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Inventory and distribution of hard ticks (Ixodidae), ectoparasites of cattle and domestic dogs, in the Djelfa region, search for pathogens in the *Rhipicephalus sanguineus* species, and control trials.

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DEDICATION

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Your love, sacrifices, and wisdom have shaped every step of this journey.

Your words — *“Petit à petit, l’oiseau fait son nid”* — guided me through.

May this humble work reflect your dreams and my deep gratitude.

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To the person I was, the person I am, and the person I am becoming.

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الملخص

يقدم هذا البحث دراسة شاملة حول مجتمعات القراد الصلب والكائنات الدقيقة المرتبطة بها في منطقة الجلفة، مع التركيز على نوعي *Hyalomma excavatum* و *Rhipicephalus sanguineus*. يتكوّن العمل من أربعة محاور رئيسية:

يتناول المحور الأول جرد القراد الصلب الذي تم جمعه من الكلاب المنزلية في موقعين، ومن الأبقار في موقع واحد، وذلك على مدار سنة كاملة. تم تحديد ما مجموعه 8405 عينة من القراد، حيث كان نوع *R. sanguineus* الأكثر انتشارًا لدى الكلاب، في حين كان *H. excavatum* الأكثر وفرة لدى الأبقار. لوحظت فروقات موسمية في معدلات الإصابة، حيث سُجّلت النسب الأعلى خلال فصل الصيف. كما كشفت النتائج عن تباين في توزيع الأنواع، ونسب الذكور والإناث، وبعض المؤشرات البيئية بين المواقع، مما يسلط الضوء على تأثير العوامل البيئية وخصائص العائل.

يركز المحور الثاني على تفاعلات الكائنات الممرضة الدقيقة في *H. excavatum*. وقد تم تأكيد تحديد هذا النوع باستخدام التحاليل المورفولوجية والجزيئية تسلسل جين (16S rRNA) كشفت الفحوص عن انتشار مرتفع لبكتيريا *Rickettsia*، مع تباين واضح بين الجنسين؛ حيث سُجّلت معدلات عدوى مشتركة أعلى في الإناث، بينما أظهرت الذكور أنماط عدوى أبسط. وأوضحت تحليلات الشبكة أن كلاً من الجنس والتغيرات الموسمية يؤثران بشكل ملحوظ في تفاعلات هذه الكائنات، حيث لعبت البكتيريا التكافلية من نوع *Francisella* دورًا رئيسيًا في الحفاظ على بنية الشبكة، خصوصًا خلال فصل الصيف.

يتناول المحور الثالث ديناميكيات المجتمع المجهرية في *H. excavatum* عبر فصول الربيع، الصيف، والخريف. ورغم ثبات مؤشر التنوع ألفي (alpha diversity)، أظهرت تحاليل التنوع البيئي (beta diversity) نمطًا موسميًا مميزًا في تركيبة المجتمعات الدقيقة. كما تم تسجيل تغييرات واضحة في وفرة بعض الأنواع الأساسية مثل *Francisella* و *Candidatus Midichloria*. وقد أظهرت تحليلات تماسك الشبكة أن *Francisella* يلعب دورًا محوريًا في فصل الصيف، في حين تساهم *Rickettsia* بشكل أكبر في الترابط خلال الخريف.

أما المحور الرابع، فيستعرض التأثير السام لزيوت أساسية مستخلصة من نبات الشيح الأبيض بنسبة 48.84 في المئة من مركب الدافانول، ونبات إكليل الجبل بنسبة 43.52 في المئة من مركب الكافور، ونبات الزعتر بنسبة 18.3 في المئة من مركب الكارفكرول، ضد نوع القراد *R. sanguineus*. أظهر زيت الزعتر أعلى فعالية، حيث حقق معدل وفيات بنسبة 100 في المئة في يرقات القراد عند تركيز 5 ميكرو لتر لكل مليلتر خلال 72 ساعة، مع معدل تثبيط للفقس بلغ 90 في المئة عند نفس التركيز. كما بلغت قيم الجرعة المميتة LD90 بعد 72 ساعة ما يلي: 2.990 ميكرو لتر لكل مليلتر لزيت الشيح الأبيض، و 3.783 لزيت إكليل الجبل، و 2.677 لزيت الزعتر. وقد أكدت التحاليل الإحصائية الفعالية العالية المرتبطة بالجرعة لجميع الزيوت المدروسة، مع تميز زيت الزعتر كخيار واعد ضمن استراتيجيات مكافحة النباتية. توفّر هذه النتائج رؤى معمّقة حول بيئة القراد، تفاعلات الكائنات الدقيقة الممرضة، وديناميكيات المجتمعات المجهرية، كما تسلط الضوء على إمكانيات مكافحة البيئية باستخدام بدائل نباتية فعالة في المناطق شبه القاحلية.

الكلمات المفتاحية: القراد الصلب، تفاعلات مسببات الأمراض، ديناميكيات موسمية، المؤشرات البيئية، مكافحة النباتية.

Abstract

This study presents an integrated investigation of hard tick populations and their associated microbial communities in the Djelfa region, with particular emphasis on *Hyalomma excavatum* and *Rhipicephalus sanguineus*. The research is organized into four main sections :

The first section provides a spatiotemporal inventory of hard ticks collected from domestic dogs (two localities) and cattle (one locality) over the course of one year. A total of 8,405 ticks were identified. *Rhipicephalus sanguineus* was the predominant species in dogs, while *Hyalomma excavatum* dominated in cattle. Pronounced seasonal variations were observed, with infestation rates peaking in summer. Differences in species composition, sex ratios, and ecological indices between sites underscored the influence of environmental and host-related factors.

The second section focuses on pathogen interactions in *Hyalomma excavatum*. Species identification was confirmed through morphological analysis and 16S rRNA gene sequencing. Pathogen screening revealed a high prevalence of *Rickettsia* spp., with sex-specific patterns: higher coinfection rates in females and simpler infection profiles in males. Network analyses showed that both sex and seasonal shifts significantly shaped pathogen interaction structures, with *Francisella*-like endosymbionts playing a central role in maintaining network integrity, especially during summer.

The third section explores the microbiome dynamics of *Hyalomma excavatum* across spring, summer, and autumn. While alpha diversity remained stable, beta diversity analyses revealed distinct seasonal clustering of microbial communities. Marked seasonal changes were observed in the relative abundance of key microbial taxa, including *Francisella*, *Candidatus*, and *Midichloria*. Network robustness analyses indicated that *Francisella* contributed significantly to microbial community cohesion during summer, while *Rickettsia* played a greater role in network connectivity in autumn.

The fourth section evaluates the acaricidal activity of essential oils extracted from three medicinal plants: *Artemisia herba alba* (48.84% Davanone), *Rosmarinus officinalis* (43.52% Camphor) and *Thymus vulgaris* (18.3% Carvacrol). *Thymus vulgaris* oil exhibited the highest efficacy, achieving 100% larval mortality and 90% egg hatch inhibition at 5 µl/ml within 72 hours. The estimated LD90 values after 72 hours were 2.990 µl/ml for *Artemisia herba alba*, 3.783 for *Rosmarinus officinalis*, and 2.677 for *Thymus vulgaris*. Statistical analysis confirmed significant dose-dependent efficacy for all tested oils, highlighting *Thymus vulgaris* as a promising candidate for eco-friendly tick control. Altogether, this study offers novel insights into tick ecology, pathogen–microbiome interactions, and environmentally sustainable control strategies, contributing valuable knowledge toward improved management of tick-borne diseases in semi-arid regions.

Keywords: Hard ticks – Microbiome – Pathogen interactions– Botanical control – Seasonal dynamics – Ecological indices.

Resume

Cette étude offre une exploration intégrée des communautés de tiques dures et de leurs microorganismes associés dans la région de Djelfa, avec un accent particulier sur les espèces *Hyalomma excavatum* et *Rhipicephalus sanguineus*. Elle s'articule autour de quatre volets complémentaires :

Le premier volet présente un inventaire spatio-temporel des tiques dures collectées chez les chiens domestiques (deux localités) et les bovins (une localité) sur une période de douze mois. Un total de 8 405 spécimens a été identifié. *Rhipicephalus sanguineus* était l'espèce la plus abondante chez les chiens, tandis que *Hyalomma excavatum* prédominait chez les bovins. L'analyse a mis en évidence des variations saisonnières nettes, avec un pic d'infestation en été. Des différences marquées dans la composition spécifique, la répartition sexuée et les indices écologiques ont également été observées entre les sites, traduisant l'influence des conditions environnementales et des hôtes.

Le deuxième volet explore les interactions pathogènes chez *Hyalomma excavatum*. L'identification morphologique, confirmée par le séquençage du gène 16S rRNA, a validé l'espèce. Le dépistage des agents pathogènes a révélé une forte prévalence des *Rickettsia*, avec des schémas d'infection différents selon le sexe : des co-infections plus fréquentes chez les femelles, et des profils plus simples chez les mâles. Les analyses de réseaux ont montré une influence significative du sexe et des variations saisonnières sur la structure des interactions, avec un rôle clé des endosymbiotes *Francisella* dans la stabilité du réseau en été, tandis que *Rickettsia* contribuait davantage à la connectivité en automne.

Le troisième volet s'intéresse aux dynamiques du microbiome de *Hyalomma excavatum* au printemps, en été et en automne. La diversité alpha est restée relativement stable, mais la diversité bêta a mis en évidence un regroupement saisonnier des communautés microbiennes. Des variations notables ont été observées dans l'abondance relative de certains genres bactériens majeurs, notamment *Francisella*, *Candidatus* et *Midichloria*. L'analyse de la robustesse du réseau a révélé que *Francisella* joue un rôle structurant dans la cohésion du microbiome en été, tandis que *Rickettsia* assure une plus grande connectivité en automne.

Enfin, le quatrième volet évalue l'effet acaricide de trois huiles essentielles extraites de plantes médicinales locales : *Artemisia herba alba* (Davanone 48,84 %) ; *Rosmarinus officinalis* contenant (camphre 43,52%) ; et *Thymus vulgaris* contenant 18,3 % de carvacrol. L'huile essentielle de *Thymus vulgaris* a montré la plus forte efficacité, avec une mortalité larvaire de 100 % et un taux d'inhibition de l'éclosion de 90 % à la concentration de 5 µl/ml après 72 heures. Les valeurs de LD90 estimées après 72 heures étaient de 2,990 µl/ml pour *Artemisia herba alba*, 3,783 pour *Rosmarinus officinalis* et 2,677 pour *Thymus vulgaris*. Les analyses statistiques ont confirmé une efficacité dose-dépendante significative pour l'ensemble des huiles, positionnant l'huile de *Thymus vulgaris* comme une option prometteuse dans les stratégies de lutte biologique. Dans son ensemble, cette étude met en lumière des éléments clés de l'écologie des tiques, la complexité des interactions pathogènes et microbiennes, et propose des alternatives durables pour la gestion des tiques dans les zones semi-arides.

Mots-clés : Tiques dures – Microbiome – Interactions pathogènes– Lutte botanique – Dynamique saisonnières – Indices écologiques.

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LIST OF ABBREVIATIONS

APL: Average Path Length (in network analysis)

Bp: Base pairs (unit for measuring DNA length)

Clr value: Centered Log-Ratio value (used in compositional data analysis)

EP (%): Erythrocyte Percentage

EPI: Ecological Parasitic Index

FAO: Food and Agriculture Organization

HR (%): Hazard Ratio (percentage)

IBM SPSS: International Business Machines Statistical Package for the Social Sciences

LCC: Largest Connected Component (in network analysis)

REI: Relative Ecological Importance

RO (%): Reproductive Output (percentage)

SparCC: Sparse Correlations for Compositional data (method for correlation estimation in microbiome studies)

NIST: National Institute of Standards and Technology

EPA: Environmental Protection Agency

NIH: National Institutes of Health

MS: Mass Spectrometry

A260/A280: Ratio indicating DNA purity (absorbance at 260 nm and 280 nm)

Amplicon: DNA fragment resulting from PCR amplification

ANOVA: Analysis of Variance

API: Apparent Parasitic Index

ASV: Amplicon Sequence Variant

BLAST: Basic Local Alignment Search Tool

BP: Tick-Borne Pathogens

CAN: Co-occurrence Network Analysis

CI: Confidence Interval

CTAB: Cetyltrimethylammonium Bromide (DNA extraction buffer)

DNA: Deoxyribonucleic Acid

EO: Essential Oil

EtBr: Ethidium Bromide (for gel electrophoresis visualization)

FLE: Francisella-Like Endosymbionts

GC %: Guanine-Cytosine Content Percentage

GC-MS: Gas Chromatography–Mass Spectrometry

Gel Electrophoresis: Technique to check DNA quality and integrity

I: Parasitic Intensity

IC₅₀: Inhibitory Concentration at 50%

IQR: Interquartile Range

LD₅₀: Lethal Dose for 50% of the Population

LD₉₀: Lethal Dose for 90% of the Population

LT₅₀: Lethal Time for 50% of the Population

LT₉₀: Lethal Time for 90% of the Population

MIC: Minimum Inhibitory Concentration

MID: Minimum Infectious Dose

Mod.: Modularity (Network Analysis)

MTD: Maximum Tolerated Dose

N: Parasitic Abundance

Nanodrop: Spectrophotometer used to measure nucleic acid concentration and purity

NetCoMi: Network Construction and Comparison for Microbiome Data

NGS: Next-Generation Sequencing

P: Prevalence

PCA: Principal Component Analysis

PCoA: Principal Coordinates Analysis

PCR: Polymerase Chain Reaction

PERMANOVA: Permutational Multivariate Analysis of Variance

Proteinase K: Enzyme for protein digestion during DNA extraction

qPCR: Quantitative Polymerase Chain Reaction

Rf: Retention Factor (Chromatography)

RNA: Ribonucleic Acid

RNase: Ribonuclease (removes RNA during DNA extraction)

Robust.: Robustness Index

rRNA: Ribosomal Ribonucleic Acid

SD: Standard Deviation

SDS: Sodium Dodecyl Sulfate (lysis agent)

SE: Standard Error

SFG: Spotted Fever Group

VBD: Vector-Borne Disease

α -diversity: Alpha Diversity

β -diversity: Beta Diversity

χ^2 (Chi-square): Statistical test for independence or goodness-of-fit

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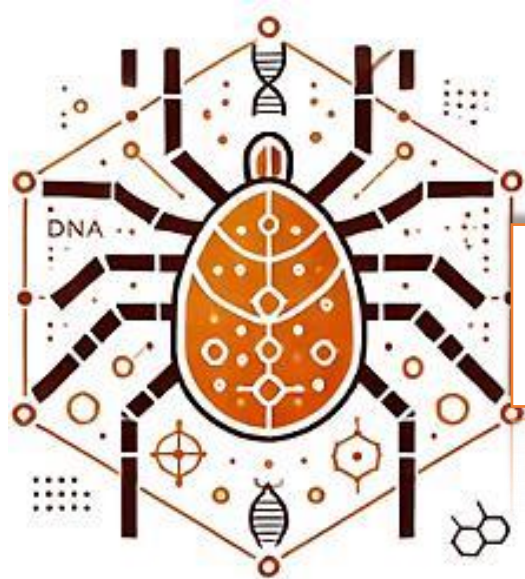
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INTRODUCTION

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Ticks are obligate hematophagous ectoparasitic arthropods that rely entirely on one or more hosts to complete their life cycle. With over 900 species worldwide (Mans and Neitz, 2004), they are among the most widespread ectoparasites. The hard tick family (Ixodidae) is particularly dominant, both in species diversity and epidemiological importance (Tsatsaris et al., 2016). Unlike mosquitoes, which primarily affect human health due to their endophilic affinity (Boulanger et al., 2019), ticks play a leading role in veterinary health by transmitting a range of pathogens, including protozoa, bacteria, and viruses, to animals during feeding (Sonenshine et al., 2002). This transmission occurs through three primary routes: transstadial (between life stages via molting), horizontal (through a host or co-feeding), and transovarial (from infected females to their offspring). The latter is particularly significant in maintaining pathogen diversity, making ticks both vectors and reservoirs of harmful vector-borne diseases (Azad and Beard, 1998; Balashov, 1999; Danielová et al., 2002; Bonnet et al., 2007). Many of these diseases are zoonotic (WHO et al., 2004), with nearly 60% capable of infecting humans as accidental hosts (Bueno-Marí et al., 2015), including Lyme disease, tularemia, granulocytic anaplasmosis, ehrlichiosis, Crimean-Congo hemorrhagic fever, tick-borne encephalitis, and rickettsioses (Ashagrie et al., 2023).

Recognizing the increasing risk posed by zoonotic diseases, the World Health Organization (WHO) has adopted and further developed the "One Health" concept, an interdisciplinary and collaborative approach to addressing zoonoses at the interface of human, animal, and environmental health. Initially formulated by Schwabe (Schwabe, 1969), this framework aims to improve public health protection by integrating veterinary and medical sciences to mitigate the risks associated with vector-borne diseases. Over the past decade, vector-borne zoonotic diseases have increased significantly, posing a global concern due to high case-fatality rates and rapid spread (Wikel, 2018; NAP, 2002). This emergence is driven by complex interactions among vectors, animal hosts, and humans (Cuervo et al., 2023), facilitating the evolution of novel pathogens with enhanced replication and dissemination potential (Weiss and Sankaran, 2022).

Ecological factors are key to zoonotic disease epidemiology (Patricia, 2021). Climate change, intensified by human activities, alters environmental conditions through extreme weather events (Allen et al., 2018), affecting host availability and tick distribution (McCoy and Boulanger, 2015). As a result, tick-borne pathogens are expanding with their vectors (Wikel,

2018). Temperature significantly influences tick life cycles, impacting egg development and metamorphosis (Estrada-Peña et al., 2021a). For instance, high temperatures accelerate *Theileria parva* replication and reduce transmission time in *Rhipicephalus appendiculatus* nymphs (Ochanda et al., 1988). Warming trends have also driven *Amblyomma americanum* and *Dermacentor variabilis* into higher altitudes and northern U.S. regions (Molaei et al., 2019).

In addition, demographic growth and urbanization have intensified human activities, including trade and landscape modifications, increasing human-pet interactions with natural environments and altering disease dynamics (NAP, 2002; Weiss and Sankaran, 2022; Daszak, 2005). In 2020, outdoor recreation in the Greater Alpine Region coincided with a surge in tick-borne encephalitis (Rubel, 2021). Likewise, socio-economic factors in Russia have increased human contact with *Ixodes ricinus* and *Ixodes persulcatus*, raising morbidity rates for tick-borne encephalitis and Lyme borreliosis (Korenberg, 2021).

The cross-border movement of animals, particularly through illegal trade, accelerates the spread of exotic tick species and their pathogens, increasing the risk of emerging zoonotic diseases (Heyman et al., 2010). Cases include the establishment of *Rhipicephalus sanguineus* in Hungary via dogs from Croatia (Hornok and Farkas, 2005) and the introduction of *Hyalomma marginatum* into the U.K. through imported horses (Jameson and Medlock, 2009). To mitigate such risks, the WHO enforces international health regulations on animal trade (Cunningham et al., 2017). Migratory birds also facilitate the urbanization of exotic ticks (Estrada-Peña et al., 2021b), with climate change altering migration routes (Hoogstraal et al., 1961) and enabling engorged female ticks to influence local populations (Kelly et al., 2021). For instance, *Hyalomma rufipes* has been introduced into Europe and Turkey through bird migrations (Patricia, 2021).

Beyond their role as pathogen vectors, ticks harbor one of the most diverse microbiomes among arthropods (Duron et al., 2017), comprising pathogens, symbionts, and commensals within a dynamic micro-ecosystem that interacts with the host (Grandi et al., 2023). Among these, endosymbionts serve vital functions, including vitamin synthesis and reproductive support (Aguilar-Díaz et al., 2021). Three genera -*Francisella*, *Midichloria mitochondrii*, and *Coxiella*- are specific to ticks, with *Coxiella* being the most widespread, found in two-thirds of tick species (Benyedem et al., 2022). More importantly, endosymbionts facilitate tick adaptation to new environments and support pathogen maintenance (Bonnet et al., 2017), underscoring the growing trend of integrating tick microbiome studies with pathogen detection (Lau et al., 2023).

In Algeria, livestock farming is a key agricultural sector contributing to economic growth and food security (Ogni et al.,2014). However, tick infestations pose a major threat, both directly through parasite burden and indirectly via the transmission of diseases such as theileriosis, babesiosis, and anaplasmosis (Gharbi and Darghouth, 2014; Benyedem et al.,2022). Tick-borne diseases incur an estimated annual loss of \$17.33 billion worldwide, with nearly 80% of global livestock at risk (Yeo et al.,2017). These parasites significantly impact productivity, causing economic losses due to antiparasitic treatments (Pazinato et al.,2016) and production setbacks, including skin damage, weight loss, reduced milk yield, and anemia (Luns et al.,2021).

Epidemiological studies on tick-borne pathogens necessitate accurate vector identification using dichotomous keys. In Africa, the most widely used keys are those by Walker et al. (2003) and Estrada-Peña et al. (2004), which enable species identification based on morphological characteristics. Extensive research on hard tick (*Ixodidae*) populations in Algeria has documented 24 species across five genera: *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus* (Senevet and Rossi, 1924; Yousfi-Monod and Aeschlimann, 1986; Boulkaboul, 2003; Boudierda and Meddour, 2006; Benchikh-Elfegoun et al.(2007) ; Bouhous et al. (2011) ; Kernif et al. (2012) ; Khaldi et al. (2012) ; Bendjeddou et al. (2016) ; Khelfaoui et al. (2018) ;. and more recent studies of Bouchama et al. (2020) ; Senaoui et al. (2020) ; Derradj and Kohil (2021) ; Derradj and Kohil (2022) ; Bedouhene et al. (2022) ; Mechouk et al. (2022)).

The absence of vaccines for many tick-borne diseases has made synthetic acaricides the primary control method, offering rapid efficacy but posing environmental and health risks (Savadojo et al., 2016). Excessive use of these chemicals has led to tick resistance, ecological contamination, and toxicity concerns (Dande, 2015). As an alternative, research is increasingly focusing on plant-based compounds (Abdelali et al., 2023). Over 2,000 insecticidal plant species have been identified (Merabti et al., 2015), with bioactive compounds such as phenols, terpenoids, and alkaloids extracted through various methods, including solvent extraction and distillation (Dubey, 2010). Essential oils, in particular, are highly valued (Aissaoui et al., 2022). While global interest in acaricidal plants is growing, studies in Algeria remain limited (Djebir et al., 2019; Alimi et al., 2022).

Given the limited in-depth studies on various aspects of tick populations in the selected study region, this research aims to enhance knowledge of the ixodid fauna in the Wilaya of Djelfa, Algeria's steppe capital. It involves compiling a species inventory from livestock in two distinct

locations, assessing their parasitic and ecological indices, and analyzing the seasonal dynamics of these ectoparasites. Additionally, the study examines how sex and seasonal variations in *Hyalomma excavatum* influence the composition and interactions of tick-borne pathogens (TBPs). Using high-throughput PCR, DNA sequencing, and network analysis, it explores seasonal shifts in the microbial communities of *H. excavatum*, particularly their impact on interactions between *Rickettsia*, *Francisella*, and surrounding microbiota. This research provides the first comprehensive analysis of TBP communities in Algeria. Furthermore, in the context of biological control against ixodid ticks, the acaricidal activity of essential oils from three plant species was tested on different life stages of the *R. sanguineus*.

This document is structured into two main parts. The first part presents a bibliographic review that provides an overview of ticks as obligate ectoparasites and their role as vectors of various pathogens. It explores the diversity of tick species, their biology, and their epidemiological importance, with a particular focus on the pathogens they transmit, including bacteria, protozoa, and viruses. Additionally, it discusses current control strategies, ranging from chemical acaricides to biological control methods, and highlights challenges related to resistance and environmental concerns.

The second part is dedicated to the experimental work and begins by describing the geographical and ecological characteristics of the study regions, emphasizing their relevance to tick populations and disease transmission. It then details the methodologies used for field sampling, laboratory analyses, and statistical approaches. The results obtained are presented and interpreted in light of previous research, providing insights into tick distribution, seasonal dynamics, pathogen interactions, microbiome composition, and the effectiveness of control strategies.

Although the official title of this thesis remains as originally registered, in accordance with administrative requirements, the scientific scope of the work evolved during the course of the research. Specifically, the study shifted toward a different tick species (*Hyalomma*) and incorporated an additional focus on pathogen interactions and microbiome analysis. This manuscript reflects those developments.



1 CHAPTER I: LITERATURE REVIEW

1.1 Tick systematics

Ticks belong to the arthropod phylum, characterized by the presence of an exoskeleton and articulated appendages (legs, antennae, mandibles, chelicerae, etc.) (Morel, 1976). They are classified into three families whose monophyly is well established: soft ticks (Argasidae; around 190 species), hard ticks (Ixodidae; around 900 species), and Nutalliellidae (a family consisting of a single genus that includes only one species, *Nutalliella namaqua*) (McCoy and Boulanger, 2015).

