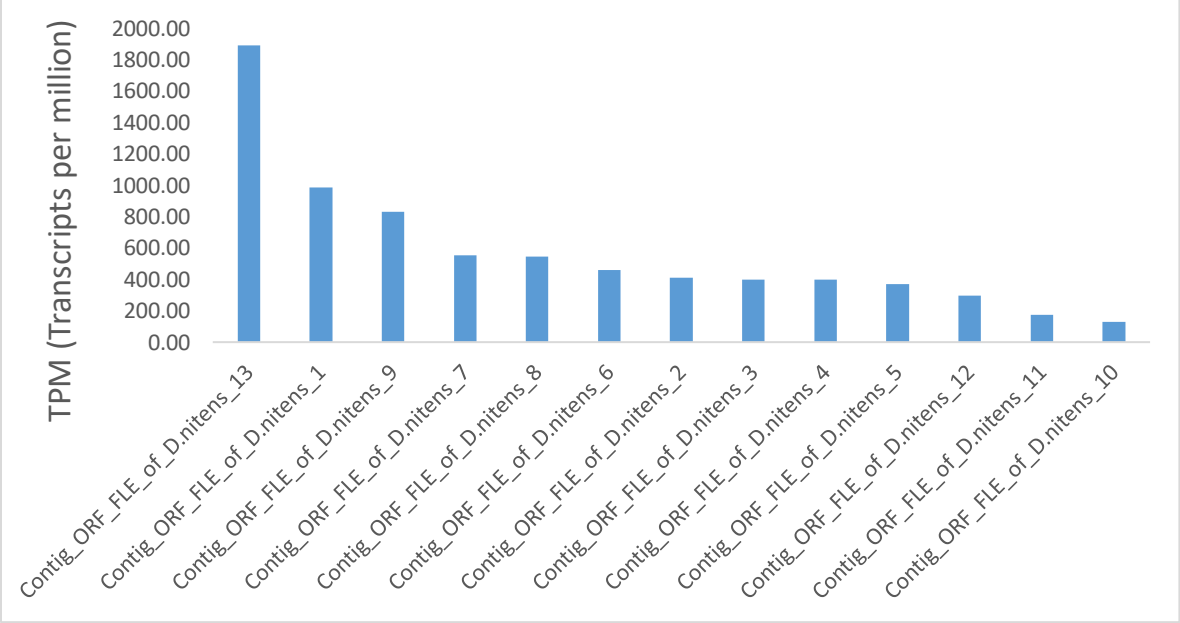
Overall, the thirteen contigs were categorized as FLE, containing presumed independent operons with an average length of 4,794 bp. Table 2.4 shows the length and coverage information, the sequence name, the gene encoded, and the putative gene size for each contig (Figure 2.5). The highest coverage of the FLE contigs was Contig_ORF_FLE_of_*D. nitens*_13, which partially encodes the mechanosensitive ion channel protein MscS with a length of 596 and 1,892.14 TPM (transcripts per million reads) (Figure 2.6 and Table 2.3). FLE putative operon sequences were submitted to GenBank and are contained in the BioProject PRJNA953638.

Figure 2.5 Graphical representation of the genes annotated for each contig identified as FLE.

Graphical representation of the genes annotated for each contig identified as *Francisella*-Like Endosymbiont (FLE) obtained from the partially fed *D. nitens* female pools. **(A)** represents the contig named Contig_ORF_*D.nitens*_FLE_1. **(B)** represents the contig named Contig_ORF_*D.nitens*_FLE_2. **(C)** represents the contig named Contig_ORF_*D.nitens*_FLE_3. **(D)** represents the contig named Contig_ORF_*D.nitens*_FLE_4. **(E)** represents the contig named Contig_ORF_*D.nitens*_FLE_5. **(F)** represents the contig named Contig_ORF_*D.nitens*_FLE_6. **(G)** represents the contig named Contig_ORF_*D.nitens*_FLE_7. **(H)** represents the contig named Contig_ORF_*D.nitens*_FLE_8. **(I)** represents the contig named Contig_ORF_*D.nitens*_FLE_9.

(J) represents the contig named Contig_ORF_*D.nitens*_FLE_10. **(K)** represents the contig named Contig_ORF_*D.nitens*_FLE_11. **(L)** represents the contig named Contig_ORF_*D.nitens*_FLE_12. **(M)** represents the contig named Contig_ORF_*D.nitens*_FLE_13. The genes represented in yellow color correspond to previously known annotated genes available on the NCBI website database, genes represented with green color are current non-annotated predicted genes obtained from the homology-based search at the NCBI databases.

Figure 2.6 Abundance of FLE contigs in the metatranscriptome of *D. nitens*.



Sequence ID	Gene name	Open reading frame (bp)	GenBank accession number
Contig_FLE_D.nitens_1, length = 9969bp, Coverage = 1628			
TRINITY_DN179725_c0_g1_Gene1	3-Oxoacyl-ACP synthase CDS	972	
TRINITY_DN179725_c0_g1_Gene2	Phosphate acyltransferase CDS	1047	
TRINITY_DN179725_c0_g1_Gene3	rpmF CDS	183	
TRINITY_DN179725_c0_g1_Gene4	Hypothetical protein CDS	504	
TRINITY_DN179725_c0_g1_Gene5	Transketolase CDS	1992	
TRINITY_DN179725_c0_g1_Gene6	Glyceraldehyde-3-phospate dehydrogenase CDS	1002	
TRINITY_DN179725_c0_g1_Gene7	Phosphoglycerate kinase CDS	1179	

