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Characterization of Tick-Associated Microbiota in Spain Using a Nanopore-based Metabarcoding Approach: Insights into Potential Zoonotic Pathogens

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Abstract

Ticks, well-known as hematophagous vectors, play a crucial role in transmitting numerous zoonotic pathogens ranging from viruses and bacteria to protozoa. To prevent and understand the transition from tick-borne disease (TBD) endemicity to epidemicity, it is imperative to focus on epidemiological aspects to develop robust surveillance methods and strategies. In this study, we developed a Nanopore-based metabarcoding approach for bacteria detection in ticks collected from Catalunya and Valencia. A total of 186 adult ticks belonging to *Rhipicephalus bursa*, *Dermacentor marginatus*, *Hyalomma lusitanicum*, *Hyalomma marginatum*, and *Ixoded ricinus* species were studied. In this study, we used PCR amplification combined with Nanopore sequencing techniques and bioinformatics pipelines to characterize the tick-associated microbiota studies. Full-length 16S rRNA gene metabarcoding sequencing demonstrated 59 species and 336 genera generated belonging to 22 phyla, 33 classes, 111 orders, and 178 families. Within this examination, we identified some novel endosymbionts bacterial species that according to the best of our knowledge were not documented previously in Spain including *Candidatus Coxiella mudrowiae, Francisella persica, Rickettsia africae, Rickettsia japonica, Rickettsia rhipicephali,* and *Unclassified Aeromonas*. *Candidatus Coxiella mudrowiae* and *Rickettsia africae* species predominated in the *Rhipicephalus bursa* tick. The *Francisella* genus and *Rickettsia rhipicephali species* dominated in *Hyalomma marginatum*. While *Rickettsia japonica* is associated with all the examined tick samples except *Rhipicephalus bursa*. The alpha diversity results, as assessed by the Shannon index (p-value > 0.05), indicate that there are no statistically significant variations in the bacterial communities within ticks, whether we consider different geographic locations or the feeding status of the ticks.

Keywords: Tick microbiome,16S rRNA metabarcoding, MinION Nanopore sequencing, TBP, Spain

List of Abbreviations

HIV: Human Immunodeficiency Virus

GHSA: Global Health Security Agenda

I. ricinus : Ixodes ricinus

Rh. sanguineus: Rhipicephalus sanguineus

Rh. bursa: Rhipicephalus bursa

H. marginatum: Hyalomma. marginatum

H. lusitanicus: Hyalomma. Lusitanicus

D. reticulatus: Dermacentor reticulatus

D. marginatas: Dermacentor marginatas

M. mitochondrii : *Candidatus Midichloria mitochondrii*

TBPDs: Tick-Borne Pathogen Diseases

TBPs: Tick-borne Pathogens

SFR: Spotted Fever Rickettsioses

SFGR: Spotted fever group rickettsiae

MSF, Mediterranean spotted fever

JSF: Japanese spotted fever

TG : Typhus group

FLEs: Francisella-like endosymbionts

CCHFV: Crimean-Congo Haemorrhagic Fever

TBRF: Tick-Borne relapsing fever

TIBOLA: tick-borne lymphadenopathy

DEBONEL: Dermacentor-borne necrosis erythema lymphadenopathy

NGS: Next-Generation Sequencing

16S rRNA: 16S ribosomal RNA

OUT: Operational Taxonomic Units

ASV: Amplicon Sequence Variants analysis

PBS: Phosphate-buffered saline

ONT: Oxford Nanopore Technologies

RAP: Rapid Sequencing Adapters

SQB: Sequencing Buffer

LB: Loading Beads

Chapter 1

1. Introduction

1.1. Zoonosis Infection: Pathogen–Host Interactions

In recent years, there has been a rise in the occurrence of zoonotic diseases worldwide due to growing globalization, advanced agricultural methods, urbanization trends, and climatic changes. Based on surveillance data collected by the Centers for Disease Control and Prevention, the majority of zoonotic diseases are attributed to bacterial agents (41.4%), followed by viral (37.7%), parasitic (18.3%), fungal (2%), and prionic (0.8%) agents. Roughly 60% of infectious diseases stem from zoonotic pathogens that are transmitted from animals to humans [1].

Bacterial zoonotic pathogens in humans can be transmitted via either ingestion or bacterial invasion of the skin's epidermal layer. These bacterial infections can be induced by alterations in the human ecological surroundings, animal rearing, animal by-products, and exposure to contaminated animal excretions, which encompass saliva, blood, urine, and feces. The effectiveness of a viral infection is contingent upon factors such as the host's ability to facilitate adequate virion production, the accessibility of the virus to enter the host, the presence of compatible host receptors, and tropism (Fig.1) [2].

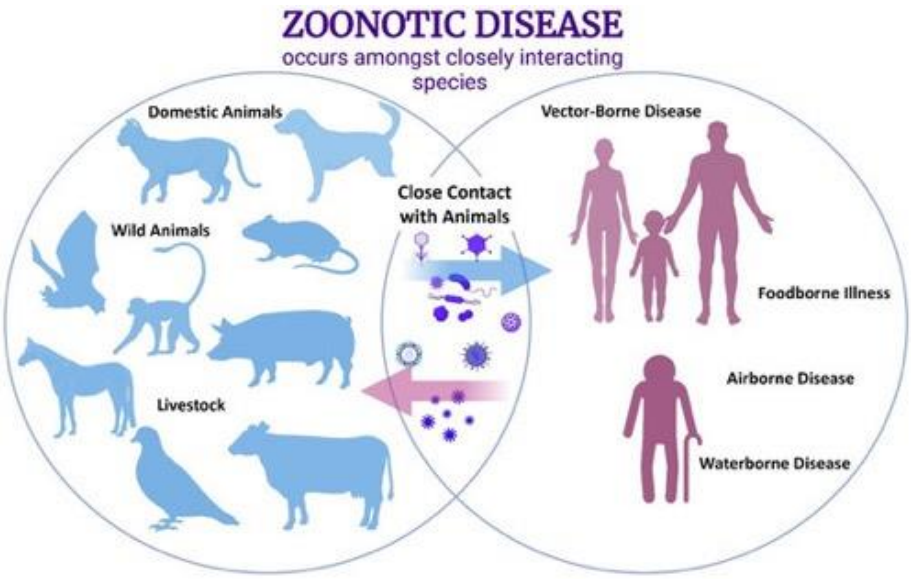


Figure 1. The zoonotic transfer of pathogens takes place when intimate contact facilitates the transmission of diseases between animals, including domestic, wild, or livestock, and humans [3].

Over the last few decades, incursions originating from animal and livestock reservoirs have resulted in significant epidemic outbreaks. Zoonotic incidents have resulted in considerable economic detriment to communities engaged in livestock husbandry, involving both direct and indirect expenses. In parallel, diseases originating as zoonoses have subsequently undergone genetic alterations, leading to the emergence of strains exclusively affecting humans, as observed in the case of Human Immunodeficiency Virus (HIV). Additional zoonotic diseases like Ebola, SARS-CoV-2, and salmonellosis have the potential to trigger repeated episodes of disease outbreaks. It is worth noting that numerous pathogenic bacteria (e.g., *Rickettsia parkeri*) found in ticks have closely related non-pathogenic counterparts, indicating a range of intermediary or transitional conditions from non-pathogen in humans to becoming an infected disease later [3], [4]. Hence, in 2014, the initiation of the Global Health Security Agenda (GHSA) aimed to oversee and contribute to the prevention of zoonotic diseases as part of global health security efforts [2], [5].

1.2. Ticks as the Infectious Vector: Tiny but Troublesome

There are several animals that can carry and transmit zoonotic pathogens to humans. Some examples of these animals include rodents, bats, ticks, mosquitoes, fleas, and some domesticated animals such as dogs, cats, and livestock. Ticks are identified as the second most extensive category of ectoparasites, following mosquitoes [6]. Ticks are haematophagous ectoparasites since they can feed on different hosts at different stages of their lifecycles. They act as the main vectors of zoonotic pathogens that carry a broad spectrum of microbial pathogens including viruses, bacteria, and protozoans that impact human and animal health significantly [7]. In the most recent update according to the TicksBase facilitated by the Integrated Consortium on Ticks and Tick-borne Diseases (ICTTD), a total of 889 known tick species have been classified under the class *Arachnida*, subclass *Acari*, further categorized into three families 702 *Ixodid* ticks (also known as hard ticks), 186 *Argasid* (soft ticks), and 1 *Nuttalliella* tick ((*Nuttalliella namaqua*) species [8]. Scientifically, hard ticks are categorized into five genera, namely *Ixodes*, *Rhipicephalus*, *Hyalomma*, *Haemaphysalis*, and *Dermacentor*, while soft ticks are classified into two genera, *Argas* and *Ornithodoros*. Ticks are distributed across the Old World and infect various vertebrate hosts, including birds, rodents, shrews, ruminants, bats, and humans through contact with infected humans or animals or tick bites [9].

In Spain and Europe, the most common and important species are *Ixodes ricinus* (wood tick), *Rhipicephalus sanguineus* (brown dog tick), *Rhipicephalus bursa*, *Haemaphysalis spp*., two *Hyalomma spp.* (*H. marginatum* and *H. lusitanicus*), *Dermacentor reticulatus,* and *Dermacentor marginatas* as reported by the European Centre for Disease Prevention and Control (ECDC). *I. ricinus* is the most widely distributed tick species followed by *Hyalomma* species, a hard tick species belonging to the family *Ixodida*, which are a significant focal point in public health due to their crucial role as carriers of multiple diseases [5].

1.3. Tick-borne Pathogen Diseases (TBPDs) and Transmission

The tick microbiome represents complex ecological communities comprising viruses, bacteria, and eukaryotic organisms[10]. Among the microbial entities inhabiting these arthropod vectors, Tick-borne Pathogens (TBPs) hold paramount importance in the fields of medical and veterinary research. Numerous genera and species of ticks within the families *Ixodidae* and *Argasidae* hold significant relevance in public health due to their roles as vectors for specific diseases. *Ixodid* ticks can transmit pathogens to humans and cause a range of diseases, including Lyme borreliosis, Ehrlichiosis, Spotted Fever Rickettsioses (SFR), Tularemia, Q fever, Babesiosis, viral Encephalitis, and Crimean-Congo haemorrhagic fever (CCHFV). *Argasid* ticks are a vector for diseases of lower epidemiological importance, such as Tick-borne relapsing fever (TBRF) and Q fever [4]. In Spain, the prevailing tick-borne diseases consist primarily of specific rickettsioses and Lyme disease. Additionally, occasional instances of anaplasmosis, babesiosis, tularemia, and Crimean-Congo fever have been documented. The likelihood of transmission of Tick-Borne Pathogens (TBPs) depends on both the presence of ticks in the environment and the probability of an infected tick coming into contact with a host that is vulnerable to infection[11].

The majority of TBP can potentially be transmitted through various routes, including vertical transmission: infectious agents transfer from a parent to its offspring, and horizontal transmission: through direct or indirect contact, parasites are transmitted between hosts and ticks, and vice versa (Fig. 2). For example, Ticks belonging to the genera *Rhipicephalus* and *Ixodes* are generally implicated in the transmission of bovine babesiosis Comprehending the specific transmission strategies of individual species requires insight into their phylogenetic or evolutionary lineage, which is shaped by factors such as host genetics, physiology, and environmental conditions [12], [13]. Moreover, the dispersal of the

CCHF virus, *H. marginatum* is the main vector, could have been facilitated by birds that were infected with the virus [14]. CCHF virus is known to have a broad geographic distribution, with outbreaks having been reported in several regions of Africa, Asia, the Middle East, and Europe with mortality rates ranging from 5% to 30% [10], [15]. Due to the mode of transmission and high case fatality (Category A Priority Pathogens), the absence of effective and wieldy available vaccines against CCHF, together with the wide spread of the virus, and inadequate knowledge of interactions between CCHFV and its host, the World Health Organization (WHO) listed in of the top 10 needed research and development efforts[5], [16]. Climate change and human activities drive infectious pathogens and vector proliferation. Recent research examines *H. marginatum* distribution, revealing potential expansion in Europe due to changing climates [17].

The tick functions as a carrier of the pathogen during the whole of their lifecycle stages, therefore, Information on the infection rate of pathogens in ticks, and the genetic diversity of the circulating pathogens are important variables in understanding the epidemiology and control of TBDs [4]. Otherwise, an endemic infection in many regions of the world, including Africa, Asia, and Europe could soon become an epidemic in the rest of the world.

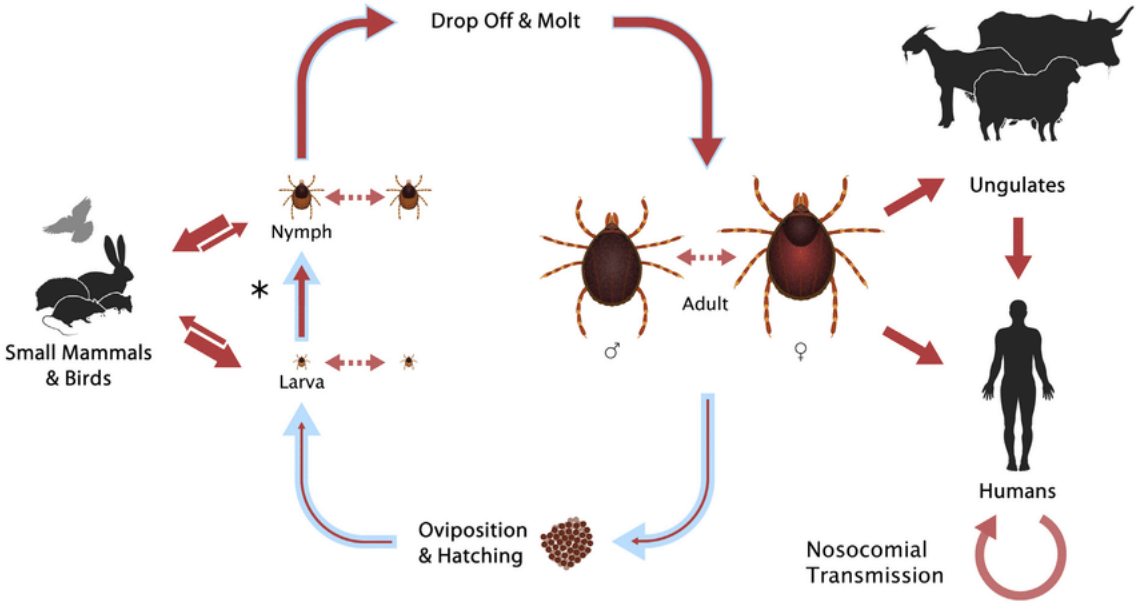


Figure 2. During the tick life cycle (blue arrows), pathogens can be transmitted between ticks and mammals (solid red arrows), and directly between ticks through co-feeding (dashed arrows) [19].

1.4. Tick Microbiome

Numerous investigations have demonstrated the close relationship between ticks and bacterial symbionts, encompassing both pathogenic and non-pathogenic varieties. Endosymbiont bacteria are classified as obligatory or facultative based on their essential role in shaping tick physiology [18]. The Obligate symbionts that are transmitted vertically, play a significant role in aspects such as host nutritional adaptation, survival, fitness, vector competence, immunity, and reproductive functions, highlighting their intricate involvement in various facets of tick biology. For example, *Coxiella* or *Francisella* Symbionts provide Vitamin B [17], [19], *Rickettsia* Symbionts are essential for tick physiology, fitness, and population dynamics, and Midichloria for nutrient biosynthesis, aiding antioxidative defense, hydric balance, *Wolbachia* for reproductive functions, vectorial competence, defense mechanisms. Reports exist indicating the presence of horizontal transmission in tick symbiont bacteria such as *Coxiella*, *Midichloria*, and *Arsenophonus* [20]. Several of the most crucial tick-borne bacteria include types belonging to various species within the *Rickettsia, Borrelia, Francisella, Anaplasma*, *Coxiella*, and *Ehrlichia* genera, which are recognized as significant threats to human and veterinary public health [17], [21]. For example, *Rickettsia* is a pathogen transmission, which can cause SFR [10]. In addition, some facultative symbionts, such as *Cardinium* and *Spiroplasma*, function as reproductive parasites, exerting substantial effects on the reproduction of arthropods. An infection of ticks with *Anaplasma* phagocytophilum disturbs the composition of the gut microbiota, resulting in an elevated presence of *Pseudomonas* and a reduction in the abundance of *Rickettsia* and *Enterococcus*. The interactions between ticks and their microbiota could be tracked by the sex and the environment. For instance, A. Portillo, et al. reported that females possess a significantly higher amount of endosymbiont compared to males, and ticks in close geographical proximity have microbial communities in common [4]. Researchers continue to study the fundamental requirement for understanding the microbial composition of ticks to uncover novel perspectives for the strategic control of ticks and TBD in the future [22].

1.5. Review of Research on TBDs

The composition of microbial communities varies and shapes depending on the examined tick species, sex, the season during which ticks were collected, geographical regions, tick-

developmental stage, tick immunity, ticks' feeding status, and the presence of pathogens [18]. There are comprehensive studies that investigate the influence of these parameters on bacterial diversity in ticks. Here, I reviewed several of them.

In 2023 A. Namina *et al.* investigated the epidemiology of *I. ricinus*, *I. persulcatus*, and *D. reticulatus* tick species of 126 tick specimens, which were field-collected in Latvia. They found 96 genus-level and top-ten abundant genera were Ca. *Lariskella*, Ca. *Midichloria*, *Corynebacterium*, *Francisella*, *Halomonas*, *Methylobacterium*, *Mycobacterium*, *Propionibacterium*, *Pseudomonas*, *Rickettsia*, and *Sphingomonas* within all the samples. Nonetheless, notable variations in the diversity and composition of microbial species were detected based on tick species, sex, and life cycle development [23].