According to Guglielmone et al. (2010), the classification of the Ixodidae is structured as follows:

Phylum	Arthropoda Latreille, 1829
Sub-phylum	Chelicerata Heymons, 1901
Class	Arachnida Cuvier, 1812
Sub-class	Acari Leach, 1817
Order	Parasitiformes Reuter, 1909
Sub-order	Ixodida Leach, 1815 (Synonym = Metastigmata Canestrini, 1891)
Family	Ixodidae Leach, 1815

In order to make taxonomic groupings of the Ixodidae, Hoogstraal (1972), based on a set of characteristics linked to morphology, ecology, host associations, and biogeographical distribution of ticks, proposed the first phylogenetic tree illustrating the kinship between hard ticks. This concept divided the Ixodidae into two groups: The Prostriata, when the anus is surrounded at the front by an anal groove, containing only the genus *Ixodes*, with almost 250 species, and the Metastriata when this groove is absent or contours the anus forwards, made up of the other existing genera of Ixodidae (Hoogstraal, 1972).



The Metastriata are subdivided into four subfamilies: *Amblyomminae*, *Haemaphysalinae*, *Hyalomminae*, and *Rhipicephalinae*. The latter includes the genera *Rhipicephalus*, *Dermacentor*, and *Boophilus*. The *Boophilus* genus had been singled out because of the particular biology of these species, which carry out their three blood meals on the same host individual (i.e. monophasic and monoxenic ticks) (Murrell et al.,2001).

Subsequently, several molecular phylogeny analyses showed that the species recognized in the genus *Boophilus* formed a group within the genus *Rhipicephalus*, making the latter group paraphyletic, therefore it was proposed that the genus *Boophilus* be integrated into the genus *Rhipicephalus*. Also, the separation of *Hyalomminae* and *Rhipicephalinae* was not justified, so they proposed a new subdivision of *Metastriata* into *Amblyomminae*, *Haemaphysalinae*, *Rhipicephalinae*, and *Bothriocrotinae* (Murrell et al.,2000) (Figure 1).

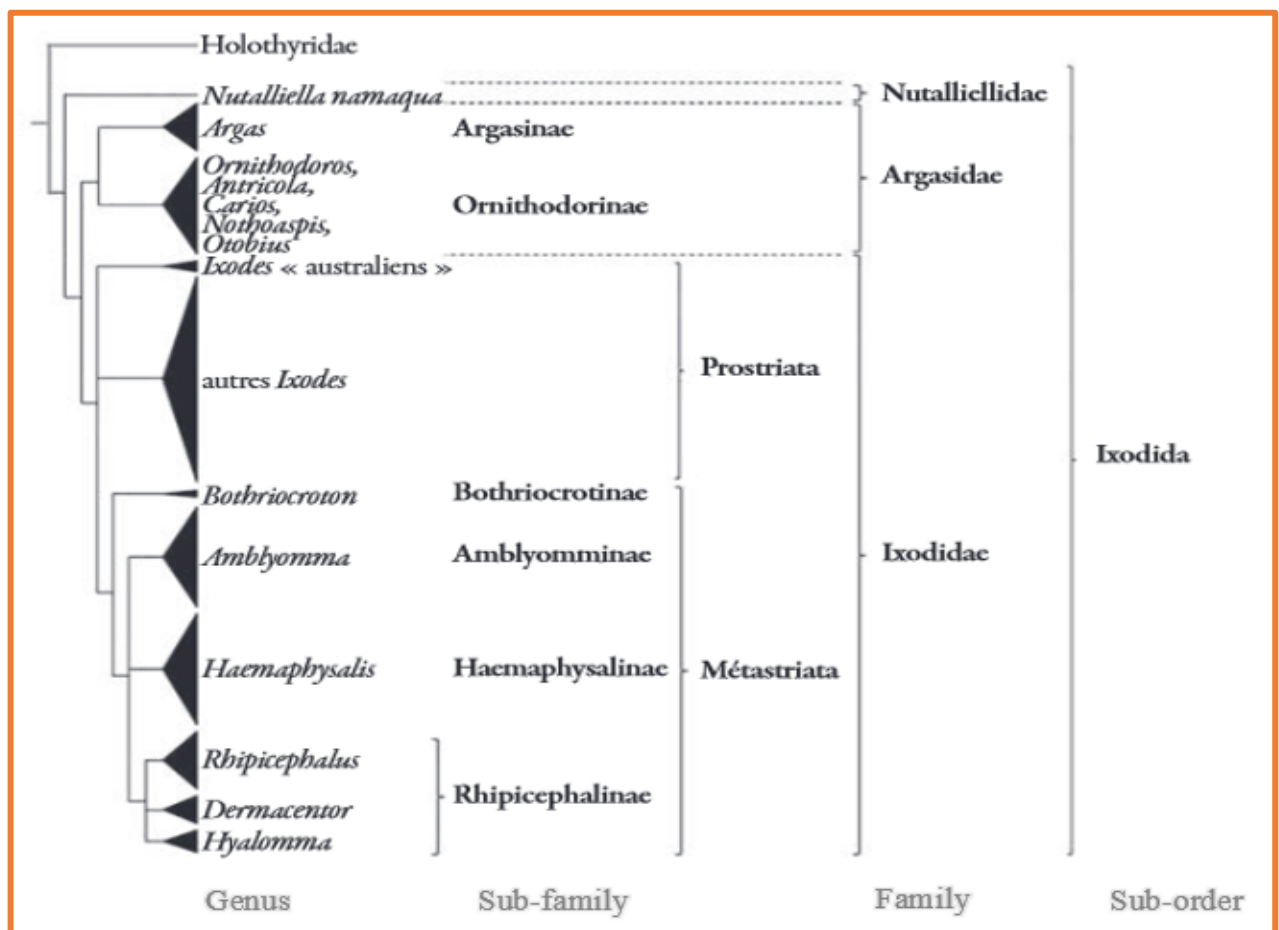


Figure 1: Phylogenetic tree detailing the relationships between the different tick genera, sub-families, and families in the sub-order Ixodida (McCoy and Boulanger, 2015)



The number of ixodidae species described to date is close to 900, belonging to 14 genera (Guglielmone et al., 2010 ; Barker and Burger, 2018) which are:

- *Amblyomma* (Koch, 1844)
- *Anomalohimalaya* (Hoogstraal, Kaiser et Mitchell, 1970)
- *Archaeocroton* (Dumbleton, 1943)
- *Bothriocroton* (Keirans, King et Sharrad, 1994)
- *Cosmiomma* (Schulze, 1919)
- *Dermacentor* (Koch, 1844)
- *Haemaphysalis* (Koch, 1844)
- *Hyalomma* (Koch, 1844)
- *Ixodes* (Latreille, 1795)
- *Margaropus* (Karsch, 1879)
- *Nosomma* (Schulze, 1919)
- *Rhipicentor* (Nuttall et Warburton, 1908)
- *Rhipicephalus* (Koch, 1844).
- *Robertsicus* (Price, 1959)

The exact number of species varies from author to author, as there are some species for which there is no consensus on synonymy. Several publications have drawn up "update" lists of ixodidae species to take account of new species descriptions and synonymies, for example:

Hyalomma anatolicum excavatum previously described as a single species is now divided by Guglielmone et al., (2009 ; 2010) into two distinct species *H.anatolicum* (Koch, 1844) and *H.excavatum* (Koch, 1844). The same author has given a new name to the species *Hyalomma detritum* (Schulze, 1919) as *Hyalomma scupense* (Schulze, 1919).



Furthermore, *Boophilus annulatus*, originally described as *Margaropus calcaratus*, is now considered to be *Rhipicephalus annulatus* (Say, 1821). Taking these new nomenclature and notions into account, published inventories of hard ticks in Algeria indicate the presence of 24 species belonging to 5 genera (Table 1).

Table 1 : Ixodidae species present in Algeria.

Genus	N° of species worldwide	N° of species present in Algeria	Species	Reference
<i>Dermacentor</i>	34	1	<i>Dermacentor marginatus</i>	a, b, c, n
<i>Haemaphysalis</i>	166	3	<i>Haemaphysalis erinacei</i>	g
			<i>Haemaphysalis punctata</i>	a, b, c, n
			<i>Haemaphysalis sulcata</i>	c, f
<i>Hyalomma</i>	27	10	<i>Hyalomma anatolicum</i>	l, m
			<i>Hyalomma excavatum</i>	a, b, c, j, m, n
			<i>Hyalomma dromedarii</i>	c, e
			<i>Hyalomma scupense</i>	a, b, c, d, e, j, l, m, n
			<i>Hyalomma impeltatum</i>	a, b, c, e, m, n
			<i>Hyalomma lusitanicum</i>	a, b, c, d, l, m, n
			<i>Hyalomma marginatum</i>	a, b, c, e, j, l, m, n
			<i>Hyalomma rufipes</i>	b, n
			<i>Hyalomma aegyptium</i>	c
			<i>Hyalomma truncatum</i>	f
<i>Ixodes</i>	243	4	<i>Ixodes ricinus</i>	a, b, c, n
			<i>Ixodes hexagonus</i>	k
			<i>Ixodes inopinatus</i>	o
			<i>Ixodes vespertilionis</i>	h, i
<i>Rhipicephalus</i>	82	6	<i>Rhipicephalus (Boophilus) annulatus</i>	a, b, c, d, j, l, m, n
			<i>Rhipicephalus bursa</i>	a, b, c, j, l, m, n
			<i>Rhipicephalus evertsi</i>	e
			<i>Rhipicephalus guilhoni</i>	e
			<i>Rhipicephalus sanguineus</i>	a, b, c, d, e, l, m
			<i>Rhipicephalus turanicus</i>	a, b, c, d, j, n

***References:**
a: Yousfi-Monod and Aeschlimann (1986) – b: Boulkaboul (2003) – c: Bouderdou and Meddour (2006) - d: Benchikh-Elfegoun et al.(2007) – e: Bouhous et al. (2011) – f: Kernif et al. (2012) -g: Khaldi et al. (2012) – h: Bendjeddou et al. (2016) – i: Khelfaoui et al. (2018) -j: Bouchama et al. (2020) – k: Senaoui et al. (2020) -l : Derradj and Kohil (2021) – m: Derradj and Kohil (2022) – n :Bedouhene et al. (2022) – o: Machouk et al. (2022)



1.2 Morphology of Ixodid ticks

Ixodidae is a large acarid with a globular body that divides into two not clearly delimited parts, a front part, the Gnathosoma, also known as the rostrum, and a globular rear part, the Idiosoma. They have four pairs of legs, a pair of chelicerae, and a pair of pedipalps (Rebaud, 2006).

The gnathosoma is located Antero-terminally in front of the body, resembling a head or pseudo cephalon (Guigen and Degeith, 2001) bearing the mouthparts and organs necessary for attaching the tick to the host (Socolovschi et al., 2008) houses the first part of the digestive tract and is largely involved in the feeding function constituent of :

- Capitulum, whose variable shape (triangle, rectangle, trapezoid, pentagonal, or hexagonal) determines the genus (Rebaud, 2006);
- Hypostome, located ventrally resulting from the fusion of two paired elements, bearing retrograde denticles (allowing solid attachment of ticks to their host, more developed in females), the number of which is important in systematics, particularly in *Boophilus* (Anderson and Magnarelli, 2008);
- A pair of chelicerae, dorsal parts made up of three parts: a swollen muscular base integrated into the capitulum, which tapers to a long tubular sheath, where tendons and nerves run, to which two terminal hooks are connected. In addition to their secondary sensory role, the chelicerae act like a pair of scissors to incise the integument, enabling penetration of the hypostome (Blary, 2004)
- A pair of pedipalps, made up of four articles covering the rest of the mouth apparatus, are lateral sensory appendages that play no role in the tick's attachment process (Yapi, 2007)
- Females also have, on the dorsal side, porous areas which correspond to the openings of the dermal glands whose secretion serves to waterproof the eggs (Socolovschi et al., 2008).

The entire rostrum and capitulum can be either square (brevirostral ticks) or elongated (longirostral ticks) (Guigen and Degeith, 2001)



The **idiosoma** corresponds to the body of the tick itself, this posterior part does not play a role in fixation, but it does contain numerous criteria for genus and species identification (Perez-Eid, 2007).

Dorsally the idiosoma has a shield (scutum), The cuticle is made up of chitin, proteins, and lipids, as well as dermal glands that help maintain the water balance of the ixodus body, even in the face of the risk of desiccation during the free phase (Knülle and Rudolph, 1982), more developed in males (conscutum), hence the name "hard tick".

Males are unable to distend, whereas females, with their reduced scutum (alloscutum), dilate considerably when feeding favoring the storage of an extreme quantity of blood sufficient for ovogenesis and oviposition Guetard, 2001), The color (yellow, brown, or black), the shape (round, oval, sub-oval), and the grooves (cervical, scapular, mediodorsal, lateral, caudal) marked or unmarked by punctation and ornamentation (bristles, hairs, striae) representing the characteristics used to distinguish tick species from one another (Meddour-Bouderda, 2000) also its posterior edge is generally cut into nine to eleven festoons that are more or less fused or even absent (Bourdeau, 1993).

laterally the concave or convex eyes are housed in orbits which, by their presence in some cases (*Hyalomma*, *Rhipicephalus*, *Dermacentor*) or absence in others (*Ixodes*, *Haemaphysalis*), help to distinguish the various genera (Meddour-Bouderda, 2000). The legs are made up of six articles, the coxa, which attaches the leg to the rest of the body and may bear internal or external spurs, their taxonomic interest based on the size and length of these spurs according to the species, followed by the trochanter, femur, patella (or genua), tibia, and tarsus which are continued by the ambulacrum equipped with two claws and a sucker or pulville (Perez-Eid, 2007). The tarsus of the first pair of legs contains a cavity known as Haller's organ, responsible for the tick's olfactory sense and containing numerous chemoreceptors, which enable it to locate its host (Haller, 1992).

The ventral face of the idiosoma features a genital opening in adult females in the anterior region, the shape of this gonopore surrounded by the genital groove is used in species diagnosis, notably in the genera *Hyalomma* and *Rhipicephalus*, they have no ventral plates, and the stigmas are less pronounced than in males (Anderson and Magnarelli, 2008). While in males the existence of sclerified ventral plates or escutcheons which, by their absence in certain genera (*Dermacentor*, *Ixodes*, *Haemaphysalis*), their arrangement in others (*Hyalomma*,



Rhipicephalus, *Rhipicephalus annulatus*), make it possible to differentiate the genera and species (Meddour-Bouderda, 2000). In most ixodids that have them, these crests include The adanals located on either side of the anus, the accessories on either side of the adanals, and, the subanals behind the adanals. An anal opening in the posterior region on the midline. It is accompanied by a groove that is either anterior (perianal groove), posterior (postanal groove), or absent. In addition, there are two respiratory stigmas (peritremes) located behind the coxae of the last pair of legs, depending on the species, the shape of the stigmas can be oval, round, ovoid, or comma-shaped (Branch, 1976; Perez-Eid, 2007).

Ticks show a clear sexual dimorphism, the female differs from the male by several elements. First, in size, the female is generally larger than the male, even when fasting (Guigen and Degeith, 2001) . Depending on the genus and the state of engorgement, the size of the female varies between 4 and 15 mm, while that of the male is between 1.5 mm and 8 mm. Also, the porous areas present on the gnathosoma exist only in females, in addition to the presence of two depressions in the middle of the dorsal surface of the idiosoma called foveas, linked to foveal glands present in certain genera (except *Ixodes*) (Boyard, 2007). The female's dorsal scutum is reduced, covering only the anterior part of the body, whereas, in the male, the scutum covers the entire dorsal surface of the idiosoma. The male also has ventral scutums, which are used in the diagnosis of genera and species but are absent in the female (Anderson and Magnarelli, 2008) (Figure 2).

1. The nymph

The nymph's morphology is largely similar to that of the female, with a comparable body structure and general appearance. However, it lacks certain distinguishing features, such as the genital pore and the porous areas present on the capitulum of adult females. In addition to these differences, the nymph is notably smaller in size, typically measuring between 1 and 2.5 mm. This stage of development plays a crucial role in the tick life cycle, serving as an intermediate phase between the larval and adult stages, during which the tick continues its feeding and growth processes before reaching maturity (Francois, 2008)(Figure 3).

2. The larva

The larva shares the same general morphology as the pupa but exhibits several distinguishing characteristics. It possesses only three pairs of legs, unlike the nymph and adult stages, which



have four. Additionally, the larva lacks stigmas, the respiratory openings found in later developmental stages. In terms of size, it typically ranges from 0.5 to 1 mm, making it the smallest stage in the tick's life cycle. This early stage is critical for the tick's development, as it must successfully locate and feed on a suitable host to progress to the next phase of its life cycle (Francois, 2008) (Figure 3).

3. The eggs

They exhibit an oval shape with a characteristic amber coloration, measuring between 460 and 650 μm in length. Their outer shells are coated with an oily, water-repellent layer, which plays a crucial role in preventing dehydration by reducing water loss. This protective adaptation enhances their resilience and ensures their survival under varying environmental conditions, particularly in arid or dry habitats where moisture retention is essential (Morel, 1976) (Figure3).

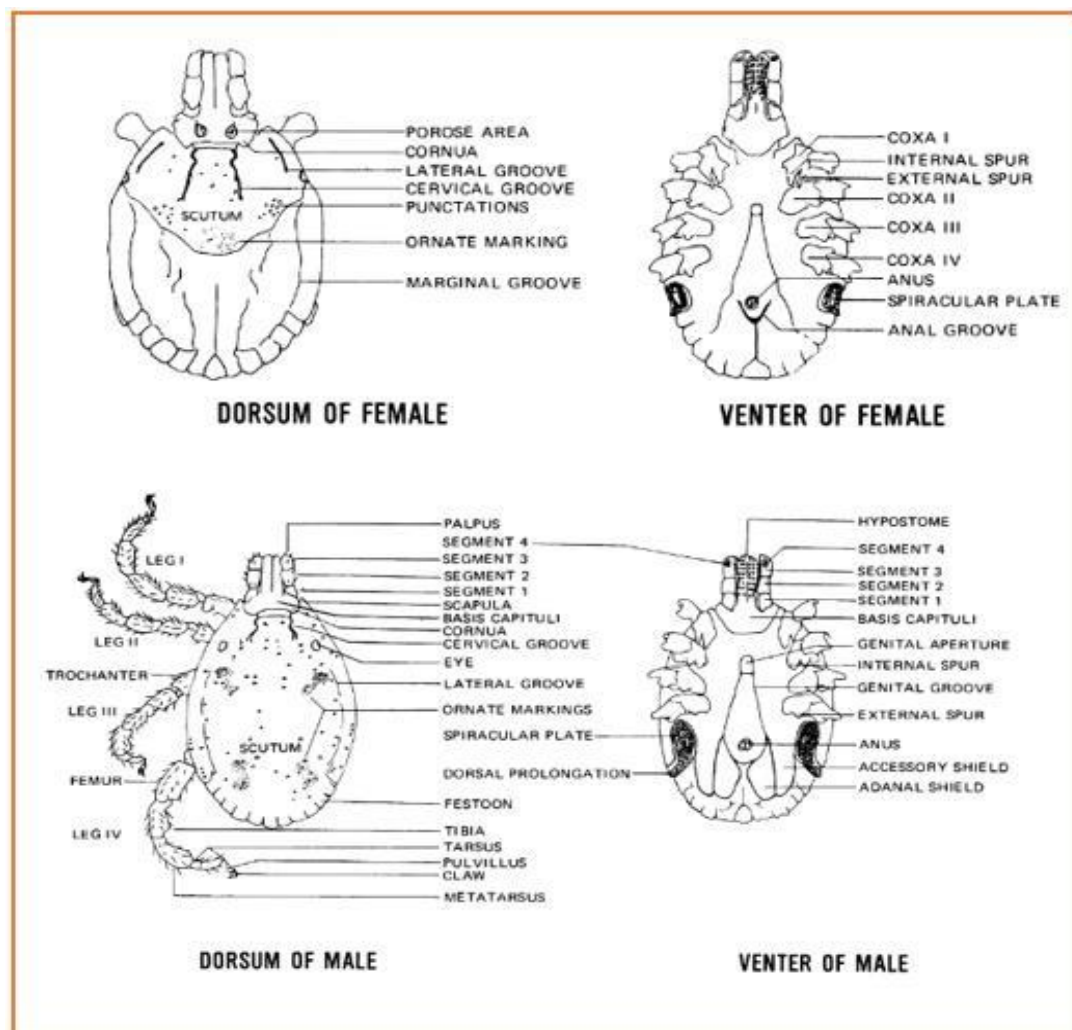


Figure 2 : Morphological Characteristics of Male and Female Hard Ticks (Branch, 1976)



Figure 3 : Different stages of hard tick (CDC, 2019)

1.3 Life cycle of Ixodid ticks

Hard ticks are temporary parasites, characterized like all acarians by four evolutionary stages: egg, larva (hexapods), nymph, adult (male or female), with development cycles alternating between parasitic (feeding) phases on the host and free phases on the ground (Yadav and Upadhyay, 2021) (Figure 4).

In order to start gorging, the adult female must be fertilized. Fertilization can either take place on the ground or on the host. Most metastriates, including the genera *Dermacentor*, *Amblyomma*, and *Rhipicephalus*, couple only on their hosts, while other genera such as *Ixodes* can copulate both in the absence of hosts and while engorging (Kiszewski et al., 2001). Once fully engorged, females leave their hosts and lay their eggs in suitable microenvironments, such as burrows, crevices, or leaf litter. The number of eggs varies according to tick species and degree of gorging (Sonenshine and Roe, 2013).



When the larva hatches, following 20 to 50 days of embryogenesis, it measures approximately 1 mm. Initially pale, soft, and swollen, it possesses three pairs of legs. Within a few days, it undergoes water loss, flattening as its cuticle hardens and darkens. Once metabolic waste from embryogenesis is expelled from the digestive tract, the larva begins searching for its first meal (Perez-Eid and Gilot, 1998). Many species exhibit negative geotropism, moving toward nearby herbaceous vegetation and forming clusters at the tips of plants under the influence of aggregation pheromones, awaiting a passing host (Lefevre et al., 2003). After feeding for several days, larvae detach and fall to the ground, where they metamorphose into pupae in a sheltered microenvironment. This transformation can take between 2 to 8 weeks, depending on species and climatic conditions (Socolovschi et al., 2008).

Pupae measure 2 to 4 mm at emergence (Perez-Eid and Gilot, 1998) and display the same activities as larvae, with the exception of aggregation. They fix onto the host, feed, and then regain the ground to metamorphose into adults, which generally last longer, up to 20 to 25 weeks under the most unfavorable conditions (Yadav and Upadhyay, 2021).

Subsequently, after a period of hardening, the adult begins to search for a host. Some ticks attack by recognizing the host visually or by receiving a chemical stimulus (carbon dioxide, water vapor, chemical constituents of urine or perspiration: acetone, butyric acid...) or a physical stimulus (moving form, shadow, touch, or heat)(Obenchain and Galun, 1982). Moreover, some of them are endophilic, like the *Rhipicephalus sanguineus* species, colonizing animal nests or burrows, and it is the hosts that fall into its trap. Unlike other exophilic species (*Hyalomma*, *Amblyomma*), which are generally long-legged, they can actively search for their host over a radius of a few meters (Morel, 1982). Adult meals are more abundant and last longer than those of previous stages, with only females taking an authentic blood meal, essential to ensure egg-laying. Males generally feed very little, if at all (James and Oliver, 1989) and usually die after mating, while females can increase their body weight 120 times and die just after laying (Cupp, 1991). The cycle lasts from a few months (around twenty weeks) to 3 or 4 years, with the parasite's life span varying in length. Ticks spend most of their lives in the environment,



and climatic factors lead to alternating periods of activity and diapause (Figure 4) (James and Oliver, 1989)

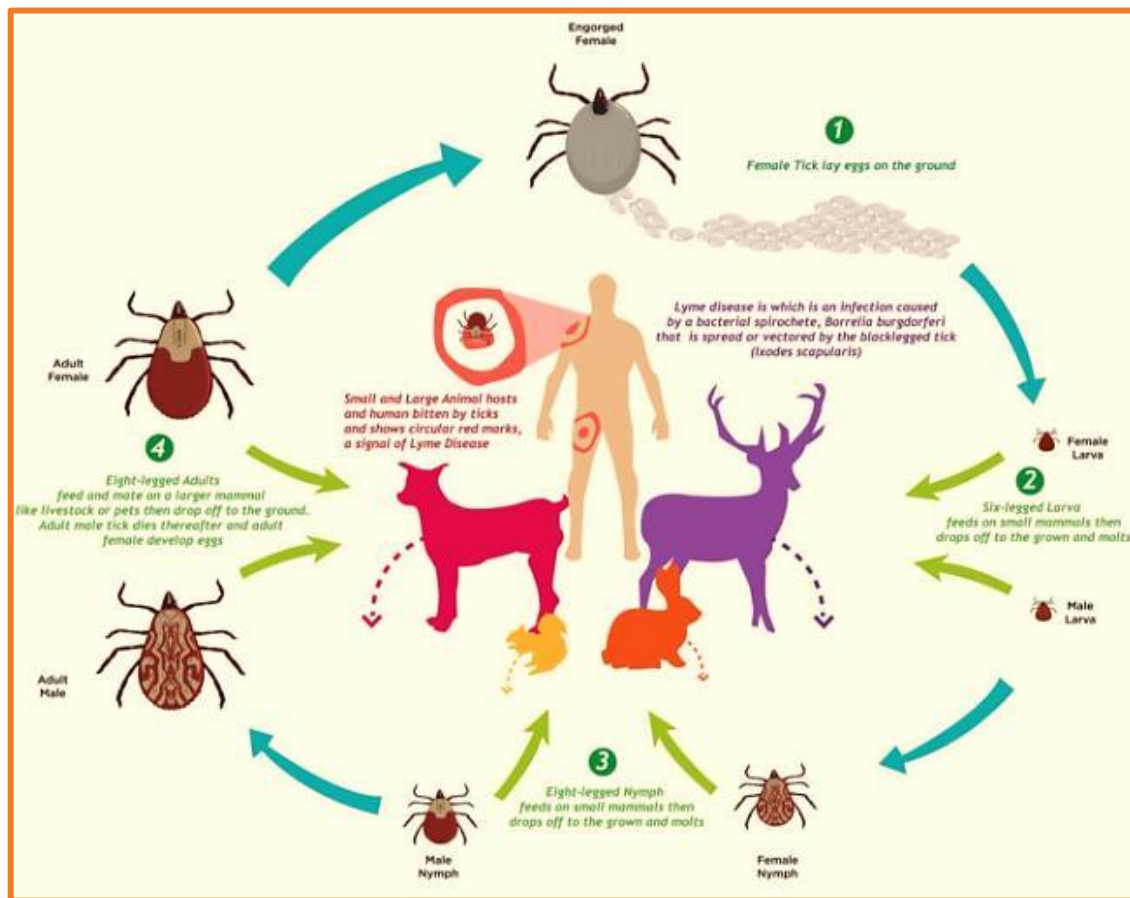


Figure 4: Life cycle of hard ticks (VCD, 2023)

1.3.1. Cycle according to the number of phases

This classification is essential for understanding the epidemiology of tick-borne infections and implementing effective tick control measures (Perez-Eid and Gilot, 1998). As intermittent ectoparasites, ticks exhibit three distinct life cycles based on the number of hosts involved (Jean-Baptiste, 2008):

1.3.1.1 Monophasic cycle (monoxenes)

In this type of life cycle, all developmental stages -larva, nymph, and adult- occur on a single host, where both metamorphic transitions (larval to nymphal and nymphal to adult) also take place (Hunter, 1994). Unlike two-host or three-host cycles, this cycle is characterized by a single parasitic phase, meaning that once the larva finds a suitable host, it remains attached



throughout its entire development. The only stages that occur off-host are oviposition, egg incubation, and the initial questing behavior of the newly hatched larvae searching for a suitable host. Once attached, the larvae feed, molt into nymphs, and subsequently develop into adults while remaining on the same host. The absence of multiple free-living stages significantly shortens the duration of the cycle and reduces the risks associated with finding new hosts, making this a highly efficient strategy for survival in stable host populations (Jean-Batiste, 2008).