TRINITY_DN179725_c0_g1_Gene8	Pyruvate kinase CDS	1437
TRINITY_DN179725_c0_g1_Gene9	Fructose-1,6-bisphosphate aldolase CDS	1065
Contig_FLE_D.nitens_2, length = 5250bp, Coverage = 696		
TRINITY_DN15830_c0_g2_Gene1	Nucleotide exchange factor GrpE CDS	588
TRINITY_DN15830_c0_g2_Gene2	Molecular chaperone DnaK CDS	1929
TRINITY_DN15830_c0_g2_Gene3	Molecular chaperone DnaJ CDS	1122
TRINITY_DN15830_c0_g2_Gene4	LysR family transcriptional regulator CDS	906
TRINITY_DN15830_c0_g2_Gene5	Hypothetical protein CDS	705
Contig_FLE_D.nitens_3, length = 8089bp, Coverage = 675		
TRINITY_DN25174_c0_g1_Gene1	Hypothetical protein CDS	1444
TRINITY_DN25174_c0_g1_Gene2	Hypothetical protein CDS	620
TRINITY_DN25174_c0_g1_Gene3	Hypothetical protein CDS	1006
TRINITY_DN25174_c0_g1_Gene4	Hypothetical protein CDS	1003
TRINITY_DN25174_c0_g1_Gene5	Membrane protein CDS	478
TRINITY_DN25174_c0_g1_Gene6	Hypothetical protein CDS	934
TRINITY_DN25174_c0_g1_Gene7	moxR CDS	962
TRINITY_DN25174_c0_g1_Gene8	Hypothetical protein CDS	444
TRINITY_DN25174_c0_g1_Gene9	pdcY CDS	853
TRINITY_DN25174_c0_g1_Gene10	Hypothetical protein CDS	345
Contig_FLE_D.nitens_4, length = 5373bp, Coverage = 660		
TRINITY_DN3539_c0_g1_Gene1	Carbamoyl phosphate synthase small subunit CDS	1167
TRINITY_DN3539_c0_g1_Gene2	Carbamoyl phosphate synthase large subunit CDS	3285
TRINITY_DN3539_c0_g1_Gene3	Aspartate carbamoyltransferase CDS	921
Contig_FLE_D.nitens_5, length = 5215bp, Coverage = 617		
TRINITY_DN112697_c0_g1_Gene1	Coproporphyrinogen III oxidase CDS	1143
TRINITY_DN112697_c0_g1_Gene2	Polysacccharide biosynthesis protein GtrA CDS	378
TRINITY_DN112697_c0_g1_Gene3	Peroxidase CDS	882
TRINITY_DN112697_c0_g1_Gene4	Aconitate hydratase CDS	2812
Contig_FLE_D.nitens_6, length = 1350bp, Coverage = 787		
TRINITY_DN1678_c0_g1_Gene1	Glutamate dehydrogenase CDS	1350
Contig_FLE_D.nitens_7, length = 2846bp, Coverage = 942		
TRINITY_DN396500_c0_g1_Gene1	Glycine dehydrogenase CDS	1381
TRINITY_DN396500_c0_g1_Gene2	Glycine dehydrogenase CDS	1465
Contig_FLE_D.nitens_8, length = 4254bp, Coverage = 880		
TRINITY_DN1569_c0_g1_Gene1	ATP synthase subunit alpha CDS	1542
TRINITY_DN1569_c0_g1_Gene2	ATP F0F1 synthase subunit gamma CDS	897
TRINITY_DN1569_c0_g1_Gene3	ATP synthase subunit beta CDS	1377

TRINITY_DN1569_c0_g1_Gene4	atpC CDS	438
Contig_FLE_D.nitens_9, length = 7945bp, Coverage = 1393		
TRINITY_DN253568_c0_g1_Gene1	Leucyl aminopeptidase CDS	1440
TRINITY_DN253568_c0_g1_Gene2	lptF CDS	1087
TRINITY_DN253568_c0_g1_Gene3	lptG CDS	1063
TRINITY_DN253568_c0_g1_Gene4	Insulinase family protein CDS	1254
TRINITY_DN253568_c0_g1_Gene5	Insulinase family protein CDS	1254
TRINITY_DN253568_c0_g1_Gene6	rsmD CDS	579
TRINITY_DN253568_c0_g1_Gene7	Trimeric intracellular cation channel family protein CDS	654
TRINITY_DN253568_c0_g1_Gene8	tRNA-(ms[2]io[6]A)-hydrolase CDS	614
Contig_FLE_D.nitens_10, length = 3170bp, Coverage = 221		
TRINITY_DN182378_c0_g1_Gene1	Amino acid transporter CDS	705
TRINITY_DN182378_c0_g1_Gene2	Oxidoreductase, short chain dehydrogenase/reductase family CDS	827
TRINITY_DN182378_c0_g1_Gene3	Hypothetical protein CDS	471
TRINITY_DN182378_c0_g1_Gene4	NAD(FAD)-utilizing dehydrogenase CDS	1167
Contig_FLE_D.nitens_11, length = 4745bp, Coverage = 306		
TRINITY_DN15837_c0_g1_Gene1	Hypothetical protein CDS	653
TRINITY_DN15837_c0_g1_Gene2	Hypothetical protein CDS	417
TRINITY_DN15837_c0_g1_Gene3	Alanine--tRNA ligase CDS	2598
TRINITY_DN15837_c0_g1_Gene4	Transporter CDS	1077
Contig_FLE_D.nitens_12, length = 3517bp, Coverage = 491		
TRINITY_DN182530_c0_g1_Gene1	Hypothetical protein CDS	537
TRINITY_DN182530_c0_g1_Gene2	rplT CDS	357
TRINITY_DN182530_c0_g1_Gene3	50S ribosomal protein L35 CDS	199
TRINITY_DN182530_c0_g1_Gene4	Translation initiation factor IF-3 CDS	519
TRINITY_DN182530_c0_g1_Gene5	Threonine--tRNA ligase CDS	1905
Contig_FLE_D.nitens_13 length = 596bp, Coverage = 3219		
TRINITY_DN15777_c0_g1_Gene1	Mechanosensitive ion channel protein MscS-Partial	596
Total coverage		12515

Table 2.4 Annotations of bacterial contigs captured in the metatranscriptome of *Dermacentor nitens*.

