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Microbiome-based surveillance of zoonotic tick-borne pathogens from urban wild boars in Barcelona, 2022–2023

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ABSTRACT

Incursions of wild animals into urban areas amplify the potential risks of zoonotic disease transmission by increasing contact between humans and animal reservoirs. Monitoring the presence of pathogens in these animals is crucial for assessing zoonotic risks but remains challenging due to the vast array of known and unknown pathogens harboured by animals. Microbiome-based approaches provide an efficient and comprehensive alternative for monitoring microbial communities and scanning the whole spectrum of bacterial pathogens. In this study, we applied this innovative conceptual framework to implement a sentinel monitoring system for investigating zoonotic tick-borne bacteria in three tick species sampled from wild boars in the Metropolitan Area of Barcelona (MAB). Using Nanopore sequencing of the full length 16 s rRNA gene, we demonstrated a fast and cost-effective approach for microbiome analysis. Our findings revealed the presence of two pathogenic genera widely documented in ticks, encompassing five species: *Rickettsia massiliae* and *R. slovaca*, both previously detected in the area, and for the first time, *Francisella tularensis* —the causative agent of tularemia— as well as *F. hispaniensis* and *Diplorickettsia massiliensis*, potentially emerging patogens. Finally, our results showed distinct bacterial compositions across the tick species examined. This study highlights the sensitivity and comprehensiveness of microbiome-based surveillance of tick-borne pathogens, enabling the early detection of emerging and low-abundance bacterial species that might otherwise go unnoticed with less sensitive techniques. Such proactive detection efforts are crucial for facilitating early identification and implementing prevention strategies to mitigate zoonotic risks.

1. Introduction

Ticks are vectors for various diseases such as Lyme disease, anaplasmosis, and tick-borne encephalitis in humans, domestic animals and wild animals. Furthermore, the incidence of tick-borne diseases is rising globally, posing a significant public health concern [1]. Monitoring the presence of pathogens can help predict and prevent outbreaks among the human population living near wildlife-livestock-human interface areas. Early identification of the presence of pathogens in ticks serves as a preventive system for potential disease transmission to humans and domestic animals.

Environmental factors, including host composition and abundance,

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influence tick ecology and epidemiology of tick-borne pathogens (TBPs) [2]. Wildlife, acting as reservoirs or tick amplification hosts, can influence TBPs epidemiology, expanding tick range and abundance [3]. Assessing the risk of TBPs transmission to humans involves studying ticks carried by sympatric species, such as the Eurasian wild boar (*Sus scrofa*). The populations of this species have severely increased across Europe [4], particularly around urbanized areas, such as the Metropolitan Area of Barcelona (MAB) in northeast Spain, where they often enter urban spaces [5]. These increasing wildlife-human interactions pose heightened risks, creating new epidemiological scenarios where TBPs can spread. In fact, hospital admissions for tick-borne diseases have increased in recent years in humans in NE-Spain [6], evidencing the urgent need for TBPs surveillance in ticks from one of the most densely populated area in the Mediterranean. Monitoring ticks from wild boars in this region is crucial to evaluating the risk of zoonotic diseases in Barcelona's urban population and can contribute to preventing future outbreaks.

Traditionally, most studies aimed at detecting TBPs in ticks have relied on conventional molecular techniques, such as PCRs, which are limited to detecting a predefined set of pathogens. In contrast, the recent development of next-generation sequencing (NGS)-based methods has enabled the simultaneous identification of the full diversity of bacteria in a sample by amplifying and sequencing universal bacterial genetic targets, such as the 16S rRNA gene [7]. Studies with a greater microbial community approach are especially important for ticks, as they are associated with both pathogenic and non-pathogenic bacterial symbionts, which play crucial roles in their nutritional adaptation, survival, fitness, vector competence, immunity, and reproduction [8]. Thus, a 16S rRNA microbiome approach can reveal microbial interactions that influence tick biology and pathogen transmission, ultimately enhancing our understanding of TBPs and informing which more effective control strategies. Moreover, in contrast with conventional molecular techniques, NGS methods allow the discovery of previously unknown species of clinical importance. However, most published studies on tick microbiome are based on sequencing one to four of the nine hypervariable regions (V1-V9) of the 16S rRNA gene, leading to different bacterial diversity patterns and taxonomic resolutions, hampering direct comparisons. Furthermore, hypervariable regions of 16S rRNA are strongly preserved across some species, limiting identification at the species level [9]. Nowadays, third-generation sequencing methods, such as nanopore-based sequencing by Oxford Nanopore Technologies, enable longer read lengths, allowing for the sequencing of the entire 16S rRNA gene. This advancement provides improved taxonomic resolution, enhances cost efficiency, and offers a more comprehensive understanding of microbial diversity [10].