However, the prolonged attachment period to the host also increases the risk of host immune responses affecting tick survival, as well as the potential for the transmission of pathogens that persist within the host over extended periods. This cycle is particularly advantageous in environments where host availability is predictable, such as in livestock or wildlife populations with limited movement. Well-known examples of single-host ticks include *Dermacentor albipictus*, commonly referred to as the winter tick, which primarily infests large mammals such as moose and deer, and *Boophilus microplus*, or the cattle tick, which is a major pest in the livestock industry due to its role in transmitting tick-borne pathogens affecting cattle health and productivity (Yadav and Upadhyay, 2021) (Figure 5).

1.3.1.2 Biphasic cycle (dixene)

In the two-host life cycle, immature stages—larvae and nymphs—complete their feeding and first metamorphosis on an initial host. Once the nymph has engorged, it detaches and drops to the ground, where it undergoes molting to reach the adult stage (James and Oliver, 1989). This transition period is crucial, as environmental conditions such as temperature, humidity, and habitat characteristics can influence the survival and development of the detached nymph.

Upon reaching adulthood, the tick seeks out a second host, which may sometimes belong to the same species or even be the same individual that hosted the immature stages, depending on the host's availability and mobility. Once successfully attached, adult ticks engage in feeding, and mating occurs directly on the host. Mated females then detach from the host and drop to the ground to lay eggs in the surrounding environment, thus completing the cycle. This type of cycle offers an intermediate balance between the efficiency of the single-host cycle and the greater host diversity of the three-host cycle. It reduces the number of host transitions, limiting exposure to external environmental risks, while still allowing for the exploitation of different host species if necessary (Yadav and Upadhyay, 2021) (Figure 5).



1.3.1.3 Triphasic cycle (trixene)

In the three-host life cycle, each developmental stage—larva, nymph, and adult—feeds on a separate host, which may or may not belong to the same species. This cycle consists of three distinct parasitic phases, with each feeding stage followed by a free-living phase on the ground, during which molting occurs. After hatching from eggs, larvae actively seek out a host, attach, and feed before detaching to molt into nymphs in the environment. The newly developed nymphs then find a second host, where they feed and later drop off to undergo the final molting process into adults (James and Oliver, 1989). Adult ticks then attach to a third host, where they feed and mate. Once engorged, females detach and drop to the ground to lay eggs, while males typically die after reproduction. This life cycle, characteristic of most *Ixodes* species, requires greater host availability but provides the advantage of dispersal across multiple hosts, reducing competition and enhancing survival opportunities in diverse environments (Guiguen and Degeilh, 2001) (Figure 5).

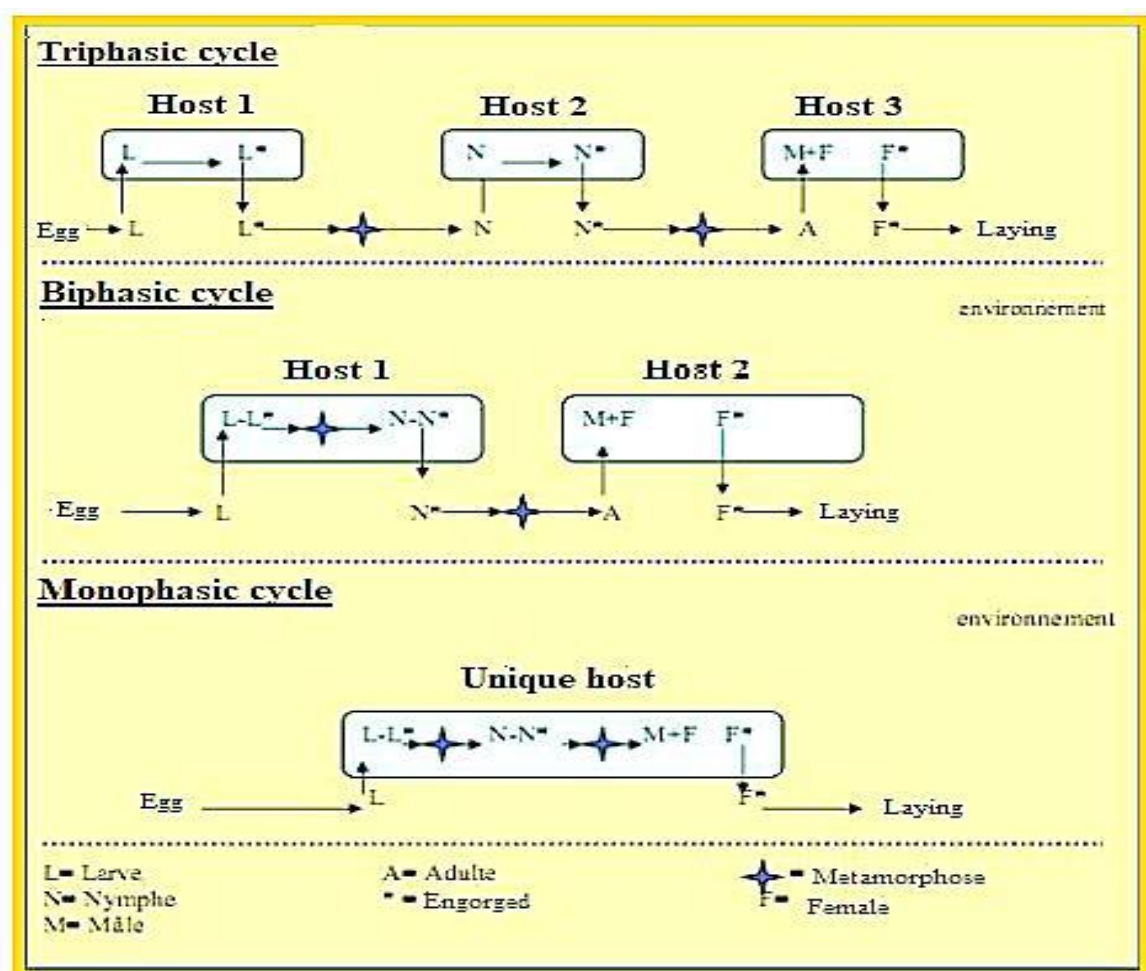


Figure 5: Types of life cycles based on the number of hosts involved (Francois, 2008)



1.3.2. Cycle classification based on tick host selectivity

Understanding the different cycle types is essential for elucidating pathogen transmission dynamics in ticks (Perez-Eid and Gilot, 1998). Tick life cycles can be categorized into three types based on the degree of host specificity exhibited across developmental stages, reflecting variations in host preference and selection.

1.3.2.1. Monotropic Cycle

In a monotropic cycle, all three developmental stages-larva, nymph, and adult—feed exclusively on hosts belonging to the same species. This high degree of host specificity is a key feature of certain tick genera, influencing both their ecological niche and vectorial capacity.

An example of this cycle is observed in *Boophilus* species, which exhibit a strong preference for cattle as their primary host throughout their entire life cycle (Sonenshine and Roe, 2013). Similarly, *Rhipicephalus sanguineus*, commonly known as the brown dog tick, demonstrates a distinct affinity for canines, making domestic and stray dogs its principal hosts (Table 2) (Chartier et al., 2000).

1.3.2.2 Ditropic Cycle

The ditropic cycle is characterized by the utilization of two distinct host species during different developmental stages (Sonenshine and Roe, 2013). In this cycle, immature stages -larvae and nymphs- typically feed on small vertebrates such as rodents, birds, or reptiles, while adult ticks seek out larger mammals as their primary hosts.

This host shift is particularly evident in *Hyalomma marginatum*, where juveniles parasitize small animals (Table 2), facilitating their dispersion in the environment, whereas adults prefer large ungulates, including livestock and wild herbivores (Chartier et al., 2000).

1.3.2.3Telotropic Cycle

The telotropic cycle is characterized by a marked shift in host preference between developmental stages, where each stage parasitizes a host belonging to a different zoological group. In this cycle, immature stages -larvae and nymphs- exhibit low host specificity, attaching to a wide range of available vertebrates, including small mammals, birds, and reptiles. In



contrast, adult ticks demonstrate a more selective host preference, typically parasitizing larger vertebrates such as carnivores or ungulates (Chartier et al., 2000).

This host transition is observed in species like *Ixodes ricinus* and *Haemaphysalis punctata* (Table 2), where juveniles exploit diverse host communities for dispersal, while adults focus on more stable, long-lived hosts that facilitate reproductive success (Sonenshine and Roe, 2013).

Table 2: Different types of tick species life cycles (Marzak, 1974)

Ticks	Cycle type	Host		
		Larvae	Pupae	Adults
<i>Ixodes ricinus</i>	Biphasic	Rodents	Rodents	Ungulates
	Telotropic	Insectivores	Insectivores	Carnivores
<i>Haemaphysalis punctata</i>	Triphasic	Birds Rodents	Birds Rodents	Ungulates
	telotropic	Hares Ungulates	Hares Ungulates	Carnivores
		Carnivores	Carnivores	
<i>Haemaphysalis sulcata</i>	Triphasic	Lizards Birds	Lizards Birds	Ungulates
	telotropic		Ungulates	carnivores
			Carnivores	
<i>Dermacentor marginatus</i>	Triphasic	Rodents Hares	Rodents Hares	Ungulates
	Ditropic			carnivores
<i>Hyalomma excavatum</i>	Triphasic	Rodents Hares	Rodents Hares	Ungulates
<i>Hyalomma dromedarii</i>	Ditropic			carnivores
<i>Hyalomma impeltatum</i>				
<i>Hyalomma lusitanicum</i>	Triphasic	Rabbit Garenne	Rabbit Garenne	Ungulates
	Ditropic			Carnivores
<i>Hyalomma marginatum</i>	Diphasic		Birds-hares	Ungulates
	Ditropic			
<i>Hyalomma scupense</i>	Diphasic		Ungulates	Ungulates
	Monotropic			
<i>Rhipicephalus sanguineus</i> (domestic population)	Triphasic	Dog	Dog	Dog
	Monotropic			
<i>Rhipicephalus sanguineus</i> (wild population)	Triphasic	Rodents	Rodents	Hares
	Ditropic			Hedgehogs
<i>Rhipicephalus turanicus</i>				



			Ungulates
			Carnivores
<i>Rhipicephalus bursa</i>	Diphasic	Ungulates	Ungulates
	Monotropic		
<i>Rhipicephalus annulatus</i>	Monophasic	Ungulates	
	Monotropic		

1.4 Distribution of Ixodid Ticks

Ixodidae are very closely associated with their environment. Numerous ecological factors condition the distribution and development of different species in their natural environment. These include climatic, hydrometric, and plant cover factors, as well as human action (Morel, 1969). Climate change resulting from certain human activities gives rise to increased frequency and severity of heat waves, heavy rains, and droughts inducing a modification of environmental conditions (Allen et al., 2018) that influences the distribution and availability of animals, which automatically affect the biology and the redistribution of ticks (McCoy and Boulanger, 2015).

Nevertheless, TBD epidemiology has changed radically in many parts of the world (Dantas-Torres et al., 2012; Ashagrie et al., 2023). Predictive models on the distribution of these ectoparasites assume that new tick species will invade new geographical areas where they have never been seen before (Nadolny and Gaff, 2018; Cox et al., 2021; Kopsco et al., 2022) or establish ranges in common localities, giving rise to epidemics and epizootics around the world (Morand et al., 2018). Examples include *Hyalomma marginatum* (Koch, 1844) permanently established in southern France and other Mediterranean regions of Europe, increasing the risk of spreading Crimean-Congo hemorrhagic fever (CCHF), a serious disease in humans (Stachurski and Vial, 2018), as well as *Haemaphysalis longicornis* Neumann, 1901, an Asian native currently dispersed in the USA (Raghavan et al., 2019).

However, in Algeria, there is a huge lack of knowledge about the diversity and distribution of ticks in most of the country, as most of the studies reported on the distribution of ixodid fauna have focused on the northern part of the territory (between 35°N and 36°N) (Mechouk et al., 2022).



1.4.1. *Dermacentor* Genus

The *Dermacentor* genus includes 32 (or 33, depending on the author) known species, mainly in North and Central America and Eurasia, with a few species in South America and Africa. In North Africa, *D. marginatus* shares the same habitat as *Ixodes ricinus* (Walker et al., 2003) and is distributed from the Mediterranean basin to the Asian steppes.

It is a quite thermophilic tick, found in open environments where temperatures are relatively high, as well as in forest environments, exclusively in clearings or along sun-dappled roads (Estrada-peña et al., 2004 ; Perez-Eid, 2007), moving mainly from holm oak stages at 300 meters to areas at 1200 meters where hazel and alder vegetation persists (Gilot et al., 1976). Being exophilic, the adults of this tick are commonly known as "horn and bun ticks", attaching themselves mainly to domestic ungulates (cattle, horses, small ruminants) and wild ungulates (wild boar), while the larvae and nymphs parasitize small mammals (McCoy and Boulanger, 2015).

In Algeria, it is the only species of this genus reported in the Tlemcen region on cattle, and more frequently on wild suids in the regions of Ain Témouchent, Oran, Tlemcen, Sénia and Mostaganem (Yousfi-Monod and Aeschlimann, 1986). It has also been found in Tiaret (Boulkaboul, 2003), Annaba, Skikda, Azzaba, El Harrouch, Guelma, Grarem, Constantine, El Khroub and Sétif (Bouderda and Meddour, 2006), as well as in Blida, Boumerdes, Bouira and Tizi Ouzou (Bedouhene et al., 2022) (Figure 6).

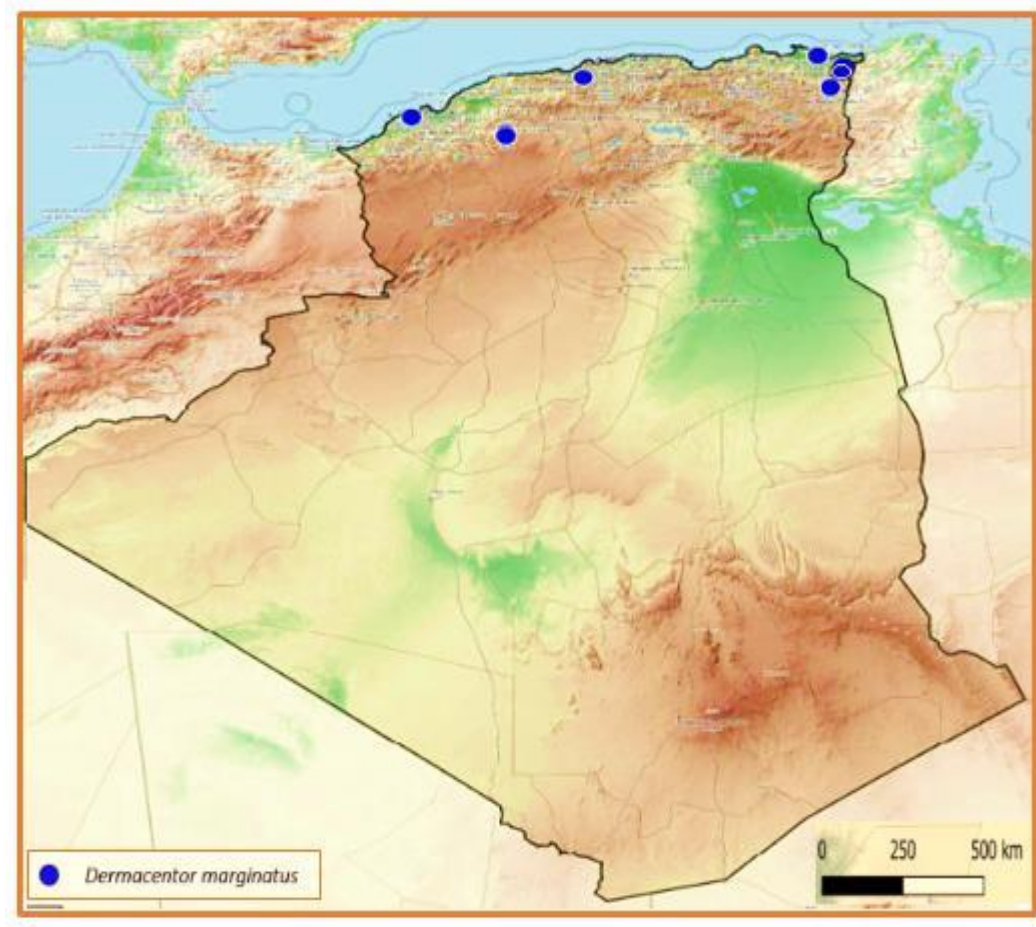


Figure 6: Geographical distribution of *Dermacentor marginatus* in Algeria [Adapted from Mechouk et al., (2022)]

1.4.2. *Haemaphysalis* Genus

Three species of the genus *Haemaphysalis* have been identified in Algeria (Figure 7):

-*Haemaphysalis punctata* is a triphasic tick that mainly infests cattle and sheep. It is distributed throughout the Mediterranean basin, Europe and Western and Central Asia (Bailly-Choumara et al., 1976). In Algeria, it is found in the northern part of the country, including Oran, Annaba and Tizi Ouzou, as well as in Tiaret, where *Haemaphysalis punctata* has been reported by Boulkaboul (2003) on cattle.

- *Haemaphysalis sulcata* is also a triphasic tick, infesting mainly sheep. It is widely distributed in North Africa, Europe and Asia (Pomerantzev, 1950 ; Estrada-peña et al., 2004). In Algeria, it is particularly localized in Setif (Bouchama et al., 2020).



-*Haemaphysalis erinacei* is a tick infesting mainly hedgehogs, In Algeria it was reported from a single inventory by Khaldi et al. (2012), collected from the desert hedgehog *Paraechinus aethiopicus* and the North African hedgehog *Atelerix algirus*, within the northern part of the country

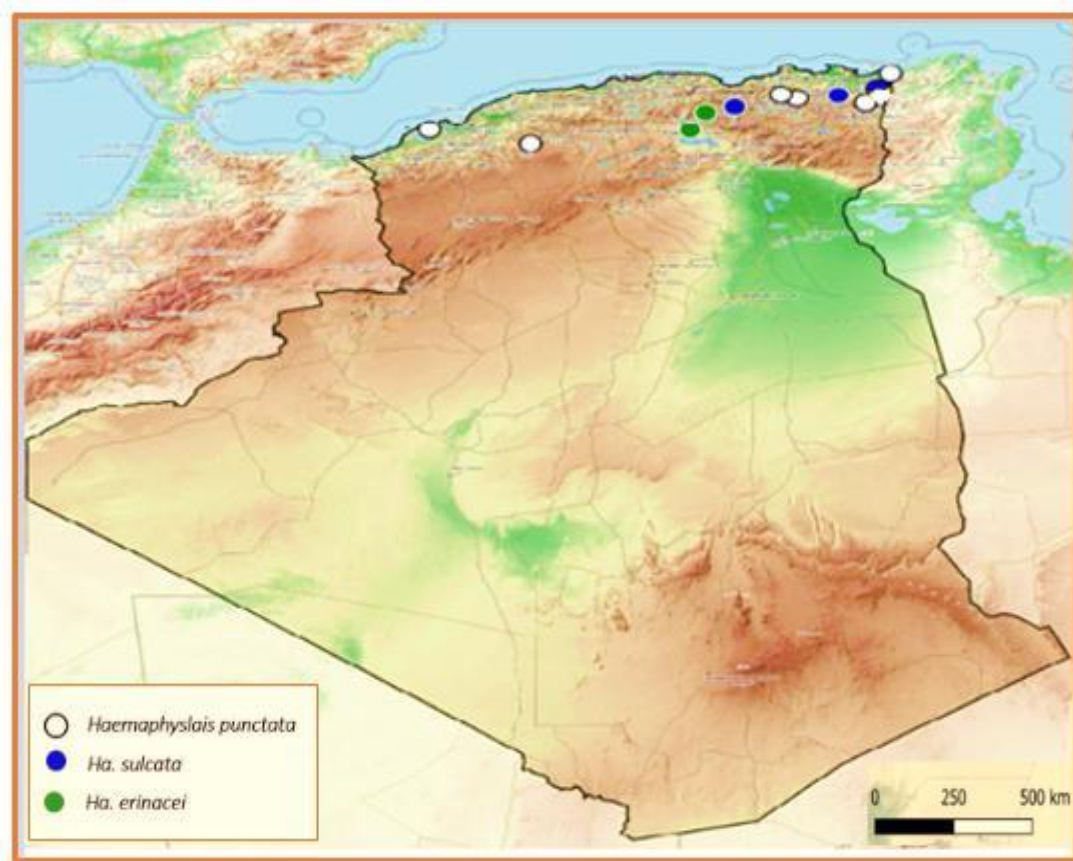


Figure 7: Geographical distribution of the *Haemaphysalis* genus in Algeria [Adapted from Mechouk et al., (2022)].

1.4.3. *Hyalomma* Genus

Hyalomma is a genus of ticks present in Asia, Europe, North Africa as well as South Africa. The genus is thought to have originally appeared in the region of Iran or the southern part of the former Soviet Union, then spread to Asia, including the Middle East, southern Europe, then Africa (Roberts et al., 2009). This genus is the widest in terms of the number of species recorded in Algeria, with 10 species (Figure 8).

Hyalomma aegyptium is a triphasic tick, for which turtles of the genus *Testudo* are the main hosts at all stages of development. It is widespread in the Mediterranean basin and the Black Sea (Široký et al., 2006), in humid, sub-humid, semi-arid and arid bioclimatic zones. This tick



has been identified in northern and interior regions such as Annaba, Skikda, Constantine and Guelma (Bouderda and Meddour, 2006), its range corresponding with that of terrestrial turtles (Mihalca et al., 2012).

Hyalomma anatolicum is a diphasic, sometimes triphasic, endophilic tick that parasitizes large ungulates, mainly cattle, horses, camels, sheep and goats (Bakheit et al., 2012). This tick is widespread in Africa, especially in eastern Libya, Egypt, the Near East and Asia (Estrada-peña et al., 2004). In Algeria, *H. anatolicum* has been reported in northern regions such as Annaba, Skikda and Jijel (Bouderda and Meddour, 2006 ; Derradj and Kohil, 2021), in the interior at Constantine, Guelma et Tadjnont (Bouderda and Meddour, 2006 ; Derradj and Kohil, 2022) and in the south of the country at Adrar (Bouhous et al., 2011).

Hyalomma dromedarii is a diphasic or triphasic tick. it mainly infests camels, even occasionally domestic mammals. its range extends to Mediterranean, steppe, and desert climates (Walker et al., 2003), which closely correlate with the geographical distribution of its main host, the dromedary. in Algeria, it has been reported in the south of the country (Bouhous et al., 2011).

Hyalomma excavatum is a diphasic, sometimes triphasic, exophilic tick which, in the adult stage, parasitizes domestic mammals, especially cattle and camels, and, in the immature stage, insectivores, lagomorphs and rodents. This tick species is known from North Africa, East Africa, Southern Europe, the Middle East and Central Asia (Estrada-peña et al., 2004 ; Bakheit et al., 2012). It is also found in the semi-desert steppes north of the Sahara and around the Arabian-Persian desert (Morel, 1969 ; Bailly-Choumara et al., 1976). In Algeria, it has been reported on cattle in the semi-desert and Saharan bioclimatic zone in the Biskra region (Bouderda and Meddour, 2006), in the north in Boumerdes, Bouira, and Tizi Ouzou (Bedouhene et al., 2022), in Tadjnanent (Derradj and Kohil, 2022), Setif (Bouchama et al., 2020), Oran (Yousfi-Monod and Aeschlimann, 1986), and Tiaret (Boulkaboul, 2003).