In addition, in China J. Zhang et al. studied the tick-associated Microbiota diversity of 191 adult ticks from five tick species. The tick species included *I. ovatus, I. acutitarsus*, *I. granulatus, Rh. microplus,* and *Haemaphysalis kolonini* from host-seeking ticks. The results demonstrated 11 phyla and 126 genera of bacteria, including pathogenic *Anaplasma*, *Ehrlichia*, *Candidatus Neoehrlichia*, *Rickettsia*, *Borrelia*, and *Babesia.* Their conclusion states that it is uncertain whether all the microbial DNA identified originated solely from the ticks, or if the findings could have been influenced by the ticks' previous blood meals [22].

Extensive research was conducted by G. Grandi *et al.* who studied the microbial composition in a total of 2000 Swedish ticks, including whole tick *I. ricinus*, *I. persulcatus* , and *I. trianguliceps* species, and tick organs (midguts, Malpighian tubules, ovaries, salivary glands) from vegetation and engorged *I. ricinus* female ticks. The predominant bacteria were found within the *Proteobacteria* phylum, (*Candidatus Midichloria mitochondrii* and *Candidatus Lariskella*). As a result, they recognized that analyzing tick organs especially when the organs were obtained from engorged ticks yielded valuable insights into the diversity of microbial communities associated with *I. ricinus* ticks. Notably, bacteria genera are linked to a host blood meal and the existence of TBPs [24].

A total of 776 adult ticks of *Hyalomma* species were examined by H. Benyedem *et al.* utilizing Next-Generation Sequencing of the hypervariable V3-V4 region of the bacterial 16S rRNA gene in Tunisian. According to their study, in *Hyalomma* ticks, *Francisella*, *M. mitochondrii*, and *Rickettsia* endosymbionts were the most prevalent within 16 bacterial families and *Proteobacteria* was the most dominant phylum. Furthermore, they concluded that microbial diversity and composition vary depending on the tick's life stage and sex in the specific case of *H. scupense*. The male showed *Rickettsiaceae* 22.9% and *Francisellacea* 2.56%. In contrast,

78.8% of *Francisellacea* was the main family in the females followed by *Rickettsiaceae* with 19.9% [19].

In Spain, ticks can transmit a variety of diseases to humans and animals, including Lyme disease, Mediterranean spotted fever (MSF), Q fever, Anaplasmosis, and Tularemia. The distribution of TBDs in Spain may vary depending on the geographic region and the tick species present. Recently, several surveys have reported many TBPs in many provinces in Spain, like *Francisella-like endosymbionts (FLEs)* such as *Francisella tularensis* cause tick-borne tularemia and *F. tularensis subsp. Holarctic* [25], *Francisella hispaniensis* [26]–[28], and *Coxiella burnetii* caused Q fever [29], [30]. A total of 12 *Rickettsia* species that caused the MSF group have been demonstrated in Spain [31] those are common: *R. conorii* [32], [33], *R. Helvetica* [34], *R. monacensis* [35], [36], *R. felis* [37], [38], *R. slovaca* [39], *R. raoultii*, *R. sibirica*, *R. aeschlimannii* [34], [40], *R. rioja*, *R. typhi*, and *R. prowazekii*, *R. massiliae* [34], [41], [42].

1.6. Bacterial Community Detection in Ticks

Ticks are known to carry a wide variety of bacteria and viruses that can be harmful to humans and animals. Conventional methods of identifying this microbiome generally include microscopy, culture, antigen detection, serology modalities covering ELISA, and tagged immune microscopy. In addition, the culture of organisms in a laboratory can be time-consuming and may not always yield accurate results. However, advances in sequencing technology have made it possible to rapidly and accurately identify the microorganisms present in a tick sample. The previous methods have their limitations, for instance, microscopy is not reliable in identifying bacteria taxa, and the serology test for the detection of some diseases like TBRF is impractical due to bacterial antigenic variation and cross-reactivity [43], and only 1% of bacteria is culturable. PCR amplification to detect the target DNA or RNA is another method but it is often problematic. PCR can introduce challenges such as achieving a lower taxonomic resolution because of identifying just one gene or species in every single reaction and/or primer interaction and reduced sensitivity for individual targets [44]. To address limitations in PCR-based pathogen detection, researchers often employ a combination of strategies such as high-throughput sequencing primarily focused on the PCR amplification study followed by bioinformatics tools [45]. This approach is revolutionizing the research in

the fields of epidemiology and diagnosis of infectious diseases, among others, overcoming the limitation of detecting only one or few microorganisms at a time.

1.7. Sequencing Approaches for Identifying Tick Pathogens and Data Analysis

Genome sequencing analysis is a method that is used to determine the order of nucleotides (A, T, C, G) that make up a DNA or RNA molecule. Genome sequencing has evolved through three generations. Sanger and Maxam-Gilbert sequencing technologies were categorized as the First Generation Sequencing Technology used for shorter DNA fragments, providing accurate but relatively slow and expensive sequencing. The second generation is also called Next-Generation Sequencing (NGS) or High Throughput Sequencing Technology [45]. The second generation utilizes a variety of techniques, including Illumina, 454 pyrosequencing, and SOLiD, notable for generating massive amounts of short DNA sequences (reads) in parallel. Currently, 454 and SOLiD sequencing technologies are not in common use, while Illumina stands as the gold standard for NGS. The third Generation Sequencing generally known under the name of Single-Molecule Sequencing Technology or Long-Read Sequencing provides longer DNA fragments compared to NGS, enabling the sequencing of single DNA molecules directly without the need for PCR amplification. Technologies like Pacific Biosciences (PacBio) and Oxford Nanopore that introduced Nanopore sequencing like MinION sequencer fall into this category and are valuable for applications requiring the sequencing of entire genomes, resolving complex regions, and studying epigenetic modifications. The second and third overcome the limitations of the first generation in terms of, high throughput simultaneously sequencing millions to billions of DNA fragments from multiple samples, speed, cost-effectiveness, resolution, scalability, and clinical application [46]. These techniques are well-suited for whole-genome sequencing, metagenomics, metatranscriptomics, targeted sequencing, metabarcoding, and viral transcriptomics. Short-read sequencers such as Illumina and 454 have historically been employed for shotgun sequencing of tick metagenomes, transcriptomes, and viromes. However, there is a current shift towards increased usage of amplicon sequencing using nanopore due to its advantages, including shorter processing times, cost-effectiveness, longer read lengths, and easy library preparation. It's important to note that, despite these advantages of amplicon sequencing, shotgun sequencing remains advantageous due to its ability to alleviate the issues related to PCR bias commonly

observed in amplicon sequencing [45]. Fig. 3 illustrates the first, second, and third-generation sequencing process.

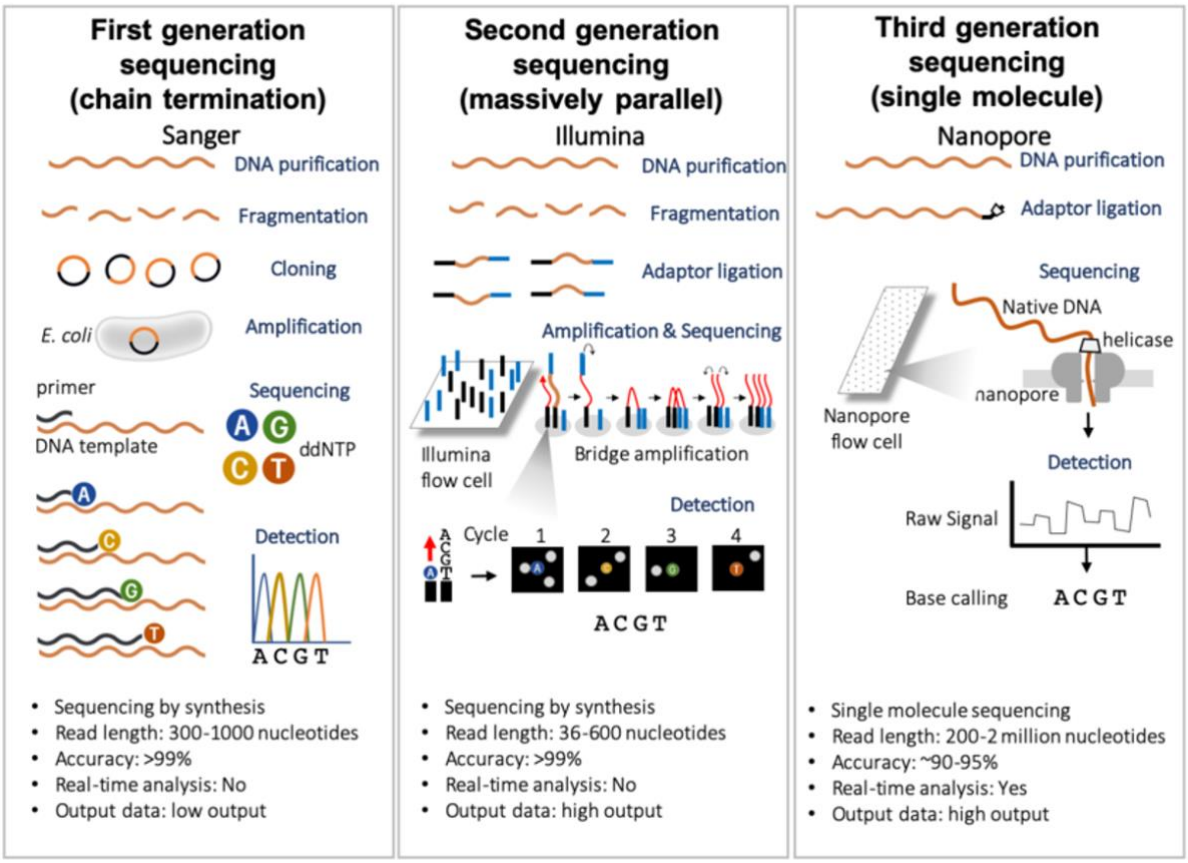


Figure 3.The first, second, and third-generation sequencing process [48].

1.8. Metabarcoding Technique

Today taxonomic identification of bacteria is becoming increasingly by analyzing their DNA sequence information, most commonly the gene that codes for the small subunit of the ribosome (16S rRNA gene). The ribosome is an essential piece of the cell's protein-making machinery. All bacteria have the 16S rRNA gene, the central gene about ~1500 bp long with V1-V9 hypervariable conserved regions. In other words, these nine regions of the 16S gene are unique to each species and are used as a molecular fingerprint to exhibit varying degrees of sequence diversity to distinguish between all bacterial species [22]. Although 16s rRNA PCR amplification is applicable for discovering vector microbes under diverse conditions drawbacks such as overinflate diversity estimates, limited information about the microbial function, and the possibility of taxa unspecific variation among strains [47]. To address these issues, the 16S

rRNA metabarcoding sequencing is currently the prevailing approach to microbiome classification.

Metabarcoding is defined as a DNA-based technique that involves PCR amplification and sequencing of a specific tagged region of DNA. The metabarcoding methodology involves amplifying the 16S rRNA gene using primers with a unique barcode which are generally short nucleotide sequences via PCR, loading samples onto a flow cell for sequencing, and bioinformatics approaches. Sample barcoding enables the simultaneous sequencing of multiple samples by combining them in a single pool (multiplexing). Fig. 4 represents the prevailing metabarcoding sequencing methods for each generation of sequencing technology. The species or genus level and phylogenetic tree can typically be achieved by matching sequence data with reference data in the bioinformatics pipeline. Closely related organisms have more similarities in their rRNA genes than distantly related organisms. Sequencing of universal genes with NGS also allows for novel species identification. Although regions V3-V4 have typically been preferred targets in tick microbiome investigation, sequencing full-length 16S rRNA genes offers the most comprehensive estimates of bacterial families. Conversely, the highest estimated diversity was identified by the V4 region [45].

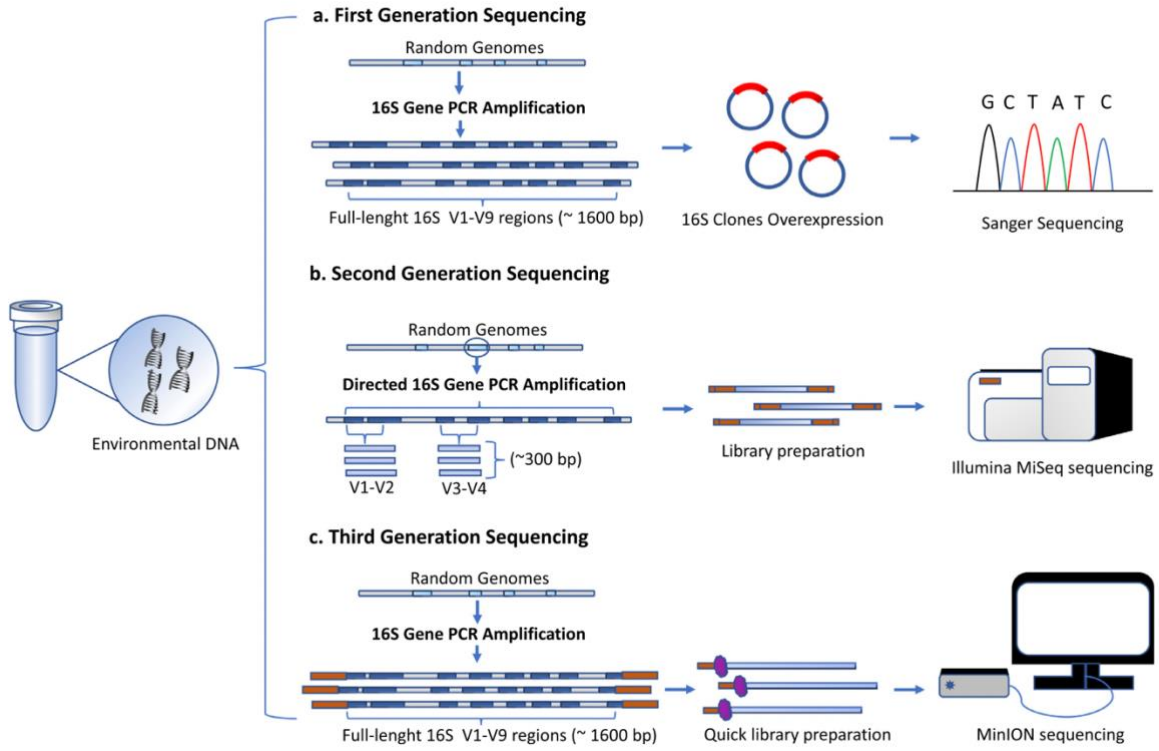


Figure 4. The prevailing metabarcoding sequencing methods for each generation of sequencing technology for each sequencing technology generation. (a) First-generation sequencing (Sanger). (b) Second-generation sequencing (Illumina). (c) Third-generation sequencing (Nanopore) [50].

1.9. Current Limitations of Tick Microbiomes Based on Sequencing

Although high throughput sequencing approaches fundamentally alter the way researchers assess microbial diversity and ecology, there are some disadvantages of these techniques that require consideration. The output of the Sanger platform is low (1.9–84 Kb) and is not suitable for the comprehensive assessment of bacterial diversity. The limited read length (75–300 bp) of the high-accuracy Illumina sequencing method makes it possible to sequence certain variable regions of the 16S rRNA gene rather than its entire length. However, this constraint prevents researchers from achieving a complete taxonomic resolution of the microbiome community. Even though the Pac-Bio and nanopore sequencing examine a long single DNA strand in real-time and the outputs are long, the error rate falls, and the quality of the reads generated are drawbacks compared to the illumine technique.

Taxonomic identification of biological specimens based on DNA sequence information is becoming increasingly common in biodiversity science. There are many parameters that are widely recognized for their significant influence on the results and interpretations of sequencing analyses. For instance, the quality and purity of extracted DNA, differential Lysis, DNA shearing and fragmentation, storage conditions, and incomplete removal of inhibitors are known to be some of the influential parameters in the downstream assessment of microbial composition [48], [49]. Isolation of DNA from ticks due to the hard chitinous exoskeleton and the small amount of microbial nucleic acid present is problematic [50]. Furthermore, tick DNA is susceptible to degradation and PCR can be challenged by inhibitors [50]–[52].

1.10. Microbiome Analysis

Bioinformatics pipelines and tools are continually advancing and being enhanced to effectively handle the growing complexity of datasets for microbiome community analysis. In the present day, numerous potent tools are accessible, facilitating the effective integration of diverse data types. The common process for 16S metagenomic studies, standard analysis packages, and pipelines typically includes, demultiplexing, sequence quality control, Operational Taxonomic Units (OUT) clustering that creates an OUT table, and/or Amplicon Sequence Variants analysis (ASV) analysis followed by diversity analysis, taxonomic differential abundance, followed by data visualization and exploration [56], [50]. Measuring microbial diversity using 16S rRNA

sequencing is dependent on sequencing depth. By chance, a sample that is more deeply sequenced is more likely to exhibit greater diversity than a sample with a low sequencing depth. Therefore, the rarefaction curve is used as a primary method for correcting differences in read depth prior to diversity analyses. There are two types of diversity explored in microbial ecology: alpha diversity and beta diversity [17]. Analyzing the data through mining and visualization from nanopore technology has the potential to achieve elevated taxonomic resolution, especially utilizing nucleotide sequence of the 16S rRNA gene that is specific to 96% of bacterial genera and for some, 87.5% of bacteria species. This trend leads to detecting pathogenic bacteria in clinical research or environmental samples that play a crucial role in providing valuable information for guiding public health interventions in the field of infectious disease prevention and control [53], [54].

