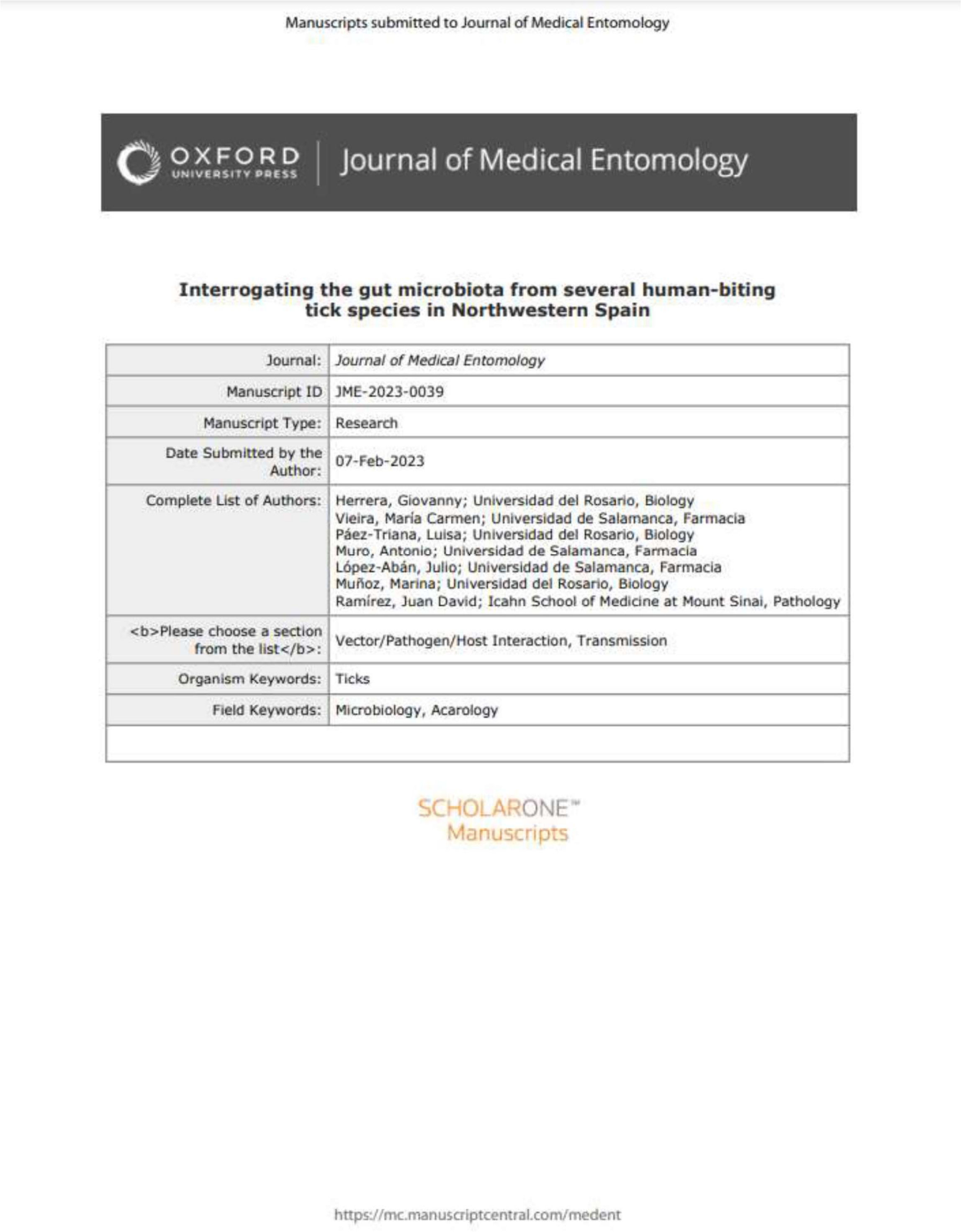


OXFORD UNIVERSITY PRESS | Journal of Medical Entomology

Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain

Journal:	*Journal of Medical Entomology*
Manuscript ID	JME-2023-0039
Manuscript Type:	Research
Date Submitted by the Author:	07-Feb-2023
Complete List of Authors:	Herrera, Giovanny; Universidad del Rosario, Biology Vieira, María Carmen; Universidad de Salamanca, Farmacia Páez-Triana, Luisa; Universidad del Rosario, Biology Muro, Antonio; Universidad de Salamanca, Farmacia López-Abán, Julio; Universidad de Salamanca, Farmacia Muñoz, Marina; Universidad del Rosario, Biology Ramírez, Juan David; Icahn School of Medicine at Mount Sinai, Pathology
Please choose a section from the list:	Vector/Pathogen/Host Interaction, Transmission
Organism Keywords:	Ticks
Field Keywords:	Microbiology, Acarology

Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain

Giovanny Herrera[1,2], María Carmen Vieira Lista[2], Luisa Páez-Triana[1], Antonio Muro[2], Julio López-Abán[2], Marina Muñoz[1], Juan David Ramírez[1,3*]

[1] Centro de Investigaciones en Microbiología and Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia

[2] Infectious and Tropical Diseases Research Group (e-INTRO), Biomedical Research Institute of Salamanca-Research Centre for Tropical Diseases at the University of Salamanca (IBSAL-CIETUS), Faculty of Pharmacy, University of Salamanca, 37007 Salamanca, Spain

[3] Molecular Microbiology Laboratory, Department of Pathology, Molecular and Cell-Based Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

* Corresponding author: juand.ramirez@urosario.edu.co, juan.ramirezgonzalez@mountsinai.org