The objective of this study was to conduct a microbiome-based initial assessment of zoonotic tick-borne pathogens from different tick species infesting urban wild boars from the MAB. This study introduces Nanopore sequencing of the full-length 16S rRNA gene as an effective and broad screening tool for zoonotic infectious diseases in urban wildlife-human interface areas.

2. Methods

2.1. Sample collection, pool separation and DNA extraction

Ticks were opportunistically collected between 2022 and 2023 from wild boars distributed within the Metropolitan Area of Barcelona (Catalonia, Spain), one of the most human-populated areas in southern Europe (3,303,927 inhabitants in 636 km^2). Wild boars were found dead by hunters or Rural Agents and submitted for a systematic necropsy and diagnosis investigation within the Wildlife Passive Surveillance of Catalonia (https://agricultura.gencat.cat/ca/ambits/ramaderia/sanitat-animal/programes-sanitaris/pla-vigilancia-sanitaria-fauna-salvatge/programa-departament).

Ticks were identified at the species level using taxonomic keys [11] according to morphological features such as the basis of the capitulum, shape and colour of the scutum, palpi and chelicerae, and grouped into pools (3–5 ticks/pool), based on individual wild boar host and species. A total of 37 adult ticks feeding from 9 wild boars were selected for this study and were divided into 9 pools.

Tick pools were washed three times with ethanol 70 % and once with sterile Phosphate-Buffered Saline (PBS). A total of 500 µl of PBS were added to each pool and ticks were mechanically disrupted using a 5 mm steel bead in a TissueLyser II (Qiagen, Hilden, Germany) at an oscillation frequency of 30 Hz for 10 min. DNA extraction was performed on 250 µl of the tick supernatant using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

2.2. Sequencing through nanopore technology

Initial DNA concentration was quantified using the Qubit Fluorometric Quantification High Sensitivity Assay (Invitrogen, California, USA). The 16S rRNA gene was selectively amplified by PCR following the protocol of the 16S Barcoding Kit 1–24 (SQK-16S024) from Oxford Nanopore Technologies (ONT, Oxford, United Kingdom) [12,13], producing amplicons of approximately 1500 bp. The final DNA concentration was also analysed using Qubit Fluorometric Quantification. Each tick pool was assigned a unique barcoded library and, after adapter ligation and library clean up, sequencing was performed using a MiniON sequencer (ONT) in a single sequencing run using a brand new R9.6 flow cell.

2.3. Data processing and statistical analysis

Fastq raw reads were trimmed and quality-filtered through Porechop software [14]. Quality check was performed using FastQC and MultiQC software tools [15,16]. Taxonomic assignment to the species level was carried out with Emu v3.2.0 [10], using Emu 16S bacterial Database (rrnDB v5.6 and NCBI 16S RefSeq) (Fig. 1).

Alpha diversity was estimated using Observed species richness, Shannon, Inverse Simpson, and Pielou indices. Statistical differences between tick species were assessed using the Kruskal–Wallis test [17], applying a significance threshold of $p < 0.05$. Additionally, relative abundance tables were obtained from count tables (Supplementary Material 1), and beta diversity was then determined based on Bray–Curtis distances. Statistical differences in beta diversity were assessed using multivariate homogeneity of groups dispersion (Beta disper) and non-parametric multivariate permutation analysis (PERMANOVA), both with a significance threshold set at $p < 0.05$. Rarefaction curves, alpha diversity boxplots, non-metric multidimensional scaling (NMDS) plots, and bar plots depicting the 50 most abundant bacterial species were then generated at the species level using the R package 'vegan' [18]. Finally, core microbiomes (taxa shared by 100 % of samples), accessory microbiomes (taxa shared by samples from 2 or 3 tick species, and exclusive taxa unique to one species) were identified and a Venn diagram illustrating these results was obtained using the 'VennDiagram' package [19]. All statistical analyses were performed using R version 4.1.1.