1.11. Hypothesis, objectives, and goals

The main hypothesis of this study is that ticks' feeding status and geographic locations significantly influence the composition and diversity of bacterial communities in ticks. Moreover, there are specific bacterial taxa associated with ticks that may have implications for TBDs.

Objectives:

Primary objective: Characterize the bacterial communities in ticks from different tick species and various geographic locations using 16S rRNA metabarcoding sequencing.

Second objective: utilizing statistical analysis to assess the diversity of bacteria species within tick bacterial communities to understand the richness and evenness of bacterial taxa present.

Third objective: Investigate potential associations between tick bacterial communities and TBDs.

Goals:

Primary goal: Collect tick samples from a range of tick species and geographic locations and optimize the DNA protocol to obtain high-quality nucleic acid and solve DNA degradation issues.

Second goal: Employing the full-length 16S rRNA method by PCR, along with metabarcoding NGS techniques to analyze tick samples.

Third goal: Use a statistical pipeline to analyze the significance of differences in bacterial community composition between ticks' feeding status groups and geographic locations.

Chapter 2

2. Research Methodology

2.1. Study Area and Sample Collection

Sampling was performed during the period spanning April to June 2023, when most of the tick species are active [8]. A total of 186 adult ticks belonging to five species of *Rh. bursa*, *D. marginatus*, *H. lusitanicum*, *I. ricinus*, and *H. marginatum* were collected from four distinct sites in eastern Spain, Barcelona, Girona, Tarragona, and Valencia. The tick collection sites are shown in Fig. 5, and all information related to the samples is outlined in Table 1. Most of the tick specimens were collected from Ports de Tortosa i Beseit (Baix Ebre) where Coxiella burnetii (Cb) ELISA seropositivity and PCR-positive results were detected in wildlife and Muela de Cortes (Vall de Cofrents) in eastern Spain [55], plus additional samples from other regions from Catalunya, north-eastern Spain. The sample collection method involved utilizing a standard $1m^2$ flannel cloth, which was flagging along the forest floor or along low vegetation and CO_2-trap were placed at least 200 m away from each dragging transect and left for 2 h [56] in areas proximate to various infected animal habitats (unengorged) or directly gathering from hunted wild animals with tweezers (engorged). Using taxonomic reference keys, the morphology, life stage (larva, nymph, adult), sex and feeding degree, and species of collected samples were identified and recorded at the laboratory of the Faculty of Veterinary (CRESA department) at Universidad Autónoma de Barcelona (UAB) and storage at – 80 ^{0}C for further experiments. According to tick species and engorged or unengorged, samples were grouped into 16 pools of 10 individuals each, except pools 9-11 containing 20 individual ticks per pool. In our study, *Rh. Bursa* was the most prevalent species (55%, 110/200) and was distributed across Tarragona and Valencia sampling sites [40]. These pools were subjected to microbiology techniques, and a subset of these pools was conducted for sequencing analysis at the laboratory of the Faculty of Genetics at the University of UAB.

Table 1. Details about collected engorged and unengorged tick specimens from different locations.

Pool No.	Tick species	engorged /unengorged	Province	Comarca	Region	Animal host
P1	*Rh. bursa*	U	Valencia	Valle de Cofrentes	Muela de Cortes	-
P2	*Rh. bursa*	U	Valencia	Valle de Cofrentes	Muela de Cortes	-
P3	*Rh. bursa*	U	Valencia	Valle de Cofrentes	Muela de Cortes	-
P4	*D. marginatus*	E	Barcelona	Osona	Sora	Wild boar
P5	*H. lusitanicum*	E	Barcelona	Vallès Occidental	Sentmenat	Wild boar
P6	*I. ricinus*	E	Girona	Osona	Vidrà	Roe deer
P7	*D. marginatus*	E	Barcelona	Vallès Occidental	Sentmenat	Wild boar
P8	*Rh. bursa*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-
P9	*I. ricinus*	E	Girona	Ripollès	RNC Freser-Setcases	Pyrenean chamois
P10	*Rh. bursa*	E	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	Wild boar
P11	*Rh. bursa*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-
P12	*D. marginatus*	E	Barcelona /Girona	Osona/Garrotxa	Rupit/ Sant Aniol de Finestres	Wild boar
P13	*Rh. bursa*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-
P14	*H. marginatum*	E	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	Wild boar
P15	*Rh. bursa*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-

P16	*H. marginatum*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-

E: engorged, U: unengorged

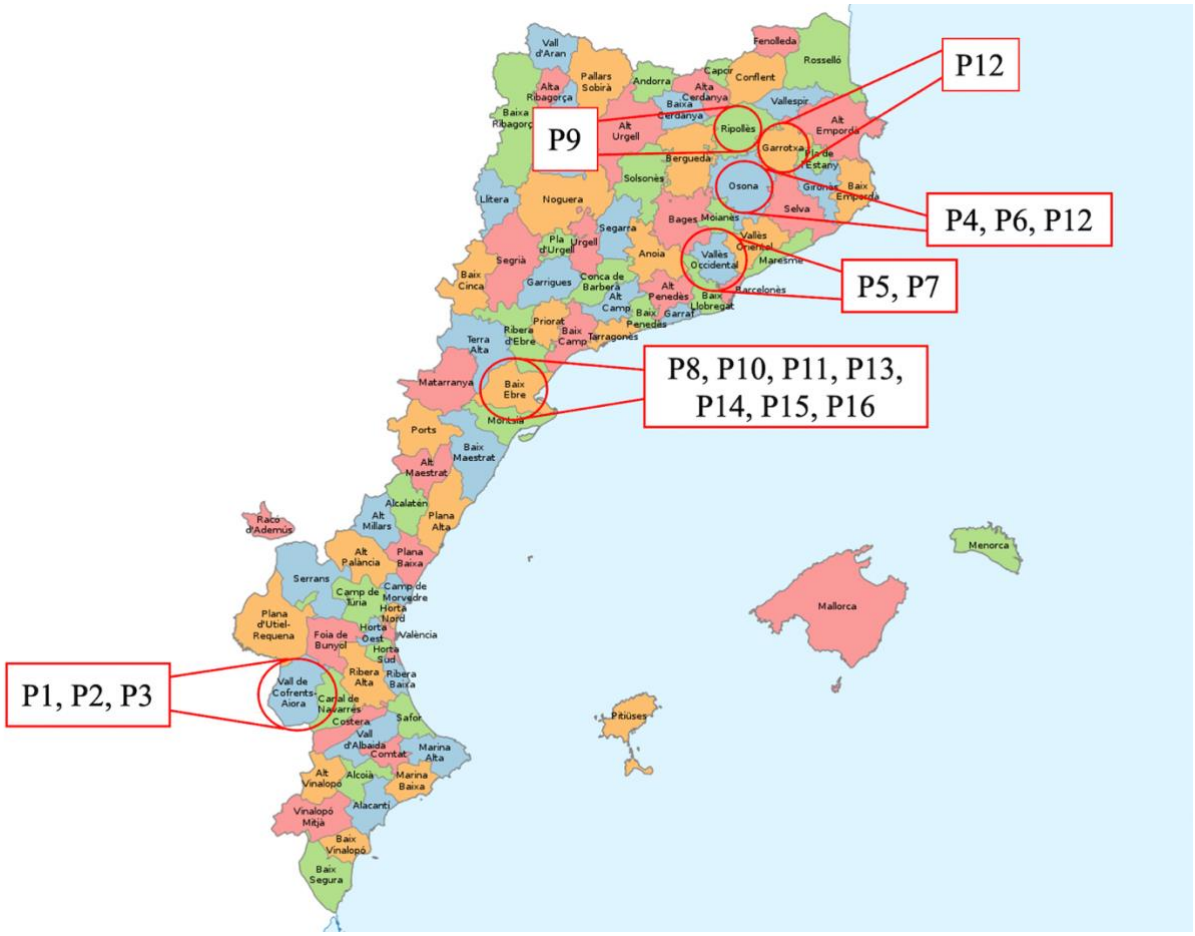


Figure 5. The study area where ticks were collected from four distinct sites in eastern Spain, Barcelona, Girona, Tarragona, and Valencia provinces.

2.2. Sample Processing and DNA Extraction

As mentioned above, a total of 186 adult ticks were pooled based on tick species and engorged or unengorged groups. 16 pools containing 30 engorged *D. marginatus*, 110 *Rh. bursa* containing 90 unengorged ticks and 20 engorged ticks, 16 *H.marginatum* involving 6 unengorged ticks and 10 engorged ticks, 10 engorged H. lusitanicum and 30 engorged I. ricinus ticks were analyzed. Isolating nucleic acid from ticks is often problematic due to the chitinous exoskeleton of ticks that must be eliminated before the extraction process, engorged ticks may contain substances that lead to inhabit Taq-polymerase, and in addition, DNA extracted from ticks appear to degrade easily, and the exact reasons for this susceptibility are not well understood [1][57]. In this study, to overcome this problem and rapid DNA extraction we modified some steps in extraction procedures. Generally, the DNA extraction process involves initially breaking down the exoskeleton through the mechanical machine, followed by the enzymatic degradation of Proteinase K, before utilizing a commercial kit for DNA extraction following a modified version of a previously documented procedure [1]. Prior to DNA extraction, ticks were surface-sterilized by immersion in 70% ethanol in three washes and then rinsed one time through Phosphate-buffered saline (PBS) buffer to avoid environmental contamination. Each individual tick was divided into two equal parts with a sterile scalpel (blade size 12) [58]. One of these halves was placed back in RNA later solution (Invitrogen™, Thermo Fisher Scientific) and then stored in a -80°C freezer for future studies, while the other half in screw-cap tubes was employed for DNA extraction. Three modified methods of DNA extraction from different pools were examined [59]. Fig. 6 represents flow diagram for pre-extraction and DNA extraction.

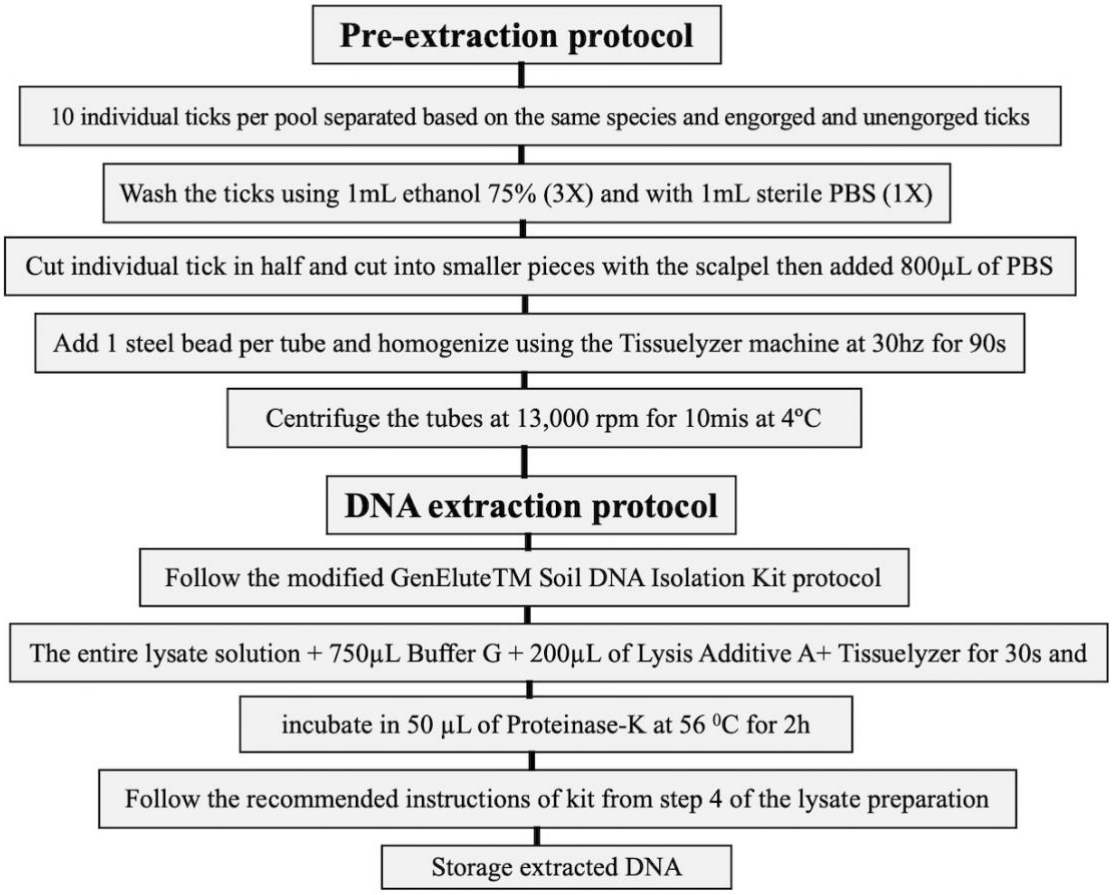


Figure 6. Flow diagram for DNA pre-extraction followed by DNA extraction.

2.3. Standardization of DNA Isolation Methods

A standardized and efficient DNA extraction method is mandatory to address the extraction issues and achieve high-quality nucleic acid. In this study, we proposed three different DNA extraction procedures coupled with a bead beater, protein enzyme digestion, and increasing the number of ticks. The protocol involves Method-1: bead-based physical disruption of the tick exoskeleton by tissuelyzer for 10 mins and DNA extraction using a commercial kit, Method-2: bead-based physical disruption of the tick exoskeleton by tissuelyzer for 10 mins, followed by Proteinase K incubation, and DNA extraction using a commercial kit, Method-3: bead-based physical disruption of the tick exoskeleton by tissuelyzer for 2 mins, followed by Proteinase K incubation, and DNA extraction using a commercial.

2.3.1. Method-1: Bead-Based Tissuelyzer Approach with Commercial Kit

For this purpose, we studied frozen ticks of P1-P8, 10 individual halved ticks per pool and to establish the most efficient approach to physical disruption of the hard and chitinous shell of ticks were cut into smaller pieces in a 1.5mL microtube [50], then added 1 sterile steel microbead and mechanically homogenized by using a tissuelyzer II (QIAGEN) at 30 Hz frequency for 10 min in 800 µl of PBS. The centrifuge of the tubes was performed at 13,000 rpm for 10 min at 4 ^{0}C in a benchtop microcentrifuge (5415R, Eppendorf). The lysate with pellet was used for DNA extraction by GenElute^{TM} Soil DNA Isolation Kit based on recommended protocols (Sigma-Aldrich) [58].

2.3.2. Method-2: Bead-Based Tissuelyzer Approach with Proteinase K, Increasing the number of ticks and DNA Commercial Kit

In this experiment, the number of ticks in P9-P11 was increased, to 20 individual ticks per pool. The tubes were filled with 800 µl of PBS containing a steel bead, crushed by the tissuelyzer at 10 min, and incubated 50 µL of Proteinase K (22 mg/ml) at 56 ^{0}C for 2 h on thermomixer comfort (Eppendorf ThermoMixer® C, 2mL). Subsequently, the process followed the DNA-recommended protocol kit.

2.3.3. Method-3: Bead-Based Tissuelyzer Approach with Proteinase K and Commercial Kit

Isolation was performed on P12-16 by this method. in this method, we decreased the tissuelyzer time to 90 seconds in the pre-extraction step, and then the lysate was suspended in 750 µL Buffer G and 200 µL of Lysis Additive A and was homogenized by tissuelyzer at 30 Hz frequency just for 30 secs. Next, 50 µL of Proteinase K (22 mg/ml) was added to the lysate and incubated at 56 ^{0}C for 2 hours, centrifuged for 2 mins at 14,000 rpm. Clean supernatants were transferred into another DNase-free tube, following the DNA manufacturer's instructions.
For all procedures, the final elution volume was 100 µL for the DNA extraction methods. the quantity, purity, and visual integrity of extracted DNA were evaluated using a Qubit 3.0 Fluorometer (Qubit 3.0 DNA High Sensitivity Assay Kit and Qubit Broad-Range Assay Kit: Thermo Fisher Scientific), absorbance 260/280 measurement Nanodrop 2003 (Thermo Fisher

Scientific) using ultra-pure water as a blank, and gel electrophoresis detection (PowerPac Basic, BIO-RAD), respectively.

2.4. Gel electrophoresis

The isolated nucleotide and PCR products were observed using 1% (w/v) agarose gel with (AgaPure™ Agarose LE, Canvax), 6X BX-Loading Buffer (Canvax), and TE, 10X Sterile Solution (Tris-Acetate- EDTA, Canvax), incorporating 2 µl of GreenSafe DNA Gel Stain (Canvax) and 2 µl of DNA samples, alongside a DNA ladder (BrightMAX™, Canvax) for molecular size reference which ranges from 100-1000 bp in size. UV transilluminator facilitated gel visualization which revealed the outcome lanes at voltages of 100 for 50 minutes.