Hyalomma impeltatum is a diphasic or triphasic tick. In the adult stage, it parasitizes camelids and bovids, while the immature stages prefer leporids and murids (Guglielmone et al., 2014). It is widespread in the Palearctic region (Apanaskevich and Horak, 2009), inhabiting arid regions where rainfall conditions do not exceed 500 mm per year (Morel, 1965). In Algeria, it has been reported on livestock, especially dromedaries, in a few localities such as Oran, Tiaret, Tadjnont and Blida, but it is highly prevalent in southern Algeria, precisely in Biskra and Adrar (Bouderda and Meddour, 2006 ; Bouhous et al., 2011).



Hyalomma lusitanicum is a triphasic tick, parasitizing cattle and other domestic and wild ungulates. It is mainly localized in the western part of the Mediterranean sub-region of the Palaearctic zoogeographical region (Apanaskevich and Horak, 2009), in the sub-humid, humid and semi-arid zones (Bailly-Choumara et al., 1976). In Algeria, it has been found mainly in the north and interior of the country.

Hyalomma marginatum is a diphasic tick, parasitizing mammals in the adult stage, while immature forms prefer migratory birds ensuring transport across the Mediterranean basin (Hoogstraal and Kaiser, 1958 ; Jaenson et al., 1994). As a result, it has a wide geographical distribution that includes southern Europe and North Africa (Apanaskevich and Horak, 2009). In Algeria, *H. marginatum* has been recorded in almost all the surveys carried out.

Hyalomma rufipes is a diphasic exophilic tick. Adults parasitize cattle, sheep, goats, horses and camels, while immatures infest birds and leporids (Apanaskevich and Horak, 2009). In Algeria, it has been recorded in the work of Bedouhene et al. (2022) in Blida, Boumerdes, Bouira and Tizi Ouzou, and in that of Boulkaboul (2003) in Tiaret.

Hyalomma scupense mainly parasitizes cattle, as well as sheep and horses (Chauvet, 2004), and even dromedaries. It is widespread in the Mediterranean basin, the semi-desert steppes of the Near East and Central Asia (Bailly-Choumara et al., 1976). In Algeria, it has been recorded in almost all previous works in the country. *Hyalomma truncatum*, parasitizing domestic herbivores in the adult stage, while the immature stages infest hares and rodents (Walker et al.,



2003). In Algeria, this tick species was only reported in the work of Kernif et al. (2012) in the northern parts of the country.

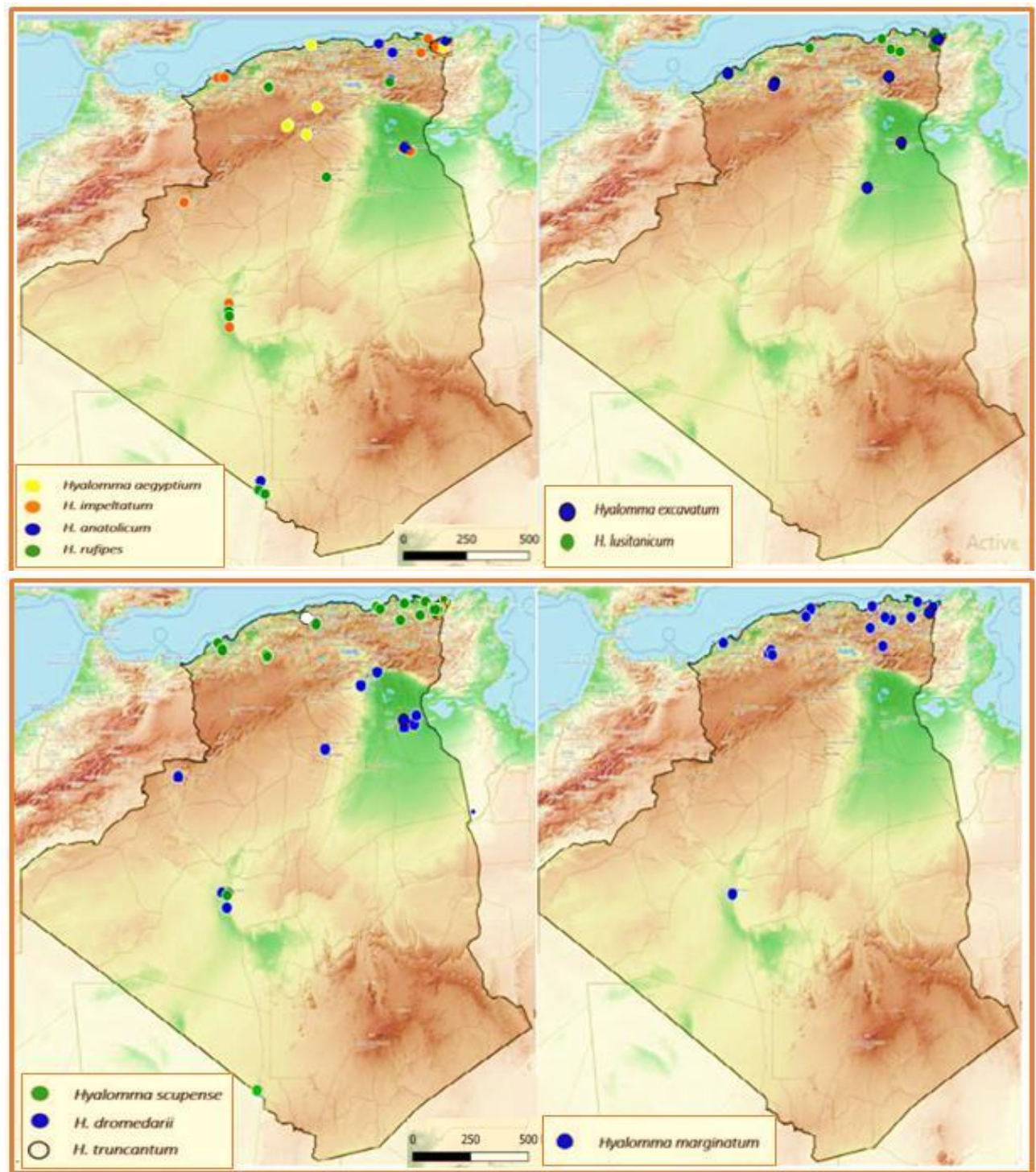


Figure 8: Geographical distribution of the *Hyalomma* genus in Algeria [Adapted from Mechouk et al., (2022)].



1.4.4. *Ixodes* Genus

The *Ixodes* genus is the most abundant of the hard ticks, with 243 species spread over all continents. These species are present in a variety of ecological niches and parasitize a very wide range of hosts, its geographic distribution is also spreading rapidly as a result of environmental change (Leger et al., 2013). This exophilic tick is generally associated with protected biotopes with abundant vegetation, at least for part of the season (Agoulon et al., 2012). In Algeria, four species of the genus *Ixodes* have been recorded (Figure 9):

Ixodes ricinus, which is a telotrophic tick. Immature stages parasitize birds and lizards, while mammals are the preferred hosts of adults (Estrada-Peña et al., 2018). Its distribution is limited to the northern part of Algeria, mainly in mountainous regions, sharing the same habitats or biotopes as *D.marginatus* (Mechouk et al., 2022).

Ixodes hexagonus is also a telotrophic species whose main hosts are carnivorous mammals and hedgehogs. Only two published studies are known about this tick, which has been collected on dogs and hedgehogs (Mechouk et al., 2022). In Algeria, its geographical distribution is in the north-east (Senaoui et al., 2020). However, given the lack of studies, it may be widespread at least in the northern region of the country.

Ixodes vespertilionis is a ditropic endophilic tick that parasitizes bats widely distributed in Europe (Hornok et al., 2014 ; Burazerović et al., 2015), China (Busha and Robbins, 2012), Iran (Hassan et al., 2010) and Turkey (Bursali et al., 2015), with a large variety of bat species as hosts for both stages. In Algeria, few studies have reported tick infestation of troglodyte bats (Bendjeddou et al., 2017) effectively localizing to the northeast of the country (Bendjeddou et al., 2016 ; Khelfaoui et al., 2018).

Ixodes inopinatus sensu Estrada-Peña et al. (2014) is a new species presenting in allopatry with *I. ricinus* in Spain and Portugal. Its geographical distribution reaches Morocco and Tunisia (Estrada-Peña et al., 2014), however recent studies indicate that it extends to central Europe, sometimes in sympatry with *I. ricinus*, *Ixodes inopinatus* is a telotrophic exophilic tick. Immature stages feed on lizards and adults on red foxes and sheep (Petney et al., 2015; Chitimia-Dobler et al., 2018).

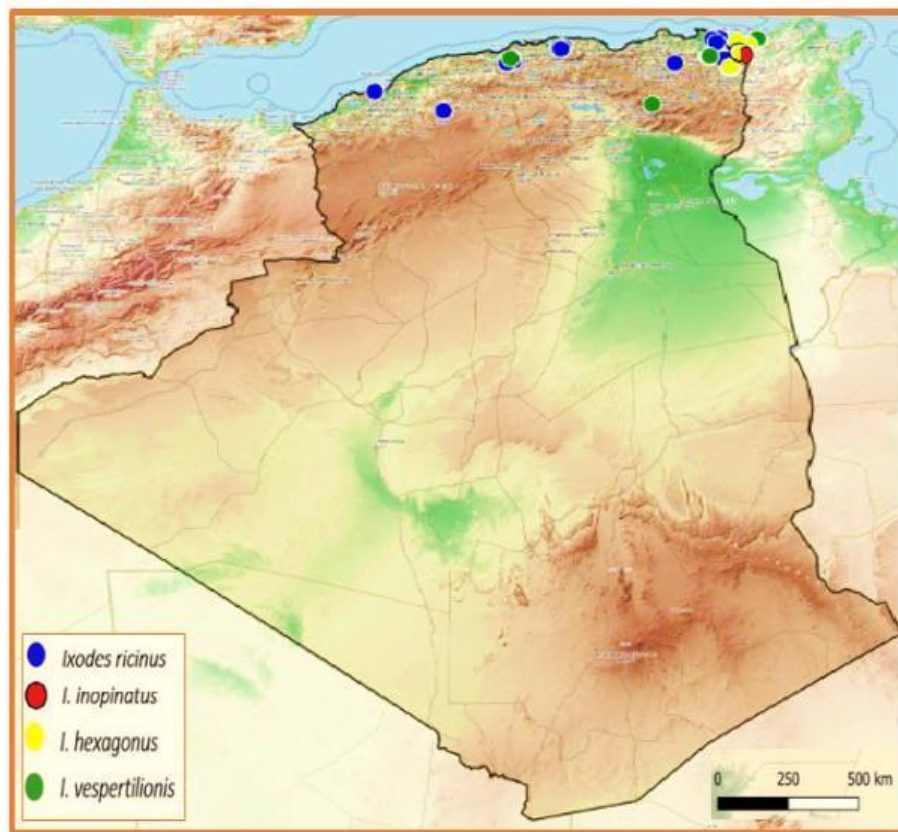


Figure 9: Geographical distribution of the *Ixodes* genus in Algeria [Adapted from Mechouk et al. (2022) modified].

1.4.5. *Rhipicephalus* Genus

The genus *Rhipicephalus* is distributed worldwide, predominantly between latitudes 50°N and 35°S (Walker et al., 2000), in regions with warm climates and mild winters. It is thus found mostly in Mediterranean, tropical and subtropical regions (Dantas-Torres, 2010). In Algeria, there are six species, all of which are relatively abundant in almost as many domestic hosts. This tick is monotropic at all stages of development, its preferred hosts being ruminants, but occasionally infestations of wild boar and cats are also often cited (Walker et al., 2003).

Rhipicephalus annulatus is mainly cited in most of the inventories carried out in the country, especially in the northern region, which is characterized by a predominantly Mediterranean climate (Mechouk et al., 2022).

Rhipicephalus sanguineus is an endophilic tick typically cohabiting in close proximity to its principal host, the dog, hence the name "brown dog tick". It may therefore colonize urban or rural areas, or even the very inside of homes, most often climbing walls, facades, or furniture,



hiding under rocks or carpets, or lying in wait on blades of herb (Uspensky and Ioffe-Uspensky, 2002). In Algeria, it is the most species cited of the inventories (Mechouk et al., 2022).

Rhipicephalus turanicus are morphologically very close to *R. sanguineus*. These species are therefore likely to be found in sympatry in the Mediterranean basin (Walker et al., 2003). In Algeria, this species shares almost the same habitats with *R. sanguineus*.

Rhipicephalus bursa is a diphasic tick that feeds on various mammals. It is found around the Mediterranean Sea (Walker et al., 2000). In Algeria, its distribution seems to be almost limited to the northeastern region of the country, reported in Skikda, Tébessa, Batna, Guelma, Constantine (Bouderda and Meddour, 2006), Jijel (Benchikh-Elfegoun et al., 2007), and even in Tiaret where the semi-arid climate is favorable (Boulkaboul, 2003).

Rhipicephalus evertsi and *Rhipicephalus guilhoni* are telotrophic ticks that feed on cattle and wild ungulates. They are widespread in all African countries, with a preference for the Afrotropical zoogeographical region (Walker et al., 2000). In Algeria, *R. evertsi* and *R. guilhoni* have been reported sporadically on sheep and camels in the south of the country by Bouhous et al., (2011).

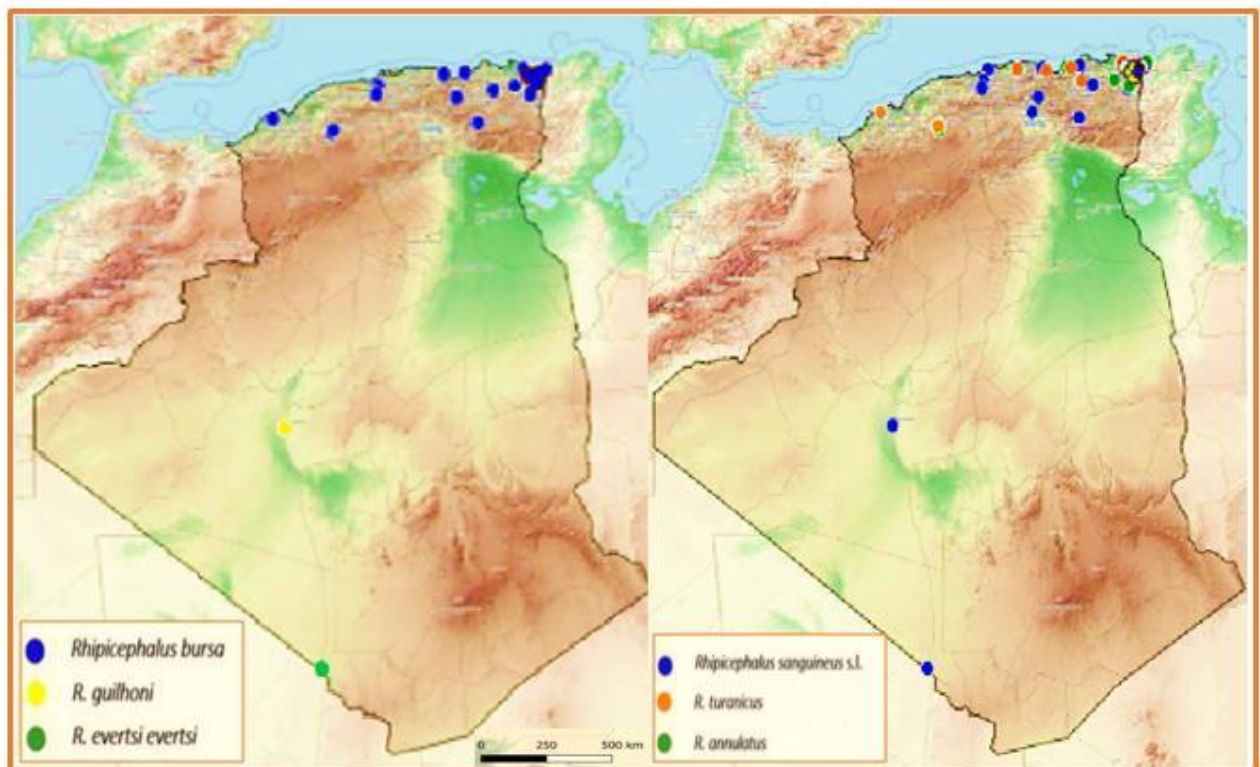


Figure 10: Geographical distribution of the *Rhipicephalus* Genus in Algeria [Adapted from Mechouk et al. (2022)]



1.5 The pathogenic impact of Ixodid ticks

1.5.1. Direct pathogenic role of Ixodid ticks

Tick infestations impact host organisms through various mechanisms. The initial penetration of tick mouthparts involves a mechanical action, where the chelicerae lacerate the epidermis, followed by tissue degradation at the bite site due to anticoagulant saliva (Laamri et al., 2012). The bite itself is caused by the rostrum, which includes a toothed hypostome. Often, tick bites go unnoticed as their saliva contains anesthetic and antihistaminic compounds. Consequently, most bites do not provoke a noticeable local inflammatory response (Mutz, 2009). However, in some cases, tick bites can lead to localized reactions such as papules, ulcers, or eschars. Additionally, they can cause hypersensitivity, irritation, inflammation, and physical damage, particularly in domestic animals (Mercier, 2016).

Some tick species are capable of inflicting toxic bites. In certain cases, females produce neurotoxic substances during ovogenesis, leading to "tick toxicosis," which should be distinguished from false paralysis caused by edema-induced muscle pain or nerve compression at the attachment site (Mercier, 2016). According to Mutz (2009), over 40 tick species worldwide can induce paralysis, which can be fatal, particularly in livestock such as cattle and sheep. Tick paralysis manifests as an ascending flaccid paralysis that appears 2 to 7 days after tick attachment, beginning with extreme fatigue, leg numbness, difficulty walking or standing, and muscle pain. If the tick is not removed, the condition may progress to affect the tongue and facial muscles. Severe complications include seizures, respiratory failure, and, in 12% of untreated cases, fatal paralysis.

Another major consequence of tick infestations is blood loss. While individual blood meals may be minimal, chronic or heavy infestations can significantly impact the host's overall health, leading to anemia and reduced productivity (Laamri et al., 2012; Wikel, 2018). Additionally, tick saliva plays a critical role in modulating host immune responses, facilitating prolonged feeding, and creating a conducive environment for pathogen transmission and establishment within the host. This field of research is advancing rapidly, shedding light on the complex interactions between ticks, their hosts, and the pathogens they transmit (Chmela et al., 2016; Wikel, 2018).



1.5.2. Indirect pathogenic role of Ixodid ticks

Ticks are the most diverse arthropod vectors of infectious diseases, transmitting a wide array of pathogens that affect both humans and animals worldwide (Jongejan and Uilenberg, 2004; Pfäffle et al., 2013). Between 1940 and 2004, over 60% of emerging human infectious diseases were classified as zoonoses, with 71.8% originating from wildlife and 22.8% being vector-borne arthropod diseases (WHO et al., 2004; Ashagrie et al., 2023). Among these, tick-borne diseases -including Lyme disease, tularemia, granulocytic anaplasmosis, ehrlichiosis, Crimean-Congo hemorrhagic fever, tick-borne encephalitis, and various rickettsioses- have a profound impact on global public health, contributing to significant morbidity, mortality, and economic burdens (Jones et al., 2008).

Zoonoses are infections naturally transmitted between humans and vertebrate animals (Schwabe, 1969), and their prevalence has been increasing over the last decade. Tick-borne diseases, in particular, have seen a resurgence, with previously known diseases re-emerging and new pathogens being identified (Socolovschi et al., 2008). The expansion of these diseases is driven by multiple intrinsic and extrinsic factors, including tick and pathogen demography, climatic variations at both micro and macro scales, human activities (travel, land use changes, and habitat fragmentation), economic and political shifts, and population dynamics (Baneth, 2014; Dantas-Torres, 2015). Given their complexity, the World Health Organization (WHO) has advocated for the "One Health" approach, an interdisciplinary strategy integrating human and veterinary medicine to enhance zoonotic disease control (Schwabe, 1969).

The epidemiology of tick-borne pathogens (TBPs) is particularly intricate due to the involvement of multiple hosts and environmental factors. Ticks undergo a three-stage life cycle -larva, nymph, and adult- each requiring a blood meal, which facilitates pathogen acquisition and transmission. Wildlife, domestic animals, livestock, and humans all play roles in maintaining and spreading these pathogens (Madison-Antenucci et al., 2020).

Tick-borne diseases impose substantial economic losses, particularly in livestock production. Theileriosis, babesiosis, and anaplasmosis are among the most significant vector-borne diseases affecting cattle, leading to reduced milk production, weight loss, reproductive issues, and mortality (Hove et al., 2018; Okafor et al., 2018). In North Africa, bovine theileriosis is primarily caused by *Theileria annulata*, while bovine babesiosis is associated with *Babesia bovis*, *B. bigemina*, and *B. divergens* (Gharbi et al., 2006). In Algeria, ovine babesiosis is mainly



due to *Babesia ovis* and *B. motasi*, leading to clinical manifestations such as fever, anorexia, anemia, jaundice, and high mortality rates (Darghouth, 2004; Aouadi et al., 2017; Esmailnejad et al., 2014; Sevinc et al., 2018). The primary vectors of these parasites include *Rhipicephalus bursa*, *R. annulatus*, and *Hyalomma scupense*, with over half of diagnosed piroplasmosis cases attributed to tropical theileriosis (Sergent et al., 1945; Ziam and Benaouf, 2004; Benchikh Elfegoun et al., 2018).

Anaplasmosis and ehrlichiosis, caused by intracellular bacteria from the *Anaplasmataceae* family, affect a broad range of hosts, including cattle, sheep, dogs, and humans. The predominant species in bovine anaplasmosis is *Anaplasma marginale*, a pathogen widely distributed in tropical and subtropical regions (Kocan et al., 2003; Hanzlicek et al., 2016). Other species such as *A. phagocytophilum*, *A. centrale*, and *A. bovis* also infect cattle. In Algeria, *Ehrlichia canis*, a major cause of canine ehrlichiosis, has been reported in domestic animals (Dahmani et al., 2015; Bessas et al., 2016; Rjeibi et al., 2018).

Rickettsial diseases are among the oldest known vector-borne infections. In Algeria, nine *Rickettsia* species from the spotted fever group (SFG) have been identified, including *R. conorii* subsp. *conorii*, *R. aeschlimannii*, *R. massiliae*, *R. monacensis*, *R. africae*, *R. sibirica* subsp. *mongolitimonae*, *R. slovaca*, *R. helvetica*, and *R. felis* (Bitam et al., 2006; Mouffok et al., 2009; Kernif et al., 2012; Mokrani et al., 2012; Dib et al., 2019). Mediterranean spotted fever, a well-documented tick-borne illness, is endemic to the region (Dib et al., 2019).

Q fever, caused by the intracellular bacterium *Coxiella burnetii*, is another globally distributed zoonosis with significant public health implications. The primary reservoirs of *C. burnetii* are domestic ruminants, including cattle, sheep, and goats (Arricau-Bouvery and Rodolakis, 2005). Although ticks play a role in the ecology of this pathogen, they are not recognized as primary vectors. In Algeria, Q fever was first identified in French soldiers in 1948, and subsequent serological studies have confirmed high infection rates among residents (Pierrou et al., 1956; Lacheheb and Raoult, 2009).

Despite the high socio-economic burden of tick-borne diseases and their impact on public and animal health, research on ticks and TBPs in Algeria remains limited. Most studies focus on the northeastern region of the country (Bitam et al., 2006; Azzag et al., 2015; Bessas et al., 2016; Benchikh Elfegoun et al., 2018; Abdelkadir et al., 2019; Ziam et al., 2019; Sadeddine et al., 2020; Foughali et al., 2021). Effective control programs require a comprehensive understanding



of the epidemiology of these infections, including their geographic distribution, seasonal variations, host preferences, and vector competence. Expanding research efforts and surveillance in Algeria and neighboring regions is essential for mitigating the impact of tick-borne diseases on human and animal populations.

1.6 Strategies for tick control

Effective tick control requires robust acarological surveillance and a comprehensive strategy that targets all stages of the tick life cycle while actively involving local communities. When selecting the most suitable control method or combination of methods, several factors must be considered, including the ecological characteristics and behavior of the target species, available resources, cultural context, public perception and participation, feasibility of implementation, and the necessary coverage level (Dolan, 1991; Deken et al., 2012; Bishop et al., 2023).

Tick control measures can target either larvae or adults (Carnevale, 1995). However, controlling immature ticks is particularly effective in preventing tick-borne disease transmission, as larvae tend to be more localized, less mobile, and occupy smaller habitats than adult ticks (Elsheikha, 2017).

According to WHO (1999), tick control methods can be categorized as follows:

1.6.1. Environmental management

Environmental management plays a crucial role in reducing tick populations by altering their natural habitats and limiting favorable conditions for their proliferation. This approach focuses on modifying landscapes, managing host populations, and implementing targeted interventions to disrupt tick life cycles and reduce their density in specific environments (Eisen and Stafford, 2021; Piesman and Eisen, 2008).

One of the key strategies in environmental management is habitat modification, which involves altering or eliminating areas where ticks thrive. Since ticks rely on specific microhabitats for survival, particularly those with high humidity and dense vegetation, modifying these environments can significantly reduce their abundance. For instance, clearing dense vegetation, trimming grass, and removing leaf litter in grazing and residential areas can decrease the humidity required for tick survival, thereby limiting their ability to establish populations (Maupin et al., 1991; Ginsberg and Stafford, 2014).