Abstract

Tick-borne diseases have increased significantly in Europe and Spain in recent years. One strategy explored for tick surveillance and control is the study of the microbiota. The focus is on understanding the relationships between pathogens and endosymbionts within the microbiota and how these relationships can alter these arthropods' vectorial capacity. Thus, it is pivotal to depict the bacterial communities composing the microbiota of ticks present in specific territories. This work aimed to describe the microbiota present in 29 individuals of 5 tick species collected from 4 provinces of Castilla y Leon in northwestern Spain from 2015 to 2022. DNA extraction and sequencing of the V4 hypervariable region of 16S-rRNA was performed, with subsequent analysis of diversity, taxonomic composition, and correlations between genera. There were no differences in the alpha diversity by species, nor were compositional changes evident at the phylum level. However, inter-individual differences at the genus level allowed spatial differentiation of the five species included in the study. Correlation analyses showed complex interactions between different genera of pathogens and endosymbionts with other microbiota members. These findings provide an initial insight into the composition of the gut microbiota of various tick species in northwestern Spain, which can contribute to establishing surveillance and control measures to reduce diseases such as rickettsiosis, Lyme disease, and Crimean-Congo hemorrhagic fever.

Keywords: Tick microbiota, Tick-borne diseases, Ixodidae, Spain, emerging diseases.

Introduction

Ticks are hematophagous ectoparasitic arthropods able to transmit several pathogens, such as viruses, bacteria, and protozoa, to humans and animals, generating health risks and becoming a worldwide concern (Estrada-Peña et al., 2008; Ghosh et al., 2007; Parola & Raoult, 2001). Among the main microorganisms transmitted by ticks are *Rickettsia* spp., *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp., *Babesia* spp., and the Crimean-Congo virus (Dantas-Torres et al., 2012; Rochlin & Toledo, 2020; Wu-Chuang et al., 2021), having significant economic effects due to morbidity and mortality rates, as well as severe implications for healthcare systems (Ghosh et al., 2007; Heyman et al., 2010).

More than 890 ticks species have been described worldwide, where the Ixodidae family has most of the vectors found in Europe, reaching 70 different species throughout the continent and five main genera: *Ixodes*, *Dermacentor*, *Haemaphysalis*, *Rhipicephalus* and *Hyalomma* (Estrada-Peña et al., 2018a; Gilbert, 2010). In the Iberian Peninsula, these five genera are potential vectors of the *Anaplasma, Rickettsia*, and Crimean-Congo virus (Fernández Soto, 2003). Likewise, a recent study showed fluctuating distribution patterns of ticks with their potential to transmit several pathogens throughout northwestern Spain. These findings demonstrate the importance of monitoring the dispersion of these arthropods and the transmitting pathogens (Vieira Lista et al., 2022). In this regard, it is worth noting that tick-borne diseases have increased in southern Europe, especially in Spain, where rickettsiosis, Lyme disease, and tick-borne encephalitis are considered emerging health problems with incidences of up to 0.36 cases/100,000 individuals (Dantas-Torres et al., 2012; Portillo et al., 2015) and Crimean-Congo hemorrhagic sporadic cases (Lorenzo Juanes et al., 2023)

Ticks are hosts to a broad range of commensal and symbiont microorganisms in their microbiota, involved in various physiological processes such as nutrition, reproduction, development, vectorial ability, and immunity (Bonnet et al., 2017; Dantas-Torres et al., 2011;

Pollet et al., 2020). The tick's microbiota exhibits substantial variation in its composition and diversity according to different factors such as tick species, sex, life stage, and environmental factors (Aivelo et al., 2019; Ponnusamy et al., 2014; Sperling et al., 2017; Van Treuren et al., 2015). Despite these variations, the ability of some tick-borne pathogens to modulate their microbiota stand out (Abraham et al., 2017; Adegoke et al., 2020; Narasimhan et al., 2017). Interactions with specific key taxa play a crucial role in modifying the microbiota, potentially leading to significant alterations in its structure and further affecting the ticks' vectorial ability (Maitre et al., 2022). Therefore, studying tick microbiota is considered fundamental for preventing and managing tick-borne diseases due to the impact that changes in the microbiota could produce on the transmission of pathogens or commensals/symbionts.

Despite the importance of this in both epidemiological surveillance and vaccine development, limited investigations have been conducted in the Iberian Peninsula to explore variations in the microbiota among different species of ticks. Thus, this study aimed to describe the composition and diversity of the microbiota of 5 ticks found in the Castilla y León between 2015 and 2022. This is the first study that reports the composition and diversity of the microbiota of ticks from northwestern Spain. The role of endosymbionts in the structure and development of interactions with other pathogenic microorganisms present in the microbiota.

Materials and methods

Tick Collection, Identification, and DNA extraction

A total of 29 adult ticks were included in the present study, stored at the University of Salamanca, Spain. The samples were collected and preserved using the protocol described by Vieira et al. (Vieira Lista et al., 2022). Briefly, the ticks were gathered between 2015 and 2022 from individuals who attended primary healthcare centers and hospital services for tick removal. After removal, the ticks were transported to the laboratory for coding and morphological identification using taxonomic keys (Apanaskevich et al., 2008; Estrada-Peña et al., 2004, 2018b; Gil-Collado et al., 1979). Once the ticks were identified, DNA extraction was performed using the NucleoSpin Tissue kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's instructions.

DNA quality control and sequencing process

The extracted DNA was subjected to quality control by 2% agarose gel electrophoresis to verify the integrity of the DNA. Additionally, the concentration was evaluated using a NanoDrop/2000/2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). A 260/280 relationship between 1.8 and 2.0 and a minimum concentration of 20 ng/µL was verified. Paired-end sequencing was performed on the Illumina NovaSeq platform (PE 250 Platform) with 0.1 million reads per sample at the facilities of Novogene Corporation Inc. (Shanghai, China) using primers targeting the hypervariable V4 region of the 16S-rRNA marker specific for bacteria and Archaea 515-F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806-R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011).