2.5. Bacteria analysis: DNA Metabarcoding, library preparation, and sequencing process for bacterial taxonomic identification

Detection of endosymbiotic bacteria performed using 16S Barcoding Kit 1-24 (SQK-16S024) from Oxford Nanopore Technologies (ONT, United Kingdom) standard protocol following the manufacturer's instruction that provides a technique for amplifying the V1-V9 the entire ~1,500 base pairs of the 16S rRNA gene derived from extracted DNA, followed by sequencing library preparation. The amplification of the 16S rRNA for the sequencing analysis was carried out by PCR. The universal primer for the 16S rRNA gene, namely 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') that contain 5' tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters (RAP), was used to amplify and analyze the sequence of the 16S rRNA gene. The PCR reaction was carried out in a 50 µl PCR reaction comprising 25 µl LongAmp Taq 2X master mix, 10 µl DNA template with initial concentration, 10µl of 16S Barcode, and 5µl ultra-pure water. PCR amplification was performed in a Thermal Cycler (MJ Mini™ Gradient) under conditions such as initial denaturation at 95°C for 1 min, followed by 25 cycles of denaturation at 95°C for 20 secs; annealing at 55°C for 30 secs, and extension at 65°C for 2 mins, then followed by 1 cycle of final extension step at 65°C for 5 mins and hold at 4°C. The primer was set in ~1500 bp PCR products. Amplicons were then purified using AMPure XP beads**,** 10 µl of final elution by ultra-pure water, and transferred into a 1.5 ml Eppendorf tube. 1µl eluted sample quantified by Qubit 3.0 Fluorometer and 2µl of each PCR product run in 1% (w/v) agarose to visual bands.

The positive PCR pools were subjected to the sequencing process in accordance with the 16S Barcoding Kit 1-24 (SQK-16S024) manufacturer's instruction to identify present bacteria in each tick species. As a summary of the sequencing process (Fig. 7), 1 µl of RAP was added to the 10 µl Barcoded DNA (16S PCR positive pools) to prepare the DNA library and incubated the reaction for 5 mins at room temperature to bind the RAP to the Barcoded DNA. First, the MinION sequencer (ONT, United Kingdom) using a brand new R9.6 flow cell was prepared by loading 1000 µl of priming mix into the flow cell via the priming port SpotON. Subsequently, 75 µl of prepared library was loaded into the flow cell which contained 34 µl Sequencing Buffer (SQB), 25.5 µl Loading Beads (LB), 4.5 µl ultra-pure water, and 11µl DNA library, and the sequencing process run for 24 hours.

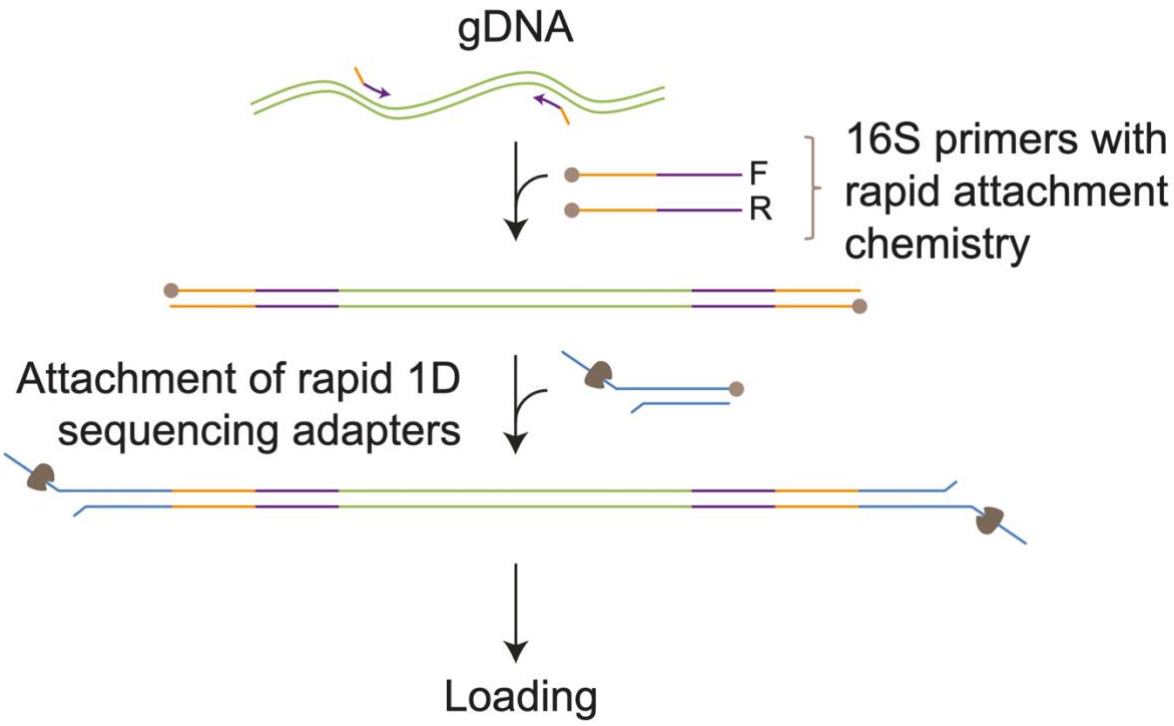


Figure 7. The DNA is amplified by PCR using specific 16S primers (27F and 1492R) that contain 5' tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters.

2.6. Statistical Microbiome Analysis

In this study, the FAST5 data of 16S rRNA metabarcoding results were converted into FASTQ sequences through the Min-KNOW and basecalled with Guppy 3.0. (ONT) on the MinION device. Before downstream statistical analysis, amplified 16S rRNA census data were filtered in length (>1500 bp) and quality (>10) using NanoFilt 1.1.0 adapters, and barcodes were clipped with qcat-1.1.0 (ONT) [60]. Visual graphical outputs, abundance, diversity, and statistical analysis of tick microbiome samples were performed by marker data profiling module on the MicrobiomeAnalyst 2.0 online server (https://www.microbiomeanalyst.ca/). To handle low-abundance taxa, we applied a 20% prevalence filter containing at least 4 counts associated with the inter-quantile range (IQR) variance to focus our analysis on more consistently detected taxa while acknowledging the presence of rare taxa in the tick microbiome. Rarefaction curves were calculated prior to all analytical techniques in order to determine sufficient sequencing depth based on the number of OTUs (species richness) from all samples. Filtered data was normalized using the total sum scaling (TSS) method. Subsequently, Normalized OTU data, aggregated at the species and genus level, were utilized to generate bar plots. Alpha diversity is used to measure the richness and evenness of species abundances in a given sample by the Chao1 and Shannon index, respectively.

Chapter 3

3. Results and Discussion

3.1. Gel Electrophoresis Results of DNA Extracted

To obtain DNA extracted efficiently, we studied extraction techniques for DNA using a bead beater as our mechanical disruption instrument. We also compared the results in the presence and absence of protein digestion, utilizing the mentioned commercial kit. Table. 2 shows information about DNA concentration (ng/µl) and A_{260}:A_{280} absorbance ratio. Fig. 8 depicts the results of DNA electrophoresis gel in each pool extracted by different methods. As can be seen, using various methods has resulted in different DNA quality and fragment length. The initial step of tick washing with ethanol and PBS is essential to achieve the bacteria composition inside the ticks and eliminate all bacteria from the surrounding environment, including soil, hosts, and other sources. This is due to the non-target organisms on ticks' surfaces which can potentially produce undesired amplicons with primers, leading to contamination and false-positive results in PCR analysis [61].

According to the results obtained by the extraction of P1-P16, interesting interpretations can be claimed that in what follows we will delve into them but before that let us point out some explanations about the experiments. The experiments have been done in three different conditions; the first one is, an initial fine crushing by tissuelyzer device for 10 mins with a pool containing 10 ticks (method 1). P1- P8 has been carried out by this method. In P9- P11 the same amount of time was spent on tissuelyzer, but the number of ticks was increased to 20 and the proteinase-K was utilized for digesting in each pool (method 2). In the third method, (P12-P16) all the procedure is like in method 2 except in P12-P16 tissuelyzer was used for 2 mins, not 10 mins. Note that in Fig. 8 due to the similar DNA electrophoresis gel result of samples, only some pools have been shown. Tissuelyzer caused physical disruption of the hard, chitinous exoskeleton of ticks. Although lysing of the polysaccharide chains of the chitin of the microorganism exoskeleton through mechanical bead-beating is a very quick and effective step, the result demonstrated the DNA degradation ~100-200 bp during the tissuelyzer prolonged time (Fig. 8, lanes 1-6 and lane 8). Therefore, extended bead-beating times have not influenced the total nucleic acid yield (DNA concentration < 20 ng/µl) [59] and it is essential that efficient time as a critical role in preventing DNA fragment shearing [1]. In Table. 2 using

this method a low concentration of each pool can be extracted. Enhanced DNA extraction yield from homogenized ticks in P9-P11, (Fig. 8 lanes 5, 6, and 7) was related to the increase in the number of tick specimens from 10 to 20 per pool [62] and in addition, incubation of the lysate in Proteinase K. Comparing the obtained results from P1- P8 to P9- P11 reveals that the DNA concentration of P9- P11 is considerably increased. Note that although in method 2 the number of ticks doubled, the DNA concentration increased much more than double which can be interpreted as the effect of digesting by Proteinase K. The absence of discrete bands and the appearance of a smear of products in lanes 5-12 signify the effective action of Proteinase K in digesting each pooled sample [52]. As a result, enzymatic protein degradation before DNA extraction was sufficient for maximum isolation of DNA ~240 ng/µl for engorged species and ~50 ng/µl for unengorged with a 260: 280 ratio of approximately 1.9 [59]. We must note that although in P9 to P11 the concentration of the DNA is very good, DNA degradation happened, and the length of the fragments is not proper, and this could be due to the long bead beating as mentioned above. As we can see in Fig. 8 lanes 7 and 8 the high yield and purity of DNA and long-length fragments were prepared via method 3 using freshly collected ticks (pools 12 and 13) and decreased bead-beating process time to 2 mins. Considering the advantages of method 3 and the results of the current evaluation, the use of this protocol for DNA extraction from engorged ticks could be useful as well ~180 ng/µl for engorged species and ~134 ng/µl for unengorged. Indeed, the quantities of nucleic acids originating from arthropods, the host's blood, and the tick's microbial community can influence the total genetic material obtained [58]. As a result, in P12 and P13 we expected to have more appropriate lengths of DNA fragments by reducing the tissuelyzer time from 10 to 2 mins. It can also be expected to have a lower concentration, but the extracted concentration is still appropriate.

Table 2. Information about DNA concentration (ng/µl) and A260:A280 absorbance ratio.

Pool No.	Tick species	engorged /unengorged	DNA	
			Concentration (ng/µl)	260/280
P1	*Rh. bursa*	U	4.12	1.96
P2	*Rh. bursa*	U	17.7	1.96
P3	*Rh. bursa*	U	6.21	1.83
P4	*D. marginatus*	E	14.1	1.74
P5	*H. lusitanicum*	E	7.10	1.90
P6	*I. ricinus*	E	3.96	1.68
P7	*D. marginatus*	E	59	2.09
P8	*Rh. bursa*	U	20.5	1.63
P9	*I. ricinus*	E	231	1.97
P10	*Rh. bursa*	E	252	1.84
P11	*Rh. bursa*	U	52	1.95
P12	*D. marginatus*	E	180	1.93
P13	*Rh. bursa*	U	134	1.84
P14	*H. marginatum*	E	3.97	1.98
P15	*Rh. bursa*	U	5.63	1.89
P16	*H. marginatum*	U	23.5	1.92

E: engorged, U: unengorged

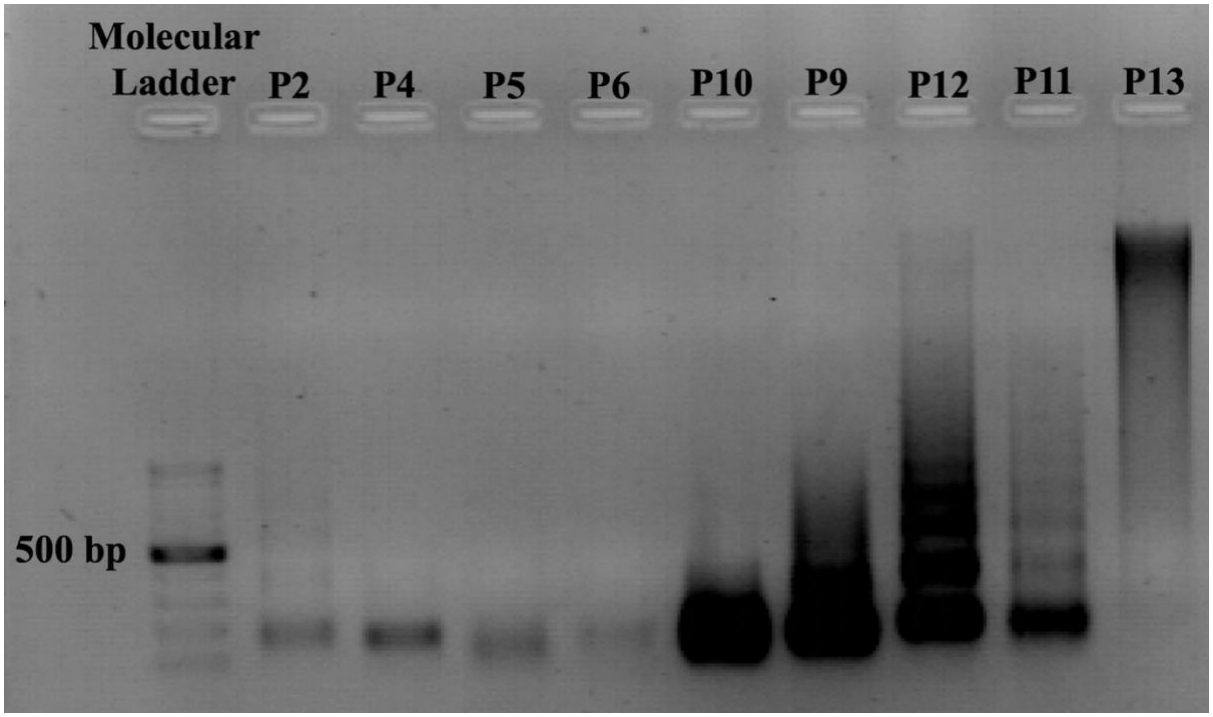


Figure 8. The results of DNA electrophoresis gel in each pool that extracted by different methods. DNA extraction of P2, P4, P5, and P6 was followed by method 1, DNA of P9, P10, and P11 was extracted by Method 2, and P12 and P13 DNA extraction was based on method 3.

3.2. Detection of Bacteria by 16S rRNA Amplification from Samples

the PCR amplification method is employed for detecting the DNA of pathogens which is a crucial step in microbiology [57]. This approach enables users to visualize all organisms within the sample without the need to sequence extraneous genomic regions, resulting in a more expedient and cost-effective identification process. The results shown in Fig. 9 are related to 46 (25%) of the 186 ticks analyzed were positive for bacteria. All the positive pools displayed a band around ~1500 bp in the 1% (w/v) agarose gel electrophoresis because of the tick 16s rRNA metabarcoding that conforms to the presence of bacteria in samples. Moreover, using the amplification of a segment from the tick mitochondrial 16S rRNA gene served as a reliable control to validate the effectiveness of the DNA extraction process. It is important to know that the choice of which region to amplify can impact the taxonomic resolution of the analysis. The negative PCR product does not mean there are no bacteria in these pools, it can be due to ineffective DNA extraction with degraded DNA fragmentation (P1-P8) that leads to insufficient DNA templates to amplify the conserved and variable regions within the 16S rRNA gene [57]. This result can be seen in P14 and P16 (Fig.9), even though the DNA extracted concentration is low, they were able to amplify variable regions. The result indicates that if the 16S rRNA gene is fragmented at the DNA level, amplification will not occur. This is due to that short DNA fragments may not cover enough variable regions, while long DNA fragments produce superior coverage of the targeted region, and enhance taxonomic classification, often to the genus or species level. Therefore, the length of the DNA molecule has an impact on amplification and sequence analysis and could potentially result in diverse interpretations [58]. Pools related to the 16S PCR-Positive results which were subjected to the sequencing technique are outlined in Table 3.

Table 3. 16s rRNA PCR-Positive Pools that were subjected to the sequencing process.

Pool No.	Tick species	engorged /unengorged	Province	Comarca	Region	Animal host
P7	*D. marginatus*	E	Barcelona	Vallès Occidental	Sentmenat	Wild boar
P12	*D. marginatus*	E	Barcelona /Girona	Osona/Garrotxa	Rupit/ Sant Aniol de Finestres	Wild boar
P14	*H. marginatum*	E	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	Wild boar
P15	*Rh. bursa*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-
P16	*H. marginatum*	U	Tarragona	Baix Ebre	Ports de Tortosa i Beseit	-

E: engorged, U: unengorged

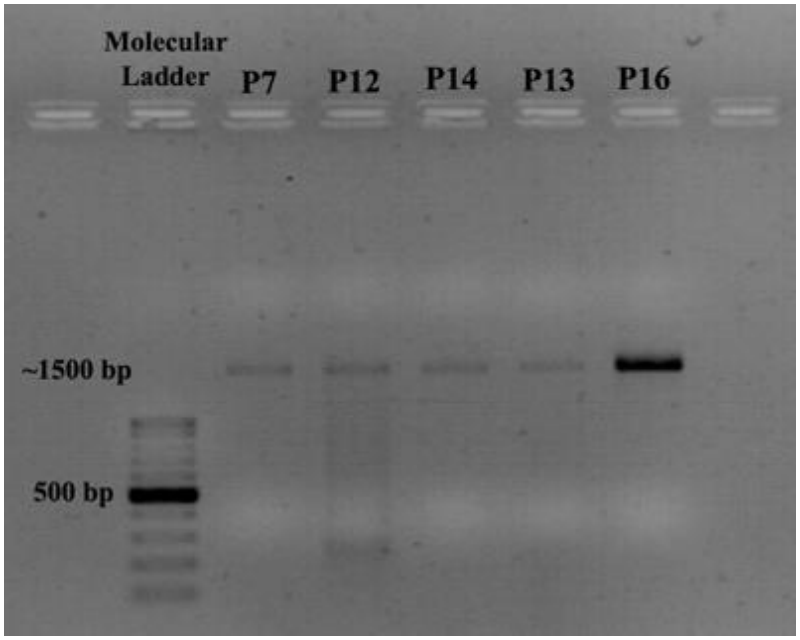


Figure 9. Positive PCR metabarcoding amplification of the tick 16S rRNA gene. The gel was loaded thus: lanes 1, 100 bp-ladder; lanes 2 and 3 engorged *D. marginatus*; lanes 4, engorged *H. marginatum*; lane 5, unengorged *Rh. bursa*; lane 6, unengorged *H. marginatum*.