3. Results

Three different adult tick species were identified and divided into 9 pools: *Dermacentor marginatus* ($n = 3$), *Hyalomma lusitanicum* ($n = 5$), and *Rhipicephalus sanguineus* ($n = 1$). A variety of pathogenic and non-pathogenic bacteria were identified in all tick pools, demonstrating that bacterial composition varied between tick species. The vector-borne pathogenic bacteria *Rickettsia massiliae, R. slovaca*, and *Francisella tularensis* were found among the 50 most detected bacteria species (Fig. 2). Moreover, other bacteria of pathogenic relevance to humans, though not confirmed as tick-borne, were also detected (Table 1). *Hyalomma lusitanicum* exhibited the highest microbial diversity, with

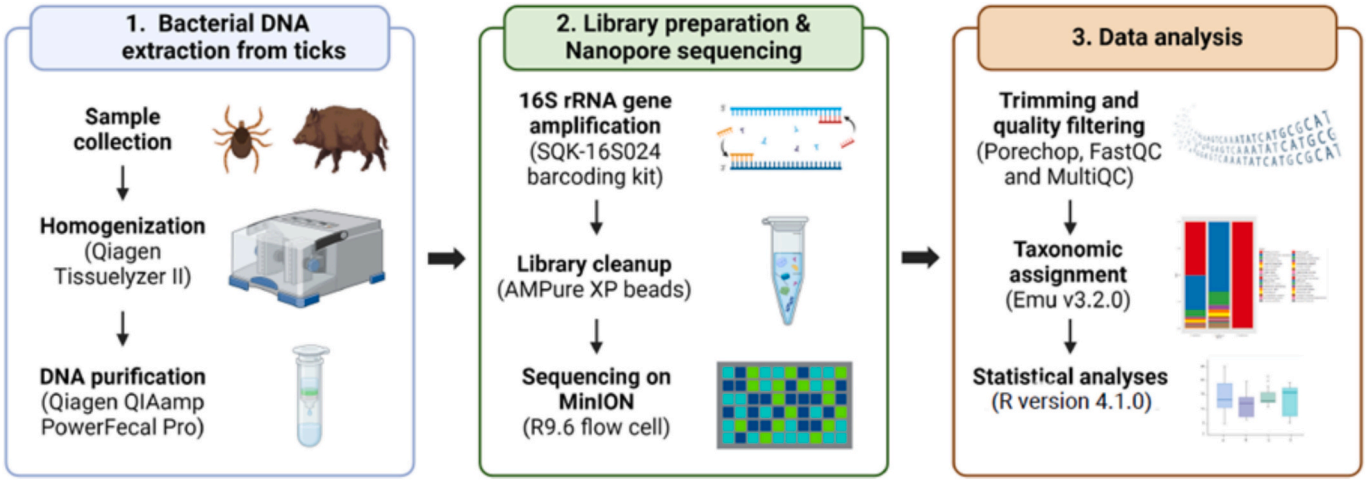

Fig. 1. Workflow for describing bacterial microbiome in ticks from wild boars in the Metropolitan Area of Barcelona (MAB), including DNA extraction, Nanopore sequencing, and data analysis.