Taxonomic assignment

Initially, a quality control step was performed to assess the quality of the sequencing reads, including the average number of reads per sample, Phred score, frequency of unassigned bases, and presence of adapters in the samples. Subsequently, the barcodes and primers were removed using the QIIME2 tool (Bolyen et al., 2019, p. 2) before proceeding with taxonomic assignment using the DADA2 tool in R studio (R Core Team, 2013), following the default pipeline (Callahan et al., 2016, p. 2). The sequences were assigned, comparing the sequences obtained for the 16S-rRNA marker versus the 16S-rRNA using SILVA database version 138 (Quast et al., 2013) until obtaining the Amplicon Sequence Variants (ASVs). The ASVs are defined as each sequence obtained in the sequencing process, which may vary by a single nucleotide.

Taxonomical composition, diversity analysis, and correlation plot

The phyloseq package (McMurdie & Holmes, 2013) was used for importing, storing, analyzing, and graphically displaying already clustered phylogenetic sequencing data. With the resulting ASVs, the relative abundance of the different phyla was plotted for each tick genus. Subsequently, alpha (Shannon and Simpson) and beta diversities were analyzed using the ASVs by tick genera using a main coordinate plot (PCoA) based on Bray-Curtis dissimilarity. Permutational multivariate analysis of variance (PERMANOVA), i.e., adonis (analysis and partitioning of sums of squares using dissimilarities), and vegan functions (descriptive statistical package related to community ecology) (Oksanen, 2011) were used to assess differences between centroids. Finally, the pathogens transmissible to humans and tick symbionts found in the samples were plotted for each tick genus using a chord diagram using the cyclize package (Gu et al., 2014).

Statistical analyzes

Statistically significant differences between the studied groups were evaluated using the Kruskal–Wallis test with respective *post hoc* analyses with the Dunn test using Benjamini–Hochberg correction with a confidence level of 95%. Likewise, correlogram graphs were obtained between ASVs corresponding to genera of the most abundant phyla (*Actinobacteriota*, *Bacillota*, *Bacteroidota*, and *Pseudomonadota*). Based on these, a filter of sub-represented data was carried out, eliminating all those ASVs corresponding to genera whose sum of reads was less than 1,000 and those ASVs corresponding to genera whose reads were not present in at least 25% of the samples. These filters were carried out to reduce potential technical bias and ensure that comparisons were conducted between ASVs in the groups and not in single samples. The correlation matrix was constructed using the psych package (Revelle, 2020) on R software, applying the spearman method with Benjamini–Hochberg correction. We considered only strong correlation values greater than 0.7 and less than − 0.7 (Spearman Rho strong correlation) and selected statistically significant ($p < 0.05$) during the process of establishing a correlation between the ASVs evaluated.

Results

Geographical distribution of collected ticks.

Of the 29 ticks studied, 28% were *H. marginatum* (6 male, two female), 24% were *D. marginatus* (5 female, two male), 17% to *I. ricinus* (5 female), 17% were *R. bursa* (4 female, one male), and 14% were *R. sanguineus sensu lato* (4 female). Most of the ticks were gathered in the provinces of Ávila (n=17) and Salamanca (n=5), most of them from the southern and western of Castilla y León (Figure 1).

Taxonomical composition and alpha and beta diversity

The alpha diversity analyses showed no statistically significant differences between tick species, with low average values of Shannon and Simpson's indices (Figure 2A). The opposite occurred when analyzing PCoA since spatial differences between tick species were evident (*p*-value = 0.017) (Figure 2B). In terms of the microbiota composition, there were no differences between groups, with the phylum *Pseudomonadota* as the most abundant, followed by the phyla *Bacillota* and *Bacteroidota* (Figure 2C). The analysis of tick symbionts and pathogens transmissible to humans showed an increase in the genus *Francisella* (K-W test=11.598, *p*-value=0.00087) in ticks corresponding to the genus *Hyalomma* versus individuals of the genera *Dermacentor* (*p*-value= 0.0086) and *Rhipicephalus* (*p*-value= 0.0275) (Figure 2D). Likewise, the years of the collection did not impact the results of beta diversity or the taxonomic composition (Figure S1). Also, it is noteworthy that the taxonomic assignment of microbiota members was limited to the genus level.

Complex correlation between bacterial genera in ticks

Finally, correlation analyses showed distinctive patterns for each tick genus (Figure 3). The genera *Hyalomma* (Figure 3B) and *Ixodes* (Figure 3C) showed the minor complex interactions between the different bacterial genera found, with a predominance of positive correlations, while the genera *Dermacentor* (Figure 3A) and *Rhipicephalus* (Figure 3D) displayed the most complex interactions with the presence of many negative correlations. The interactions between the genera of tick endosymbionts, pathogens transmissible to humans, and the other bacteria present in the microbiota are striking and characterized by positive correlations, with *Rickettsia, Coxiella,* and *Stenotrophomonas* as the genera with the highest number of interactions.

Discussion

The tick microbiota analyzed presented diverse profiles. The absence of differences in alpha diversity between the different tick genera contrasting with that reported by Portillo et al. (Portillo et al., 2019), who observed diverse alpha diversity between *I. ricinus* and *D. marginatus* collected in northern Spain. These discrepancies could be due to the presence of various pathogens in the samples, food preferences, and even the sex of the tick, which can affect the diversity of the microbiota of these arthropods (Adegoke et al., 2020; Narasimhan et al., 2021; Swei & Kwan, 2017). Despite statistically significant differences in centroids, the spatial groupings of the different tick genera showed some overlap between *Rhipicephalus, Dermacentor,* and *Ixodes* (Figure 2B). Such spatial overlap was reported previously, suggesting that the composition of the microbiota of these genera is usually quite similar and is altered by sex and geographic distribution (Portillo et al., 2019).