3.3. 16S rRNA Metabarcoding Sequencing Analysis

The results based on genus-level classification are a total of 656,247 read counts were obtained involving p7 engorged *D. marginatus*: 96470, P12 engorged *D. marginatus*: 72816, P14 engorged *H. marginatum:* 357936, P16 unengorged *H. marginatum*: 98621, and P15 unengorged *Rh. bursa*: 30384 counts, with an average of 131,249 counts per sample. Taxonomic resolution using the non-specific reference base identified 336 genera generated belonging to 22 phyla, 33 classes, 111 orders, and 178 families.

A total of 657,691 taxonomically assigned reads were obtained at the species level including P7 which is related to engorged *D. marginatus*: 100006, P12, engorged *D. marginatus*: 77614, P16 unengorged *H. marginatum:* 348918, P14 engorged *H. marginatum*: 100062, and P15 unengorged *Rh. bursa*: 30551 counts, with an average of 131,538 counts per sample. Taxonomic classification using the non-specific reference base identified 30 genera generated from tick species 59 belonging to 4 phyla, 7 classes, 13 orders, and 23 families. A standard MinION Flow Cell is expected to produce more than 20 Gb or roughly 100,000 reads per barcoded sample is sufficient sequence depth [63].

3.4. Rarefaction Curve

As illustrated in Fig. 10, the rarefaction curves for all tick species exhibit rapid initial growth, mainly encompassing the most frequently encountered bacteria species. Following this initial phase, except for unengorged *Rh. bursa* (P15), the curves finally reach a plateau, indicating that only the rare species remain to be sampled. The objective is to determine the smallest sample size at which the count of OTUs reaches a plateau. This signifies that further increasing the number of reads will no longer lead to an increase in the count of OTUs and the diversity in the data has been fully captured [64].

Sample P16, unengorged *H. marginautm*, showed more species (42 bacteria species) than in other samples, with an increasing number of sequences (348918 reads). In contrast, unengorged *Rh. bursa* (P15) shows a low species richness (27 bacteria species), as the number of species does not increase very much regardless of the sequencing depth is insufficient. Based on the rarefaction curves, our finding result demonstrated that the sufficient sequence depth in our studied tick samples was about 100,000 reads to detect common and novel bacteria species except in P15.

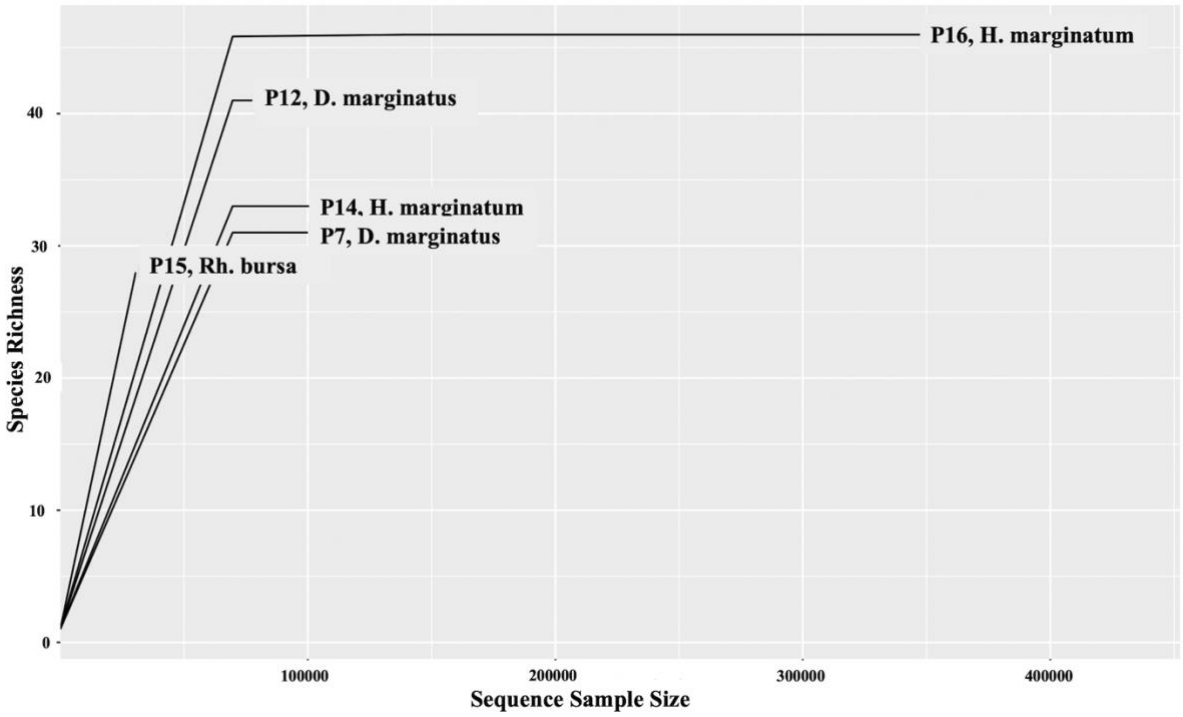


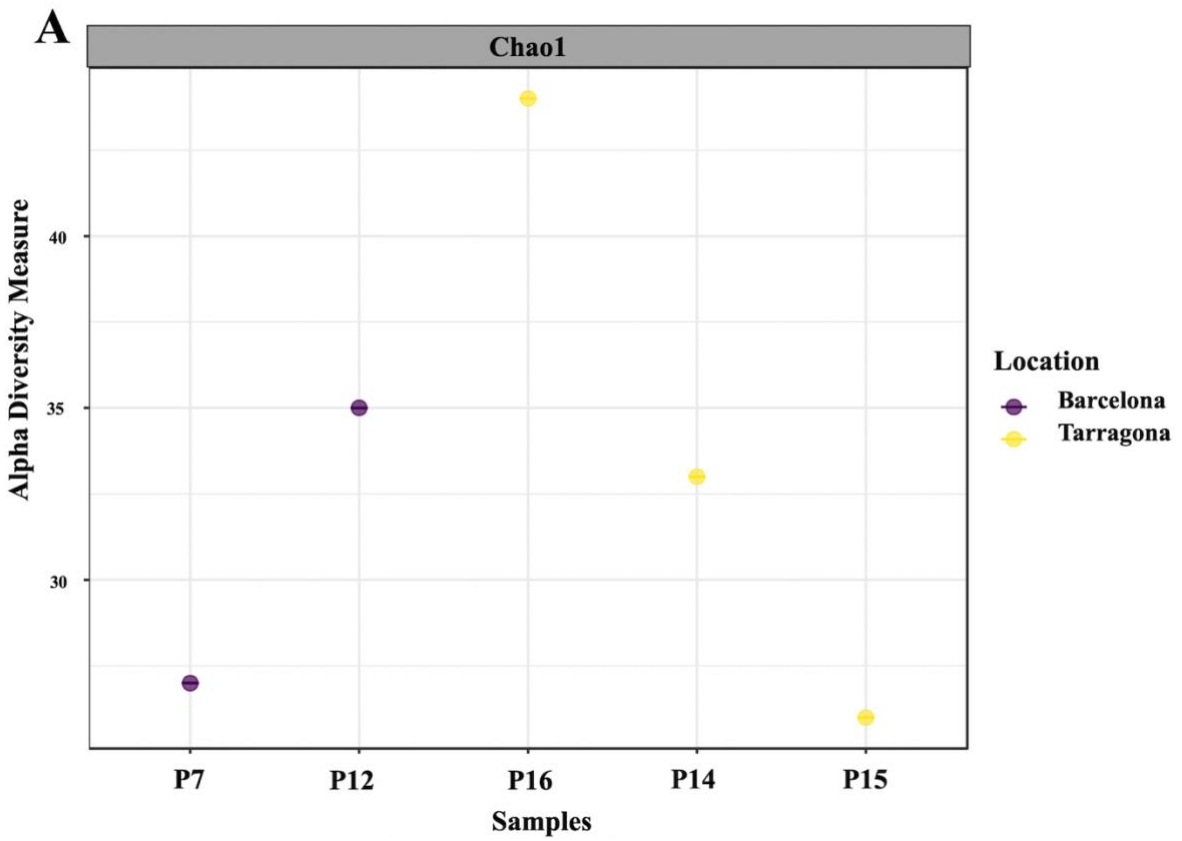
Figure 10. Rarefaction curves of ticks bacterial 16s rRNA amplicon sequences. The sequence sample size of P7 which is related to engorged *D. marginatus*: 100006, P12, engorged *D. marginatus*: 77614, P14 engorged *H. marginatum:* 348918, P16 unengorged *H. marginatum*: 100062, and P13 unengorged *Rh. bursa*: 30551 counts.

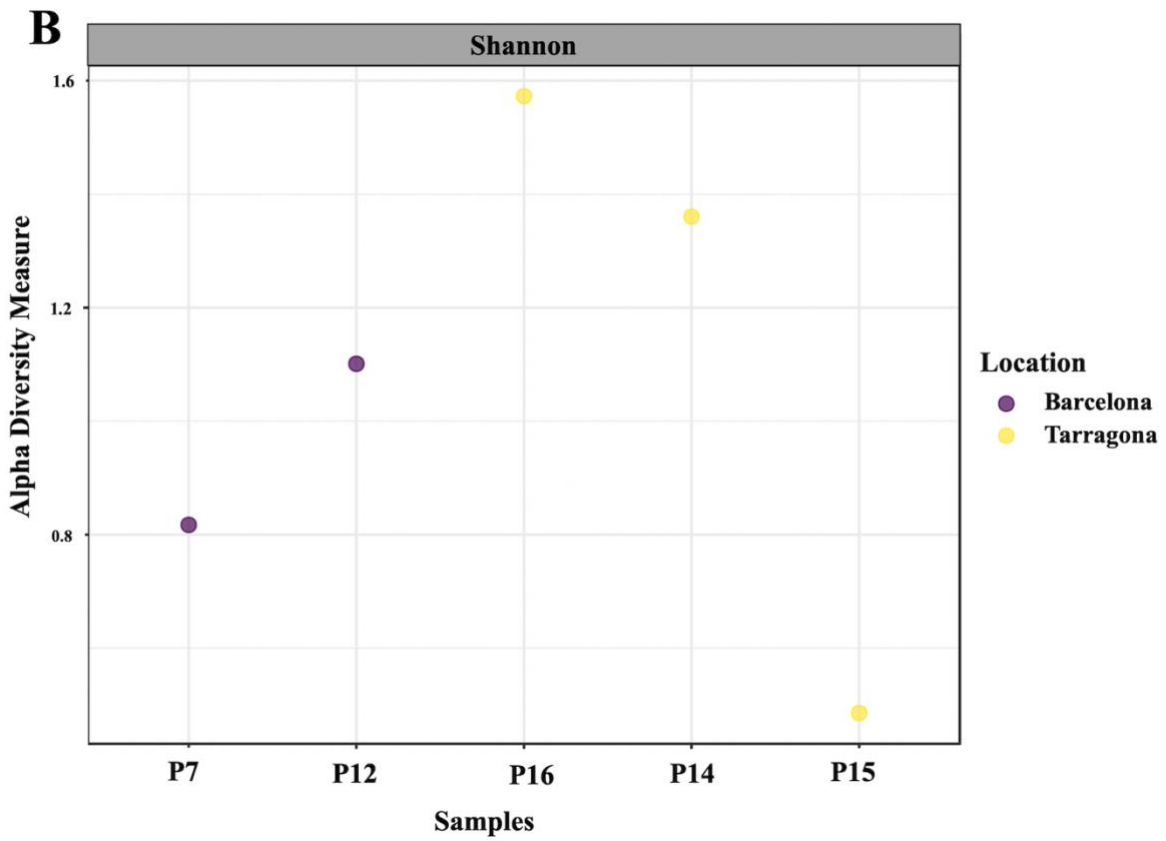
3.5. Alpha Diversity, Microbial Richness and Evenness

Alpha diversity metrics are typically classified into two categories: measures of richness and evenness. Chao1 Index is the most prevalent metric for assessing richness, while Shannon is the most widely utilized index for evaluating evenness. In alpha diversity analysis, scatter plots are a more commonly utilized visualization method when depicting metrics across multiple samples and box plots if the objective is to compare different sample cohorts [65]. In this study, we compared the results of the box plots for two conditions, the ticks' feeding status of ticks (engorged and unengorged), and the geographical location (Barcelona and Tarragona provinces) where the ticks' samples were collected, as these are two important parameters that influence on the microbial community. Fig. 11A and B demonstrate the scatter plots of alpha diversity analyses by Choa1 and Shannon Indexes for two conditions. The index's values range between 25 and 50 for Choa1 (Fig. 11A, Fig. 12D) and 0.2 and 1.6 for Shannon (Fig. 11B, Fig. 12E). The indexes revealed differential diversity patterns for the examined conditions.

3.5.1. Alpha diversity Comparison Based on Geographic Location as a Factor

Here, we aimed to evaluate the impact of the geographical location of sample collection on the diversity of tick species. Two commonly used diversity indexes, Chao1 and Shannon, were employed to assess both species richness and diversity within the collected samples, respectively. Fig. 11A provides a visual representation of the Chao1 index for tick species diversity across different locations. As we can see in Fig. 11A-B, the P16, unengorged *H. marginatus* from the Tarragona zone exhibited the highest richness, indicating a greater number of tick species present in the samples collected from this region. Conversely, the lowest richness was observed in the same Tarragona zone (P15, *Rh. bursa* ticks). These findings suggest a notable variation in species richness and diversity within the Tarragona province itself. This was followed by ticks collected from Barcelona P12 and p14 from Tarragona showed more richness, implying a more balanced distribution of tick species. On the other hand, samples from Tarragona (P14) and Barcelona (P12) were found to have higher diversity in species distribution according to the Shannon index (Fig. 11C). It is important to note that in our study, the p-values obtained, which exceed 0.05, indicate there is no significant difference in diversity indices between the examined locations.


A
Chao1
Alpha Diversity Measure
40
35
30
P7
P12
P16
P14
P15
Samples
Location
Barcelona
Tarragona


B
Shannon
Alpha Diversity Measure
1.6
1.2
0.8
P7
P12
P16
P14
P15
Samples
Location
Barcelona
Tarragona