Discussion

Hard ticks harbor a considerable diversity of bacteria, many of which are significant pathogens to humans or domestic animals (Cabezas-Cruz et al., 2018; CDC, 2022; Dantas-Torres et al., 2012; Duron et al., 2017; Jongejan & Uilenberg, 2004; Madison-Antenucci et al., 2020; Narasimhan et al., 2021). A comprehensive survey of tick microorganisms may allow us to uncover the spectrum of the vectorial capacity of ticks for known pathogens and yield novel potential pathogenic microorganisms. In addition, it may provide a better understanding of the interactions among microorganisms under different environmental conditions. Thus, identifying symbiotic microorganisms and their effects on the vectorial capacity is critical for predicting future outbreaks caused by febrile diseases of unknown etiology (Prasad et al., 2015). In this study, metatranscriptome and bacterial 16S rRNA sequencing enriched the sequence database with newly uncovered *Francisella*-like Endosymbionts (FLE) in the blood-fed *D. nitens* originating from three different geographical areas in Colombia.

Differences in the bacterial compositions of ticks collected from animals coming from Bolivar, Antioquia, and Cordoba populations were found with either inclusion or exclusion of the FLE sequences. (Figures 2.2C and 2.2D). The NMDS plot for 16S sequences revealed clusters for tick geographical origin with a unique bacterial assortment. Geographically separated populations of ticks have previously been shown to have distinctive microbial compositions in several tick species (Cotes-Perdomo et al., 2020; Gurfield et al., 2017; D. Kumar, Downs, et al., 2022; Santodomingo et al., 2019; Van Treuren et al., 2015). Microbial compositions could be influenced by other factors, such as the degree of tick engorgement (Clay et al., 2008; Clow et al., 2018; Moreno et al., 2006). The capacity of ticks to acquire and spread pathogens may be significantly impacted by these variations in microbial composition.

We found that the most abundant bacterium was FLE (80% of classified reads), which is phylogenetically related to the pathogenic bacteria *F. tularensis* and causes tularemia in humans (Park et al., 2021). While *Dermacentor variabilis* and *Dermacentor andersoni*, are known to carry this pathogen and are common in the northern hemisphere, the effect of interactions of FLE with pathogens and their role in disease transmission remain unknown (Ahantarig et al., 2013; Bonnet et al., 2017; Cabezas-Cruz et al., 2019; Gurfield et al., 2017; Yeni et al., 2021). Previous results have shown a positive association of vertically-transmitted FLE against pathogenic *Francisella novicida* artificial infection in *D. andersoni*; however, *F. novicida* is not considered a tick-borne pathogen, which means this interaction is unlikely to happen in natural conditions (Gall et al., 2016).

Our result shows that the microbial composition of *D. nitens* appears to vary depending on the geographic location of the species' population. We observed an overall higher proportion of FLE compared to those previously reported in *D. variabilis* (62%), and *D. occidentalis* (41%) in the Americas (Gurfield et al., 2017; Travanty et al., 2019). This high abundance of these FLE was in accordance with previous 16S rRNA sequencing studies on whole-body samples obtained from partially or fully-engorged adult females of *D. variabilis*, *D. marginatus*, *D. reticulatus*, *D. silvarum*, and *D. albipictus* (Duan et al., 2020; Sperling et al., 2020; Travanty et al., 2019; Y.-K. Zhang et al., 2019). Metatranscriptomic analysis suggested high levels of FLE coverage (*i.e.*, transcript per million reads TPM) for Cordoba samples, but without statistical significance in all pairwise comparisons by Student t-test. 16S rRNA analysis, showing the relative abundance, also suggested that the Cordoba population is richer in FLE. The department of Cordoba, an agricultural stronghold in northern Colombia, has a constant flow and exchange of animals. Thus, the associated ticks may be exposed to a more diverse bacterial environment, which may

explain the increased detection frequency of both endosymbiont and transient bacteria, through mechanisms such as horizontal transfer (Bonnet et al., 2017; Van Treuren et al., 2015). These tendencies of small differences in the communities of endosymbionts related to the geographical origin of the ticks have also been reported for *D. occidentalis* (Gurfield et al., 2017).

In other tick species, such as *Ixodes scapularis*, the endosymbiont population has been shown to impact pathogen infection processes. An unaltered intestinal microbiota favored colonization of *Borrelia burgdorferi s.l.*, whereas an induced microbial dysbiosis environment showed a negative effect by blocking the colonization of *Anaplasma phagocytophilum* (Bonnet et al., 2017; Bonnet & Pollet, 2021). In *D. nitens*, the transmission of human pathogens is yet unknown; however, *D. nitens* ticks collected from equines in Brazil were found positive for *B. burgdorferi s.l.*, the complex known as the causal agent of Lyme disease in the Americas (Gonçalves et al., 2013). Whereas *D. nitens'* potential as a Lyme disease vector and the roles of the FLE population have not been documented, the initial characterization of the FLE population may provide insights into their involvement in tick vector competence.

Our FLE sequence analysis revealed three different *D. nitens* FLE variants, OTU001, 002, and 010, with relatively large variations (8 to 21 bp or 1.7 to 4.5% difference) in the V3-V4 region. The source of these variants are likely derived from different strains that occurred in all three geographical locations. While the genus *Francisella* contains three 16S rRNA copies, we exclude the possibility of intra-genomic variations from these copies based on a study that described a 99.65% minimum similarity average in 1374 Proteobacteria genomic sequences of 16S rRNA (Ibal et al., 2019). These results are comparable to our previously reported study in *Amblyomma americanum*, where at least two different strains of *Coxiella*-like endosymbionts were found at the individual tick level (Maldonado-Ruiz et al., 2021). Three *D. nitens* FLE

OTUs were monophyletic and clustered while this cluster is also grouped with the FLE of other *Dermacentor* FLEs (Fig. 2). However, FLEs of *R. microplus* and *I. scapularis* were also grouped in this clade (Scoles, 2004), indicating, that endosymbionts are more diverse than previously thought. This data also suggest that relatively recent independent invasions or transfers of FLEs frequently occurred, as it has been shown that the FLE initially evolved from the pathogenic *Francisella* species (Bonnet et al., 2017; Díaz-Sánchez et al., 2019; Duron et al., 2017, 2018; Gerhart et al., 2016, 2018; D. Kumar, Sharma, et al., 2022; Scoles, 2004; Travanty et al., 2019).