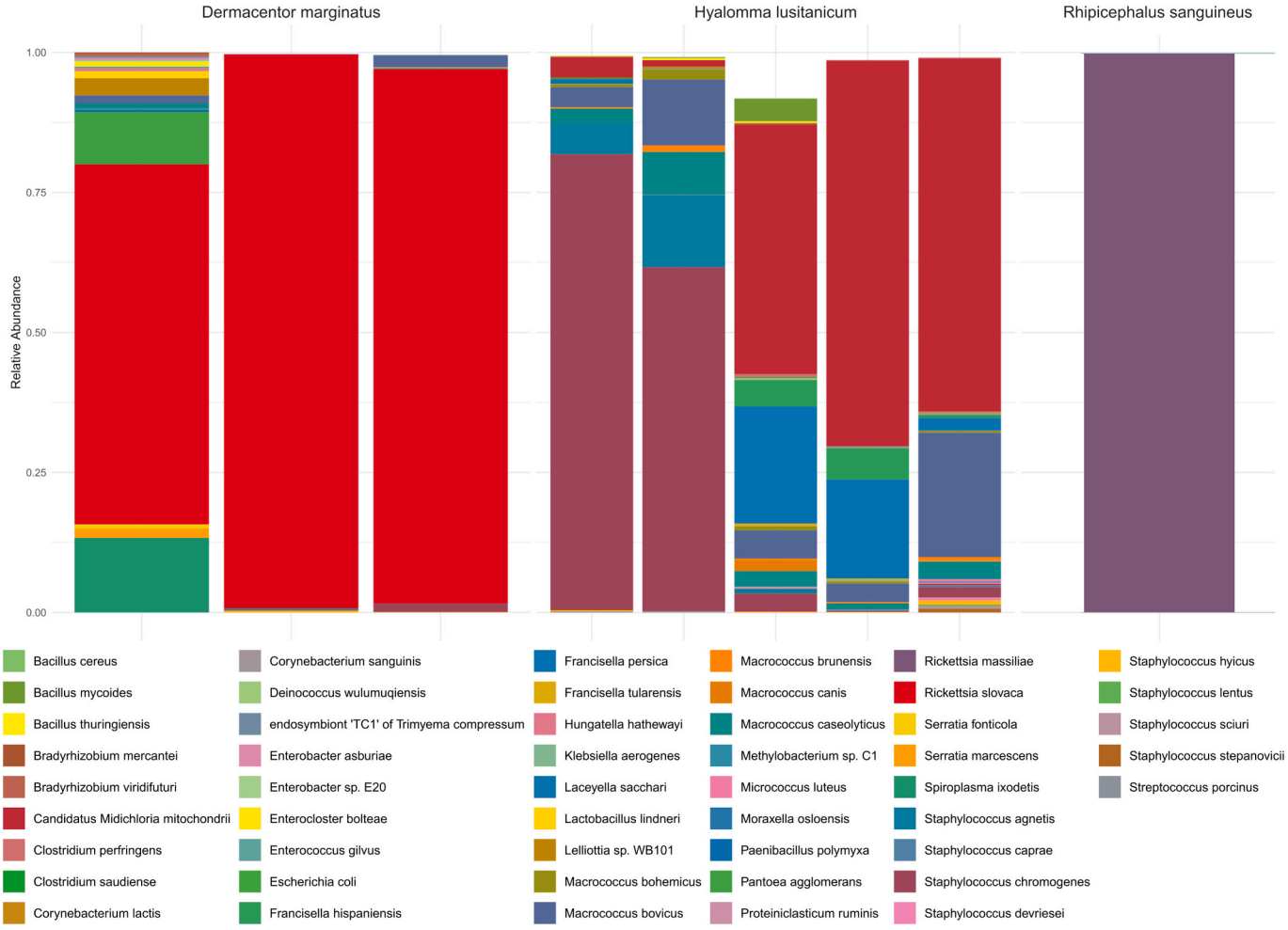

Fig. 2. Stacked bar plot of the 50 most abundant bacterial species found in *Dermacentor marginatus*, *Hyalomma lusitanicum*, and *Rhipicephalus sanguineus* tick pools.

significantly higher values in the Observed, Shannon, and Inverse Simpson alpha diversity indices (Fig. 3), and a considerably larger exclusive microbiome, comprising 360 bacterial species (Fig. 4). On the contrary, *Rhipicephalus sanguineus* exhibited the lowest microbial diversity, with only nine bacterial species assigned and an exclusive microbiome of only two bacterial species, intensely dominated by *Rickettsia massiliae* (Fig. 2). Core microbiome showed that only 2 microbial species were shared between all 3 species of ticks: *Staphylococcus chromogenes* and *Laceyella sacchari* (Fig. 4).

Moreover, NMDS showed a significantly differential bacterial composition within tick species (Fig. 5), with a *p*-value of 0.002 and a R^2 of 0.653. The raw sequencing data have been deposited in the NCBI

Table 1
Bacterial species of recognised pathogenic importance to humans, found among the 50 most detected bacterial species in the microbiome of ticks feeding on wild boars.