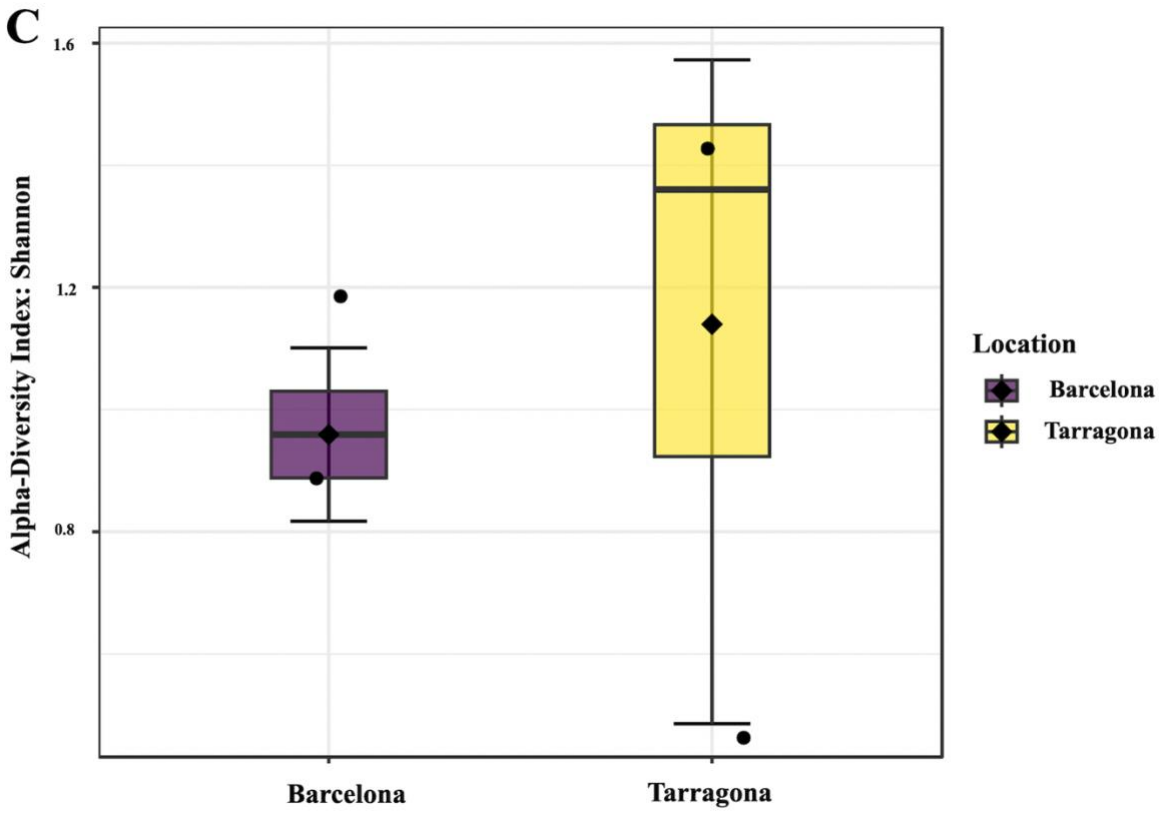
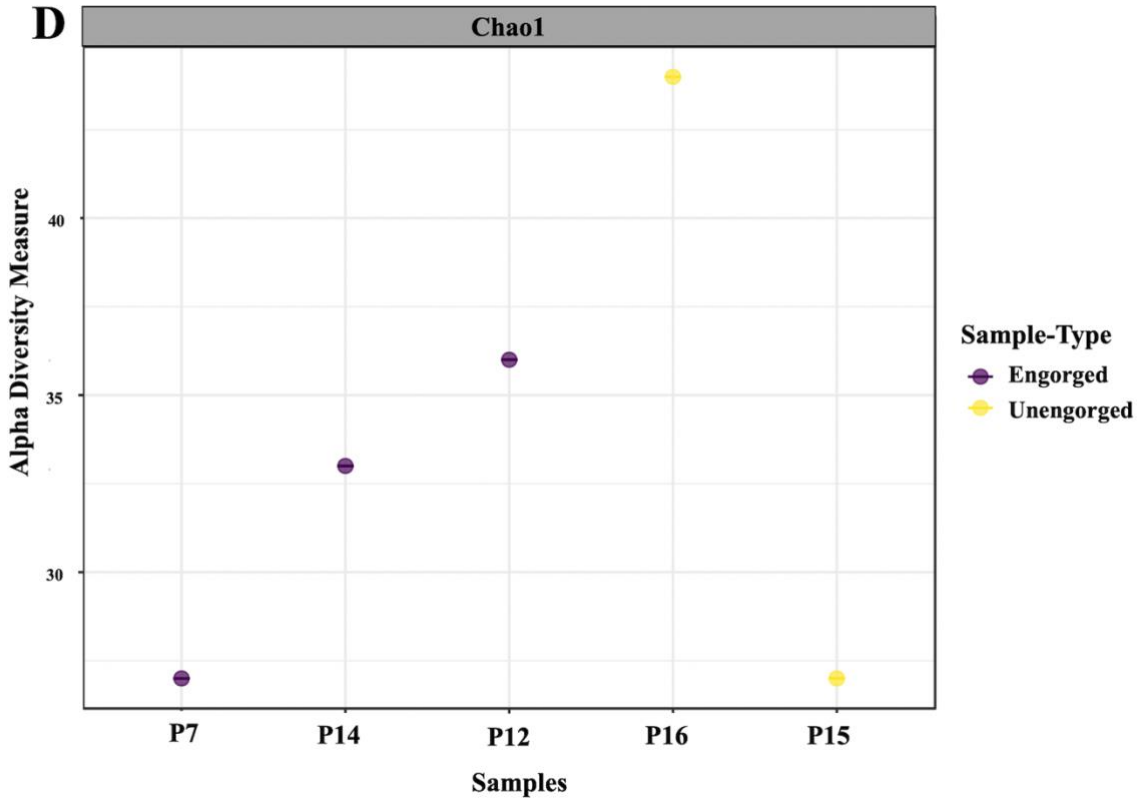


Figure 11. Alpha diversity analysis, scatter plot of Chao1 index. (A) and Shannon index (B) in each sample community. The box plot presents the comparison of the Shannon index between the two provinces of Barcelona and Tarragona over-studied tick species (C).

3.5.2. Alpha Diversity Comparison Based on Tick's Feeding Status as a Factor

Another factor we examined is the ticks' feeding status of tick samples. Fig.12 D-F depicted the Chao1 and Shannon index analyses for host-meal status scenarios, in both index the highest levels of richness and diversity were observed in pool 16, consisting of unengorged H. marginatus species, with 47 different bacteria species and a Shannon index value of 1.6. This was followed by the P12 engorged *D. marginatus* and the P14 engorged *H. marginatus* ticks. In contrast, the lowest richness was observed in the P7, which featured engorged *D. marginatus* ticks, as well as in the P15, comprising unengorged *Rh. bursa* ticks, both with a Chao1 index value of 27 (Fig. 12D). The lowest diversity in the tick community was observed by the Shannon index in P15, which involved unengorged *Rh. bursa* ticks (Fig. 12E). In summary, it is evident that there exists a linear relationship between richness and evenness diversity. Tick species with higher evenness tend to exhibit greater diversity in terms of bacteria species, encompassing both common and rare bacteria

Fig. 12F illustrates a comparison of Shannon values through pairwise analysis between two groups of tick samples: the unengorged group, consisting of two tick species (P16 *H. marginatus* and P15 *Rh. bursa*), and the engorged group, comprising ticks from P7 and P12 of the *D. marginatus* and P14 *H. marginatus* species, which have fed on wild boar. It is evident from the box plot that the unengorged group exhibits a higher richness in the bacteria community compared to the engorged group. However, the calculated p-value (>0.05) indicates that there are no statistically significant differences in the bacteria community between the engorged and unengorged groups, and the same bacteria species have been detected in both.



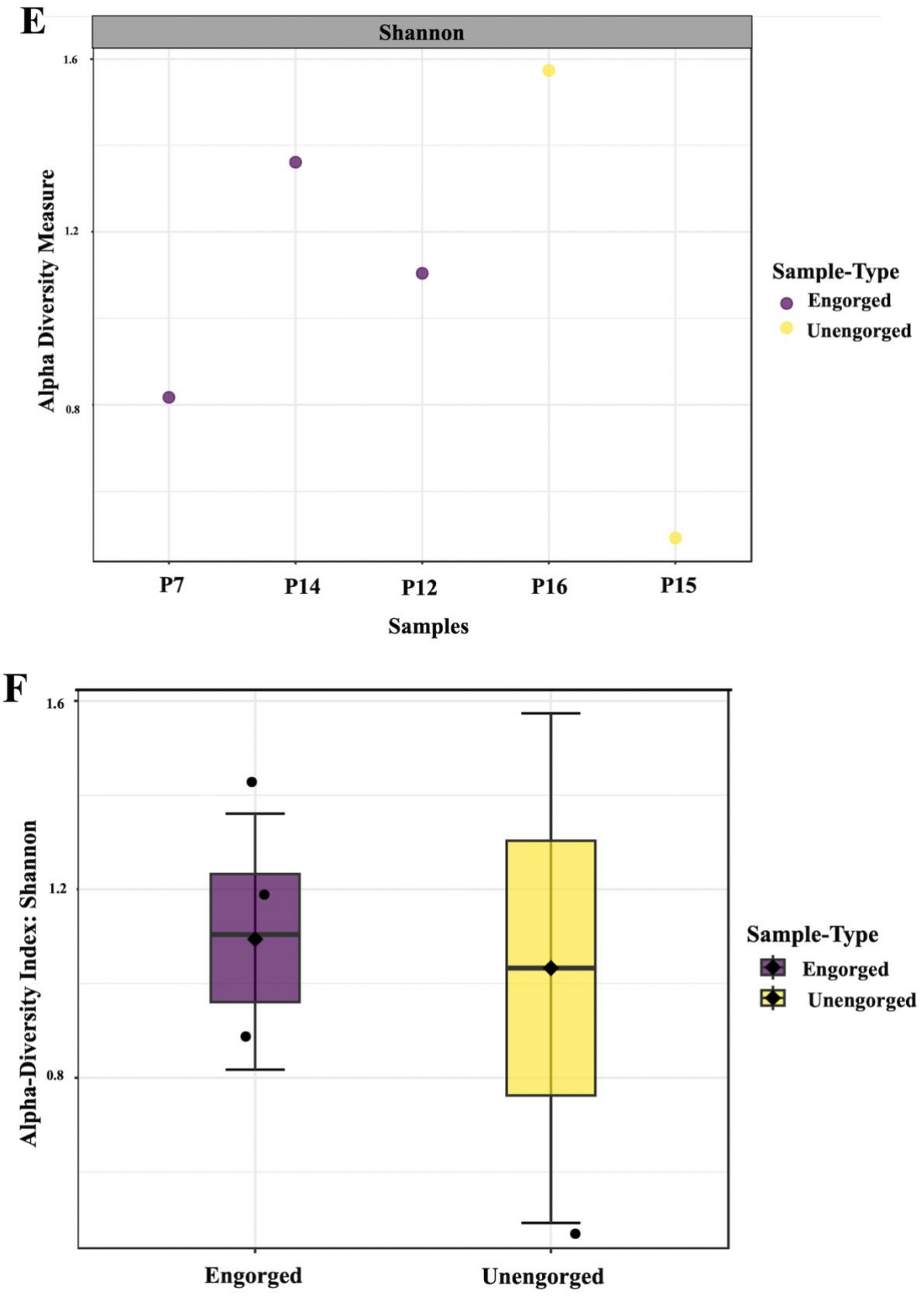


Figure 12. Alpha diversity analysis, scatter plot of Chao1 index (D) and Shannon index evenness (E) in each sample community. The box plot presents the comparison of the Shannon index between engorged and unengorged over-studied tick species (F).

As a result, the p-values greater than 0.05 in both the analysis of tick species diversity based on collection geographical location and host-blood meal condition indicate a lack of statistically significant differences in these respective factors. In other words, our data suggest that there are no significant variations in tick species diversity when considering different collection locations or host blood meal conditions.

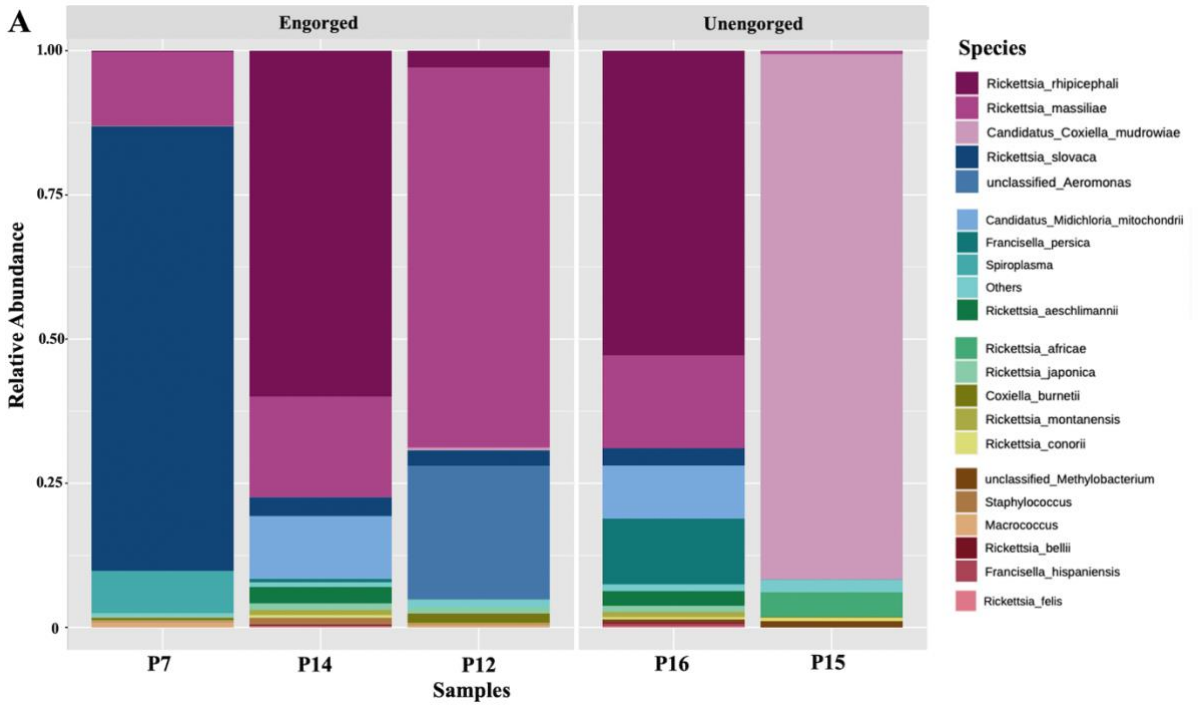
3.6. Bacteria Relative Abundance

We conducted additional analysis to examine the relative distribution of bacteria across various taxonomic classifications within three different tick species *D. marginatus, H. marginatum*, and *Rh. bursa*. Fig. 13A and 13B illustrate the taxa derived from the taxonomic classification of identified bacterial OTUs at the species and genus taxonomic levels, respectively. The predominant phylum Proteobacteria, Firmicutes, Planctomycetota, Cyanobacteria, Myxococcota, and Acidobacteriota were observed to be present within all tick samples. The phylum Proteobacteria was detected in similar abundances at an average of 95% in all tick species in two engorged and unengorged gropes, while Firmicutes phylum was only identified in the engorged group at about 10% abundance in *D. marginatus* (P7, P12) and *H. marginatum* species (P14). Furthermore, the abundance of Planctomycetota in the engorged ticks' pool of *D. marginatus* species was more than in the other pools. Coxiellaceae (88%), Rickettsiaceae (5.2%), and Beijerinckiaceae (2%) constituted approximately 95% of the bacteria family identified within P15, unengorged *R.bursa* ticks, while the *Rickettsiaceae* family is the predominant family in both groups of *D. marginatus* and *H. marginatum* species. Several bacteria species inherited maternally such as *Rickettsiella*, *Coxiella*, *Arsenophonus*, *Francisella*, and *Spiroplasma*, were abundant, and their relative abundance varied depending on tick species. Among the observed bacteria species (Fig 13. A), *Rickettsia* showed high abundance in the two species, *D. marginatus* and *H. marginatus*, and Coxiella at an abundance of 87% in the *Rh. bursa* (P15), about 2% in *D. marginatus* species and it was not present in *H. marginatus* tick species. In contrast, the *Francisella* genus which is in the *Candidatus Midichloria* family was just detected at a relative abundance of ~11% in unengorged and about 1% in engorged *H. marginatum* species respectively. Moreover, in unengorged of this tick's species the *Achromobacter* and *Methylobacterium Methylorubrum* genera (<1%) were identified at similar abundance. About 10% of bacteria are not assigned in both groups of engorged and unengorged *H. marginatum* tick species. *Spiroplasma* was just detected in

engorged ticks of *D. marginatum* species at an abundance of 7.5%. *Rickettsiaceae*, *Spiroplasmataceae*, *Coxiellaceae*, and *Spiroplasmataceae* families were composed of *D. marginatum* tick species composition.

Stenotrophomonas bacteria and *Methylobacterium Methylorubrum* genera were identified in unengorged *R.bursa* and *H . marginatum* tick samples. Similar distinctions in bacterial abundance were further identified when examining the taxonomic classifications at the genus level, demonstrating consistency with the patterns observed at the family and phylum levels.

As a result, two major phyla were related to the Proteobacteria and Firmicutes, which detected 99.5% of the bacteria in unengorged ticks and 95% in engorged ticks were identified from *D. marginatus*, *Rh. bursa*, and *H. marginatum* tick's species. The relative abundance of bacteria species in each sample is reported in Table 4.



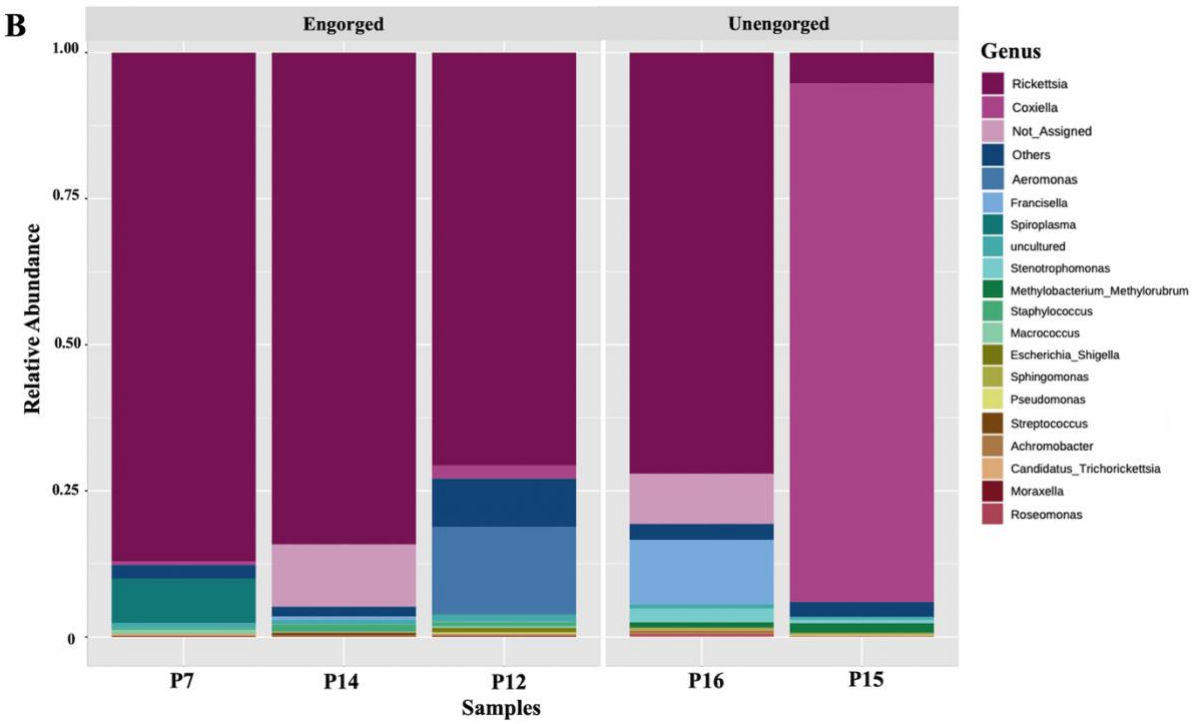


Figure 13. Stacked bar plot of relative abundances of the top 20 most abundant genera from D. marginatus, H. marginatus, and Rh. bursa species. (A) Bacteria abundance at the species-level, and (B) at the genus-level.