Another essential aspect of environmental management is breeding site control, which involves strategies to disrupt tick reproduction and prevent population expansion. In livestock areas, managing breeding sites by improving drainage, maintaining clean stable walls, and regularly removing organic waste can reduce the number of suitable locations for tick larvae and nymphs to develop. Additionally, slow-burning of waste near stable walls can help eliminate tick eggs and immature stages, decreasing their overall numbers in farming environments (Yadav and Upadhyay, 2021).

Rotational grazing and pasture management are also effective in controlling tick populations. By alternating and rotating grazing between different fields, livestock exposure to tick-infested areas can be minimized, allowing time for natural tick predators or environmental factors to reduce tick numbers in the resting pastures. Burning pastures is another widely used strategy that helps eliminate ticks present in the vegetation, particularly in areas where high infestations occur seasonally. However, the ecological impact of pasture burning must be carefully evaluated to avoid unintended consequences, such as soil degradation or loss of beneficial organisms (Duffy et al., 1994; McKay et al., 2020).

A promising natural approach to environmental management is the cultivation of tick-repellent plants, which act as biological deterrents. Certain plant species produce volatile compounds that repel ticks or interfere with their ability to locate hosts. For example, neem (*Azadirachta indica*), lantana (*Lantana camara*), and certain aromatic herbs like rosemary and lavender have been reported to possess acaricidal properties that can help reduce tick abundance in livestock and residential areas. Integrating such plants into grazing pastures or around animal enclosures may serve as a sustainable, eco-friendly method of tick control (Gareh et al., 2022 ; Gaid et al., 2024)

By implementing a combination of these environmental management strategies, tick populations can be effectively suppressed while minimizing reliance on chemical acaricides. A well-planned approach considers local ecological conditions, the behavior of host animals, and the long-term sustainability of intervention measures to ensure effective tick control while preserving environmental balance (Benjamin et al., 2002 ; Lindsay et al., 2015)



1.6.2. Biological control

Biological control involves leveraging natural predators, parasites, or pathogens to regulate tick populations in a sustainable manner. This method aims to reduce reliance on chemical acaricides and mitigate their associated environmental and resistance-related concerns (Samish et al., 2004).

One approach is the introduction of natural tick predators, such as certain species of ants and birds, which have been observed preying on ticks in various ecosystems. For instance, birds like oxpeckers (*Buphagus spp.*) consume ticks from livestock, while some ant species actively hunt ticks on the ground. However, the effectiveness of these biological agents is limited by potential drawbacks. Some predatory ants can inflict painful stings, causing distress to livestock and humans, while oxpeckers may inadvertently cause skin injuries on their hosts, increasing the risk of secondary infections and attracting myiasis-causing agents (Ostfeld et al., 2006; Mvumi et al., 2021).

Another promising strategy involves the use of entomopathogenic fungi and bacteria that infect and kill ticks. Several fungal species, including *Metarhizium anisopliae* and *Beauveria bassiana*, have demonstrated pathogenicity against ticks by penetrating their exoskeleton and disrupting physiological processes. Similarly, bacterial pathogens such as *Bacillus thuringiensis* (Bt) have shown potential in tick control. Bt produces toxic proteins that are lethal to various arthropods. However, unlike insects that ingest these bacterial spores, ticks feed exclusively on blood, making the application of Bt challenging in natural settings. The need for direct exposure to these microbial agents limits their field effectiveness compared to their well-established role in controlling other pests (Manjunathachar et al., 2014; Nwanade et al., 2022).

Despite these challenges, biological control remains an area of active research. Future advancements may focus on optimizing microbial formulations, enhancing delivery mechanisms, and integrating these methods into broader tick management strategies to improve long-term efficacy while minimizing environmental impacts (Samish et al., 2004).



1.6.3. Genetic control

Genetic control strategies for managing tick populations encompass two primary approaches: the release of sterilized male ticks and the induction of infertility through natural hybridization between closely related species (Deken et al., 2012).

Sterile insect technique (SIT): This method involves mass-rearing ticks in laboratory settings, followed by sterilization commonly achieved through gamma irradiation. The sterilized males are then released into the wild, where they mate with wild females, resulting in no offspring and a subsequent decline in the tick population. This technique has been explored in various studies, including applications on the tick *Hyalomma excavatum* (Serkan et al., 2013). Additionally, research has demonstrated that silencing specific genes, such as subolesin, can render male ticks sterile, preventing successful mating and reducing tick populations (De La Fuente et al., 2006).

Natural hybridization: In regions where closely related tick species coexist, natural hybridization can occur, leading to hybrids with reduced fertility or viability. For instance, hybridization events have been documented between *Dermacentor andersoni* and *Dermacentor variabilis* in North America (Araya-Anchetta et al., 2013), as well as between *Ixodes persulcatus* and *Ixodes pavlovskyi* in Western Siberia. While these occurrences are primarily natural, understanding the mechanisms and outcomes of such hybridization events could inform potential genetic control strategies (Kovalev et al., 2015).

Advancements in genetic research, including the identification of sex-determining genes in ticks, pave the way for innovative pest control methods. For example, recent studies have identified genetic markers associated with sex determination in cattle fever ticks, opening avenues for targeted genetic interventions (Kimberly, 2025). These developments hold promise for more effective and sustainable tick management strategies in the future.

1.6.4. Chemical control

Chemical control remains one of the most widely employed strategies for tick management, primarily through the use of acaricides chemical agents specifically designed to eliminate ticks. These substances belong to several major classes, including organochlorines,



organophosphates, pyrethroids, and carbamates, all of which disrupt the tick's nervous system by interfering with neurotransmission (Mouchet, 1980).

Acaricides can be applied through various methods, depending on the target and the environment. Direct application to animals includes topical treatments such as powders, sprays, lotions, medicated baths, and impregnated collars designed to provide prolonged protection. Systemic acaricides, administered through the bloodstream, work by affecting ticks that feed on treated hosts. Environmental control measures involve the use of fumigants in enclosed spaces to eliminate tick populations in animal housing, while powders, liquid suspensions, and granules are commonly applied in open areas where ticks thrive (Mvumi et al., 2021; Bishop et al., 2023).

Despite their effectiveness, the widespread use of acaricides raises significant ecological and health concerns. These chemicals tend to persist in the environment, leading to contamination of soil and water, which can have detrimental effects on non-target organisms, including beneficial arthropods and vertebrates. Moreover, prolonged exposure to acaricides accelerates the emergence of resistant tick populations, making future control efforts increasingly challenging. To mitigate these risks, the integration of chemical control with other management approaches, such as biological and environmental strategies, is essential for sustainable tick control programs (Agwunobi et al., 2021).

1.6.4.1.Synthetic acaricides and their mechanisms of action

Since the early 1890s, various synthetic acaricides have been developed and widely used for tick control. These compounds belong to multiple chemical classes, including arsenicals, chlorinated hydrocarbons, organophosphates, carbamates, formamidines, pyrethroids, macrocyclic lactones, and, more recently, insect growth regulators (Adenubi et al., 2018). Typically administered through spraying, pouring, or injection, these treatments have imposed significant costs, particularly on livestock farmers (Abbas et al., 2018). Arsenic-based compounds were among the first acaricides introduced and were extensively used between the late 19th and early 20th centuries. While they were affordable, water-soluble, and relatively stable, their short residual efficacy, high toxicity, and the emergence of resistance in *Boophilus* ticks led to their eventual prohibition (George et al., 2004). This ban prompted the introduction of organochlorine acaricides in the mid-20th century, including DDT, BHC, lindane, dieldrin, and toxaphene in the 1940s. Although these compounds were biodegradable and rapidly



metabolized, their efficacy diminished quickly, and they had severe ecological consequences, particularly in disrupting predator populations within the food chain (Sager et al., 2018; Heath and Levot, 2015). By the 1960s, organophosphates and carbamates replaced organochlorines as primary tick control agents due to their improved biodegradability. However, their high acute toxicity to livestock and the rapid development of resistance, first reported in 1963, necessitated the introduction of new compounds, such as formamidines like amitraz, in the 1970s (George et al., 2004). From the 1980s onwards, the demand for acaricides with lower mammalian toxicity led to the widespread adoption of pyrethrins and certain biopesticides, including macrocyclic lactones like avermectins and milbemycins (Mitchell, 1996). Pyrethrins, derived from natural sources, were soon replaced by synthetic pyrethroids, which underwent successive modifications to enhance stability and residual effect. Pyrethroids are classified into four generations: the first generation (allethrin), the second generation (tetramethrin, resmethrin, bioresmethrin, biolaethrin, and fontarin), the third generation (fenvalerate and permethrin), and the fourth and current generation (Matsuo, 2019). By the late 20th century, growth regulators such as benzoyl-phenyl urea derivatives like fluazuron and phenylpyrazoles like fipronil emerged as newer acaricide classes. Spinosad, a natural acaricide derived from the fermentation of *Saccharopolyspora spinosa*, also gained prominence due to its unique mode of action (Abbas et al., 2018; Sager et al., 2018). More recently, isoxazolines have been introduced into the ectoparasiticide market, particularly for companion animals (McTier et al., 2016). Despite continuous development, synthetic acaricides face major challenges, primarily due to the rapid evolution of tick resistance. Resistance mechanisms can lead to cross-resistance, reducing the efficacy of acaricides even in tick populations that have never been directly exposed to certain compounds (Waldman et al., 2023). Additionally, the number of novel acaricides entering the market remains limited (Asahi et al., 2018). Beyond resistance, acaricides pose significant environmental and toxicological risks. Many of these compounds persist in soil, water, and air, contributing to widespread contamination. Furthermore, their neurotoxic effects can disrupt physiological processes such as growth, reproduction, and metabolism in non-target organisms, including humans (Heath and Levot, 2015; Benelli et al., 2016). Prolonged exposure has been linked to developmental disruptions in embryos, fetuses, and children (Kolaczinski and Curtis, 2004; Air Parif, 2016).

As a result of these concerns and the growing interest in organic farming practices, several acaricides, including organochlorines, organophosphates, and pyrethroids, have been increasingly restricted on the global market (Figure 11) (Table 3) (Ellse and Wall, 2014).

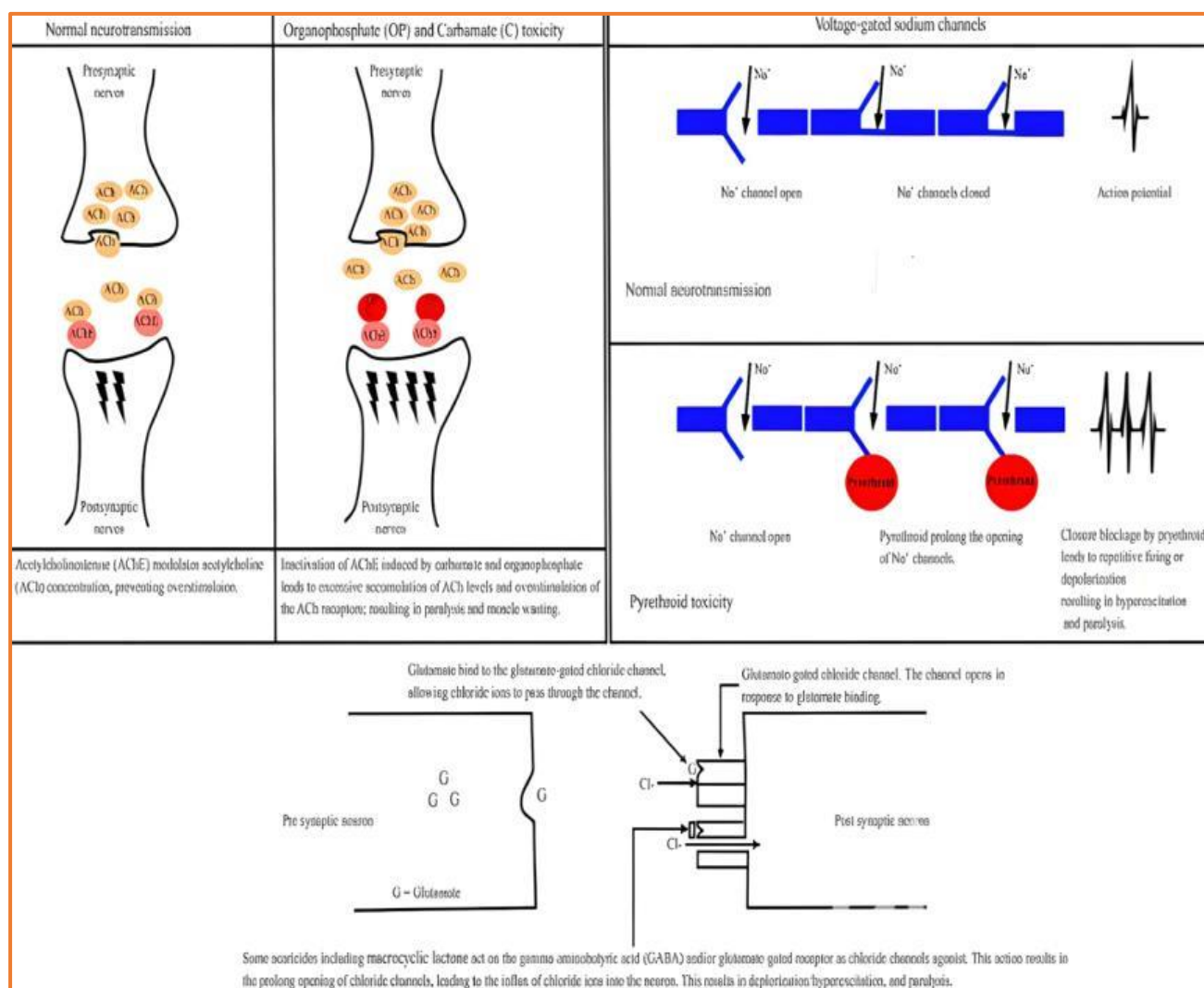


Figure 11: Mechanisms of action of organophosphate, carbamate, pyrethroid, and macrocyclic lactone (Nwanade et al., 2022)

Table 3: Chemical classes of acaricides and their modes of action.

Acaricidal Class	First Year of Use	Mode of Action	References
Arsenic	1893	They inhibit pyruvate dehydrogenase, compete for phosphate, and uncouple oxidative phosphorylation, thereby inhibiting energy-dependent reduction of nicotinamide adenine dinucleotide, mitochondrial respiration, and adenosine triphosphate synthesis, leading to death.	(Reshma and Prakasan, 2020)
Organochlorines	1946	GABA-gated chloride channel antagonists Bind at the	(Abbas et al., 2014)



Organophosphates	1955	microtoxinin site in the γ -aminobutyric acid (GABA) chloride ionophore complex. Acetylcholinesterase Inhibits the action of acetylcholinesterase Competes with octopamine for its receptor site, guanosine diphosphate is replaced with guanosine triphosphate, inducing	(Singh et al., 2014) (Abbas et al., 2014)
Formamidines (Amitraz)	1975	the production of cyclic adenosine monophosphate leading to inhibition of attachment and ultimately blood feeding with eventual death.	
Pyrethroids	1977	GABA-gated chloride channel antagonists Prolong the opening of sodium channels in nerves, muscles, and other excitable cells.	(Matsuo, 2019)
Macrocyclic lactones	1981	Bind to GABA and glutamate-gated chloride channels (GluCl) thereby opening chloride channels in nerves, resulting in disruption of activity and loss of function in these cells leading to paralysis and death.	(George et al., 2004)
Phenylpyrazoles (Fipronil)	1993	Blocks the gamma-Aminobutyric acid-gated chloride ion channel (GABA-C)	(Sparks and Nauen, 2015)
Benzoylphenyl urea (Fluazuron)	1990	Disturbs cuticle formation Blocks the incorporation of radiolabeled N-acetylglucosamine	(Sparks and Nauen, 2015)
Spinosad (Tetracyclicmacrolide compounds)	2001	Nicotinic acetylcholine receptors (nAChRs) γ -amino-butyric acid (GABA) receptors Hyperexcitation and disruption of an insect's nervous system	(Nwanade et al., 2022)
Isoxazolines	2014	Inhibits GABA-gated chloride ion channels	(McTier et al., 2016)

1.6.4.2. Tick resistance to synthetic acaricides

Tick resistance to synthetic acaricides is a growing concern in livestock and public health, as pesticide resistance leads to repeated failures in tick control despite proper application (Rodriguez-Vivas et al., 2017; IRAC, 2018). This resistance arises from the excessive or improper use of acaricides, driving the selection of resistant individuals and the evolution of resistant populations. The first documented case dates back to 1936 when *Rhipicephalus*



(Boophilus) microplus, the primary tick species affecting livestock, developed arsenic resistance due to its extensive use between 1890 and 1910 (Legg, 1947) (Figure 12). Since then, resistance has been increasingly reported worldwide, with frequent studies identifying multiple acaricide-resistant strains (Fernández-Salas et al., 2012; Muyobela et al., 2015; Fular et al., 2018). Resistance development is particularly concerning as most acaricides rely on single active compounds, making them more vulnerable to resistance selection (Mvumi et al., 2021). Reports from various countries confirm resistance in major tick vector species, with cases documented against nearly all synthetic acaricides (Abbas et al., 2014).

In parasitology, four main types of resistance are recognized. Natural resistance, which is not induced by acaricide exposure, results from the tick's impermeable cuticle and is not heritable (Githaka et al., 2022). Acquired resistance occurs through a hereditary decrease in acaricide sensitivity over time (Abbas et al., 2014; Adebe and Kebede, 2018). Cross-resistance arises when resistance to one acaricide extends to others with similar modes of action (Heath and Levot, 2015), whereas multiple resistance refers to resistance against acaricides with different mechanisms of action (Waldman et al., 2023). The emergence of resistance and subsequent cross-resistance has contributed to the resurgence of disease-transmitting tick populations in many regions. Several factors drive resistance development, including the frequency of acaricide application, dosage, and product persistence. Additionally, ticks possess biological and genetic traits that facilitate resistance selection, such as high mutation rates and ecological adaptability, while operational factors such as poor management practices further exacerbate the issue (Mvumi et al., 2021).

Resistance mechanisms in ticks fall into three main categories. Cuticular resistance occurs when thickening or chemical modifications of the cuticle reduce acaricide penetration. Metabolic resistance results from changes in tick enzymatic systems that enhance detoxification or sequestration of the acaricide. Target site resistance arises when genetic mutations alter the sites where acaricides bind, reducing their efficacy. Among these, metabolic and target site resistance are the most studied, as they play a crucial role in acaricide failure (Waldman et al., 2023). Understanding these mechanisms is essential for developing effective tick control strategies and mitigating the impact of acaricide resistance.

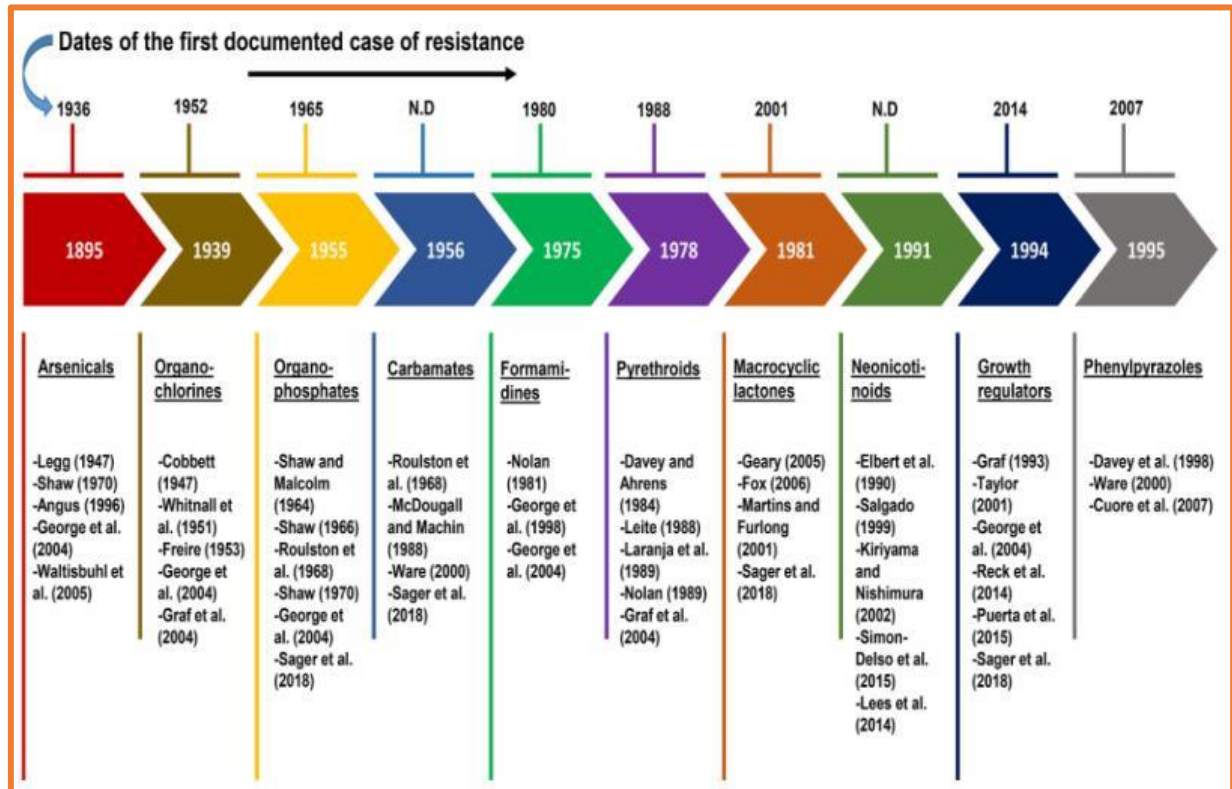


Figure 12: Chronology of chemical acaricide development and the onset of tick resistance.

N.D: Not determined (Agwunobi et al., 2021).

According to Sparks and Nauen (2015), a total of 586 arthropod species have developed resistance to pesticides, with resistance reported for 325 different pesticide molecules:

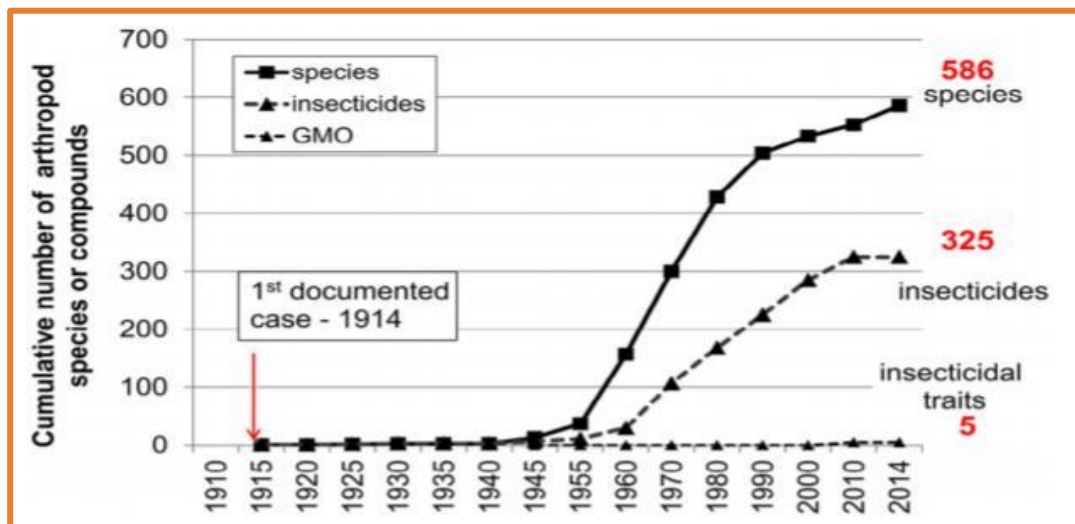


Figure 13: Cumulative number of pesticide-resistant arthropod species, resistant pesticides, and GMOs with reported resistance (Sparks and Nauen, 2015).



1.6.5. Plant-based tick control

Resource-limited farmers have traditionally relied on plant materials for the treatment and control of parasitic, viral, and bacterial diseases in livestock (Radsetoulalova et al., 2017; Sharifi-Rad et al., 2017). The knowledge of plant-based treatments has been passed down through generations, particularly in rural areas, with variations depending on local flora availability (Abbas et al., 2018). However, the scientific validation of pesticidal plants in veterinary parasitology remains a relatively recent research field (Figure 14). Expanding the range of plant extracts for ectoparasite control could not only enhance treatment options but also reduce the risk of overharvesting endangered plant species (Ellse and Wall, 2014).

Plants have developed diverse chemical and physical defense mechanisms against pathogens and pests, primarily through the production of secondary metabolites such as phenols, polyphenols, terpenoids, and alkaloids. These bioactive compounds can be extracted using various methods, including maceration, organic solvent extraction, supercritical fluid extraction, and distillation techniques (Dubey, 2010 ; Selles et al., 2021). The growing interest in plant-derived biopesticides is reflected in the publication of 7,177 research articles between 1980 and 2011 on neem and essential oils, with 13.63% of these studies focused on their efficacy against ticks, reporting effectiveness ranging from 5% to 100% (Isman and Machial, 2006; Isman and Grieneisen, 2014; Ntalli et al., 2019). More than 200 plant species have demonstrated acaricidal properties, with 55 species from 22 botanical families exhibiting both direct toxicity and growth inhibition against ticks. The most frequently studied families include Lamiaceae (20%), Asteraceae (13%), Rutaceae and Fabaceae (9%), and Solanaceae (7%). Additionally, 27 plant species from 18 families possess repellent properties, particularly those from Asteraceae (15%) and Lamiaceae (11%). Regarding larvicidal activity, 40 plant species from 19 families have been identified, with Lamiaceae (25%) being the most represented, followed by Asteraceae and Poaceae (10% each) (Adenubi et al., 2016; Mvumi et al., 2021; Selles et al., 2021). Many of these plant species also affect tick reproduction by inhibiting oviposition and egg hatching. Essential oils, primarily extracted from aerial plant parts (63%), along with ethanol (22%), methanol, and hexane extracts (7%), have shown promising acaricidal activity (Adenubi et al., 2016; Mvumi et al., 2021; Selles et al., 2021).