Bacteria species	Evidence of tick-borne transmission	Disease	Reference
Bacillus cereus	No	Food-borne gastrointestinal disease, also systemic and local infections reported	[20]
Clostridium perfringens	No	Food-borne and non-foodborne gastroenteritis	[21]
Escherichia coli	No	Intestinal and systemic infections	[22]
Francisella hispaniensis	Unknown	Dermatitis and systemic infection	[23]
Francisella tularensis	Yes	Tularemia	[24]
Klebsiella aerogenes	No	Systemic infection, opportunistic pathogen	[25]
Laceyella sacchari	No	Bagassosis (rare hypersensitivity pneumonitis)	[26]
Rickettsia massiliae	Yes	Mediterranean Spotted Fever (MSF)	[24]
Rickettsia slovaca	Yes	Scalp eschar and neck lymphadenopathy (SENLAT)	[24]
Serratia fonticola	No	Multi-systemic opportunistic infections	[22]
Serratia marcescens	No	Multi-systemic opportunistic infections	[22]

Sequence Read Archive (SRA) under the Bioproject ID PRJNA1219035.

4. Discussion

Nowadays, emerging bacterial tick-borne pathogens of human importance in southern Europe comprise *Borrelia* genus (*B. burgdorferi* sensu lato complex and Tick-borne relapsing fever group), *Anaplasma* spp., *Francisella* spp., *Rickettsia* spp., *Ehrlichia* spp., and *Neoehrlichia mikurensis* [1,27]. In the present study, we identified two different *Rickettsia* spp. of human clinical importance, along with *Francisella tularensis* and *F. hispaniensis*. These findings are in accordance with the study by Castillo-Contreras et al. [28] results, that only detected *Rickettsia* spp., despite screening for *Ehrlichia* spp., *Anaplasma* spp., *Borrelia burgdorferi* s.l., and *Coxiella burnetii* via real-time PCR and/or reverse line blot hybridization assay in a total of 180 tick pools and 167 spleens from wild boars from the Metropolitan Area of Barcelona (MAB).

Rickettsia was the most abundant genus in all *D. marginatus* and *R. sanguineus* pools, as previously reported in Spain [29]. This reinforces the hypothesis that *Rickettsia* spp. are key endosymbionts of these tick species, playing an essential role in tick physiology, as described by several authors [30–32]. The two pathogenic *Rickettsia* species detected in this study, *R. massiliae* and *R. slovaca*, belong to the Spotted Fever Group rickettsiae. Furthermore, *R. massiliae* was detected in the only pool of *R. sanguineus* s.l. analysed in this study, with a high dominance within the microbiome. This bacteria has also been described in *Rhipicephalus sanguineus* s.l. from northern Spain [33] and in carnivores and wild boars within the MAB [28,34].

Rickettsia massiliae, along with *R. conorii,* are the etiological agents of Mediterranean Spotted Fever (MSF), a disease characterized by fever, maculopapular rash, and an inoculation eschar ("tache noire") at the tick bite site [24]. Even though *R. conorii* is endemic in Spain, with Catalonia ranking as the second Spanish region reporting the highest number of MSF cases between 2005 and 2015 [35], it has never been detected in ticks from the MAB [28]. One plausible explanation is the absence of its main vector, *Rhipicephalus sanguineus* sensu stricto. Therefore, we hypothesise that MSF human cases in the MAB might be related with *R. massiliae* transmitted by *R. sanguineus* sensu lato, rather than *R. conorii*. On the other hand, *Rickettsia slovaca* is the etiological agent of scalp eschar and neck lymphadenopathy (SENLAT), previously referred to as tick-borne lymphadenopathy/*Dermacentor*-borne necrotic erythema and lymphadenopathy (TIBOLA/DEBONEL) syndrome, and it was only detected in *Dermacentor marginatus* ticks. SENLAT manifests as a necrotic skin lesion at the tick bite site (eschar), accompanied by lymphadenomegaly near the bite area, fever, and headache [36]. In this study, wild boars carrying *R. slovaca*-infected ticks were captured near urban parks frequented by children, whichis concerning as children are particularly prone to tick bites. In fact, a significant proportion of SENLAT cases in southern Europe are diagnosed in children and women [36]. *Dermacentor* spp. ticks, the main vectors of *R. slovaca,* show high