P7 and P12 belong to the engorged *D. marginatum* ticks which fed on wild board. The difference between these pools is their location, P7 tick samples were collected from Barcelona while in P12, half of the ticks are from Barcelona and half from Girona province. As a result, both P7 and P12 share several common bacteria, but their dominant species and some unique bacterial species differ in varying proportions. *R. slovaca, R. massiliae, Spiroplasma, R. rhipicephal, Coxiella burnetiid, R. japonica, Macrococcus, Staphylococcus, R.conorii. Rickettsia spp*. obligate intracellular bacteria from the *Rickettsia* genus are the main species in P7 and P12 samples. P7 is dominated by *R. slovaca* (77.07%) followed *R. Massiliae* (13%), and *Spiroplasma* (7.3 %), while in P12, the dominant bacterium is *R. massiliae* (65.88%), followed by *unclassified Aeromonas* (23.12%). The differences in bacterial composition and proportion may be due to the different areas where the tick samples were collected, host blood meal, or other environmental factors.

Both P14 unengorged *H. marginatum* and P16, engorged *H. marginatum* samples exhibit overlapping bacterial species, including *R. rhipicephali, R. massiliae, Candidatus Midichloria mitochondrii, R. slovaca, R. aeschlimannii, R. montanensis, R. japonica, R. conorii,* and *R. bellii.* Within P14 and P16, *R. rhipicephali* (60%), *R. massiliae* (17.5%), *Candidatus,*

Midichloria mitochondrii (11%), *R. slovaca* (3.26%), *R. aeschlimannii* (2.8%), are the prevailing bacterial communities. P16 has a similar bacteria composition but a slightly lower species proportion than P14. The *Francisella persica* by 11.41% showed another main bacteria species in P16 while the P14 has 0.6 % species abundance of this bacteria. Both samples have unique bacterial species not found in the other. P14 has *Staphylococcus* (1%), while P16 has *Francisella_hispaniensis* and *unclassified Methylobacterium* (<1%). This could be possibly influenced by factors such as ticks' feeding status, or host interactions.

Within P15, unengorged *Rh. bursa* the bacteria with high abundance include *Candidatus Coxiella mudrowiae* 91.11% belonging to the genus and *R. africae* 4.25%, *unclassified Methylobacterium* 1.10%, and ~ 4% remain of abundance for *R. conorii, R. massiliae*, Coxiella *burnetii*, *Methylobacterium goesingense*, *Variovorax paradoxus*, *R. parkeri*, *unclassified Sphingomonas*.

Several research studies have reported limited correlations between tick microbiomes and the microbial flora found on the skin or in the blood of their hosts, indicating that the source of the host's blood meal may not significantly impact the composition of the tick's microbiome. Other researchers, however, have found the inverse [66]. The common bacteria species in engorged ticks' samples contain *Macrococcus*, *Romboutsia*, and *Staphylococcus* and within unengorged ticks are *Lichenibacterium ramalinae*, *Methylobacterium brachythecii*, *Methylobacterium_bullatum*, *Methylobacterium cerastii*, *Methylobacterium durans*, *Methylobacterium phyllosphaerae*, *Rhizobacter gummiphilus*, *Roseomonas elaeocarpi*, *Sphingomonas melonis*. As a result, in our study, there is no significant relationship between tick-feeding status and diversity in the bacteria community. Therefore, the bacterial diversity in vegetation adult tick samples is greater than that in fed ticks.

The shared bacteria species in Barcelona are *Candidatus Coxiella mudrowiae*, *Clostridium*, Spiroplasma, Terrisporobacter, unclassified Aeromonas, and in the Tarragona region are *R. africae, R. parkeri, R. aeschlimannii, R. bellii, Rickettsia endosymbiont of Bemisia tabaci, R. hoogstraalii, R. peacockii, Candidatus Midichloria mitochondrii, Francisella hispaniensis, Francisella Lichenibacterium ramalinae, Massilia,* Methylobacterium brachythecii, *Methylobacterium bullatum, Methylobacterium cerastii, Methylobacterium durans, Methylobacterium phyllosphaerae, Rhizobacter gummiphilus, Roseomonas elaeocarpi, Sphingomonas melonis, Variovorax boronicumulans*.

Table 4. Bacteria abundance at the species taxonomy level and tick-born pathogen in humans.

Tick's / Bacteria	*D. marginatus*	*D. marginatus*	*H. marginatum*	*H. marginatum*	*Rh. bursa*	Pathogen in human
Sample_type	Engorged			unengorged		
Candidatus_Coxiella_mudrowiae	0.057%	0.499%	-	-	91.109%	Unknown
Candidatus_Midichloria_mitochondrii	-	-	10.886%	9.181%	-	Lyme disease
Clostridium	0.058%	0.174%	-	-	-	-
Coxiella_burnetii	0.486%	1.703%	-	-	0.171%	Q fever
Francisella_hispaniensis	-	-	0.028%	0.488%	-	Unknown
Francisella_persica	-	-	0.609%	11.414%	-	Unknown
Jeotgalibaca	-	0.028%	0.030%	-	-	-
Laceyella	0.136%	0.081%	0.124%	0.118%	0.128%	-
Lichenibacterium_ramalinae	-	-	-	0.005%	0.112%	-
Macrococcus	0.917%	0.417%	0.061%	-	-	Non-pathogen
Massilia	-	-	0.025%	-	0.059%	
Methylobacterium_brachythecii	-	-	-	0.020%	0.046%	Facultatively bacteria
Methylobacterium_bullatum	-	-	-	0.072%	0.105%	-
Methylobacterium_cerastii	-	-	-	0.030%	0.075%	-
Methylobacterium_durans	-	-	-	0.053%	0.052%	-
Methylobacterium_goesingense	0.010%	0.017%	0.019%	0.145%	0.292%	-
Methylobacterium_phyllosphaerae	-	-	-	0.040%	0.148%	-
Rhizobacter_gummiphilus	-	-	-	0.018%	0.062%	-
Rickettsia_aeschlimannii	-	-	2.857%	2.560%	-	Rickettsia aeschlimanni i infection
Rickettsia_africae	-	-	0.032%	0.027%	4.247%	African tick bite fever
Rickettsia_bellii	-	-	0.273%	0.246%	-	Unknown
Rickettsia_conorii	0.072%	0.106%	0.462%	0.421%	0.590%	Boutonneuse fever
Rickettsia_endosymbiont_of_Bemisia_tabaci	-	-	0.013%	0.015%	-	-
Rickettsia_felis	0.021%	0.030%	0.227%	0.206%	-	Flea-borne spotted fever
Rickettsia_honei	0.018%	0.018%	0.187%	0.153%	-	Flinders Island spotted fever
Rickettsia_hoogstraalii	-	-	0.022%	0.026%	-	SFGR
Rickettsia_japonica	0.282%	1.073%	1.071%	1.006%	-	Japanese spotted fever

Rickettsia_massiliae	**12.836%**	**65.880%**	**17.448%**	**16.085%**	**0.558%**	**Human tick-borne spotted fever, rifampin-resistant**
Rickettsia_montanensis	**0.023%**	**0.021%**	**0.923%**	**0.921%**	**-**	**SFGR**
Rickettsia_parkeri	**-**	**-**	**0.013%**	**0.012%**	**0.138%**	**American tick bite fever**
Rickettsia_peacockii	**-**	**-**	**0.046%**	**0.042%**	**-**	**Non-pathogen**
Rickettsia_rhipicephali	**0.221%**	**2.935%**	**59.985%**	**52.858%**	**0.039%**	**unknown**
Rickettsia_rickettsii	**0.023%**	**0.019%**	**0.062%**	**0.053%**	**-**	**Rocky Mountain spotted fever**
Rickettsia_slovaca	**77.073%**	**2.685%**	**3.250%**	**3.035%**	**-**	**TIBOLA or DEBONEL**
Romboutsia	**0.068%**	**0.215%**	**0.047%**	**-**	**-**	**-**
Roseomonas_elaeocarpi	**-**	**-**	**-**	**0.008%**	**0.049%**	**-**
Singulisphaera	**0.012%**	**0.045%**	**0.014%**	**0.076%**	**-**	**-**
Sphingomonas_melonis	**-**	**-**	**-**	**0.043%**	**0.105%**	**Non-pathogen**
Spiroplasma	**7.344%**	**0.090%**	**-**	**-**	**-**	**Non-pathogen**
Staphylococcus	**0.171%**	**0.143%**	**1.090%**	**-**	**-**	**Non-pathogen**
Streptococcus	**-**	**0.106%**	**0.089%**	**-**	**-**	**Can be Pathogen or Non-pathogen**
Terrisporobacter	**0.019%**	**0.097%**	**-**	**-**	**-**	**-**
unclassified_Aeromonas	**-**	**23.122%**	**-**	**-**	**0.056%**	**life-threatening diseases**
unclassified_Bradyrhizobium	**-**	**0.046%**	**-**	**0.021%**	**0.112%**	**-**
unclassified_Methylobacterium	**-**	**0.034%**	**-**	**0.439%**	**1.102%**	**=**
unclassified_Sphingomonas	**0.013%**	**-**	**-**	**0.010%**	**0.148%**	**Non-pathogen**
unclassified_Variovorax	**-**	**0.019%**	**-**	**0.012%**	**-**	**-**
Variovorax_boronicumulans	**0.016%**	**0.019%**	**-**	**-**	**-**	**-**
Variovorax_paradoxus	**0.032%**	**0.027%**	**-**	**0.059%**	**0.341%**	**-**
Vicinamibacter_silvestris	**0.018%**	**0.044%**	**0.028%**	**0.003%**	**-**	**-**

3.7. Pathogen or non-pathogenic bacterium

Ticks, aside from their role as pathogen carriers, host a diverse array of commensal and symbiotic microorganisms upon which tick biology depends. Table 4 provides an overview of human pathogens transmitted by ticks. *Rickettsia spp.* including both non-pathogenic and pathogenic strains. The SFR group that contains more than 20 *Rickettsiae* that cause mild to severe rickettsioses in humans includes *R. massiliae, R. aeschlimanni, R. helvetica, R. monacensis, R. akari,* and others. Whereas the typhus group (TG) involves *R. typhi* and *R. prowazekii* members, which are pathogen groups within the *Rickettsia* genus. *R. belli*, is the most significant variation species within the *Rickettsiae* [67]. In our study, we identified the presence of various Rickettsia spp. Among the analyzed samples, some of them were novel species like *R. rhipicephali, R. japonica*, and *R. Africa*, which had not been previously reported in Spain. Notably, *R. rhipicephali,* constituting approximately 60% of the identified bacteria, is a newly discovered member of the SFGR group and is exclusively associated with *H. marginatum* species. Importantly, *R. rhipicephali* has not been recognized as a human pathogen thus far. Additionally, *R. japonica* is known as the causative agent of Japanese spotted fever (JSF) and is classified as a highly pathogenic species [68], was detected in all examined samples except for one (P15, Rh. buras species). It is noteworthy that José A. Oteo et al. have previously documented the detection of *R. Africa* through PCR in three patients who contracted African tick bite fever after traveling to South Africa. While, in our study, we identified *R. Africa* in our tick samples, with a prevalence of 4% in *Rh. bursa* and 1% in *H. marginatum* species [69]. The research conducted by J. Márquez et al. found that *R. slovaca* was present in 24.7% of *D. marginatus* ticks, but there were no instances of rickettsiae identified in ticks of the *Hyalomma spp.* or *Rh. bursa* species that were studied. The confirmation of the presence of *R. slovaca* at a rate of 77% in our sample of *D. marginatum* ticks aligns with their finding. while, in our study, we detected about 3% of R. slovaca in *H. marginatum* species [41]. *R. massiliae* was the main bacteria species in all tick samples, except in p15, *Rh. bursa* and appeared to cause rifampin-resistant cases of rickettsiosis in Catalonia, Spain [70].

Spiroplasma gram-positive bacteria are facultative symbionts and are transmitted vertically in ticks and other arthropods [71]. Certain *Spiroplasma* species, function as male killers in arthropods and significant influences on the reproductive processes of arthropods, while others provide pathogen resistance to their host. Although *Spiroplasma* is present in ticks, its specific function within this context remains unclear [72], [68]. We found approximately 8% of

Spiroplasma only in engorged *D. marginatus* which confirmed the previous study that isolated from Spanish *D. marginatus* [73]. *Macrococcus* is a type of Gram-positive spherical bacteria categorized within the Staphylococcaceae family. It is typically regarded as a non-pathogenic microorganism that does not induce illnesses in humans or animals. The *Coxiella genus, part of the Coxiellaceae family, encompasses Coxiella burnetii, C. cheraxi, Candidatus Coxiella mudrowiae, and numerous similar endosymbionts known as Coxiella-like endosymbionts (CLEs)* [74]. Within our sample, we observed *Candidatus Coxiella mudrowiae* species in *Rh. bursa*, (P15), where it constituted a dominant presence at 91.11%. Notably, this bacterium had not been previously documented in Spain. We found this novel bacterium in *D. marginatus*, albeit at a lower proportion of approximately 1%. Furthermore, *Coxiella burnetii*, a pathogen bacterium, causes a Q fever in humans found within *Rh. bursa* and *D. marginatus* species.

In many Spanish ticks Francisella-like endosymbionts (FLEs) were identified such as *Francisella tularensis* which serves as the agent responsible for causing tularemia or rabbit fever. In our research, from the *Francisellaceae* family, we detected *Francisella persica* and *Francisella hispaniensis* in *H. marginutum* tick samples. However, the function of *Francisella* species could potentially supply vital nutrients for the ticks, some *Francisella* species are pathogenic. *Francisella hispaniensis* has been reported previously in the Iberian Peninsula. While facultative intracellular *Francisella persica* which is a reclassified of obligate intracellular *Wolbachia persica* has not been documented in Spain [75].

The presence of *unclassified Aeromonas* species has not been previously reported in Spain that we identified in P12, engorged *D. marginatus* of 23% from Barcelona and 0.05% in P15, unengorged *Rh. bursa* from Tarragona Providence. Tick-borne *Aeromonas* species commonly lead to critical diseases, including necrotizing fasciitis, which can progress to septic shock and mortality. As per the research conducted by Kondo et al., A. hydrophila, along with another species of *Aeromonas*, was identified in specific circumstances. These conditions include regions with a high population of *Rickettsia spp.*, areas endemic for JSF, the existence of ticks in their larval stage, and during the summer season [76].

Chapter 4

4. Conclusion

In this research, the bacterial communities belonging to *Rh. bursa*, *D. marginatus*, *H. lusitanicum*, *H. marginatum*, and *I. ricinus* tick species have been studied. The analyzed samples were collected from several areas in eastern Spain from the animals and the field. The standardized DNA extraction method using bead-beater and Proteinase k provided us with long DNA fragments that were compatible with the MinION Nanopore sequencing technique (long reads). The advantages of the full-length 16s rRNA gene metabarcoding sequencing method, led to the detection of 59 species and 336 genera in our samples. Several novel endosymbiotic bacteria such as *Candidatus Coxiella mudrowiae, Francisella persica, R. africae, R. japonica, R. rhipicephali,* and *Unclassified Aeromonas* were detected utilizing the 16s rRNA metabarcoding sequencing approach resulted in the high taxonomic resolution. It is worth mentioning that the above bacteria species have not been reported in Spain previously. Furthermore, multiple other bacteria species have been found in our investigation which are typically recognized as extremely virulent vertebrate pathogens such as *Candidatus-like* species *Coxiella burnetii, R. aeschlimannii, R. massiliae, R. slovaca, R. rickettsii*. Alpha diversity by Shannon index ($p < 0.05$), showed no significant relation between the two different scenarios of geographical locations or ticks' feeding status. These results highlighted the potential of MinION nanopore sequencing over traditional PCR, Illumina, or other methodologies for bacterial surveillance in ticks. it's worth emphasizing that while ticks are recognized as crucial vectors of pathogens (TBP), it is also important to conduct research on non-pathogenic bacterial species associated with ticks as these organisms might also have a role in influencing the transmission of TBD. This could be regarded as a potential avenue for future research.

Reference

[1] S. J. Salyer, R. Silver, K. Simone, and C. B. Behravesh, "Prioritizing zoonoses for global health capacity building—themes from One Health zoonotic disease workshops in 7 countries, 2014–2016," *Emerg Infect Dis*, vol. 23, no. Suppl 1, p. S55, 2017.

[2] G. G. D. Suminda *et al.*, "High-throughput sequencing technologies in the detection of livestock pathogens, diagnosis, and zoonotic surveillance," *Comput Struct Biotechnol J*, 2022.