Considering that the ticks herein included presented somewhat restrictive geographical distribution patterns (Figure 1), it is likely that this factor is a determinant of both the environmental conditions and the hosts on which they feed (Adegoke et al., 2020; Narasimhan et al., 2021). Although the study did not have samples of each species for each year, the temporal analysis showed no spatial clustering patterns (Figure S1), suggesting that annual seasonality does not generate significant differences beyond the inter-individual differences in tick microbiota. This contrasts with that reported by Lejal and collaborators (Lejal et al., 2021), who described recurrent differences in the microbiota of *I. ricinus* promoted by temporal dynamics. Such discrepancies may be caused by the difference in the seasonality of each of the individuals included in the different studies (monthly vs. annual and intermittent), the tick species (*Ixodes ricinus vs*. five different species), as well as the stage (nymphs *vs*. adults). This is supported by Pollet and collaborators, who argue that variability in the microbiota is associated with short periods (Pollet et al., 2020). Although both studies point to the strong influence of environmental factors in these differences, further research on the impact of temporal variations in the tick microbiota is required. In addition, we must highlight that the number of samples was low to support this statement.

The predominance of *Pseudomonadota* microorganisms in the taxonomic composition of the ticks evaluated in the present study agrees with previous studies carried out in Spain and Tunisia (Benyedem et al., 2022; Portillo et al., 2019). This microbiota composition is probably related to environmental factors, considering that most of the endosymbiont genera were identified, including *Coxiella* and *Francisella*, which are of great importance for the development of the ticks (Ben-Yosef et al., 2020; Gerhart et al., 2016), as well as some tick-borne pathogens, belonging to this phylum (Figure 2D). In this sense, the geographic distribution of ticks becomes essential, as it could influence both the composition and diversity of the microbiota since many of the microorganisms found in these ticks are usually associated with the soils and water of the ecosystems where these arthropods develop (Narasimhan et al., 2021; Portillo et al., 2019). Likewise, it is worth noting the marked increase of the genus *Francisella* in ticks of the genus *Hyalomma*. A finding of great importance considering the role of this bacterium in the metabolism of vitamin B, folic acid, biotin, and riboflavin, which play a fundamental role in the tick's survival (Gerhart et al., 2016; Sjödin et al., 2012). This finding could denote the effect of competition among *Coxiella*, *Francisella*, and *Rickettsia* endosymbionts within the microbiota in northwestern Spain and their limited diet. However, further research with larger samples and temporal scales is required for a comprehensive understanding of the impact of these changes at the community level and to explore the potential causes.

The complex interactions observed within the microbiota of all the ticks analyzed suggest the development of close relationships between the different members of the microbiota, especially between endosymbionts and some pathogens. Our findings contrast with previous reports that have suggested the ability of some tick-borne pathogens to modify the microbiota structure (Adegoke et al., 2020; Narasimhan et al., 2021). Despite the above, these interactions could account for endosymbionts' fundamental role in the tick's vectorial capacity by regulating pathogen populations and generating suitable environments for their development. Understanding these processes is of great importance, considering that searching for crucial taxa is one of the strategies currently used to develop vaccines against tick-borne diseases (Mateos-Hernández et al., 2020).

Likewise, it is worth highlighting the large number of negative correlations observed in *Dermacentor*, *Hyalomma*, and *Rhipicephalus*, especially between some endosymbionts/pathogens transmissible to humans and other components of the microbiota, suggesting an increase in competition among the members of this ecosystem, in agreement with Lejal et al. (Lejal et al., 2021). Also, the few correlations found within the genus *Ixodes* are noteworthy, considering that *I. ricinus* is a notable species in Europe, known to transmit a wide range of pathogens, such as *Borrelia burgdorferi* (Aivelo et al., 2019). This low number of interactions between members of the microbiota could be due to the low number of individuals included in the present study and the low spatiotemporal variability in their collection. It is worth noting that the present research, due to its exploratory nature, has some limitations, such as the low sample size and the absence of some species per year, the limited geographical area of collection, and the resolution of the taxonomic assignment.

Our results are of great relevance, considering that the region of Castilla y León has increased the number of reports of tick bites in humans in recent years (Vieira Lista et al., 2022). This region has geographical and climatological characteristics that favor the proliferation of multiple tick species and also have been reported the presence of *Francisella tularensis, Rickettsia* spp., *Borrelia* spp., and Crimean-Congo hemorrhagic fever virus (Mínguez-González et al., 2021; Monsalve Arteaga et al., 2020; Portillo et al., 2018). Considering both the taxonomic composition and the complex relationships established among the members of the tick microbiota exposed in the present study, the development of biological control strategies, as well as active epidemiological surveillance, could significantly impact the number of cases of tick-borne diseases, as well as could contribute to the immediate and adequate care of patients, considering the spatial distribution of pathogens in the territory. The results suggest that compositional differences allow spatial clustering of tick species, despite the absence of differences in diversity. Likewise, complex interaction networks between microbiota members with endosymbionts and tick-borne pathogens were observed.

Acknowledgments

AM is founded by ISC-III PI22/01721. JLA receives funds form projects MINECO PID2021-127471OB-I00 and ISC-III PI22/01721.

Conflict of interest

Authors declare no conflict of interest.

Data Availability

The data are publicly available at the European Nucleotide Archive (ENA) repository under accession number PRJEB59487.