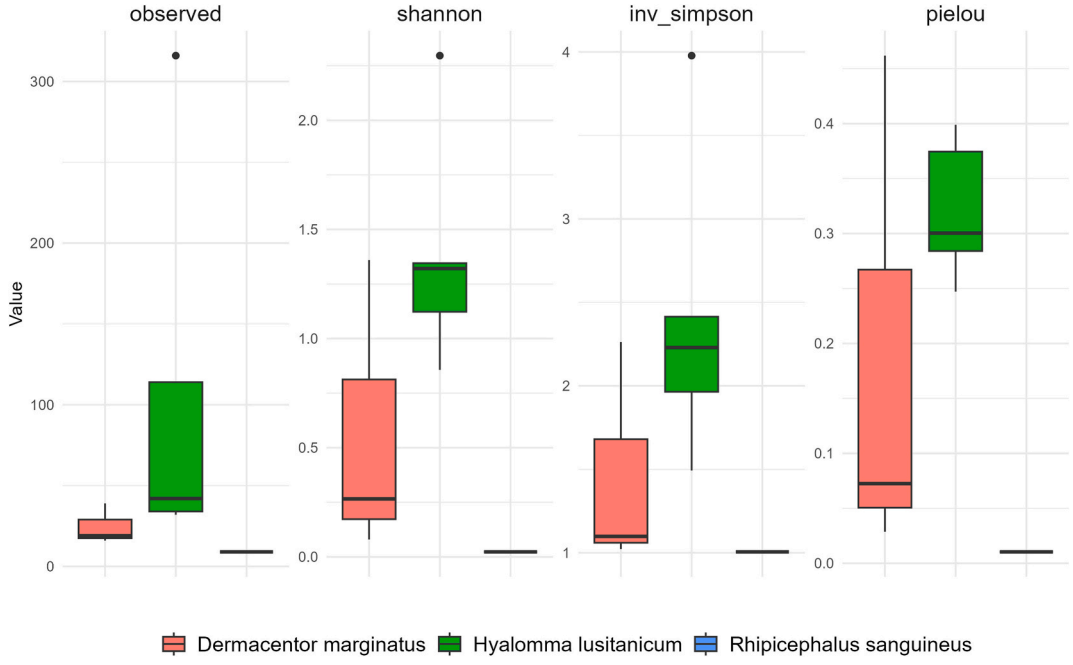

Fig. 3. Alpha diversity (Observed, Shannon, Inverse Simpson and Pielou) indices boxplots of bacterial microbiomes in *Dermacentor marginatus, Hyalomma lusitanicum* and *Rhipicephalus sanguineus* collected from urban wild boars in Barcelona.

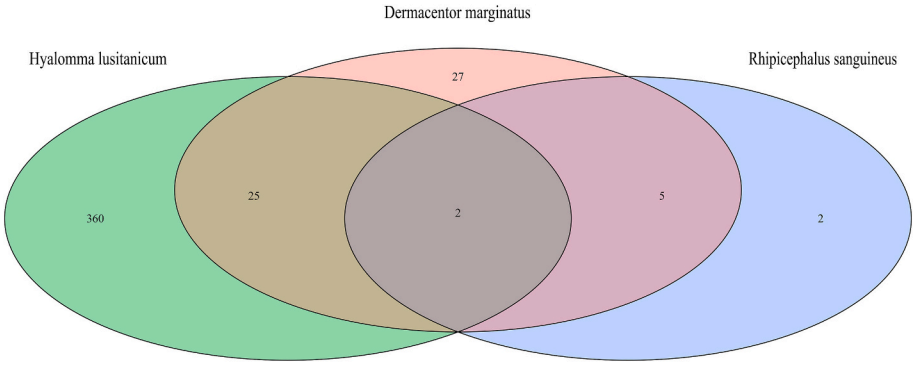

Fig. 4. Venn Diagram of core, accessory, and exclusive microbiome within three tick species collected from urban wild boars: *Hyalomma lusitanicum* (green), *Dermacentor marginatus* (red), *Rhipicephalus sanguineus* (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