Essential oils contain a complex mixture of volatile compounds, including acids (e.g., geranic acid, benzoic acid), hydrocarbons (e.g., pinene, limonene), alcohols (e.g., linalool,



sandalwood), aldehydes (e.g., cuminal, citral), esters (e.g., geranyl acetate), phenolic ethers (e.g., anethole), phenols (e.g., eugenol), oxides (e.g., 1,8-cineole), ketones (e.g., camphor), and lactones (e.g., bergapten) (Andrade et al., 2011). Due to their lipophilic nature, these compounds can penetrate cutaneous membranes and the blood-brain barrier, exerting various biological effects such as repellent activity, inhibition of chitin formation, nervous system disruption via acetylcholinesterase inhibition, interference with the octopaminergic system, anti-feeding effects, hormonal dysregulation, and reproductive interference (Adorjan and Buchbauer, 2010; Benelli et al., 2016).

The acaricidal potency of essential oils is primarily attributed to their bioactive constituents, which act synergistically or antagonistically depending on their concentrations (Boldbaatar et al., 2014; Abbas et al., 2018). According to Katoch et al. (2007), combining bioactive phytochemicals from multiple plant species enhances efficacy by reducing the LD50 and minimizing the likelihood of resistance development. Unlike synthetic pesticides, plant extracts contain a complex array of compounds, which may slow the evolution of resistance in arthropods. Vollinger (1987) reported that insects rapidly develop resistance to synthetic pesticides, yet after 42 generations of selection, *Plutella xylostella* failed to develop resistance to neem extract, likely due to the diversity of its active compounds and multifaceted mode of action.

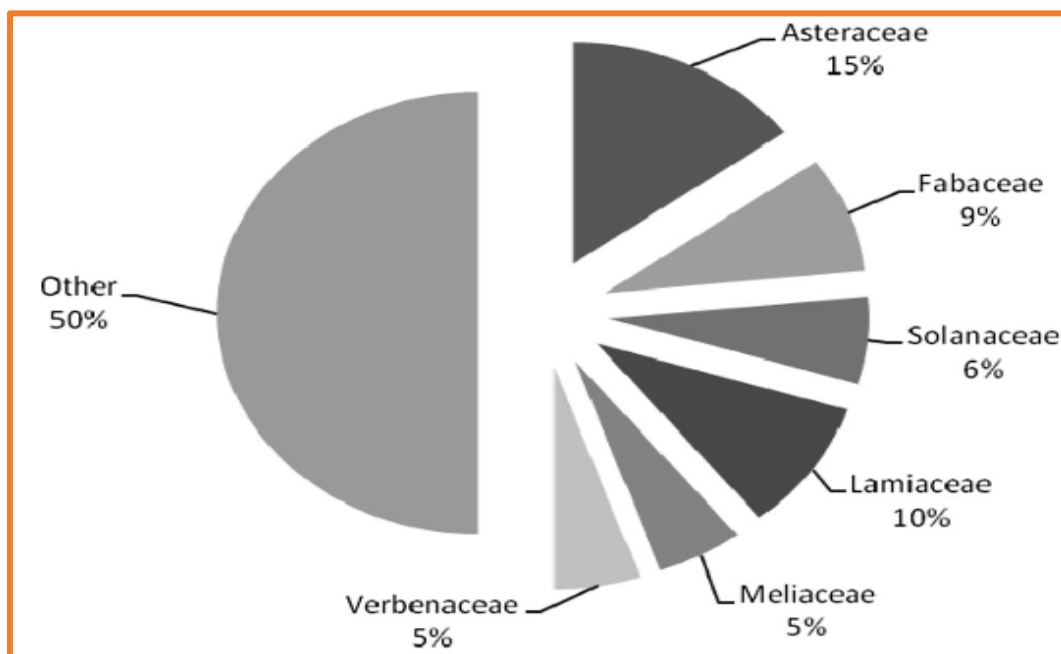


Figure 14: The most commonly utilized botanical families for tick control (Benelli et al., 2016).



2. CHAPTER II: MATERIALS AND METHODS

2.1. Section 1: Tick inventory and sampling

This section outlines the procedures employed for the inventory and sampling of ticks, detailing the study area, host selection, and collection methods. The approach was designed to capture seasonal variations, host-specific patterns, and differences across localities, ensuring a comprehensive representation of tick populations in the study region. Additionally, the application of findings through parasitic indices—such as prevalence, abundance, and intensity—alongside ecological indices, provided key insights into tick-host dynamics. Relevant statistical analyses were conducted to assess variations across seasons, hosts, and localities, ensuring robust interpretation of the collected data.

2.1.1. General characteristics of the study area

The Djelfa Province, located in central Northern Algeria, covers an area of 32,256.35 km², primarily characterized by vast steppe areas comprising 66.24% of its total area. It is bordered by several provinces to the north, east, west, and south. The province's relief features four heterogeneous zones, including the Ouled Nail mountain range. Djelfa experiences a semi-arid climate with cool winters in the central and northern regions and arid conditions in the south. Precipitation is more abundant in autumn and spring, with a dry period starting from June. Temperature variations are significant, with the hottest months being June, July, and August. Snowfall is seasonal, and white frosts occur for about 40 to 60 days during winter and early spring (Climatic data for the sample collection year (2021–2022) are available in Appendix 1). The province experiences intense winds, including the sirocco, a hot, dry desert wind, for about 20 to 30 days annually. Vegetation primarily consists of arid high steppes and forests, with Aleppo pine, evergreen oak, and Phoenician juniper being dominant species in the mountainous regions (Roubet and Amara, 2016; ANIREF, 2021).

2.1.2. Selection of study localities

The selection of study sites was guided by multiple criteria to ensure comprehensive and reliable results. Key considerations included the presence of the host species, which is critical for the study's focus, and the logistical ease of accessing and examining livestock in the area. These criteria were essential to facilitate thorough and accurate data collection.



In this study, two distinct sites were carefully chosen within the Djelfa province to capture a diverse range of environmental and biological variables.

Locality 1: Sidi Baizid

Located in the northern part of Djelfa, Sidi Baizid is situated at approximately 35°45'0" North latitude and 2°22'60" East longitude, with an average elevation of 830 meters above sea level. This region is characterized by a Mediterranean climate, featuring hot, dry summers and mild, wet winters. The area supports a variety of vegetation types, including shrublands and woodlands, providing suitable habitats for the target host species (ANAT, 2021 ; ONM, 2021)

Locality 2: Faidh El Botma

Situated in the southern part of the province, Faidh El Botma lies at approximately 34°31'40" North latitude and 3°46'55" East longitude, with an average elevation of 1,200 meters above sea level. This area experiences a semi-arid climate, characterized by low annual precipitation and significant temperature variations between day and night. The landscape is dominated by steppe vegetation, which influences the distribution and behavior of the host species (ANAT, 2021 ; ONM, 2021).

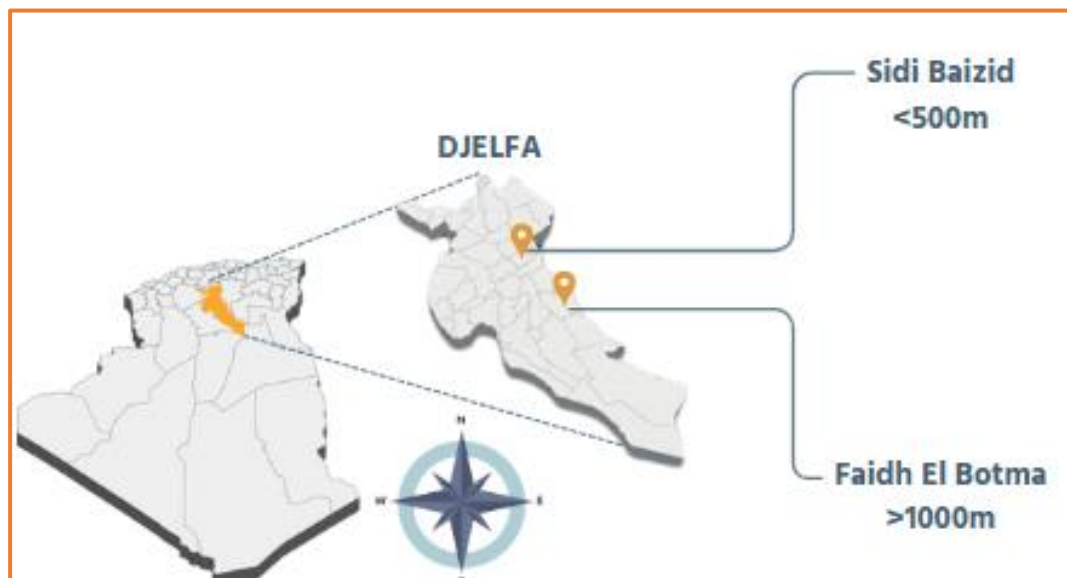


Figure 15 : Geographical sites of the study.



2.1.3. Collection method

Tick collection was conducted in two localities: Sidi Baizid (S) and Faidh El Botma (F). Two host species were selected: domestic dogs and cattle. The tick collection involved only non-invasive removal from owned animals and did not require formal ethical clearance, but was conducted with the full consent of livestock owners and in line with institutional standards for animal welfare. A total of 900 dogs and 720 cattle were examined. The examined dogs were all adult males, belonging to various breeds, including *Atlas Mountain Dog (Aïdi)*, *Sloughi*, *Labrador Retriever*, *German Shepherd*, *Staffordshire Bull Terrier*, and *Belgian Malinois*. Among them, 480 dogs were sampled in locality S and 420 in locality F. Cattle were only sampled in locality S, consisting of 720 adult females from a local breed raised under extensive farming conditions.

The collection process began with securely restraining each animal to ensure the safety of both the animal and the handler. The animals were then systematically examined in areas prone to tick infestation, including the anal region, ears, head, and neck. Ticks were carefully removed using entomological forceps and immediately placed in labeled dry tubes filled with 70% ethanol for preservation. Each tube was marked with the sample number, sampling location, date of collection, host species, breed, age, sex, and the number of ticks collected.



Figure 16 : Ticks collection. A : Tick removal tweezer B : Specimen collected from a dog (Original photograph)

2.1.4. Identification procedure



Tick identification was carried out using a systematic approach combining a binocular magnifier and the taxonomic keys of Walker et al. (2003) and Estrada-Peña et al. (2018). Sex differentiation was determined by examining the scutum, which covers only the anterior third in females but extends across the entire dorsal surface in males.

Genus identification was based on key morphological characteristics, including the structure of the rostrum, the presence of a spur on the first coxa, and the nature of the eyes. Species-level identification involved a detailed assessment of additional traits such as the presence of festoons, spiracle structure, caudal appendages, and adanal plates (Walker et al., 2003 ; and Estrada-Peña et al., 2018). Each morphological criterion was carefully examined to ensure precise classification and reliable species identification.



Figure 17 : Tick identification process (Original photograph).

2.1.5. Application of findings through indices

2.1.5.1. Application of findings through parasitic indices

2.1.5.1.1. Abundance (N)

It is a measure indicating the total count of individuals, which have been either collected or observed within the designated sampling period for each specific habitat. It provides valuable



insights into the population density and distribution of organisms within their respective environments (Johnson and Smith, 2019).

2.1.5.1.2. Prevalence (P)

Serves as a crucial metric in parasitology, delineating the extent of infestation within a given population. It quantifies the proportion of infested hosts (N) relative to the total number of hosts examined (H). This metric, expressed as a percentage through the formula:

$P = N / H \times 100$, aids in understanding the prevalence and spread of parasites within host communities (Brown and Jones, 2018).

2.1.5.1.3. Intensity (I)

Offers a deeper perspective into the parasitic burden experienced by the host population. It represents the ratio of the total number of individuals belonging to a particular parasite species (n) to the number of hosts that are infested (Np). This index, denoted by the formula:

$I = n / Np$, provides valuable information on the severity and impact of parasitic infections within host populations, offering insights crucial for ecological and epidemiological studies (Miller et al., 2020; Anderson and Miller, 2021).

2.1.5.2. Application of findings through ecological indices

2.1.5.2.1. Ecological composition indices

2.1.5.2.1.1. Total richness (S)

Richness serves as a fundamental parameter in population analysis (Ramade, 1984). Total richness (S) denotes the cumulative number of species encountered at least once across N surveys (Ramade, 1984 ; Aissaoui, 2004).



2.1.5.2.1.2. Relative abundance (RA %)

Understanding relative abundance is pivotal for comprehending population dynamics, particularly in elucidating species distribution and fluctuations (Ramade, 1984). This index expresses the proportion of individuals belonging to a specific species (n_i) relative to the total individuals (N) across all encountered species (Dajoz, 1971).

$$RA \% = n_i \times 100/N$$

RA %: Centesimal Frequency;

n_i : Number of individuals of species i encountered across N surveys;

N : Total number of individuals across all encountered species in N surveys.

2.1.5.2.1.3. Shannon-Weaver diversity index

The Shannon and Weaver diversity index (1963) provides insights into the population structures sampled and the distribution patterns of individuals across various species. It is calculated as follows:

$$H' = - \sum [P_i \log_2 P_i] \text{ where } P_i = n_i/N$$

H' : Specific diversity, expressed in Bits per individual (Binary digit).

P_i : Relative frequency of species i within the population.

n_i : Abundance of species i .

N : Total abundance of the population.

$\log_2$: Natural logarithm with base two.

Evenness (Equitability)

The Pielou evenness index (Huston, 1994, Dajoz, 2003, Frontier et al., 2008) is employed to assess if a specific species type is significantly overrepresented. Evenness (E) is derived from



the ratio of Shannon's information function (1948), H , for occurrences and the maximum theoretical value (H'_{max}).

Evenness represents another vital aspect of diversity (Ramade, 1984). It is determined by the relationship between specific diversity (H') and maximum diversity (H_{max}), expressed as follows:

$E = H' / H_{max}$, where $H_{max} = \text{Log}_2 (S)$, and S represents the number of species.

The evenness index E ranges from 0 to 1, with higher values indicating a more balanced distribution of species abundances within the population (Ramade, 1984).

2.1.5.3. Seasonal variations and host-locality influences on tick infestation prevalence

To assess the seasonal variation in tick infestation prevalence and the influence of localities (altitude) and hosts on these variations, statistical analyses were performed on tick infestation data collected from two localities (Sidi Baizid and Faifh El Botma) with varying altitudes, and two hosts (cattle and dogs) across the spring, summer, autumn, and winter seasons. The infestation prevalence was calculated as the proportion of hosts infested with ticks within each season.

For each season, the Chi-Square Test was used to evaluate differences in tick infestation prevalence between the two localities and across the two host types for the spring, autumn, and winter seasons, as the data met the assumptions for this test (expected frequencies greater than 5). The Fisher's Exact Test was applied to the summer season due to small sample sizes or low expected frequencies, making the Chi-Square Test unsuitable.

The tests were conducted separately for both the locality comparison (Sidi Baizid vs. Faifh El Botma) and the influence of host factors (cattle vs. dogs) within each season. For the locality analysis, the test evaluated differences in tick prevalence between the two localities. For the host analysis, the test assessed whether infestation prevalence varied across cattle and dogs. A significance level of 0.05 was set for all tests, with p-values below 0.05 indicating a significant difference in tick infestation prevalence between localities or hosts for each season. P-values lower than 0.001 were considered highly significant.



2.2. Section 2: Pathogen interactions in *Hyalomma* ticks

This section describes the methodologies used to investigate pathogen–pathogen interactions within *Hyalomma* tick populations. Emphasis was placed on understanding how co-occurring pathogens influence each other's presence and dynamics. Molecular techniques, including high-throughput real-time PCR and DNA sequencing, were employed to detect and identify a broad range of tick-borne pathogens. Network analysis approaches were then applied to explore patterns of positive and negative associations among pathogens, providing insights into the complexity of microbial communities. The influence of seasonal variations, biological characteristics on these interactions was also examined through relevant statistical analyses.

2.2.1. Nucleic acid extraction

Before extracting nucleic acids, each tick was meticulously washed with sterile milli-Q water to ensure cleanliness. A total of 166 ticks (winter: 14 females and 24 males; spring: 15 females and 23 males; summer: 25 females and 23 males; autumn: 18 females and 24 males) were extracted using the NucleoSpin tissue kit for Genomic DNA from tissue (Macherey-Nagel, Düren, Germany). The manufacturer's "Standard protocol for human or animal tissue and cultured cells" was employed with minor modifications tailored to our specific requirements. After disinfecting the ticks, they were carefully sectioned into quarters on a sterile Petri dish using a sterile scalpel blade and then transferred to the extraction tube that contained the provided lysis buffer. The lysis process was completed in these pre-filled tubes, followed by centrifugation to separate the DNA-containing supernatant. This supernatant was then used for DNA quantification, performed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) at an absorbance ratio of A260/A280 to ensure purity. Finally, the extracted DNA was stored at -20°C for subsequent analyses, maintaining its integrity for future genetic examinations.

2.2.2. DNA pre-amplification for microfluidic real-time PCR

To enhance the detection of the pathogen's genetic material relative to the host's, the DNA was pre-amplified using the Standard BioTools pre-amplification kit (Standard BioTools, CA, USA). Following the manufacturer's guidelines, the process began by preparing a 0.2x pool and then conducting PCR pre-amplification. Primers were combined in equal volumes to create a pooled primer mix with a final concentration of 200 nM. The pre-amplification reaction was



performed in a 5 µl volume, comprising 1 µl of PreAmp Master Mix, 1.25 µl of the pooled primer mix, 1.5 µl of distilled water, and 1.25 µl of DNA. The thermocycling program initiated with an initial cycle at 95 °C for 2 minutes, followed by 14 cycles of 95 °C for 15 seconds and 60 °C for 4 minutes. After completion, the amplification products were diluted to a 1/10th concentration and stored at -20°C to minimize contamination risks, ensuring the integrity of the samples for subsequent analysis.

2.2.3. Microfluidic real-time PCR assay

Michelet et al. (2014) extensively detailed the techniques utilized in their study, which focused on detecting tick-borne microorganisms. The primary method employed 48.48 Dynamic Array™ IFC chips (Standard BioTools, CA, USA) used within the BioMark™ real-time PCR system. These chips allow for the separation of 48 PCR assays and 48 samples into individual wells where real-time PCR reactions occur in separate chambers thanks to an on-chip microfluidics assembly. Each chip also includes a negative water control (Milli-Q water) to ascertain the absence of contaminants, and DNA from the *Escherichia coli* strain EDL933 (Milli-Q water and DNA diluted to 1/10) serves as an internal inhibition control in the assay plate to validate the absence of PCR inhibitors, using specific primers and a probe targeting the *E. coli* gene.

Once loaded, the BioMark™ real-time PCR system was programmed with parameters as reported in earlier studies (Melis et al., 2023). Throughout this process, stringent sterility measures are maintained to ensure accurate results. Post-run analysis was conducted using the "Fluidigm Real-Time PCR Analysis" software, and results were annotated in Excel. The genes targeted and the primer sequences employed for amplification are detailed in Appendix 2. This investigation cataloged a comprehensive range of tick-borne microorganisms, including 27 bacterial species such as *Borrelia burgdorferi*, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, *B. bissetii*, *B. miyamotoi*, *Anaplasma marginale*, *A. platys*, *A. phagocytophilum*, *A. bovis*, *A. centrale*, *A. ovis*, *Ehrlichia canis*, *N. mikurensis*, *R. conorii*, *R. slovaca*, *R. massiliae*, *R. helvetica*, *R. aeschlimannii*, *R. felis*, *Bartonella henselae*, *Francisella tularensis*, *Francisella*-like endosymbionts (FLE), *Coxiella*-like endosymbionts, and *Coxiella burnetii*. Additionally, 7 parasite species were identified, including *Babesia microti*, *B. canis*, *B. ovis*, *B. divergens*, *B. bovis*, *B. caballi*, and *Babesia* sp. EU1. The bacterial genera included were *Bartonella*, *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, and *Mycoplasma*, and parasite taxa



encompassed Apicomplexa, *Theileria*, and *Hepatozoon*, providing a thorough overview of the pathogens present in tick populations.

2.2.4. Confirmation of pathogen presence using conventional PCR

TBPs were detected through conventional and nested PCR assays, with the cycling conditions and primers detailed in Appendix 3. Additional PCR assays, utilizing species-specific primers, further confirmed the presence of certain target TBPs identified in the initial analysis. This crucial confirmation step strengthens the accuracy and reliability of the findings by providing an additional layer of validation (Díaz-Corona et al., 2024).

2.2.5. DNA sequencing analysis

The PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany), and the sequences were assembled using BioEdit software from Ibis Biosciences in Carlsbad, CA, USA. Our findings were then compared against publicly available sequences in GenBank using the online BLAST tool provided by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), available at <http://www.ncbi.nlm.nih.gov/blast>.

2.2.6. Phylogenetic analysis

A phylogenetic analysis of TBPs associated with *Hyalomma* species was performed, grouping them into 10 guilds based on collection seasons and sexes (M: males, MW: males collected in winter, MSP: males collected in spring, MSU: males collected in summer, MA: males collected in autumn, F: females, FW: females collected in winter, FSP: females collected in spring, FSU: females collected in summer, FA: females collected in autumn). The details about pathogens identified in these Tick-Borne Pathogen Guilds (TBPGs) are provided in Appendix 4. For this purpose, reference sequences of the 16S rRNA (bacterial pathogens) and 18S rRNA (eukaryotes) genes fragments were searched in the National Library of Medicine database; NCBI (accessed 13 June 2024). Then all sequences of particular species showing similarity to the reference ones were downloaded from the Blast database. Finally, sequences of up to 1800 nucleotides in length, excluding redundant ones underwent initial alignment using the online MAFFT tool (Katoh, 2002). Next, obtained set of sequences was analyzed using the MUSCLE algorithm in MEGA 11 (Tamura et al., 2021). Phylogenetic trees were then constructed using



the Tamura-Nei model with Gamma distribution (TN93+G) and the Tamura 3-parameter model (T92) for the 16S rRNA and 18S rRNA gene, respectively.

Moreover, our aim was to investigate whether there is a consistent pattern of genetic distances between TBPs within each guild and whether this pattern holds across guilds. To this end, the pairwise distance between sequences within each guild was calculated (as p-distance) in MEGA 11. Furthermore, the statistical significance of differences in p-distance between the studied groups (guilds) was calculated using the Mann Whitney U test, while the significance of differences in p-distance within particular guilds was calculated using the Wilcoxon test. Statistical calculations were performed using GraphPad 8.0 (Prism, Massachusetts, USA).

2.2.7. Statistical analysis of pathogen interactions

The gathered data were assembled using Microsoft Excel 2016. Prevalence rates and 95% binomial confidence intervals (CI) for each TBP infection and co-infection were calculated based on microfluidic real-time PCR amplification results. Chi-square tests (χ^2) were conducted to compare TBP prevalence between males and females, a p value < 0.05 was considered significant, the calculations were performed using SPSS software version 22.

2.2.8. Co-infections and network interactions between microorganisms

Investigations into pathogen associations within ticks have utilized a modeling approach based on binary presence/absence data, In the dataset, ticks are represented in columns and the microorganisms tested are represented in rows, where 0 indicating the absence and 1 indicating the presence of pathogen. This analysis employed Yule's Q statistic, defined for 2x2 contingency tables as:

$$\text{Yule's } Q = (ad + bc)/(ad - bc)$$

'a' and 'd' denote the number of concordant pairs (where both microorganisms are either present or absent), while 'b' and 'c' represent the number of discordant pairs (where one pathogen is present while the other is absent). Statistical analysis was conducted using the igraph package (Nepusz and Csardi, 2006) implemented in R version 4.3.3. (R Core Team, 2023) and performed using RStudio (RStudio Team, 2020).



Interaction networks were constructed using results from high-throughput microfluidic analyses, allowing simultaneous detection of multiple pathogens in ticks. The presence of some of these pathogens was confirmed by nested PCR. Only edges with weights of 1 and -1 were included. The resulting association networks, visualized as Rplots, were constructed and refined using Gephi (Bastian et al., 2009). In each network, node color and size were indicative of modularity class and eigenvector centrality. The network's spatial layout was optimized using Yifan Hu and Fruchterman Reingold parameters within Gephi. Positive and negative interactions were determined from the correlation coefficients of abundance data. Network complexity was evaluated by examining the number of nodes, edges, and overall interaction patterns. Nodes within the network represent microorganisms, while blue and red edges denote positive and negative associations, respectively. An R script detailing the calculation of Yule's Q and the construction of the co-occurrence network is provided as Additional material.

2.3.Section 3: Microbiome dynamics in *Hyalomma* ticks

This section details the approaches used to investigate the microbiome composition of *Hyalomma excavatum* ticks collected from cattle across different seasons. It outlines the methodologies applied to assess microbial diversity, community structure, and potential functional roles. Particular emphasis is placed on alpha and beta diversity analyses to evaluate microbial richness and compositional differences among samples.