Overall, this study offers a description of the diversity of bacterial communities of partially fed *D. nitens* female ticks collected in animals originating from three Colombian regions based on our 16S rRNA sequences and transcriptomic analysis. In addition to the differentiated geographical populations in the bacterial composition, we also found multiple co-existing strains of FLE in *D. nitens*, which provides the foundation for future studies aiming to examine the differences of these endosymbionts. A deeper understanding of the microbial communities hosted by ticks can be utilized to develop future measures to mitigate tick pathogen transmission.

Chapter 3 - Metatranscriptomic-based phylogeny of viral communities in *D. nitens*

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Abstract

Ticks are second only to mosquitoes in transmitting pathogens to humans and are considered the primary vectors of pathogens for livestock, companion animals, and wildlife. Ticks can colonize a wide range of ecological niches, from the tropics to the polar areas. In recent decades, increasing concerns about the threat of viruses transmitted by ticks have arisen based on the discoveries obtained from transcriptomic data. The compositions of viral communities are highly diverse in ticks, but the factors driving this diversity are not well understood. The tropical horse tick, *Dermacentor nitens*, is distributed throughout the Americas and is recognized as a natural vector of *Babesia caballi* and *Theileria equi*, the causal agents of equine piroplasmosis. We characterized the viral communities associated with partially fed *D. nitens* females collected by a passive survey on horses from field sites representing three distinct geographical areas in Colombia (Bolivar, Antioquia, and Cordoba). RNA-seq was performed using the Illumina-Miseq platform. A total of nine contigs corresponding to six different viruses were identified in three viral families: Chuviridae, Rhabdoviridae, and Flaviviridae. Metatransctiptomics revealed the contigs containing viral proteins have a trend of regional differences. These findings suggest regional distinctions among the viral communities in ticks.

Introduction

Ticks have been identified as vectors of viral, protozoan, and bacterial agents that can cause diseases. Tick-borne encephalitis virus, Powassan virus, and Crimean-Congo hemorrhagic fever virus are the most prevalent tick-borne viral infections.(Bonnet et al., 2017; Madison-Antenucci et al., 2020). Viruses form a major constituent of the microbial composition in ticks, but the viruses that are considered medical and veterinary pathogens are only a small part of the total viral community hosted by the ticks. Less than ten percent of tick species are known to be virus vectors (Mansfield et al., 2017)

Viruses are present in all domains of life, but are particularly rich in Arthropoda, which includes ticks (Pettersson et al., 2017). Metatranscriptomics is a widely used tool to investigate RNA viruses in ticks. Despite considerable insights into bacterial diversity, our understanding of tick-associated viruses is still limited, and largely unexplored compared with bacterial diversity (Tokarz et al., 2014). Improvements in sequencing technology and metagenomics data have allowed researchers to approximate the composition of viral communities in a few tick species (Brinkmann et al., 2018; Gómez et al., 2020; Orozco Orozco et al., 2021; Sameroff et al., 2019; Tokarz et al., 2014, 2018; Xu et al., 2021). Virome studies of ticks collected in Asia, Europe, and North America have revealed the emergence of novel pathogenic tick-borne viruses as well as the dearth of data on tick viromes which suggest a need for viral surveillance and discovery in this group of arthropods (Brinkmann et al., 2018; Li et al., 2015; Shi et al., 2018). In addition, more information from different species may be an efficient strategy to mitigate potential threats of tick-borne disease to public health (Madison-Antenucci et al., 2020; Prasad et al., 2015; Shi et al., 2018; Xu et al., 2021).

The tropical horse tick, *Dermacentor nitens*, is a one-host tick, with three to four generations per year (Labruna et al., 2002). Although equines are the primary host, natural infestations have been reported in other domestic, and companion animals, as well as in wild animals (Borges & Silva, 1994; Martins et al., 2015; Nelson et al., 2017). Occurrence of human pathogenic agents in this tick species have been previously reported, but the diversity of viruses has not been extensively studied. (Cotes-Perdomo et al., 2020; Santodomingo et al., 2019).

By performing a comprehensive metatranscriptomic analysis of the viral communities associated with partially fed *D. nitens* collected in different geographical populations, we gained substantial insight into tick virome composition and can begin to assess the potential role of these viruses in the tick life cycle. These results provide large numbers of sequences annotated as tick viruses and revealed a trend of differences among the three geographical populations. Therefore, tick surveillance is an indispensable tool to understand spatial-temporal virus activity and predict probable outbreaks.

Materials and methods

Sample collection and nucleic acid extraction

Tick collection was carried out by passive survey at “La Rinconada” slaughterhouse (06°11'26.0"N; 75°22'43.4" W) in the municipality of Rionegro, Antioquia, Colombia in July, and September 2019. A total of 45 blood-fed *D. nitens* adults were obtained from three horses native to each region, Bolivar, Antioquia, and Cordoba. The three departments are in the northwest of Colombia and share borders with the Department of Antioquia. Live ticks were transported to the Universidad de Antioquia facilities, where taxonomical identification was made following morphological keys (Barros-Battesti et al., 2006), and specimens were subsequently stored at -20 or -80°C until shipment to Kansas State University facilities. Blood-

fed female *D. nitens* collected from horses were pooled and processed based on the host (individual animal) and region (Bolivar, Antioquia, and Cordoba). From a total of three horses per region, one pool of five ticks per horse were chosen by using the random selection method, thus sampling a total of 45 ticks (nine pools). RNA was extracted independently following manufacturer instructions using the Zymo™ RNA extraction kit (Irvine, California, US) from the pools previously separated from the tick-exoskeleton.

RNA-seq library preparation and data processing

RNA-seq library preparation was done with the NEB Next Stranded RNA library kit without PolyA selection of the mRNA, and the nine pooled RNAs were sent to the Genome Sequencing Core at the University of Kansas. For the metatranscriptomics analysis, the RNA-seq reads were processed for removal of Illumina adaptor sequences, trimmed, and quality-based filtered using Fastp software v.0.20.0 (Chen et al., 2018). The high-quality reads (Phred-score >30) were removed by mapping onto the reference genome of *D. silvarum* (assembly ASM1333974v1) and *Equus caballus* (assembly EquCab3.0) using STAR v.2.7 (Dobin et al., 2013). The unmapped reads (Table 3.1) were used to perform the assembly and annotation of the viral transcriptome by using Trinity and Blast2GO suite in OmicsBox v.2.0.36 software (Götz et al., 2008; Grabherr et al., 2011; Langmead & Salzberg, 2012). Contigs annotated in Blast2GO were reexamined manually by BLASTn and BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the results and eliminate potential false positives. Empirical Bayes estimation and Fisher's exact tests ($\alpha = 0.05$) by pairwise comparison based on the negative binomial distribution analysis were done with edgeR by using the Galaxy platform to test statistically significant differences in abundance among the viral sequences annotated with the geographic location for the blood-fed *D. nitens*.