References

Abraham, N. M., Liu, L., Jutras, B. L., Yadav, A. K., Narasimhan, S., Gopalakrishnan, V., Ansari, J. M., Jefferson, K. K., Cava, F., & Jacobs-Wagner, C. (2017). Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proceedings of the National Academy of Sciences*, *114*(5), E781–E790.

Adegoke, A., Kumar, D., Bobo, C., Rashid, M. I., Durrani, A. Z., Sajid, M. S., & Karim, S. (2020). Tick-Borne Pathogens Shape the Native Microbiome Within Tick Vectors. *Microorganisms*, *8*(9), Art. 9. https://doi.org/10.3390/microorganisms8091299

Aivelo, T., Norberg, A., & Tschirren, B. (2019). Bacterial microbiota composition of Ixodes ricinus ticks: The role of environmental variation, tick characteristics and microbial interactions. *PeerJ*, *7*, e8217.

Apanaskevich, D. A., Santos-Silva, M. M., & Horak, I. G. (2008). The genus Hyalomma Koch, 1844. IV. Redescription of all parasitic stages of H.(Euhyalomma) lusitanicum Koch, 1844 and the adults of H.(E.) franchinii Tonelli Rondelli, 1932 (Acari: Ixodidae) with a first description of its immature stages. *Folia parasitologica*, *55*(1), 61–74.

Benyedem, H., Lekired, A., Mhadhbi, M., Dhibi, M., Romdhane, R., Chaari, S., Rekik, M., Ouzari, H.-I., Hajji, T., & Darghouth, M. A. (2022). First insights into the microbiome of Tunisian Hyalomma ticks gained through next-generation sequencing with a special focus on H. scupense. *PLOS ONE*, *17*(5), e0268172. https://doi.org/10.1371/journal.pone.0268172

Ben-Yosef, M., Rot, A., Mahagna, M., Kapri, E., Behar, A., & Gottlieb, Y. (2020). Coxiella-Like Endosymbiont of Rhipicephalus sanguineus Is Required for

Physiological Processes During Ontogeny. *Frontiers in Microbiology*, *11*, 493. https://doi.org/10.3389/fmicb.2020.00493

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., & Asnicar, F. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature biotechnology*, *37*(8), 852–857.

Bonnet, S. I., Binetruy, F., Hernández-Jarguín, A. M., & Duron, O. (2017). The Tick Microbiome: Why Non-pathogenic Microorganisms Matter in Tick Biology and Pathogen Transmission. *Frontiers in Cellular and Infection Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fcimb.2017.00236

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High resolution sample inference from Illumina amplicon data. *Nature methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the national academy of sciences*, *108*(Supplement 1), 4516–4522.

Dantas-Torres, F., Chomel, B. B., & Otranto, D. (2012). Ticks and tick-borne diseases: A One Health perspective. *Trends in parasitology*, *28*(10), 437–446.

Dantas-Torres, F., Figueredo, L. A., & Otranto, D. (2011). Seasonal variation in the effect of climate on the biology of Rhipicephalus sanguineus in southern Europe. *Parasitology*, *138*(4), 527–536.

Estrada-Peña, A., Martinez, J. M., Sanchez Acedo, C., Quilez, J., & Del Cacho, E. (2004). Phenology of the tick, Ixodes ricinus, in its southern distribution range (central

Spain). *Medical and Veterinary Entomology*, *18*(4), 387–397. https://doi.org/10.1111/j.0269-283X.2004.00523.x

Estrada-Peña, A., Mihalca, A. D., & Petney, T. N. (2018a). *Ticks of Europe and North Africa: A guide to species identification*. Springer.

Estrada-Peña, A., Mihalca, A. D., & Petney, T. N. (2018b). *Ticks of Europe and North Africa: A guide to species identification*. Springer.

Estrada-Peña, A., Venzal, J. M., Kocan, K. M., & Sonenshine, D. E. (2008). *Overview: Ticks as vectors of pathogens that cause disease in humans and animals*.

Fernández Soto, P. (2003). *Garrapatas que parasitan a las personas en Castilla y León, determinación por serología de su parasitismo y detección moleculaza de los patógenos que albergan*.

Gerhart, J. G., Moses, A. S., & Raghavan, R. (2016). A Francisella-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. *Scientific reports*, *6*(1), 1–6.

Ghosh, S., Azhahianambi, P., & Yadav, M. P. (2007). Upcoming and future strategies of tick control: A review. *Journal of vector borne diseases*, *44*(2), 79.

Gilbert, L. (2010). Altitudinal patterns of tick and host abundance: A potential role for climate change in regulating tick-borne diseases? *Oecologia*, *162*(1), 217–225.

Gil-Collado, J., Guillén-Llera, J. L., & Zapatero-Ramos, L. M. (1979). Claves para la identificación de los Ixodoidea españoles (adultos). *Rev Iber Parasitol*, *39*, 107–118.

Gu, Z., Gu, L., Eils, R., Schlesner, M., & Brors, B. (2014). Circlize implements and enhances circular visualization in R. *Bioinformatics*, *30*(19), 2811–2812.

Heyman, P., Cochez, C., Hofhuis, A., Van Der Giessen, J., Sprong, H., Porter, S. R., Losson, B., Saegerman, C., Donoso-Mantke, O., & Niedrig, M. (2010). A clear and present danger: Tick-borne diseases in Europe. *Expert review of anti-infective therapy*, *8*(1), 33–50.