Additionally, the section explores networking and sub-networking analyses to understand the interactions between microbial taxa and their potential influence on pathogen presence. Robustness analyses are also conducted to assess the stability of microbial networks under varying ecological conditions. Together, these approaches provide comprehensive insights into the dynamic nature of tick microbiomes and their role in shaping disease transmission patterns.

2.3.1. 16S rRNA amplicon sequencing and processing of raw sequences

DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany), following the manufacturer's protocol. The extracted DNA was quantified using a NanoDrop spectrophotometer and assessed for quality by electrophoresis on a 1.5% agarose gel, with visualization under UV light. High molecular weight DNA bands (~10–20 kb) were expected, confirming the integrity of the genomic DNA. The bacterial community was characterized by



amplifying the V3–V4 region of the 16S rRNA gene using Illumina-tailed universal primers [341F(5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Amplicons were sequenced on an Illumina MiSeq platform (Illumina, USA) with paired-end reads (2x250 bp) at Eurofins Genomics. All raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under the project number [PRJNA1214082]. Raw sequences were processed using QIIME2 (Quantitative Insights Into Microbial Ecology, version 2023.2). Quality filtering, chimera removal, and feature clustering were performed to obtain amplicon sequence variants (ASVs). Taxonomic assignments were conducted using the SILVA database (v138). Alpha-diversity metrics and beta-diversity measures were calculated to assess microbial richness and composition. Co-occurrence network analysis was applied to explore interactions between bacterial taxa, with a particular focus on the roles of *Rickettsia* and *Francisella*.

2.3.2. Microbial diversity, composition, and taxonomic differential relative abundance

To investigate changes in the microbiome across three seasons, both alpha and beta diversity metrics were analyzed using QIIME 2 software (Bolyen et al., 2019). Alpha diversity was evaluated to measure microbial richness and evenness. Richness was determined using metrics such as observed features (DeSantis et al., 2006) and Faith's phylogenetic diversity index (Faith, 1992), while evenness was assessed using Pielou's index (Pielou, 1966). Group comparisons for alpha diversity metrics were performed using the Kruskal-Wallis test ($p \leq .05$). Beta diversity was calculated using the Bray-Curtis dissimilarity index (Bray and Curtis, 1957) and assessed through PERMANOVA ($p \leq .05$) to compare community structures. Additionally, beta dispersion was analyzed with the betadisper function in the Vegan package (Oksanen et al., 2021) in RStudio, and statistical significance was tested with ANOVA ($p \leq .05$).

Cluster analyses were carried out using the Jaccard similarity coefficient in Vegan, and unique versus shared taxa among the three conditions were visualized using Venn diagrams generated with the Venn Diagram web tool (<https://bioinformatics.psb.ugent.be/webtools/Venn/>).



Differential taxonomic abundance was assessed using the ALDEx2 package in RStudio (Fernandes et al., 2013), and significant differences between groups were identified with the Kruskal-Wallis test ($p \leq .05$). Relative abundance data were transformed using the centered log-ratio (clr) method. Heatmaps illustrating differentially abundant taxa were created using the "Heatplus" package in RStudio. These analyses provided insights into the dynamic composition of the microbiome and highlighted taxa that varied significantly across the tested conditions.

2.3.3. Inference of bacterial co-occurrence networks

To explore interactions within microbial communities, co-occurrence networks were built using taxonomic profiles at genus levels. The Sparse Correlations for Compositional Data (SparCC) method (Friedman & Alm, 2012) was employed in R version 4.3.3 (R Core Team, 2023). Empirical p-values were calculated using a bootstrap approach with 1,000 iterations and adjusted for multiple testing using the False Discovery Rate (FDR) method. Only statistically significant correlations (adjusted $p < 0.05$) with an absolute value ≥ 0.75 were retained. A conservative threshold of $|r| \geq 0.75$ was chosen to minimize spurious associations and highlight the most reliable interactions. In the resulting networks, nodes represent individual taxa and edges indicate significant positive ($r \geq 0.75$) or negative ($r \leq -0.75$) associations.

Key topological metrics were computed and visualized using Gephi version 0.9.5 (Bastian et al., 2009). These metrics included the total number of nodes and edges, network diameter (measuring the shortest path between the most distant nodes), modularity (quantifying the degree of division into modules), average degree (mean number of edges per node), weighted degree (sum of edge weights per node), and clustering coefficient (indicating the likelihood of nodes forming tightly connected groups) (Abuin-Denis et al., 2024).



2.3.4. Subnetwork analysis of *Rickettsia* and *Francisella* associations

To explore the interactions of *Rickettsia* and *Francisella* within the microbial community, their direct associations with other bacterial taxa were analyzed. Sub-networks were constructed to highlight these positive and negative connections, offering a focused visualization of their immediate relationships. The analyses were performed using Gephi version 0.9.5 (Bastian et al., 2009), with edge strengths represented by SparCC correlation weights to quantify the association between taxa.

2.3.5. Microbial network robustness analysis

To evaluate the robustness of microbial co-occurrence networks under perturbations, the impact of node removal or addition on network connectivity was examined. The proportion of nodes that needed to be removed to reduce connectivity by 80% was determined. This analysis included random and directed node removal based on betweenness centrality (removing nodes with the highest betweenness centrality), degree centrality (removing nodes with the highest degree), and cascading removal (recalculating betweenness centrality after each node removal). The Network Strengths and Weaknesses Analysis (NetSwan) package (Lhomme, 2015) in R version 4.3.3 (R Core Team, 2023) was used for this assessment, conducted within the RStudio environment (RStudio Team, 2020).

Additionally, a node addition analysis was performed to simulate ecologically relevant scenarios, such as the introduction of new microbial taxa over time or across different environmental conditions. In each simulation, 100, 300, 500, 700, or 1000 nodes were randomly added to the existing network, and their integration patterns were analyzed. The size of the largest connected component (LCC)- i.e., the number of nodes in the biggest cluster- and the average path length (APL) -the average number of steps along the shortest paths for all possible node pairs- were calculated to assess the effects of increasing microbial richness on network



structure and resilience. The results were visualized using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

2.3.6. Statistical analysis of microbial diversity and network structure

Alpha diversity differences between groups were evaluated using the Kruskal-Wallis test ($p < 0.05$) in QIIME2 version 2023.2 (Bolyen et al., 2019). The Bray-Curtis dissimilarity index was used to compare group differences, with statistical significance determined by a PERMANOVA test ($p < 0.05$). Beta dispersion was assessed with an ANOVA test ($p < 0.05$). Taxa abundance differences were examined using the Kruskal-Wallis test ($p < 0.01$) with the ALDEx2 package version 1.28.1 (Fernandes et al., 2013) within R version 4.3.3..

The standard error for connectivity loss was calculated, incorporating variability with a threshold of 0.9. Node addition analysis were conducted in RStudio (version 2023.06.1) used Wilcoxon signed-rank tests to assess whether the mean size of the largest connected component (LCC) and average path length (APL) significantly differed from 0. p -values p -values were adjusted using the Benjamini–Hochberg procedure, and bootstrapping was applied in node removal analyses to estimate confidence intervals.



2.4. Section 4: Botanical control of *Rhipicephalus sanguineus*

This section focuses on the evaluation of botanical extracts' efficacy against *Rhipicephalus sanguineus*, particularly essential oils from selected plant species. The methodology includes assessing their impact on larval mortality, reproductive parameters, and egg-hatching inhibition. Toxicological parameters such as LD₅₀, LD₉₀, LT₅₀, and LT₉₀ are estimated to determine lethal doses and exposure times. Additionally, statistical analyses (e.g., ANOVA and Tukey's test) are applied to compare the efficacy of different doses and treatments.

2.4.1. Selection and description of selected plants

The selection of plants was guided by:

- Their traditional uses and recognized pharmacological properties.
- The abundance of steppe vegetation in the Djelfa region, ensuring easy accessibility in the field.

The plant material used in this study consists of the aerial parts of *Artemisia herba-alba*, *Rosmarinus officinalis*, and *Thymus vulgaris*.

The plants were collected in May from various locations within Djelfa. Following collection, the aerial parts were thoroughly cleaned and naturally dried at room temperature (approximately 24°C) for 15 days, protected from light and moisture to preserve the integrity of their molecular components.

2.4.1.1. *Artemisia herba alba* Asso, 1779

The genus *Artemisia*, belonging to the Asteraceae family, comprises around 400 species, distributed across the Mediterranean, North Africa, Western Asia, Southwest Europe, and the Arabian Peninsula. The Algerian flora includes 13 species of *Artemisia*, including the most reported species, *A. herba-alba* Asso. (Rekkab et al., 2016), commonly known as white wormwood or desert wormwood (Arabic name Chih) (Belhattab et al., 2014). It is a dwarf, silvery perennial shrub that grows in arid zones and semi-arid climates. With rapid growth in dry, hot climates and muddy areas (Tilaoui et al., 2015).



In Algeria, it alternates with Alfa formations occupying around three million hectares and represents an important forage resource (Belhattab et al., 2014). The different species are morphologically different from one another depending on their geographical, environmental, and climatic situation (Tilaoui et al., 2015). *A. herba alba* is a greenish-silver perennial grass, that grows 20-40 cm tall; it is a chamaephyte, i.e. the buds giving rise to new growth each year are borne close to the ground. Stems are rigid and erect. The gray leaves of the sterile shoots are petiolate, oval to orbicular in outline, while the flowering leaves are much smaller, and are characterized by a strong aromatic scent. Flowering heads are sessile, oblong, and tapered at the base. Flowering and harvesting take place around May-June and continue until October in some regions (Houmani et al., 2004 ; Mohamed et al., 2010).



Figure 18 : *Artemisia herba alba* (Original photograph).

2.4.1.2. *Rosmarinus officinalis* Linnea, 1753

Rosmarinus officinalis belongs to the Lamiaceae family (Wang et al., 2012). Rosemary is a common domestic plant cultivated in many parts of the world. It is widespread in Algeria (Boutekedjiret et al., 2003).

Rosemary is a bushy, aromatic shrub, about 1 meter high, very fragrant, growing wild or cultivated. With a lignified stem, it has evergreen, rolled leaves up to 3 cm long and 4 mm wide; green on the upper surface and white on the underside. They are narrow, opposite, and thick.



The mauve-blue flowers appear in January in symmetrical clusters, and the fruit is a camphor-scented achene (Fadi, 2011; Aouati, 2016). Rosemary is a spice and medicinal plant widely used throughout the world. It is an important medicinal and aromatic plant that has been cultivated for a long time (Elyemni et al., 2022). It is cultivated for the precious oil, which can be extracted from plants harvested when the flowers are in the bud (Hannour et al., 2017).



Figure 19 : *Rosmarinus officinalis* (Original photograph).

2.4.1.3. *Thymus vulgaris* Linnea, 1753

Thymus vulgaris (Zaatar in Arabic) is a species of flowering plant in the Lamiaceae family, native to southern Europe, from the western Mediterranean to southern Italy (Miraj and Kiani, 2016). Thyme is represented by over 300 species worldwide, 12 of which are located in Algeria, and 9 of which are endemic (Benmadhi and Abida, 2018).

The name "Thymus" derives from the Greek word "thymos" meaning "to perfume" because of the pleasant scent the plant gives off, the species *Thymus vulgaris* is a characteristic element of the Mediterranean flora, known above all for its aromatic qualities (Benmadhi and Abida, 2018). It is an herbaceous plant of the section, that thrives in mountainous regions growing well in a temperate to warm, dry, and sunny climate characteristic of the garrigue, and wherever plants do not appear to be shaded (Charles, 1989 ; Dauqan and Abdullah, 2017). Thyme is a tiny perennial shrub with ground-covering evergreen foliage that rarely reaches (40 cm)



horizontal and upright habits, the stems become woody with age (Dauqan and Abdullah, 2017). The leaves of *T. vulgaris* are oval to rectangular in shape depending on the variety, each species having a fairly completely different fragrance, with revolute margins, are linear or narrowly lanceolate and variable in size, punctate, glandular, and green to blue-gray on the upper surface, they are hairy and whitish on the underside. Flowers are violet or pink in early summer and vary in size depending on the gyroidal sex. Leaves, flowers, and herbaceous stems contain glandular hairs containing the essential oil, and other protective hairs, forming the villi of leaves and stem, protect them from evaporation (Assouad and Valdeyron, 1975 ; Charles, 1989).



Figure 20 : *Thymus vulgaris* (Original photograph).

2.4.2. The methodology adopted for essential oil extraction

The essential oil was obtained after 4 main stages: hydrodistillation, liquid-liquid extraction, water removal, and solvent removal.

2.4.2.1. Hydrodistillation

50 g of the cut-dried plant is introduced into a 1000 ml flask, followed by 500 ml of distilled water, and stirred. The flask is then placed in a hydro-distillation set-up using a Clevenger-type



apparatus (Clevenger, 1928) as recommended by the Hellenic Pharmacopoeia (Hellenic Pharmacopoeia, 2002).

The principle is to bring them to the boil. The plant cells burst, releasing the odorant molecules, which are then carried away by the steam created. In contact with the walls of the cooler, the hot vapors condense and drip into a container, where they form the distillate. The distillate is a mixture of two immiscible phases (essential oils + water) (Tour, 2015).

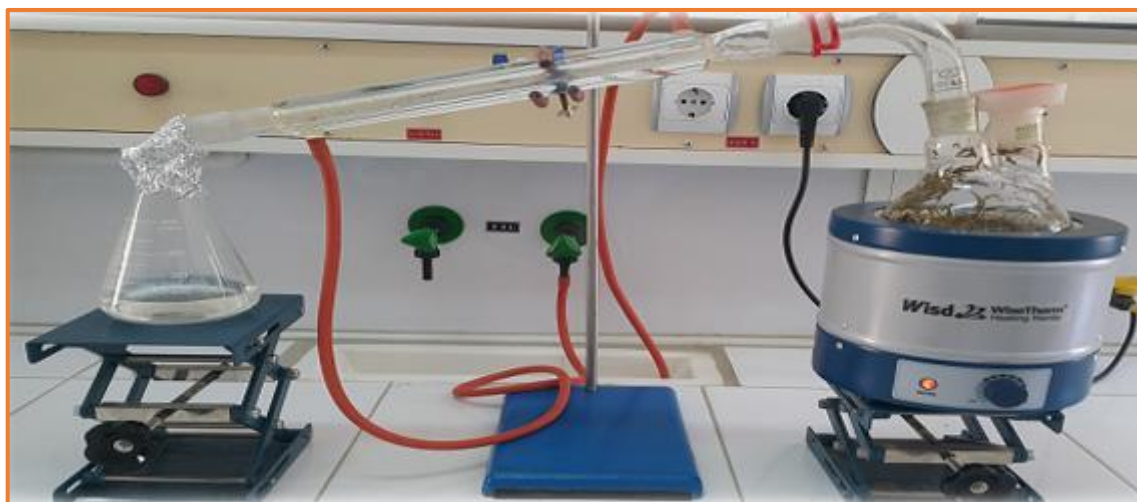


Figure 21 : Hydrodistillation assembly (Original photograph).

2.4.2.2.Liquid-liquid extraction

Extraction is straightforward, and involves extracting as much of the solute initially present in the distillate as possible with a solvent in a single step (Abe et al., 2010). The solvent is chosen on the basis of its ability to dissolve essential oils (diethyl ether in our case). The distillate is placed in a separating funnel, the solvent is added and the funnel is closed, the funnel is shaken vigorously for the time required to establish a concentration equilibrium between the two phases, then degassed and fixed to support by removing the liquids. At the end, each phase is recovered in a suitable container (Abe et al., 2010).



Figure 22 : Liquid-liquid extraction (Original photograph).

2.4.2.3. Water removal

To remove all traces of water, the organic phase is dried by adding a few grams of anhydrous magnesium sulfate MgSO_4 , then filtered using filter paper (Feknous et al., 2006).

2.4.2.4. Solvent removal

The liquid obtained in the previous step is poured into a suitable flask, then attached to a rotary evaporator to perform a simple distillation under reduced pressure with a temperature of 37°C . The solvent evaporates and the vapors thus formed are condensed by the condenser and liquefied in a different vessel (Mecquenem et al. 2018). The resulting oil is stored in hermetically sealed sterile glass vials, protected from light, and at a temperature of 4°C .

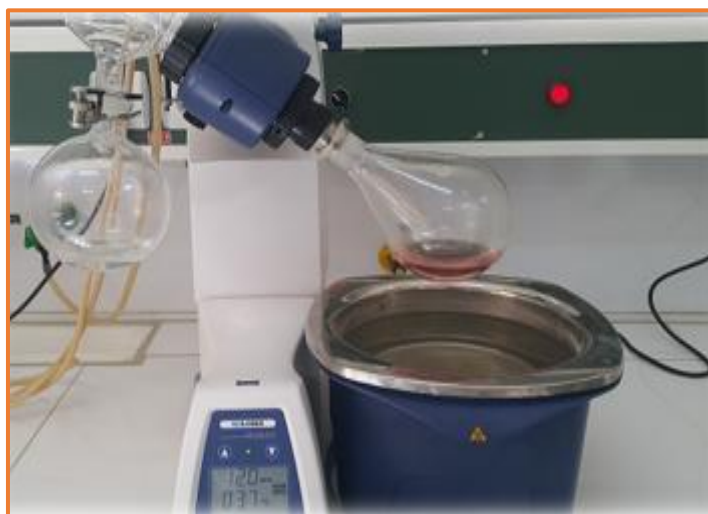


Figure 23: Rotavapor Device (Original photograph).



2.4.3. Essential oil yield

The essential oil yield of each plant is the ratio between the weight of the extracted oil and the weight of the plant material used. The yield expressed as a percentage (%) is calculated by the following formula (Laib, 2012):

$$R(\%) = (M'/M) \times 100$$

R is the yield in %.

M' is the mass of the extract (in g) after evaporation of the solvent.

M is the dry mass of the plant sample (in g).



Figure 24 : Essential oils (Original photograph).

2.4.4. Analysis of chemical composition by gas chromatography-mass spectrometry coupling

The chemical composition of the essential oil was analyzed by gas chromatography-mass spectrometry (GC/MS), enabling both qualitative and quantitative determination of the major compounds. The analyses were carried out in June of the current year. An analysis report for each essential oil was sent to the applicants after the interpretation of the chromatograms and clarification of the chemical compositions of the samples in question.

A portion of the sample (2-5 µl) was transferred to a GC vial, diluted in hexane (1-2 ml), then sealed with a high-performance septum (Delazar et al., 2004).



Constituents were identified using a Clarus 680 Perkin Elmer gas chromatograph coupled to a Clarus SQ 8 T mass spectrometer. The fused silica Rtx-5MS (30 mx 0.25 mm ID, 0.25 μ m df, RESTEK, USA) was directly coupled to the mass spectrometer (Delazar et al., 2004).

The carrier gas was helium (1 ml/min). The program used was 2 min isothermal at 60°C, then 3°C / min at 160°C, then 6°C / min at 240°C for 2 min. The injection port temperature was 250°C and the detector temperature was 240°C. Sample components were ionized in EI mode (70 eV). The MS scan range was from 30 to 300 amu (Delazar et al., 2004).

Individual constituents were identified by comparing their mass spectra with spectra stored in the NIST / EPA / NIH mass spectral database. Version 2.0 g, dated May 19, 2011.

2.4.5. Collection and breeding of *Rhipicephalus sanguineus* engorged females

Engorged females of *R. sanguineus* were collected from naturally infested domestic dogs living with other livestock (cattle, sheep) just after they started to drop off the host to ensure uniformity, this hosts had not received any acaricidal treatment for at least 45 days to avoid any negative interference, on many farms of the municipality of Ain Maabed (34° 48' 17" N, 3° 07' 46" E), Djelfa, Algeria.

The ticks were stored in cooled plastic boxes ($\approx 15^\circ\text{C}$) to reduce their activity and immediately transported to the laboratory then thoroughly washed with distilled water and dried in paper toweling. Species identification was made under a binocular magnifier according to keys and descriptions provided by Walker et al. (2003).

Then placed in Petri dishes, which were kept in an oven at 27 °C and over 80% humidity for 15 days. After oviposition had begun, the eggs were placed in tubes under the same conditions as the females to obtain larvae (Daemon et al., 2009).



Figure 25 : Breeding of *R. sanguineus* (Original photograph).

2.4.6. Toxicological test preparation

This test was carried out on 2 stages, the engorged females and the larvae using an immersion test (AIT/LIT). *Thymus vulgaris* essential oil was dissolved and serially diluted in 1 ml ethanol, then preliminary tests with different doses were carried out to select a range of concentrations before starting the toxicity test. Four concentrations (1 $\mu\text{l/ml}$, 2 $\mu\text{l/ml}$, 10 $\mu\text{l/ml}$, 30 $\mu\text{l/ml}$) have been chosen for the AIT, and five concentrations (0.5 $\mu\text{l/ml}$, 1 $\mu\text{l/ml}$, 2 $\mu\text{l/ml}$, 3 $\mu\text{l/ml}$, 5 $\mu\text{l/ml}$) for LIT. For each concentration, three replications were maintained as well as for the control.



Figure 26 : Immersion test (Original photograph).



2.4.6.1. Adult immersion test

The AIT was performed as described in the literature (Drummonds et al., 1973; FAO, 2004) with minor modifications. In the groups of fifteen engorged female ticks, each was individually weighed in order to obtain groups with similar weights (0.5 ± 0.1 g). The different groups of ticks dipped in 10 ml of each concentration for five minutes. All tests were replicated three times. After exposure, the engorged females were removed, dried then placed in Petri dishes that were incubated for fifteen days at 27 ± 2 °C and 80% relative humidity. Ticks were confirmed dead based on signs of hemorrhagic skin lesions, cuticular darkness, and lack of Malpighian tube movement. After 2 weeks, the eggs were weighed and transferred to tubes then placed in the incubator under the same conditions for larval hatching.

The egg production index (EPI), the reduction in hatching (HR), the reduction in oviposition (RO), the reproduction efficiency index (REI), and the efficiency of the extract (EP) were calculated according to the following formulas:

$EPI (\%) = (\text{weight of eggs} / \text{weight of engorged female}) \times 100$ (Bennett, 1974).

$RO (\%) = [(EPI \text{ control group} - EPI \text{ experimental group}) / EPI \text{ control group}] \times 100$ (Roulston et al. 1968).

$HR (\%) = [(\text{hatching rate in control group} - \text{hatching rate in experimental group}) / \text{hatching rate in control group}] \times 100$ (Gonzales, 2003).

$REI = (\text{egg mass weight} \times \% \text{ egg hatching} / \text{engorged females weight}) \times 20,000$ (Drummonds et al., 1973).

$EP (\%) = [(REI \text{ control} - REI \text{ treated}) / REI \text{ control}] \times 100$ (Drummonds et al., 1973).

2.4.6.2. Larval immersion test (LIT)

The LIT is not recommended or standardized by FAO. Therefore, the following protocol was modified from an earlier test described by (Ribeiro et al., 2011). The larvae used in this test (LIT) came from the eggs provided by the engorged females untreated, the treatments of larvae were performed on the 15th day after total larval hatching. 100 larvae were immersed for 5 min in tubes container 10 mL of different concentrations of *Thymus vulgaris* essential oil, the tubes



were closed and shaken vigorously for some seconds and then gently for 5 min, then these larvae were transferred with a paintbrush to dry over a paper toweling. Next, they were placed to a filter paper (8.5 × 7.5 cm) (Whatman No. 1) that was folded and closed with clips forming a packet. The packets were incubated at 27–28 °C and ≥ 80% relative humidity.

Live and dead larvae were counted after 24h, 48h, and 72h of exposure (three packets per treatment) for further calculation of the LC50, LC90, LT50 and LT90 of each group.

2.4.7. Data analysis

2.4.7.1. Larval mortality rate

Moribund larvae are those which show a slow reaction to the various excitations, the aim of which is to distinguish between live and dead larvae.

It is calculated by the following ratio :

$$\text{Mortality rate \%} = (\text{Number of dead larvae} / \text{Total number of larvae}) \times 100$$

The test is considered valid if the percentage of mortality in the controls is less than 5% or between 5% and 20%.

If the percentage of mortality in controls is between 5% and 20%, post-exposure mortality must be corrected using Abbott's formula (Abbott, 1925) :

$$M_c = [(M_2 - M_1) / 100 - M_1] \times 100$$

M_c : percentage of corrected mortality;

M_1 : percentage of mortality in the control batch;

M_2 : percentage of mortality in the treated batch.



2.4.7.2. Statistical methods for analyzing bioassay data

According to Morice (1972), it is essential to check normality before using statistical tests, by applying a normality test to examine whether real data follow a normal distribution, enabling us to judge that the sampled population is probably normally distributed. The lethal dose 50 (LD_{50}) and 90 (LD_{90}) represent the doses required to induce mortality in 50% and 90% of the target population, respectively, and are calculated from the regression line of the probits ($y = ax + b$) based on mortality numbers as a function of treatment doses. Lethal time 50 (LT_{50}) and 90 (LT_{90}) represent the time needed for 50% and 90% of individuals exposed to a given dose to die (Ramade, 2007). They are also derived from the probit regression line based on mortality numbers over time. The values obtained are considered averages. These results were then analyzed using analysis of variance (ANOVA), which, depending on the level of significance, determines the influence of the studied factors or interactions between them. This study involved a balanced ANOVA with one factor (dose), as the number of replicates was the same across all doses. A probability of less than 0.05 indicates a significant effect, while a probability higher than 0.05 suggests the effect is not significant (Siegle, 2016).