Library	Location	Raw Reads (Million)	Clean reads (Million)	Mapped reads against horse and *Dermacentor* ticks (Million)	Remaining reads (Million)
Pool 01	Bolivar	15.2	14.2	1.6	12.55
Pool 02	Bolivar	14.1	13.2	5	8.18
Pool 03	Bolivar	15.9	14.8	5.2	9.67
Pool 04	Antioquia	19.2	17.8	2.5	15.37
Pool 05	Antioquia	18.1	16.8	6.1	10.58
Pool 06	Antioquia	15.7	14.7	5.1	9.54
Pool 07	Cordoba	16.6	15.6	1.9	13.7
Pool 08	Cordoba	15.3	14.4	5.9	8.4
Pool 09	Cordoba	22.1	21.3	17.2	4.19
Total		152.2	142.8	50.5	92.18

Table 3.1 List of library sequences obtained in the metatranscriptomics study.
Each pool comprises five *Dermacentor nitens* females. Refer to the Material and Methods section for more details about cleaning and mapping processes.

Phylogenetic analysis of viral contigs

Phylogenetic analyses by comparison of Bayesian inference, Maximum-Likelihood, Minimum-Evolution, and Neighbor-Joining methods were performed as an initial assessment with the viral protein sequences detected in this study compared to the reference sequences pulled out from the NCBI GenBank database by doing a homology-based search using tBLASTn search. Viral protein sequences were retrieved from the GenBank database as indicated with the GenBank accession numbers in figure X. Sequences were aligned using Muscle in MEGA-X software (S. Kumar et al., 2018). Bayesian inference analysis was done using BEAST v1.10.4 software (Suchard et al., 2018). Phylogenetic trees for the metatranscriptomic analysis of the sequences of the viral proteins were constructed using annotated and concatenated genes for each contig by using the Maximum Likelihood method with the Tamura-Nei model and 500 bootstrap replicates (Tamura & Nei, 1993).

Ethical approval

This study was approved by the Bioethics Committee of the Universidad de Antioquia (Approval record No. 15-32-436 of June 2015). It was also granted an environmental license issued by the Colombian government through the National Environmental Licensing Authority (Autoridad Nacional de Licencias Ambientales-ANLA, Resolution ANLA 00908 of May 27, 2017).

Results

Metatranscriptome analysis of viral RNA.

A total of 152.2 million raw reads were obtained from the nine pools representing the three different regions. After quality trimming and filtering out against *E. caballus* and *D. silvarum* sequences, 92.18 million reads were used for downstream analysis. *De novo* assembly was conducted using the TRINITY pipeline built in OmicsBox software. After cleaning and filtering, 16.8 million reads were assembled into 81 contigs. Homology-based taxonomic assignment and gene function for each contig was made in Blast2Go and using manual BLAST searches.

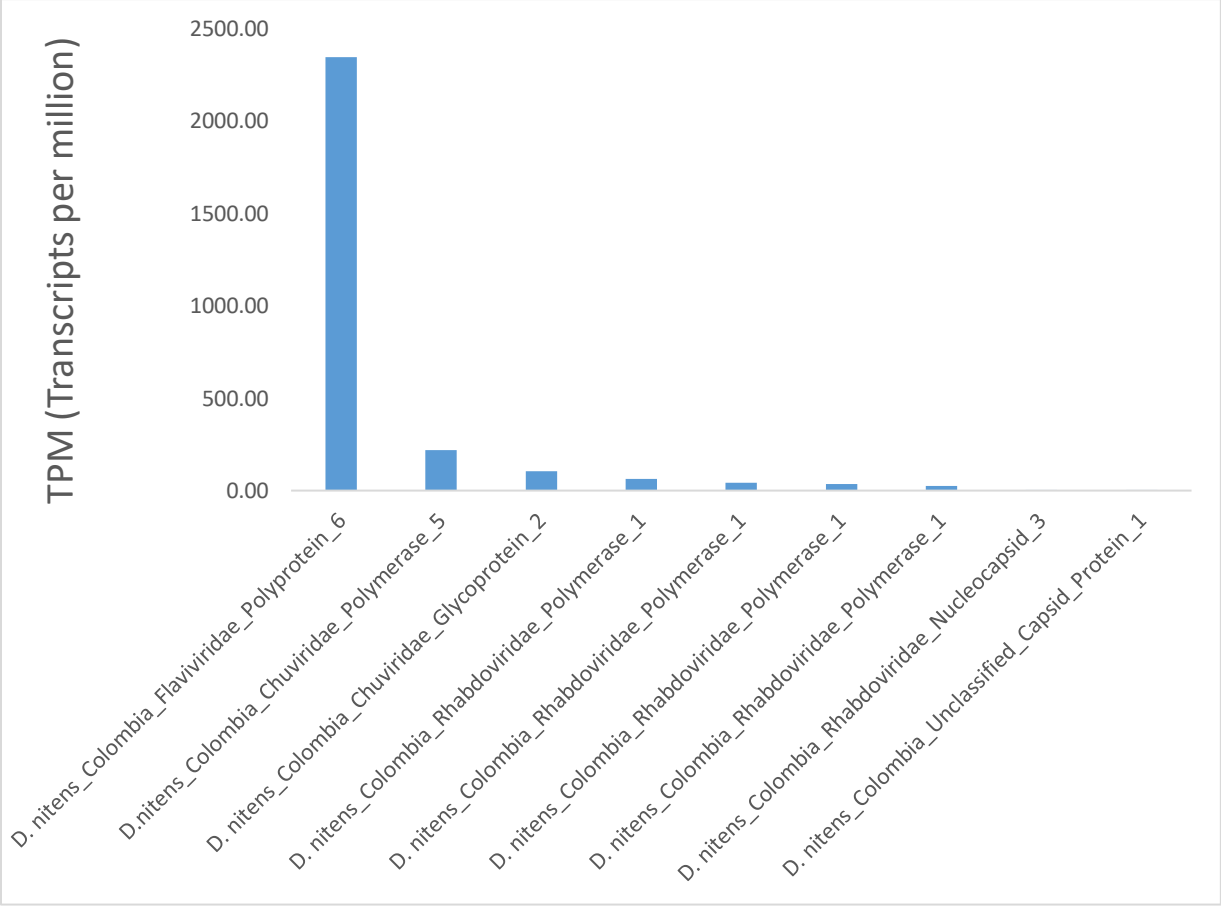
Six different putative viruses covered by nine viral contigs with an average length of 1,749 bp were identified in BLAST searches for the non-redundant protein database of NCBI and the Viral Genomes database. The sequences were manually inspected and annotated for the coding regions. Table 3.2 shows the viral contigs with the length and coverage information. The highest coverage for the viral contigs was the *D. nitens*_Colombia_Flaviviridae_Polyprotein_6 contig with a total of 2,346.25 TPM with the coverage predominantly higher in the region of Cordoba (Figure 3.1 and Table 3.3). The *D. nitens* virus contig sequences were submitted to GenBank and are contained in the BioProject PRJNA953638.