Lejal, E., Chiquet, J., Aubert, J., Robin, S., Estrada-Peña, A., Rue, O., Midoux, C., Mariadassou, M., Bailly, X., Cougoul, A., Gasqui, P., Cosson, J. F., Chalvet-Monfray, K., Vayssier-Taussat, M., & Pollet, T. (2021). Temporal patterns in Ixodes ricinus microbial communities: An insight into tick-borne microbe interactions. *Microbiome*, *9*(1), 153. https://doi.org/10.1186/s40168-021-01051-8

Lorenzo Juanes, H. M., Carbonell, C., Sendra, B. F., López-Bernus, A., Bahamonde, A., Orfao, A., Lista, C. V., Ledesma, M. S., Negredo, A. I., Rodríguez-Alonso, B., Bua, B. R., Sánchez-Seco, M. P., Muñoz Bellido, J. L., Muro, A., & Belhassen-García, M. (2023). Crimean-Congo Hemorrhagic Fever, Spain, 2013-2021. *Emerging Infectious Diseases*, *29*(2), 252–259. https://doi.org/10.3201/eid2902.220677

Maitre, A., Wu-Chuang, A., Mateos-Hernández, L., Foucault-Simonin, A., Moutailler, S., Paoli, J.-C., Falchi, A., Díaz-Sánchez, A. A., Banović, P., Obregón, D., & Cabezas-Cruz, A. (2022). Rickettsia helvetica infection is associated with microbiome modulation in Ixodes ricinus collected from humans in Serbia. *Scientific Reports*, *12*(1), Art. 1. https://doi.org/10.1038/s41598-022-15681-x

Mateos-Hernández, L., Obregón, D., Maye, J., Borneres, J., Versille, N., de la Fuente, J., Estrada-Peña, A., Hodžić, A., Šimo, L., & Cabezas-Cruz, A. (2020). Anti-Tick Microbiota Vaccine Impacts Ixodes ricinus Performance during Feeding. *Vaccines*, *8*(4), Art. 4. https://doi.org/10.3390/vaccines8040702

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Mínguez-González, O., Gutiérrez-Martín, C.-B., Martínez-Nistal, M. del C., Esquivel-García, M. del R., Gómez-Campillo, J.-I., Collazos-Martínez, J.-Á., Fernández-Calle, L.-M., Ruiz-Sopeña, C., Tamames-Gómez, S., Martínez-Martínez, S., Caminero-Saldaña, C., Hernández, M., Rodríguez-Lázaro, D., & Rodríguez-Ferri, E.-F. (2021). Tularemia Outbreaks in Spain from 2007 to 2020 in Humans and Domestic and Wild Animals. *Pathogens*, *10*(7), Art. 7. https://doi.org/10.3390/pathogens10070892

Monsalve Arteaga, L., Muñoz Bellido, J. L., Vieira Lista, M. C., Vicente Santiago, M. B., Fernández Soto, P., Bas, I., Leralta, N., de Ory Manchón, F., Negredo, A. I., Sánchez Seco, M. P., Alonso Sardón, M., Pérez González, S., Jiménez Del Bianco, A., Blanco Peris, L., Alamo-Sanz, R., Hewson, R., Belhassen-García, M., & Muro, A. (2020). Crimean-Congo haemorrhagic fever (CCHF) virus-specific antibody detection in blood donors, Castile-León, Spain, summer 2017 and 2018. *Euro Surveillance: Bulletin Europeen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin*, *25*(10), 1900507. https://doi.org/10.2807/1560-7917.ES.2020.25.10.1900507

Narasimhan, S., Schuijt, T. J., Abraham, N. M., Rajeevan, N., Coumou, J., Graham, M., Robson, A., Wu, M.-J., Daffre, S., & Hovius, J. W. (2017). Modulation of the tick gut milieu by a secreted tick protein favors Borrelia burgdorferi colonization. *Nature communications*, *8*(1), 1–17.

Narasimhan, S., Swei, A., Abouneameh, S., Pal, U., Pedra, J. H., & Fikrig, E. (2021). Grappling with the tick microbiome. *Trends in parasitology*, *37*(8), 722–733.

Oksanen, J. (2011). Vegan: Community Ecology Package. R package version 1.17-9. *http://cran. r-project. org/package= vegan*.

Parola, P., & Raoult, D. (2001). Ticks and tick-borne bacterial diseases in humans: An emerging infectious threat. *Clinical infectious diseases*, *32*(6), 897–928.

Pollet, T., Sprong, H., Lejal, E., Krawczyk, A. I., Moutailler, S., Cosson, J.-F., Vayssier-Taussat, M., & Estrada-Peña, A. (2020). The scale affects our view on the identification and distribution of microbial communities in ticks. *Parasites & vectors*, *13*(1), 1–13.

Ponnusamy, L., Gonzalez, A., Van Treuren, W., Weiss, S., Parobek, C. M., Juliano, J. J., Knight, R., Roe, R. M., Apperson, C. S., & Meshnick, S. R. (2014). Diversity of Rickettsiales in the microbiome of the lone star tick, Amblyomma americanum. *Applied and environmental microbiology*, *80*(1), 354–359.

Portillo, A., Palomar, A. M., Toro, M. de, Santibáñez, S., Santibáñez, P., & Oteo, J. A. (2019). Exploring the bacteriome in anthropophilic ticks: To investigate the vectors for diagnosis. *PLOS ONE*, *14*(3), e0213384. https://doi.org/10.1371/journal.pone.0213384

Portillo, A., Ruiz-Arrondo, I., & Oteo, J. A. (2018). Arthropods as vectors of transmissible diseases in Spain. *Medicina Clinica (English Ed.)*, *151*(11), 450–459. https://doi.org/10.1016/j.medcle.2018.10.008

Portillo, A., Santibáñez, S., García-Álvarez, L., Palomar, A. M., & Oteo, J. A. (2015). Rickettsioses in Europe. *Microbes and infection*, *17*(11–12), 834–838.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(Database issue), D590–D596. https://doi.org/10.1093/nar/gks1219

R Core Team. (2013). *R: A language and environment for statistical computing*. Vienna, Austria.