[3] O. Duron *et al.*, "The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, Coxiella burnetii," *PLoS Pathog*, vol. 11, no. 5, p. e1004892, 2015.

[4] A. Portillo, A. M. Palomar, M. de Toro, S. Santibáñez, P. Santibáñez, and J. A. Oteo, "Exploring the bacteriome in anthropophilic ticks: To investigate the vectors for diagnosis," *PLoS One*, vol. 14, no. 3, p. e0213384, 2019.

[5] E. Serretiello *et al.*, "The emerging tick-borne Crimean-Congo haemorrhagic fever virus: A narrative review," *Travel Med Infect Dis*, vol. 37, p. 101871, 2020.

[6] L. Shao *et al.*, "Detection and phylogenetic analysis of a novel tick-borne virus in Haemaphysalis longicornis ticks and sheep from Shandong, China," *Virol J*, vol. 18, pp. 1–6, 2021.

[7] L. Halos *et al.*, "Determination of an efficient and reliable method for DNA extraction from ticks," *Vet Res*, vol. 35, no. 6, pp. 709–713, 2004.

[8] M. C. Vieira Lista *et al.*, "Identification and distribution of human-biting ticks in Northwestern Spain," *Insects*, vol. 13, no. 5, p. 469, 2022.

[9] O. Poch, I. Sauvaget, M. Delarue, and N. Tordo, "Identification of four conserved motifs among the RNA-dependent polymerase encoding elements.," *EMBO J*, vol. 8, no. 12, pp. 3867–3874, 1989.

[10] D. T. Mourya *et al.*, "Characterization of a strain of quaranfil virus isolated from soft ticks in India. Is quaranfil virus an unrecognized cause of disease in human and animals?"," *Heliyon*, vol. 5, no. 3, 2019.

[11] V. Cicculli *et al.*, "Lack of Evidence for Crimean–Congo Hemorrhagic Fever Virus in Ticks Collected from Animals, Corsica, France," *Emerg Infect Dis*, vol. 28, no. 5, p. 1035, 2022.

[12] R. Ravindran, P. K. Hembram, G. S. Kumar, K. G. A. Kumar, C. K. Deepa, and A. Varghese, “Transovarial transmission of pathogenic protozoa and rickettsial organisms in ticks,” *Parasitol Res*, vol. 122, no. 3, pp. 691–704, 2023.

[13] S. Sambado, J. Salomon, A. Crews, and A. Swei, “Mixed transmission modes promote persistence of an emerging tick-borne pathogen,” *Ecosphere*, vol. 11, no. 6, p. e03171, 2020.

[14] H. V. Manjunathachar *et al.*, “Identification and characterization of vaccine candidates against Hyalomma anatolicum—Vector of Crimean-Congo haemorrhagic fever virus,” *Transbound Emerg Dis*, vol. 66, no. 1, pp. 422–434, 2019.

[15] S. D. Carter *et al.*, “Structure, function, and evolution of the Crimean-Congo hemorrhagic fever virus nucleocapsid protein,” *J Virol*, vol. 86, no. 20, pp. 10914–10923, 2012.

[16] S. Chinikar *et al.*, “Genetic analysis of crimean-congo hemorrhagic fever virus in Iran,” *J Med Virol*, vol. 73, no. 3, pp. 404–411, 2004.

[17] C. Sesmero-García, M. D. Cabanero-Navalon, and V. Garcia-Bustos, “The Importance and Impact of Francisella-like Endosymbionts in Hyalomma Ticks in the Era of Climate Change,” *Diversity (Basel)*, vol. 15, no. 4, p. 562, 2023.

[18] S. I. Bonnet, F. Binetruy, A. M. Hernández-Jarguín, and O. Duron, “The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission,” *Front Cell Infect Microbiol*, vol. 7, p. 236, 2017.

[19] H. Benyedem *et al.*, “First insights into the microbiome of Tunisian Hyalomma ticks gained through next-generation sequencing with a special focus on H. scupense,” *PLoS One*, vol. 17, no. 5, p. e0268172, 2022.

[20] S. Hussain *et al.*, “The symbiotic continuum within ticks: Opportunities for disease control,” *Front Microbiol*, vol. 13, p. 854803, 2022.

[21] A. Wiesinger, J. Wenderlein, S. Ulrich, S. Hiereth, L. Chitimia-Dobler, and R. K. Straubinger, “Revealing the tick microbiome: insights into midgut and salivary gland microbiota of female Ixodes ricinus Ticks,” *Int J Mol Sci*, vol. 24, no. 2, p. 1100, 2023.

[22] J. Zhang *et al.*, “High Diversity of Tick-associated Microbiota from Five Tick Species in Yunnan, China,” *Zoonoses*, vol. 3, no. 1, 2023.

[23] A. Namina *et al.*, “Comparative Microbiome Analysis of Three Epidemiologically Important Tick Species in Latvia,” *Microorganisms*, vol. 11, no. 8, p. 1970, 2023.

[24] G. Grandi *et al.*, “Characterization of the bacterial microbiome of Swedish ticks through 16S rRNA amplicon sequencing of whole ticks and of individual tick organs,” *Parasit Vectors*, vol. 16, no. 1, pp. 1–10, 2023.

[25] R. Escudero *et al.*, “Molecular method for discrimination between Francisella tularensis and Francisella-like endosymbionts,” *J Clin Microbiol*, vol. 46, no. 9, pp. 3139–3143, 2008.

[26] I. L. de Carvalho *et al.*, “Francisella species in ticks and animals, Iberian Peninsula,” *Ticks Tick Borne Dis*, vol. 7, no. 1, pp. 159–165, 2016.

[27] K. Köppen *et al.*, “Ex vivo infection model for Francisella using human lung tissue,” *Front Cell Infect Microbiol*, vol. 13, p. 1224356, 2023.

[28] B. Huber *et al.*, “Description of Francisella hispaniensis sp. nov., isolated from human blood, reclassification of Francisella novicida (Larson et al. 1955) Olsufiev et al. 1959 as Francisella tularensis subsp. novicida comb. nov. and emended description of the genus Francisella,” *Int J Syst Evol Microbiol*, vol. 60, no. 8, pp. 1887–1896, 2010.

[29] V. Alende-Castro *et al.*, “Q fever in Spain: Description of a new series, and systematic review,” *PLoS Negl Trop Dis*, vol. 12, no. 3, p. e0006338, 2018.

[30] R. Álvarez-Alonso *et al.*, “Stable levels of Coxiella burnetii prevalence in dairy sheep flocks but changes in genotype distribution after a 10-year period in northern Spain,” *Acta Vet Scand*, vol. 60, pp. 1–5, 2018.

[31] M. B. Labruna *et al.*, “Rickettsioses in Latin America, Caribbean, Spain and Portugal,” *Rev MVZ Cordoba*, vol. 16, no. 2, pp. 2435–2457, 2011.

[32] N. Cardeñosa, V. Roux, B. Font, I. Sanfeliu, D. Raoult, and F. Segura, “isolation and identification of two spotted fever group rickettsial strains from patients in Catalonia, Spain.,” *Am J Trop Med Hyg*, vol. 62, no. 1, pp. 142–144, 2000.

[33] Z. Herrador *et al.*, “Mediterranean spotted fever in Spain, 1997-2014: Epidemiological situation based on hospitalization records,” *PLoS One*, vol. 12, no. 3, p. e0174745, 2017.

[34] P. Fernández-Soto, R. Pérez-Sánchez, A. Encinas-Grandes, and R. Á. Sanz, “Detection and identification of Rickettsia helvetica and Rickettsia sp. IRS3/IRS4 in Ixodes ricinus ticks found on humans in Spain,” *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 23, pp. 648–649, 2004.

[35] I. Jado *et al.*, “Rickettsia monacensis and human disease, Spain,” *Emerg Infect Dis*, vol. 13, no. 9, p. 1405, 2007.

[36] F. J. Márquez, “Spotted fever group Rickettsia in ticks from southeastern Spain natural parks,” *Exp Appl Acarol*, vol. 45, pp. 185–194, 2008.

[37] J. A. Oteo, A. Portillo, S. Santibánez, J. R. Blanco, L. Pérez-Martínez, and V. Ibarra, “Cluster of cases of human Rickettsia felis infection from Southern Europe (Spain) diagnosed by PCR,” *J Clin Microbiol*, vol. 44, no. 7, pp. 2669–2671, 2006.

[38] J.-L. Pérez-Arellano *et al.*, “Human Rickettsia felis infection, Canary Islands, Spain,” *Emerg Infect Dis*, vol. 11, no. 12, p. 1961, 2005.

[39] F. J. Márquez, “Spotted fever group Rickettsia in ticks from southeastern Spain natural parks,” *Exp Appl Acarol*, vol. 45, pp. 185–194, 2008.

[40] P. Fernández Soto, A. Encinas Grandes, and R. Pérez Sánchez, “Rickettsia aeschlimannii in Spain: molecular evidence in Hyalomma marginatum and five other tick species that feed on humans,” 2003.

[41] F. J. Márquez, “Spotted fever group Rickettsia in ticks from southeastern Spain natural parks,” *Exp Appl Acarol*, vol. 45, pp. 185–194, 2008.

[42] N. Cardeñosa, V. Roux, B. Font, I. Sanfeliu, D. Raoult, and F. Segura, “isolation and identification of two spotted fever group rickettsial strains from patients in Catalonia, Spain.,” *Am J Trop Med Hyg*, vol. 62, no. 1, pp. 142–144, 2000.

[43] M. C. Domínguez, S. Vergara, M. C. Gómez, and M. E. Roldán, “Epidemiology of tick-borne relapsing fever in endemic area, Spain,” *Emerg Infect Dis*, vol. 26, no. 5, p. 849, 2020.

[44] S. Quainoo *et al.*, “Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis,” *Clin Microbiol Rev*, vol. 30, no. 4, pp. 1015–1063, 2017.

[45] T. L. Greay, A. W. Gofton, A. Paparini, U. M. Ryan, C. L. Oskam, and P. J. Irwin, “Recent insights into the tick microbiome gained through next-generation sequencing,” *Parasit Vectors*, vol. 11, no. 1, pp. 1–14, 2018.

[46] M. Kchouk, J.-F. Gibrat, and M. Elloumi, “Generations of sequencing technologies: from first to next generation,” *Biology and Medicine*, vol. 9, no. 3, 2017.

[47] L. Couper and A. Swei, “Tick microbiome characterization by next-generation 16S rRNA amplicon sequencing,” *JoVE (Journal of Visualized Experiments)*, no. 138, p. e58239, 2018.

[48] J. Sinclair, N. P. West, and A. J. Cox, “Comparison of four DNA extraction methods for 16s rRNA microbiota profiling of human faecal samples,” *BMC Res Notes*, vol. 16, no. 1, pp. 1–5, 2023.

[49] C. Elie *et al.*, “Comparison of DNA extraction methods for 16S rRNA gene sequencing in the analysis of the human gut microbiome,” *Sci Rep*, vol. 13, no. 1, p. 10279, 2023.

[50] A. D. Ammazzalorso, C. P. Zolnik, T. J. Daniels, and S.-O. Kolokotronis, “To beat or not to beat a tick: comparison of DNA extraction methods for ticks (Ixodes scapularis),” *PeerJ*, vol. 3, p. e1147, 2015.

[51] M. J. Hubbard, K. J. Cann, and D. J. M. Wright, “Validation and rapid extraction of nucleic acids from alcohol-preserved ticks,” *Exp Appl Acarol*, vol. 19, pp. 473–478, 1995.

[52] C. A. Hill and J. A. Gutierrez, “A method for extraction and analysis of high quality genomic DNA from ixodid ticks,” *Med Vet Entomol*, vol. 17, no. 2, pp. 224–227, 2003.

[53] N. S. Muhamad Rizal *et al.*, “Advantages and limitations of 16S rRNA next-generation sequencing for pathogen identification in the diagnostic microbiology laboratory: perspectives from a middle-income country,” *Diagnostics*, vol. 10, no. 10, p. 816, 2020.

[54] E. J. Kipp, L. L. Lindsey, B. Khoo, C. Faulk, J. D. Oliver, and P. A. Larsen, “Metagenomic surveillance for bacterial tick-borne pathogens using nanopore adaptive sampling,” *Sci Rep*, vol. 13, no. 1, p. 10991, 2023.

[55] L. Varela-Castro *et al.*, “On the possible role of ticks in the eco-epidemiology of Coxiella burnetii in a Mediterranean ecosystem,” *Ticks Tick Borne Dis*, vol. 9, no. 3, pp. 687–694, 2018.

[56] R. Cuadrado-Matías, L. Casades-Martí, A. Peralbo-Moreno, S. Baz-Flores, E. García-Manzanilla, and F. Ruiz-Fons, “Testing the efficiency of capture methods for questing Hyalomma lusitanicum ticks (vector of Crimean-Congo hemorrhagic fever virus),” 2023.

[57] I. Rodríguez *et al.*, “An alternative and rapid method for the extraction of nucleic acids from ixodid ticks by potassium acetate procedure,” *Brazilian Archives of Biology and Technology*, vol. 57, pp. 542–547, 2014.

[58] A. Cafiso *et al.*, “Protocol optimization for simultaneous DNA and RNA co-extraction from single hard tick specimens,” *MethodsX*, vol. 8, p. 101315, 2021.

[59] C. D. Crowder *et al.*, “Extraction of total nucleic acids from ticks for the detection of bacterial and viral pathogens,” *J Med Entomol*, vol. 47, no. 1, pp. 89–94, 2010.

[60] L. Lobato-Bailón *et al.*, “The fecal bacterial microbiome of the Kuhl’s pipistrelle bat (Pipistrellus kuhlii) reflects landscape anthropogenic pressure,” *Anim Microbiome*, vol. 5, no. 1, pp. 1–14, 2023.

[61] E. M. Elnifro, A. M. Ashshi, R. J. Cooper, and P. E. Klapper, “Multiplex PCR: optimization and application in diagnostic virology,” *Clin Microbiol Rev*, vol. 13, no. 4, pp. 559–570, 2000.

[62] M. J. Hubbard, K. J. Cann, and D. J. M. Wright, “Validation and rapid extraction of nucleic acids from alcohol-preserved ticks,” *Exp Appl Acarol*, vol. 19, pp. 473–478, 1995.

[63] A. Santos, R. van Aerle, L. Barrientos, and J. Martinez-Urtaza, “Computational methods for 16S metabarcoding studies using Nanopore sequencing data,” *Comput Struct Biotechnol J*, vol. 18, pp. 296–305, 2020.

[64] Y.-K. Zhang, Z.-J. Yu, D. Wang, V. Bronislava, P. Branislav, and J.-Z. Liu, “The bacterial microbiome of field-collected Dermacentor marginatus and Dermacentor reticulatus from Slovakia,” *Parasit Vectors*, vol. 12, pp. 1–10, 2019.

[65] J. Peeters *et al.*, “Exploring the microbiome analysis and visualization landscape,” *Frontiers in Bioinformatics*, vol. 1, p. 774631, 2021.

[66] W. J. Landesman *et al.*, “Potential effects of blood meal host on bacterial community composition in Ixodes scapularis nymphs,” *Ticks Tick Borne Dis*, vol. 10, no. 3, pp. 523–527, 2019.

[67] F. J. Márquez, “Spotted fever group Rickettsia in ticks from southeastern Spain natural parks,” *Exp Appl Acarol*, vol. 45, pp. 185–194, 2008.

[68] F. Binetruy, X. Bailly, C. Chevillon, O. Y. Martin, M. V Bernasconi, and O. Duron, “Phylogenetics of the Spiroplasma ixodetis endosymbiont reveals past transfers between ticks and other arthropods,” *Ticks Tick Borne Dis*, vol. 10, no. 3, pp. 575–584, 2019.

[69] J. A. Oteo, A. Portillo, J. R. Blanco, V. Ibarra, and S. Santibáñez, “Rickettsia africae infection. Three cases confirmed by PCR,” *Med Clin (Barc)*, vol. 122, no. 20, pp. 786–788, 2004.

[70] S. S. Long, L. K. Pickering, and C. G. Prober, *Principles and practice of pediatric infectious disease*. Elsevier Health Sciences, 2012.

[71] A. Beliavskaia *et al.*, “Spiroplasma isolated from third-generation laboratory colony Ixodes persulcatus ticks,” *Front Vet Sci*, vol. 8, p. 659786, 2021.

[72] S. Ogata *et al.*, “Spiroplasma infection among ixodid ticks exhibits species dependence and suggests a vertical pattern of transmission,” *Microorganisms*, vol. 9, no. 2, p. 333, 2021.

[73] A. Beliavskaia *et al.*, “Spiroplasma isolated from third-generation laboratory colony Ixodes persulcatus ticks,” *Front Vet Sci*, vol. 8, p. 659786, 2021.

[74] M. Lu *et al.*, “Ehrlichia, Coxiella and Bartonella infections in rodents from Guizhou province, Southwest China,” *Ticks Tick Borne Dis*, vol. 13, no. 5, p. 101974, 2022.

[75] M. A. Larson *et al.*, “Reclassification of Wolbachia persica as Francisella persica comb. nov. and emended description of the family Francisellaceae,” *Int J Syst Evol Microbiol*, vol. 66, no. 3, pp. 1200–1205, 2016.

[76] M. Kondo, Y. Matsushima, T. Nakanishi, S. Iida, K. Habe, and K. Yamanaka, “Epidemiological study of ticks harbouring Aeromonas hydrophila in areas endemic and non-endemic to Japanese-spotted fever,” *Tropical Medicine & International Health*, vol. 28, no. 2, pp. 151–156, 2023.

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