Contig ID	Length	Coverage	Sequence name	Blast result		
				GenBank ID	e-value	Name of Virus
Unclassified_Capsid_Protein_1	198	1	TRINITY_DN36539_c0_g1	QBQ65105.1	4.00E-140	Xinjiang Tick associated virus 2
Chuviridae_Glycoprotein_2	668	168	TRINITY_DN179920_c0_g1	YP_009177705.1	0	Changping Tick Virus 2
Chuviridae_Polymerase_5	2156	355	TRINITY_DN180002_c0_g1	YP_009177704.1	0	Changping Tick Virus 2
Rhabdoviridae_Nucleocapsid_3	524	4	TRINITY_DN327528_c0_g1	AUX13127.1	0	American dog tick rhabdovirus 2
Rhabdoviridae_Polymerase_1	7061	218	TRINITY_DN16706_c0_g1	QDW81034.1	0	Blanchseco virus
			TRINITY_DN399801_c0_g1	QDW81033.1	0	Blanchseco virus
			TRINITY_DN405583_c0_g1	QDW81033.1	0	Blanchseco virus
			TRINITY_DN31349_c0_g1	QDW81033.1	0	Blanchseco virus
Flaviviridae_Polyprotein_6	5140	3374	TRINITY_DN544_c0_g1	UGM45976.1	0	Flaviviridae sp.
Total coverage		4120				

Table 3.2 Viral contigs captured in the metatranscriptome of *D. nitens*

Viral contigs captured in the metatranscriptome of *D. nitens*, shown for the lengths, coverages, and Blast results.

Figure 3.1 Abundance of virus contigs in the metatranscriptome of *D. nitens*.



Sequence name	GenBank accession number	Bolivar	Antioquia	Cordoba
D. nitens_Colombia_Flaviviridae_Polyprotein_6		5.96	7.28	7.50
D.nitens_Colombia_Chuviridae_RdRp_5		1.61	3.57	1.06
D. nitens_Colombia_Chuviridae_Glycoprotein_2		0.97	2.54	0.98
D. nitens_Colombia_Rhabdoviridae_RdRp_1		0.90	0.30	2.30
D. nitens_Colombia_Rhabdoviridae_RdRp_1		0.62	0.00	2.61
D. nitens_Colombia_Rhabdoviridae_RdRp_1		0.21	0.48	2.46
D. nitens_Colombia_Rhabdoviridae_RdRp_1		0.21	0.00	2.09
D. nitens_Colombia_Rhabdoviridae_Nucleocapsid_3		0.44	0.00	0.00
D. nitens_Colombia_Unclassified_Capsid_Protein_1		0.00	0.00	0.14

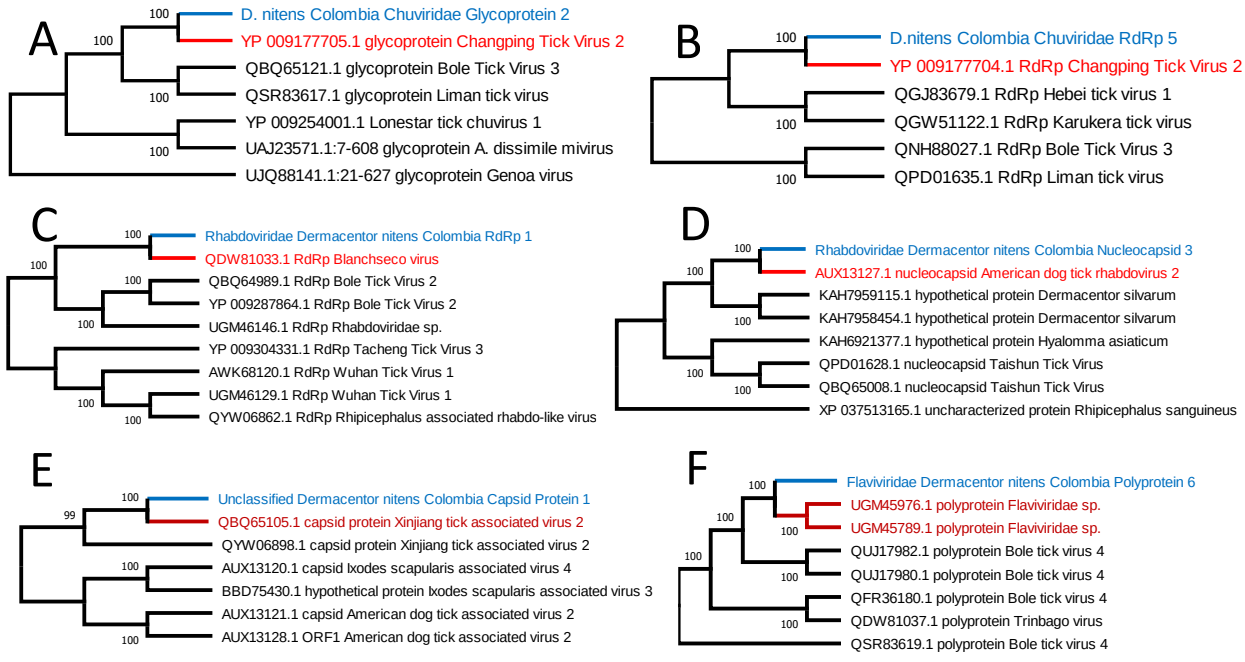
Table 3.3 Coverages of virus contigs shown by TPM for the metatranscriptome of *D. nitens*.
The geographical coverage of virus contigs are shown by $\log_{10}$(transcript per million) for the metatranscriptome of *D. nitens.* In the heat map, white is for low and red is for high frequency.