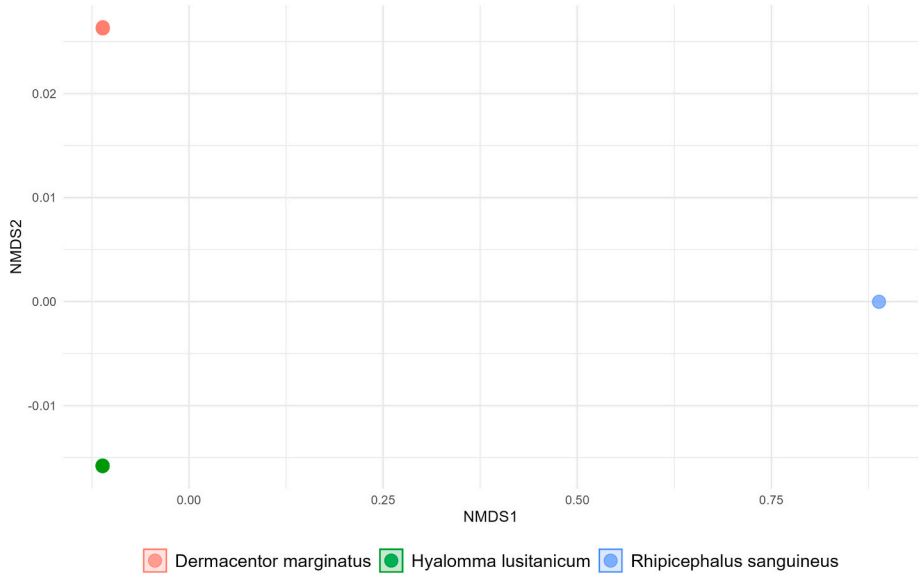

Fig. 5. Beta diversity NMDS (B) *Hyalomma lusitanicum* (green), *Dermacentor marginatus* (red), *Rhipicephalus sanguineus* (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

preference for parasitizing hairy animals, such as wild boars, allowing them to remain undetected throughout the blood-feeding process [24]. Hence, *Dermacentor* spp. would aim for the hairy skin of the head when feeding on humans [37]. The height of questing ticks aligns with children's height, increasing their exposure. Indeed, SENLAT has been diagnosed in children in the MAB for more than a decade, and it is considered a paediatric emerging infectious disease [38]. The potential relationship between the increasing presence of urban wild boars and the raise of environmental tick burden in areas frequented by children, thereby increasing the risk of tick-borne infections, warrants further investigation.

In contrast, *R. slovaca* was found in a notably low relative abundance, and *R. massiliae* was not detected in *H. lusitanicum* tick pools, despite documented detections of their presence in this species [28,39]. In addition, *Diplorickettsia massiliensis* was detected, but in minimal relative abundance. This newly described bacteria has been previously documented in humans suffering from a suspected tick-borne disease in France [40], highlighting its clinical relevance. *H. lusitanicum* presents a low relative abundance of pathogenic *Rickettsiales* compared to other *Hyalomma* species [41]. This is noteworthy for two reasons: first, the low abundance of pathogenic *Rickettsiales* in the microbiome suggests a lower likelihood of transmission through *H. lusitanicum* tick bites. And second, the detection of *Diplorickettsia massiliensis* emphasizes the sensitivity and utility of microbiome-based pathogen surveillance, which allows for the detection of low-abundance species that might otherwise go unnoticed in less sensitive approaches.

The microbiome of *H. lusitanicum* from the MAB was predominantly composed of *Candidatus Midichloria mitochondrii*, contrasting with patterns observed in southern Spain, where *Francisella*-like endosymbionts (FLEs) predominate [41]. Both Ca. *M. mitochondrii* and FLEs play an essential role in maintaining homeostasis in this tick species and, although FLEs were present in every *H. lusitanicum* pool, their relative abundance (5–32 %) was much lower in comparison (55–99 %) [41]. Among the *Francisella* spp. detected in our study, *F. tularensis* was the most clinically significant, being the causative agent of tularaemia —a potentially life-threatening zoonotic disease that can spread through multiple transmission routes, including tick bites [24]. *Francisella hispaniensis* is regarded as a pathogen of emerging importance, although its epidemiology and pathogenicity remain poorly understood, as only three cases have been documented, none are linked to tick bites [23]. Meanwhile, *Francisella persica* is considered non-pathogenic although it presents higher virulence potential compared to other FLEs [42]. The detection of these three *Francisella* species in the MAB underscores the need for continued surveillance and heightened awareness among local clinicians to better address potential health risks.