Revelle, W. (2020). *Psych: Procedures for personality and psychological research (Version 2.0. 9)[Computer software]. Northwestern University.*

Rochlin, I., & Toledo, A. (2020). Emerging tick-borne pathogens of public health importance: A mini-review. *Journal of medical microbiology*, *69*(6), 781.

Sjödin, A., Svensson, K., Öhrman, C., Ahlinder, J., Lindgren, P., Duodu, S., Johansson, A., Colquhoun, D. J., Larsson, P., & Forsman, M. (2012). Genome characterisation of the genus Francisella reveals insight into similar evolutionary paths in pathogens of mammals and fish. *BMC genomics*, *13*(1), 1–13.

Sperling, J. L., Silva-Brandão, K. L., Brandão, M. M., Lloyd, V. K., Dang, S., Davis, C. S., Sperling, F. A. H., & Magor, K. E. (2017). Comparison of bacterial 16S rRNA variable regions for microbiome surveys of ticks. *Ticks and tick-borne diseases*, *8*(4), 453–461.

Swei, A., & Kwan, J. Y. (2017). Tick microbiome and pathogen acquisition altered by host blood meal. *The ISME Journal*, *11*(3), Art. 3. https://doi.org/10.1038/ismej.2016.152

Van Treuren, W., Ponnusamy, L., Brinkerhoff, R. J., Gonzalez, A., Parobek, C. M., Juliano, J. J., Andreadis, T. G., Falco, R. C., Ziegler, L. B., & Hathaway, N. (2015).

Variation in the microbiota of Ixodes ticks with regard to geography, species, and sex. *Applied and environmental microbiology*, *81*(18), 6200–6209.

Vieira Lista, M. C., Belhassen-García, M., Vicente Santiago, M. B., Sánchez-Montejo, J., Pedroza Pérez, C., Monsalve Arteaga, L. C., Herrador, Z., del Álamo-Sanz, R., Benito, A., & Soto López, J. D. (2022). Identification and Distribution of Human-Biting Ticks in Northwestern Spain. *Insects*, *13*(5), 469.

Wu-Chuang, A., Hodžić, A., Mateos-Hernández, L., Estrada-Peña, A., Obregon, D., & Cabezas-Cruz, A. (2021). Current debates and advances in tick microbiome research. *Current Research in Parasitology & Vector-Borne Diseases*, *1*, 100036. https://doi.org/10.1016/j.crpvbd.2021.100036

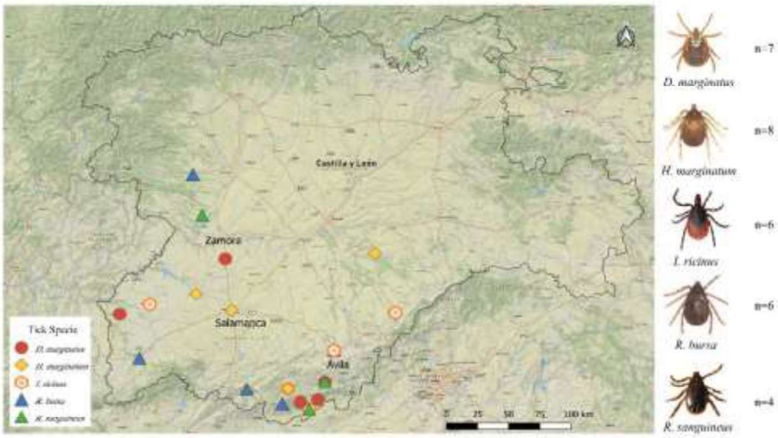


Figure 1. Geographical distribution of collected ticks. The map shows the different provinces where the samples were collected, and the number of each species included in the study

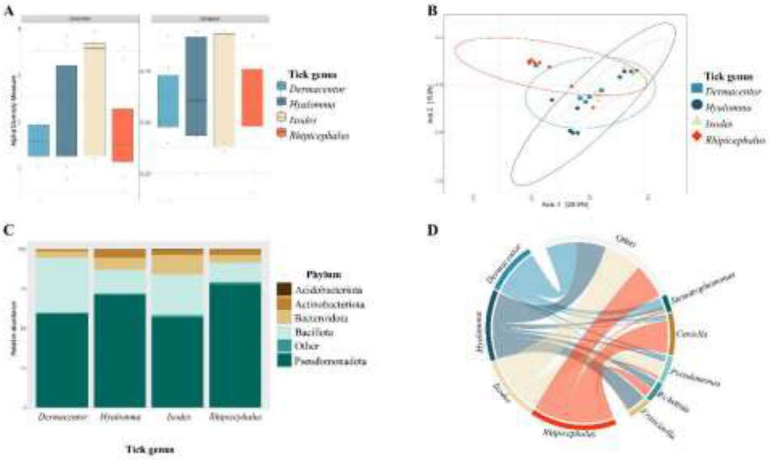


Figure 2. Diversity and taxonomical composition of ticks' microbiota. A. Shannon and Simpson indices by tick species. B. Principal Coordinate Analyses by tick species. C. Taxonomical composition of ticks' microbiota by phyla. D. Chord diagram of endosymbionts and tick-borne pathogens by tick genera.