Phylogenetic analysis of viral contigs

Phylogenetic analysis of nine viral contigs found three different families for all different viral species. The genes were capsid protein, glycoprotein, nucleocapsid, polyprotein, and RNA-dependent RNA polymerase (RdRp) (Table 3.2). Most of the putative viruses were found by identifying genes encoding RdRp with five annotated sequences and classified into two viral families, Chuviridae and Rhabdoviridae. Two different contigs, *D. nitens*_Colombia_Chuviridae_Glycoprotein_2, and *D. nitens*_Colombia_Chuviridae_RdRp_5 were grouped into the same family Chuviridae. Based on the sequence similarities and the tree pattern (Figure 3.2A and 3.2B), these contigs are likely representing two different viruses although the name of the closely related virus is the same as Changping Tick Virus 2, a virus that has been reported in China and Turkey infecting *Dermacentor* spp. and *Hyalomma asiaticum* ticks (Brinkmann et al., 2018; Li et al., 2015). These two viruses were found to be more abundant in the region of Antioquia (Table 3.3).

The Family Rhabdoviridae is represented by five sequences clustered into two putative viruses (Figure 3.2C and 3.2D). Four of them targeting RdRp were grouped in a clade with Blanchseco virus. The remaining sequence was found encoding a nucleocapsid protein and clustered with the American dog tick Rhabdovirus-2. The contig *D. nitens*_Colombia_Unclassified_Capsid_Protein_1 showed a close relationship with the capsid protein of Xinjiang tick-associated virus-2, a virus sequence that was presumably reported for the first time in the province of Xinjiang in China. This virus remains unclassified for the family, and it is grouped with other tick viruses found in *Ixodes scapularis* and *D. variabilis* (Figure 3.2E). The family Flaviviridae was found to be represented by one contig named *D. nitens*_Colombia_Flaviviridae_Polyprotein_6 (Figure 3.2F). This name was assigned due to the high similarity found with a portion of a Flaviviridae polyprotein from *Haemaphysalis longicornis* and *Rhipicephalus microplus* infesting goats (Xu et al., 2021).

Figure 3.2 Phylogenetic relationship of the RNA viruses contigs captured in the *D. nitens* samples.



Phylogenetic relationships of the contigs for the RNA viruses captured in *D. nitens* samples in this study. The maximum likelihood cladograms were constructed with complete deletion of assembly gaps. Bootstrapping percentages in 500 replications are shown at the nodes. The contig *D. nitens Colombia Chuviridae Glycoprotein 2* encodes a Glycoprotein gene with a length of 668 bp **(A)**, *D.nitens_Colombia_Chuviridae_Polymerase_5* encodes an RNA-dependent RNA polymerase with a length of 2156 **(B)**, *Rhabdoviridae_Dermacentor_nitens_Colombia_Polymerase_1* encodes an RNA-dependent RNA polymerase with a length of 7061 bp **(C)**, *Rhabdoviridae_Dermacentor_nitens_Colombia_Nucleocapsid_3* encodes a nucleocapsid with a length of 524 bp **(D)**, *Unclassified_Dermacentor_nitens_Capsid_Protein_1* encodes a capsid protein with a length of 168 bp **(E)**, *Flaviviridae_Dermacentor_nitens_Colombia_Polyprotein_6* encodes a polyprotein with a length of 5140 bp **(F)**. Names in blue correspond to the viral contigs found in this study, and red names correspond to the closest viral protein sequence in the GenBank database. The GenBank accession numbers are shown at the beginning of the names of taxa.

	Bolivar-Antioquia	Bolivar-Cordoba	Antioquia-Cordoba
Sequence name	P-value	P-value	P-value
Rhabdoviridae_Nucleocapsid_3	0.03	0.03	1
Rhabdoviridae_Polymerase_1C	0.12	0.22	0.02
Rhabdoviridae_Polymerase_1D	0.17	0.42	0.08
Rhabdoviridae_Polymerase_1B	0.30	0.78	0.21
Chuviridae_Polymerase_5	0.46	0.44	0.19
Chuviridae_Glycoprotein_2	0.48	0.79	0.36
Rhabdoviridae_Polymerase_1A	0.90	0.23	0.24
Flaviviridae_Polyprotein_6	0.98	0.98	0.99
Unclassified_Capsid_Protein_1	1	1	1

Table 3.4 Pairwise comparisons of the TPM frequencies in virus contigs.

Pairwise comparisons of the frequencies (transcript per million, TPM) in virus contigs among the ticks collected in different locations. The statistics were empirical Bayes estimation and exact tests ($\alpha = 0.05$) in the pairwise comparisons of the frequencies of the paired regions. $P < 0.05$ is highlighted.