The detection of other bacteria of pathogenic importance in humans

that are not considered to be transmitted by ticks might be due to several uncertain factors. These pathogens are ubiquitous bacteria commonly found in soil, plants, skin, or intestines. Although the ticks' exoskeletons were washed with ethanol before DNA extraction, it is plausible that some bacteria persisted in small fissures. Notably, studies that used 10 % sodium hypochlorite for tick sterilisation still detected environmental and host-associated bacteria, which suggests that these bacteria could have been ingested during feeding, especially considering that most of the ticks collected in this study were feeding in the perianal area of wild boars —a site exposed to faecal matter [9]. Nevertheless, the detection of these pathogens remains significant, since some of them, such as *Klebsiella* spp., *Serratia* spp., and *Escherichia coli,* are listed in the World Health Organization Bacterial Priority Pathogens List [22] due to their high antimicrobial resistance. This highlights the potential role of ticks and wild boars in harbouring antimicrobial-resistant bacteria, underscoring the need for further research to explore their role as carriers and assess the zoonotic risks associated with these pathogens in the MAB.

This study presented some limitations. The small sample size and opportunistic sampling may have missed other pathogenic bacteria, although a previous study with a larger sample size also failed to detect other pathogens, with the exception of *Rickettsia raoultii* [28]. However, that study analysed samples from a broader distribution, including forested areas beyond the MAB. In contrast, our study focused exclusively on wild boars captured in urban areas, where these animals have closer contact with humans, potentially increasing the risk of sharing ticks and pathogens. Additionally, the use of the 16S rRNA gene for amplification—although standard—presents challenges, as it is highly conserved within the *Rickettsia* genus, hindering species-level identification [43]. Nonetheless, our findings are consistent with those of Castillo-Contreras et al. [28] that used a different methodological approach. This consistency supports the biological relevance of our results, which align with established tick-pathogen associations. Nevertheless, to enhance robustness, future studies should consider parallel sequencing of other target regions, such as OmpB and gltA, for confirming *Rickettsia* species. Additionally, expanding sampling efforts, particularly for *Rhipicephalus sanguineus*, are needed to better define the core and exclusive microbiomes within and between tick species.

5. Conclusion

Our research provides novel insight into the microbiome of ticks collected from urban wild boars in the MAB, with a particular focus on bacterial pathogens. We report the presence *of R. massiliae* and *R. slovaca*, both previously detected in the area, and we document for the first time the presence of *F. tularensis, F. hispaniensis*, and *Diplorickettsia massiliensis*. Furthermore, our study highlights different bacterial composition (including pathogens) between tick species in the MAB and within *H. lusitanicum* individuals from another region in Spain. Finally, we were able to identify two core bacterial species shared by all three tick species studied: *Staphylococcus chromagenes* and *Laceyella sacchari*, both of which warrant ongoing monitoring within the MAB.

These findings demonstrate that full length 16S rRNA gene sequencing is a powerful tool for screening bacterial tick-borne pathogens and identifying new species of clinical importance. Our results emphasize the urgent need for intensive monitoring of tick-borne pathogens in densely populated Mediterranean regions. Proactive surveillance is essential to detect shifts in pathogen and microbiome composition, thereby predicting and mitigating potential disease outbreaks. Incorporating advanced microbiome analysis into surveillance strategies will support early detection and intervention, ultimately strengthening the prevention and control of tick-borne diseases in urban environments.

CRediT authorship contribution statement

Laura Carrera-Faja: Writing – original draft, Methodology, Conceptualization. **Elmira Ghadamnan:** Methodology, Investigation. **Iris Sarmiento:** Methodology. **Jordi Manuel Cabrera-Gumbau:** Software, Formal analysis, Data curation. **Mariette Viladomat Jasso:** Visualization, Software, Formal analysis. **Josep Estruch:** Writing – review & editing, Methodology. **Daniel Borràs:** Formal analysis. **Jaime Martínez-Urtaza:** Writing – review & editing, Supervision. **Johan Espunyes:** Writing – review & editing, Conceptualization. **Oscar Cabezón:** Writing – review & editing, Conceptualization.

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Declaration of competing interest

We declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2025.101022.

Data availability

Data will be made available on request.

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