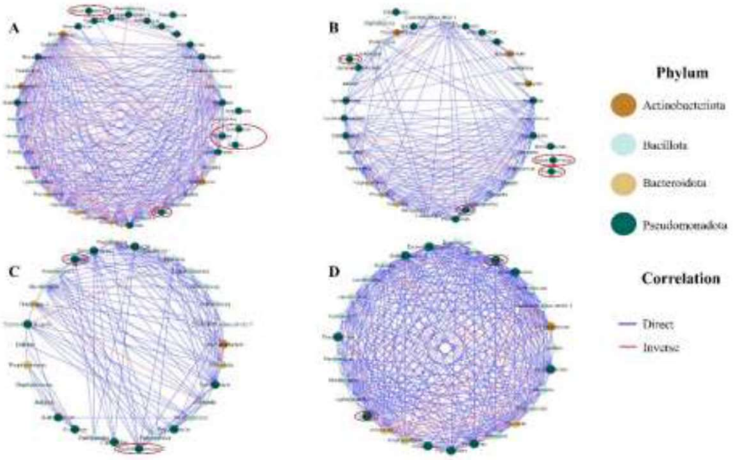


Figure 3. Complex correlations between different bacterial genera in ticks' microbiota. A. Correlation plot of *Dermacentor*. B Correlation plot of *Hyalomma*. C. Correlation plot of *Ixodes*. D. Correlation plot of *Rhipicephalus*.

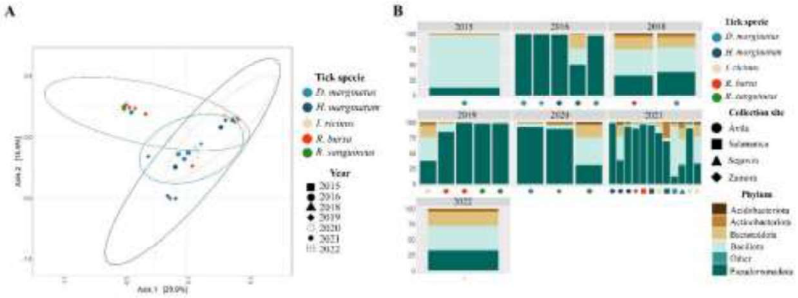


Figure S1. Influence of spatial-temporal patterns and tick sex over diversity and taxonomical composition. A) Principal Coordinate Analyses by tick species including year of collection. B) Principal Coordinate Analyses by tick species including sex C)Taxonomical composition of ticks' microbiota by phyla sorted by year and place of collection.

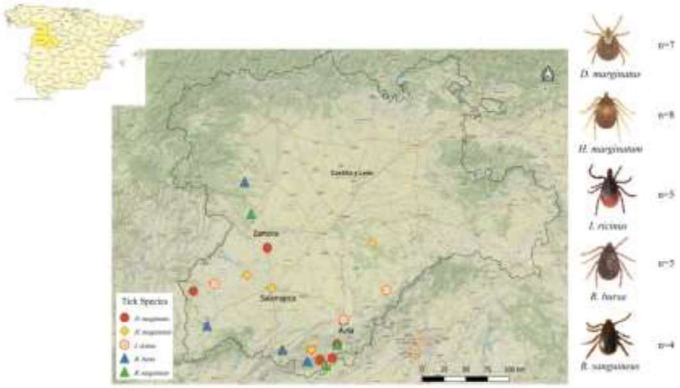


Figure 1

338x190mm (300 x 300 DPI)

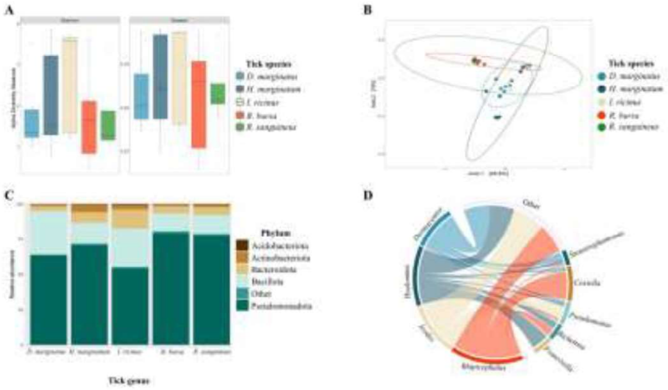


Figure 2

338x190mm (300 x 300 DPI)

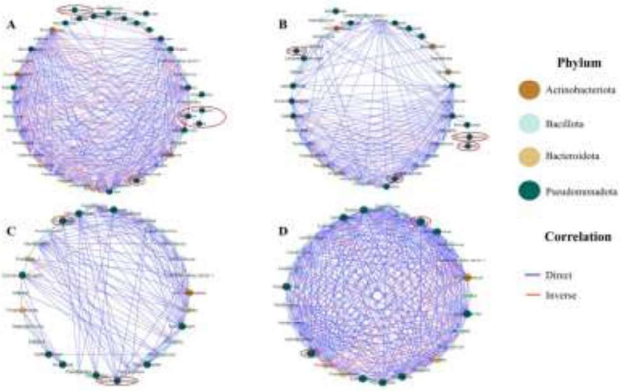
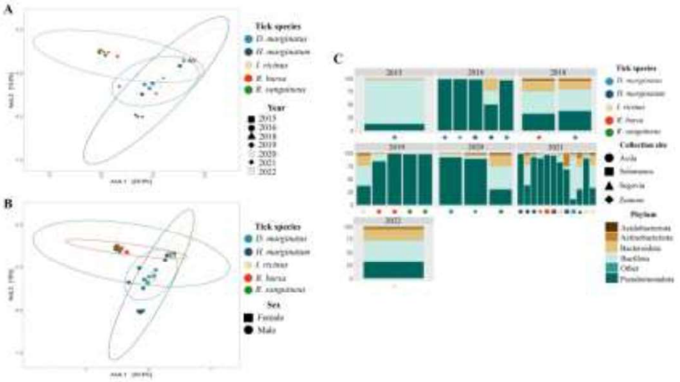


Figure 3

338x190mm (300 x 300 DPI)



338x190mm (300 x 300 DPI)