Discussion

Metatranscriptomics revealed several contigs highly similar to known viral families. The Rhabdoviridae family was found to be the most abundant and common in the pools of all sequences. This group of Rhabdoviridae viruses (Figure 3.2D) were also reported for different Ixodidae species such as *Rhipicephalus annulatus*, *R. sanguineus*, *Hyalomma marginatum*, *H.*

asiaticum, and *D. variabilis* in the United States (Brinkmann et al., 2018; Li et al., 2015; Tokarz et al., 2018). Blanchseco virus (Rhabdoviridae family) was found in one pool of *Amblyomma ovale* ticks infesting cattle and dogs in Trinidad and Tobago (Sameroff et al., 2019). Similarly, we have identified Chuviridae- related sequences in the *D. nitens* RNA pools as the second predominant viral family (Figure 3.2A). Chuviridae is a newly-proposed viral family, that constitutes a large monophyletic group, clustering in an intermediate phylogenetic branch between segmented and unsegmented negative-sense RNA viruses identified in ticks, true flies, mosquitoes, cockroaches, and crabs (Li et al., 2015). The most closely related virus (90.2% sequence identity, 11,275 out of 12,500 bp) to those found in *D. nitens* was previously identified in China (Fig. 3.2A). Similar viruses identified in samples from different continents may be a consequence of widespread historical commerce of animals.

We also found geographical differences in the Rhabdoviridae family according to the contig Rhabdoviridae_RdRp that showed differences between Antioquia and Cordoba regions ($p = 0.02$), and the sequence coverage for Rhabdoviridae_Nucleocapsid is predominant in Bolivar when compared with those in the other two regions ($p = 0.03$). The frequency data support the hypothesis of unique viral compositions in different regions (Table 3.3). The coverage of the viral gene composition among the ticks in three different populations showed statistical differences in transcripts classified into the Rhabdoviridae family (Table 3.4). A previous study with *R. microplus*, *D. nitens*, and *R. sanguineus s.l.* in the Magdalena Valley and Magdalena/Urabá ecoregions in Colombia reported the presence of Flaviviridae, Rhabdoviridae, Chuviridae, and Unclassified viruses (Orozco Orozco et al., 2021). We conclude that the core RNA virome composition appears to be poor compared with the bacterial endosymbiotic communities. However, identifying viruses by using preexisting viral sequences in the GenBank

may be limited for the discovery of novel viruses. This sequence-based survey needs further investigation to understand whether those are transiently acquired with the mammalian blood or established and vertically transmitted.

Overall, this study offers an initial description of the viral communities of partially fed *D. nitens* female ticks collected in animals originating from three Colombian regions based on our transcriptomic analysis. In addition to differentiation of viral communities by geographical region, we also found six viruses in *D. nitens.* This study provides a basic foundation for future studies that will promote a deeper understanding of the viral communities hosted by ticks which may be utilized to develop future measures to mitigate tick pathogen transmission.

Chapter 4 - Research summary and future directions

Over the past decades, research efforts focused on ticks have been exponentially increasing, and this has been reflected in the growing number of reported cases of tick-borne diseases. Despite this, our knowledge of tick microbial communities remains minimal when compared to our understanding of mosquitoes (Dantas-Torres et al., 2012). Taking into account the man-made changes in global climate and land use, especially for the livestock industry, pathogens transmitted by ticks are becoming a major threat to new populations where ticks have not been established in the past (Raghavan et al., 2016). New interactions with exotic abiotic environmental factors may be driving the diversity of pathogenic and non-pathogenic microbial communities. For this reason, molecular surveillance with bioinformatic analyses can be a powerful approach to predict and prevent future outbreaks. Understanding the current biological composition of the tick microbiome can fill the gaps in our understanding of tick biology and establish a baseline for future studies.

My thesis on the tropical horse tick contributes to the knowledge of tick-microbiome by showing typical compositions of the microbial and viral communities for this important ectoparasite of the livestock industry in the Americas. The genera *Dermacentor* has a close relationship with bacteria identified as *Francisella*-Like Endosymbionts (FLE). In chapter 2, I demonstrate that this type of endosymbiont is the predominant bacteria among regions, but its presence does not seem to affect regional microbial compositions by doing Non-Metric Multidimensional Scaling (NMDS) analysis in the presence and the absence of this bacteria from three different geographical regions. The research then was expanded to analyze the viral components of these *D. nitens* individuals, but we found a low diversity of viruses, represented by nine contigs classified in three families that have been previously reported for *Dermacentor*

ticks in China (Xu et al., 2021). Within these chapters, I described and analyzed separately two main components of the microbiome of *D. nitens.* This information will be useful as a baseline for molecular surveillance of emerging and re-emerging zoonoses in the Americas.

In Chapter 2, I described the bacterial composition of the partially fed *D. nitens* females collected from horses in three different regions in Colombia. The predominant bacteria found among all regions based on our 16s rRNA sequencing was *Francisella*/Francisellaceae, a result that was also corroborated with the metatranscriptomic analysis from the bacterial genes identified and classified based on homology-based search results and phylogenetic analyses, we found 13 genes of FLE among all three regions and most of the read coverage obtained from our RNA-seq data was associated with this endosymbiont. This endosymbiont has been related to the production of Vitamin B in ticks related phylogenetically to *D. nitens* (Duron et al., 2018; Scoles, 2004).

In chapter 3, I have expanded the analysis to explore the viral diversity of the blood-fed female *D. nitens*. The metatranscriptomic data obtained from the RNA-seq method showed us that the viral composition of ticks is poor when compared to bacterial diversity. Nine viral protein contigs were classified into three main families of RNA viruses that have been previously identified in other *Dermacentor* species.

Overall, I found that the microbial communities of *D. nitens* are mainly comprised of endosymbiotic bacteria, which are more diverse than believed in the past. The few taxonomic groups of viral communities of *D. nitens* were mainly RNA viruses that currently have not been related to any disease in humans. Further studies are required to understand the differences between sexes and developmental and feeding stages for *D. nitens*. Also, it would be interesting to analyze the bacterial and viral composition of different tissues and organs, including

endosymbionts and viruses that may be circulating in the hemolymph, which can lead to new discoveries in the vector-pathogen transmission in ticks (Yada et al., 2018; Yssouf et al., 2015; L. Zhang et al., 2011). Based on my study, the non-pathogenic, but symbiotic microorganisms are suggestive of being transmitted to the host during the blood-feeding process although further study is required. Overall, a sequence-based surveillance program is a powerful approach to understand the microbial and viral communities hosted by ticks, and these data can be utilized to develop future measures of surveillance, mitigation, and control of pathogens transmitted by ticks.

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