Before performing ANOVA and multiple comparisons, the percentage mortality values were transformed using angular (arcsine square root) transformation to meet the assumptions of normality and homogeneity of variances.

The differences between doses were assessed using the Tukey test, which allows for multiple pairwise comparisons between the various concentrations of aqueous extracts and essential oils. All statistical analyses were performed using IBM SPSS Statistics 23 on Windows, in accordance with the recommendations provided by Lefever and Moreau (2009) in their methodological guide.



3. CHAPTER III: RESULTS

3.1. Section 1: Inventory and sampling of ticks

This section presents the results of the tick inventory and sampling conducted to assess the diversity, distribution, and seasonal dynamics of hard ticks in the Djelfa region. The study involved a comprehensive monthly collection of ticks over one year from two distinct host types: dogs (across two localities) and cattle (in a single locality). This approach allowed for an in-depth examination of host-associated variations and locality-based differences in tick populations.

The analysis includes the calculation of parasitic indices, such as prevalence, abundance, and mean intensity, providing insights into infestation levels across hosts and seasons. Additionally, ecological indices were applied to evaluate species diversity, dominance, and richness, offering a broader understanding of tick community structure.

Comparisons across localities and host species were further explored through statistical analyses, identifying significant patterns and trends in tick distribution. The results obtained from this inventory form a critical baseline for understanding the epidemiological risks posed by tick infestations and serve as a foundation for subsequent molecular and ecological investigations detailed in the following sections.

3.1.1. Identification of infesting species

The collected ticks at each study site were carefully identified based on their morphological characteristics and categorized by species, sex, and collection period. Table 04 presents the ticks collected from dogs, detailing the monthly distribution of male and female specimens for each recorded species throughout the year in two localities. Similarly, Table 05 provides an overview of the ticks collected from cattle in a single locality, following the same classification criteria.



Table 4 : Systematic list of ticks collected from domestic dogs across two localities.

Sidi Baizid locality												
Date	Total	Adult		Nymph	Species							
		F	M		<i>R. bursa</i>		<i>R. camicasi</i>		<i>R. sanguineus</i>		<i>R. turanicus</i>	
					F	M	F	M	F	M	F	M
JUN	135	78	57	0	51	38	6	4	12	9	9	6
JUI	2549	1185	1066	298	444	414	169	146	314	293	258	213
AUG	736	389	288	59	173	112	46	34	92	87	78	55
SEP	277	174	82	21	67	47	27	2	44	20	36	13
OCT	0	0	0	0	0	0	0	0	0	0	0	0
NOV	0	0	0	0	0	0	0	0	0	0	0	0
DEC	0	0	0	0	0	0	0	0	0	0	0	0
JAN	0	0	0	0	0	0	0	0	0	0	0	0
FEV	23	15	8	0	0	0	3	1	5	3	7	4
MAR	93	76	17	0	24	8	16	1	20	6	16	2
AVR	29	18	11	0	4	2	3	2	7	4	4	3
MAI	20	13	7	0	8	2	1	1	3	3	1	1
Total	3862	1948	1536	378	771	623	271	191	497	425	409	297
Faidh El Botma locality												
Date	Total	Adult		Nymph	Species							
		F	M		<i>R. bursa</i>		<i>R. camicasi</i>		<i>R. sanguineus</i>		<i>R. turanicus</i>	
					F	M	F	M	F	M	F	M
JUN	486	279	207	0	57	43	88	51	40	34	94	79
JUI	1443	824	619	0	61	55	289	206	72	58	402	300
AUG	558	341	217	0	30	21	98	73	61	43	152	80
SEP	1139	699	440	0	19	10	227	180	203	100	250	150
OCT	28	20	8	0	0	0	9	3	0	0	11	5
NOV	0	0	0	0	0	0	0	0	0	0	0	0
DEC	0	0	0	0	0	0	0	0	0	0	0	0
JAN	0	0	0	0	0	0	0	0	0	0	0	0
FEV	0	0	0	0	0	0	0	0	0	0	0	0
MAR	35	25	10	0	4	0	6	3	1	1	14	6
AVR	95	65	30	0	6	2	23	11	9	4	27	13
MAI	162	111	51	0	26	19	35	18	14	6	36	8
Total	3946	2364	1582	0	203	150	775	545	400	246	986	641

F: femal / M: male



Table 5: Systematic list of ticks collected from cattle

Date	Total	Adult		Species							
		F	M	<i>H. anatolicum</i>		<i>H. excavatum</i>		<i>H. impletatum</i>		<i>H. marginatum</i>	
				F	M	F	M	F	M	F	M
JUN	52	22	30	5	7	12	15	2	3	3	5
JUI	166	67	99	17	26	35	48	5	9	10	16
AUG	81	36	45	11	12	16	20	3	5	6	8
SEP	56	21	35	7	9	10	17	2	4	2	5
OCT	44	17	27	6	8	9	14	1	2	1	3
NOV	41	15	26	5	8	8	13	1	2	1	3
DEC	38	14	24	4	6	8	13	1	2	1	3
JAN	23	8	15	2	3	6	12	0	0	0	0
FEV	0	0	0	0	0	0	0	0	0	0	0
MAR	20	8	12	2	3	4	5	1	2	1	2
AVR	32	13	19	3	5	7	9	1	2	2	3
MAI	44	19	25	4	6	10	12	2	3	3	4
Total	597	240	357	66	93	125	178	19	34	30	52

F: female / M: male

Throughout the year, a total of 8405 ticks were collected from both domestic dogs and cattle, with notable variations in abundance observed across the months. eight species were identified: *R. bursa*, *R. camicasi*, *R. sanguineus*, *R. turanicus*, *H. anatolicum*, *H. excavatum*, *H. impletatum*, and *H. marginatum*.

Among the identified species, *Rhipicephalus sanguineus* was the most abundant and remained relatively stable across both localities. In contrast, *R. camicasi* and *R. turanicus* were more prevalent in Faïdh El Botma, while *R. bursa* was more frequently recorded in Sidi Baizid, highlighting potential differences in habitat preferences. In terms of sex distribution, females were generally more abundant than males in the *Rhipicephalus* genus, whereas *Hyalomma* spp. exhibited a higher number of males than females. Interestingly, nymphal stages were only recorded in dogs from Sidi Baizid, particularly in certain months, possibly indicating



microclimatic differences or variations in the tick life cycle. Moreover, Faidh El Botma exhibited a higher total tick count than Sidi Baizid, potentially due to differences in environmental factors, host density, or management practices.

3.1.2. Seasonal trends in parasitic indices of tick infestation

The tick infestation dynamics in dogs across the two localities revealed notable variations in parasitic indices (prevalence, abundance, and intensity) with distinct differences in peak infestation periods and tick disappearance trends (Table 6). The highest infestation rates were observed in summer, where 100% of examined dogs were infested in both localities, leading to a significant increase in tick abundance (28.5 and 23.7 ticks per dog) and infestation intensity (28.5 and 23.7 ticks per infested dog). This period also recorded the highest number of collected ticks, reaching 3,420 and 2,487 specimens, highlighting highly favorable conditions for tick proliferation (Table 6).

However, differences between the two localities were evident. In the first locality, infestation peaked exclusively in summer followed by a significant decline in infestation in autumn, where only 20% of dogs were infested. In contrast in Faidh El Botma, two peaks were observed -one in summer and another in autumn- where infestation prevalence remained relatively high (48.6%) and infestation intensity (22.9 ticks per infested dog), indicating possible differences in tick survival rates between sites (Table 6).

Another important observation is the delay in tick disappearance in both localities. While infestation rates declined significantly after the peak periods, ticks did not disappear immediately. In one locality, infestations were still recorded in autumn, though at lower levels, whereas in Faidh El Botma, infestation prevalence and tick abundance remained relatively high before eventually declining. This delay in tick disappearance may be linked to microclimatic differences, variations in host-seeking behavior, or ongoing reproduction cycles within the local tick populations. During winter, infestation rates were at their lowest, with one locality recording zero infestations and the other showing minimal tick presence (10% prevalence and 0.2 ticks per dog). These findings highlight strong seasonal variations and locality-specific differences in tick infestation dynamics (Table 6).



Table 6 : Variations in parasitic indices of tick infestations in dogs across two localities

Season	Spring		Summer		Autumn		Winter		Total		
Locality	S	F	S	F	S	F	S	F	S	F	Total
Number of examined dogs	120	105	120	105	120	105	120	105	480	420	900
Number of infested dogs	68	77	120	105	24	51	12	0	224	233	457
Number of collected ticks	142	292	3420	2487	277	1167	23	0	3862	3946	7808
Infestation prevalence	56,6 7	73,3 3	100,0 0	100,0 0	20,0 0	48,5 7	10 0	0	46,6 7	55,4 8	51,0 7
Tick abundance	1,18	2,78	28,50	23,69	2,31	11,1 1	0,1 9	0	8,05	9,40	8,72
Infestation intensity	2,09	3,79	28,50	23,69	11,5 4	22,8 8	1,9 2	0	11,0 1	12,5 9	11,8 0
S: Sidi Baizid / F: Faidh El Botma											

The tick infestation dynamics in cattle from Sidi Baizid revealed significant variations in parasitic indices across different periods (Table 7). The highest infestation prevalence (96.7%) was recorded during summer, with nearly all examined cattle harboring ticks. This period also exhibited the greatest tick abundance (1.66 ticks per animal) and infestation intensity (1.72 ticks per infested animal), indicating highly favorable conditions for tick proliferation (Table 7).

In spring and autumn, infestation prevalence remained relatively high (69.4% and 71.7%, respectively), though with lower tick burdens compared to summer. Tick abundance decreased to 0.53 in spring and 0.78 in autumn, while infestation intensity was 0.77 and 1.09, respectively. Despite these fluctuations, a considerable proportion of cattle remained infested, suggesting that tick activity extended beyond the peak period (Table 7).

During winter, infestation prevalence dropped to 30.0%, with tick abundance and intensity reaching their lowest values (0.34 and 1.13, respectively). However, the presence of ticks, even at reduced levels, indicates that some individuals continued to serve as hosts, possibly supporting tick survival during unfavorable conditions (Table 7).

**Table 7:** Variations in parasitic indices of tick infestations in cattle in Sidi Baizid locality

Season	Spring	Summer	Autumn	Winter	Total
Number of examined cattle	180	180	180	180	720
Number of infested cattle	125	174	129	54	482
Number of collected ticks	96	299	141	61	597
Infestation prevalence	69,4	96,7	71,7	30,0	66,94
Tick abundance	0,53	1,66	0,78	0,34	3,32
Infestation intensity	0,77	1,72	1,09	1,13	4,71

3.1.3. Species- and sex-specific patterns in parasitic indices of tick infestations

In addition to the overall seasonal trends in infestation, a species-level analysis revealed distinct variations in parasitic indices across hosts, seasons, and sexes. Table 8 presents the prevalence, abundance, and intensity of four *Rhipicephalus* species (*R. bursa*, *R. camicasi*, *R. sanguineus*, and *R. turanicus*). *Rhipicephalus sanguineus* was the most dominant species in both localities, showing a relatively balanced distribution between males and females, with infestation rates remaining high. *R. bursa*, *R. camicasi*, and *R. turanicus* exhibited more pronounced differences in male-to-female ratios, particularly in certain infestation periods. For *R. camicasi* and *R. turanicus*, female ticks were more abundant than males, especially in Faidh El Botma, where infestation intensity for females was consistently higher. In contrast, *R. bursa* showed a more balanced distribution between males and females in Sidi Baizid, but with slight variations depending on the infestation period.

Similarly, a species-level analysis for *Hyalomma* species (*H. anatolicum*, *H. excavatum*, *H. impletatum*, and *H. marginatum*) in cattle revealed distinct seasonal and sex-specific patterns of tick infestation. Table 9 presents the seasonal and sex-specific parasitic indices for these species, showing that *H. anatolicum* and *H. excavatum* exhibited the highest infestation prevalence, particularly in summer. Among these, *H. anatolicum* had the highest overall



infestation rates, with males exhibiting a prevalence of 32.78% and females 26.11%, and infestation intensity values of 0.73 for males and 0.51 for females. Additionally, *H. excavatum* followed a similar pattern, with higher infestation prevalence and intensity in both sexes, particularly in summer. Conversely, *H. impletatum* and *H. marginatum* exhibited lower prevalence and abundance rates, though both species showed a notable increase in infestation intensity during summer. Sex-based differences were also apparent, with females generally exhibiting higher infestation rates than males across all seasons.

Table 8: Species- and sex-specific patterns of parasitic indices in tick infesting dogs across two localities

SPECIES		<i>R. bursa</i>		<i>R. camicasi</i>		<i>R. sanguineus</i>		<i>R. turanicus</i>		
		F	M	F	M	F	M	F	M	
SPRING	IH	S	15	10	15	3	18	9	12	4
		F	23	18	42	38	14	11	41	26
	P%	S	12,50	8,33	12,50	2,50	15,00	7,50	10,00	3,33
		F	21,90	17,14	40,00	36,19	13,33	10,48	39,05	24,76
	N	S	0,30	0,10	0,17	0,03	0,25	0,11	0,18	0,05
		F	0,34	0,20	0,61	0,30	0,23	0,10	0,73	0,26
	I	S	0,53	0,18	0,29	0,06	0,44	0,19	0,31	0,09
		F	0,47	0,27	0,83	0,42	0,31	0,14	1,00	0,35
	IH	S	145	134	46	38	90	89	66	61
		F	98	86	105	105	105	105	105	105
	P%	S	120,83	111,67	38,33	31,67	75,00	74,17	55,00	50,83
		F	93,33	81,90	100	100	100	100	100	100
SUMMER	N	S	5,57	4,70	1,84	1,53	3,48	3,24	2,88	2,28
		F	1,41	1,13	4,52	3,14	1,65	1,29	6,17	4,37
	I	S	5,57	4,70	1,84	1,53	3,48	3,24	2,88	2,28
		F	1,41	1,13	4,52	3,14	1,65	1,29	6,17	4,37
	IH	S	24	24	10	2	24	18	14	10
		F	10	8	39	38	35	35	40	40
	P%	S	20,00	20,00	8,33	1,67	20,00	15,00	11,67	8,33
		F	9,52	7,62	37,14	36,19	33,33	33,33	38,10	38,10
	N	S	0,56	0,39	0,23	0,02	0,37	0,17	0,30	0,11
		F	0,18	0,10	2,25	1,74	1,93	0,95	2,49	1,48
	I	S	2,79	1,96	1,13	0,08	1,83	0,83	1,50	0,54
		F	0,37	0,20	4,63	3,59	3,98	1,96	5,12	3,04
WINTER	IH	S	0	0	1	1	2	2	3	3
		F	0	0	0	0	0	0	0	0
	P%	S	0,00	0,00	0,83	0,83	1,67	1,67	2,50	2,50
		F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	N	S	0	0	0,03	0,01	0,04	0,03	0,06	0,03
		F	0	0	0,03	0,01	0,04	0,03	0,06	0,03
	I	S	0	0	0,03	0,01	0,04	0,03	0,06	0,03
		F	0	0	0,03	0,01	0,04	0,03	0,06	0,03



	F	0	0	0	0	0	0	0	0
I	S	0	0	0,25	0,08	0,42	0,25	0,58	0,33
	F	0	0	0	0	0	0	0	0

IH: infested hosts / F: female / Male

Table 9: Species- and sex-specific patterns of parasitic indices in tick infesting cattle in Sidi Baizid locality

Species		<i>H. anatolicum</i>		<i>H. excavatum</i>		<i>H. impletatum</i>		<i>H. marginatum</i>	
		F	M	F	M	F	M	F	M
Spring	IH	8,00	11,00	11,00	13,00	3,00	3	3	4
	P%	4,44	6,11	6,11	7,22	1,67	1,67	1,67	2,22
	N	0,05	0,08	0,12	0,14	0,02	0,04	0,03	0,05
	I	0,07	0,11	0,17	0,21	0,03	0,06	0,05	0,07
Summer	IH	21	28	33	41	5	6	9	16
	P%	11,67	15,56	18,33	22,78	2,78	3,33	5,00	8,89
	N	0,18	0,25	0,35	0,46	0,06	0,09	0,11	0,16
	I	0,19	0,26	0,36	0,48	0,06	0,10	0,11	0,17
Autumn	IH	14,00	15,00	15,00	20,00	3,00	4	4	7
	P%	7,78	8,33	8,33	11,11	1,67	2,22	2,22	3,89
	N	0,10	0,14	0,15	0,24	0,02	0,04	0,02	0,06
	I	0,14	0,19	0,21	0,34	0,03	0,06	0,03	0,09
Winter	IH	4,00	5,00	7,00	10,00	1,00	1	1	2
	P%	2,22	2,78	3,89	5,56	0,56	0,56	0,56	1,11
	N	0,03	0,05	0,08	0,14	0,01	0,01	0,01	0,02
	I	0,11	0,17	0,26	0,46	0,02	0,04	0,02	0,06
Total	P%	26,11	32,78	36,67	46,67	6,67	7,78	9,44	16,11
	N	0,37	0,52	0,69	0,99	0,11	0,19	0,17	0,29
	I	0,51	0,73	1,00	1,49	0,14	0,25	0,21	0,38

F: female / M: male



3.1.4. Seasonal trends in ecological indices of tick infestation

The analysis of ecological indices in ticks infesting dogs across the two localities revealed notable differences in species composition and relative abundance (Table 10). In Sidi Baizid, *Rhipicephalus bursa* was the most dominant species, representing 36.1% of the collected ticks, followed by *R. sanguineus* (23.87%), *R. turanicus* (18.28%), and *R. camicasi* (11.96%). Conversely, in Faidh El Botma, *R. turanicus* was the most prevalent species (41.2%), followed by *R. camicasi* (33.5%), while *R. bursa* was less abundant (8.9%), indicating significant locality-based variations in species distribution.

The Shannon diversity index (H') was relatively similar between the two localities (1.84 in Sidi Baizid and 1.79 in Faidh El Botma), suggesting comparable species diversity. The evenness index (E) was also high in both sites (0.92 and 0.90, respectively), indicating a relatively balanced distribution of tick species within each locality. However, the differences in relative abundance between species suggest that environmental conditions, host availability, or habitat suitability may influence species dominance (Table 10).

Furthermore, the ecological indices for *Hyalomma* species on cattle indicate a dominance of *H. excavatum*, which exhibited the highest relative abundance (50.75%) and proportion ($\pi = 0.51$). *H. anatolicum* was the second most prevalent species (26.63%), whereas *H. marginatum* (13.74%) and *H. impletatum* (8.88%) were less frequent (Table 11). As indicated in the table, the Shannon-Weaver index (H') was 1.71, reflecting a moderate diversity of tick species. The maximal diversity ($H'_{\max}$) reached 2, suggesting that while species richness was relatively high, dominance by *H. excavatum* led to lower overall diversity. Evenness (E) was 0.85, indicating a moderate distribution balance among the species, though *H. excavatum* was significantly more abundant than the others. These patterns likely reflect host preferences, environmental conditions, and seasonal factors influencing tick populations on cattle (Table 11).

When comparing the overall tick communities on dogs and cattle, diversity was slightly higher in dogs, with *Rhipicephalus* ticks exhibiting greater species richness and a more balanced distribution. In contrast, *Hyalomma* ticks on cattle showed a dominance-driven structure, particularly with the prevalence of *H. excavatum*. Evenness was comparable between the hosts, suggesting that while some species were dominant, minor species maintained a presence in both



groups. These differences may be attributed to host-specific ecological factors, including behavior, habitat use, and the varying capacity of each host to support tick populations.

Table 10: Ecological indices of tick infestations in dogs across two localities

Species	S		F	
	ni	RA%	Ni	RA%
<i>R. bursa</i>	1394	36,10	353	8,9
<i>R. camicasi</i>	462	11,96	1320	33,5
<i>R. sanguineus</i>	922	23,87	646	16,4
<i>R. turanicus</i>	706	18,28	1627	41,2
H'	1.84		1.79	
H' max	2		2	
E	0.92		0.9	

Table 11: Ecological indices of tick infestations in cattle in Sidi Baizid locality

Species	ni	RA%
<i>H. anatolicum</i>	159	26,63
<i>H. excavatum</i>	303	50,75
<i>H. impletatum</i>	53	8,88
<i>H. marginatum</i>	82	13,74
H'	1,71	
H' max	2	
E	0,85	



3.1.5. Species- and sex-specific patterns in ecological indices of tick infestations

The analysis of seasonal dynamics and sex-based differences in ecological indices of ticks infesting dogs and cattle revealed notable variations in species abundance, diversity, and evenness across different periods and between male and female ticks (Table 12 and 13).

In terms of species composition, *Rhipicephalus bursa* was among the most abundant species in Sidi Baizid, while *R. turanicus* and *R. camicasi* were more dominant in Faidh El Botma, particularly among female ticks. *R. sanguineus* displayed a more balanced male-to-female ratio, remaining one of the most prevalent species in both localities (Table 12 A). A sex-based difference in tick abundance was evident, with female ticks generally more abundant than males across most species and periods. This trend was particularly pronounced for *R. camicasi* and *R. turanicus*, where females consistently outnumbered males, suggesting potential differences in feeding duration or host attachment behavior. In contrast, *R. bursa* showed a more balanced male-to-female distribution, particularly in Sidi Baizid (Table 12 A).

The Shannon diversity index (H') revealed fluctuations in species diversity between sexes. The highest diversity was observed in spring, with $H' = 2.74$ for females and $H' = 2.77$ for males in Faidh El Botma, indicating a well-distributed tick community. However, in summer, diversity dropped significantly ($H' = 1.42$ for males and $H' = 0.17$ for females), suggesting a dominance of specific tick species, particularly among females (Table 12 B).

Evenness (E) followed a similar trend, with values being higher in spring ($E = 1.37$ for males and $E = 1.39$ for females) and lower in summer ($E = 1.36$ for males and $E = 0.15$ for females), indicating that tick communities became less evenly distributed as certain species became more dominant. During winter, infestation rates were extremely low, with no female ticks recorded in Faidh El Botma, and only a limited number of male ticks present in Sidi Baizid ($H' = 2.4$). This sharp decline in female presence suggests that sex-based survival strategies may influence tick persistence during unfavorable conditions (Table 12 B).

The relative abundance (RA%) of the four *Hyalomma* species (*H. anatolicum*, *H. excavatum*, *H. impletatum*, and *H. marginatum*) shows distinct seasonal and sex-based patterns (Table 13 A). *H. anatolicum* females are most abundant in spring (34.38%) and winter (36.07%), while males peak in winter (63.93%). *H. excavatum* females dominate in spring (65.63%) but decline sharply in winter (9.84%), with males following a similar pattern, peaking in spring (86.46%)



and decreasing through the year. For *H. impletatum*, both sexes are more abundant in winter, with 22.95% for females and 40.98% for males. *H. marginatum* remains less abundant and more stable, with the highest values for females in spring (19.79%) and for males in spring (30.21%). Overall, females dominate in warmer months, while males of *H. anatolicum* and *H. excavatum* are more prevalent in winter, reflecting possible differences in ecological roles like mating or host-seeking behavior (Table 13 A).

The Shannon diversity index (H') indicates higher species diversity for females in summer (3.39) and winter (2.95), while diversity is lower in spring (1.73) and autumn (1.49), suggesting species dominance during transitional periods. For males, winter (1.51) and spring (1.66) show the highest diversity, with lower values in summer (1.49) and autumn (0.89), reflecting seasonal shifts in species composition (Table 13 B).

The evenness index (E), which reflects how evenly species are distributed, is highest for females in summer (1.69) and winter (1.48), suggesting a more balanced species presence during these seasons. For males, winter (0.75) and spring (0.74) exhibit the highest evenness, while summer (0.45) indicates the dominance of specific species (Table 13 B).

Table 12: Species- and sex-specific patterns of ecological indices in tick infesting dogs across two localities

A										
Species			<i>R. bursa</i>		<i>R. camicasi</i>		<i>R. sanguineus</i>		<i>R. turanicus</i>	
SP	ni	S	F	M	F	M	F	M	F	M
		F	36	12	20	4	30	13	21	6
SU	RA%	S	36	21	64	32	24	11	77	27
		F	25,35	8,45	14,08	2,82	21,13	9,15	14,79	4,23
	S	12,37	7,22	21,99	11	8,25	3,78	26,46	9,28	
	F	668	564	221	184	418	389	345	274	
A	RA%	S	148	119	475	330	173	135	648	459
		F	19,53	16,49	6,46	5,38	12,22	11,37	10,09	8,01
	S	5,95	4,78	19,1	13,3	6,96	5,43	26,06	18,46	
	F	67	47	27	2	44	20	36	13	
W	RA%	S	19	10	236	183	203	100	261	155
		F	24,19	17	9,75	0,72	15,9	7,22	13	4,69
	S	6	4,21	2,42	0,18	3,94	1,79	3,23	1,16	
	F	0	0	3	1	5	3	7	4	
W	RA%	S	0	0	0	0	0	0	0	0
		F	0	0	0	0	0	0	0	0
	S	0	0	13,04	4,35	21,7	13	30,43	17,39	
	F	0	0	0	0	0	0	0	0	
B										
Seasons	Localities		H'		H' max		E			



		F	M	Total	F	M	Total	F	M	Total
SP	S	1,78	0,95	2,74	2	2	2	0,89	0,48	1,37
	F	1,66	1,12	2,77	2	2	2	0,83	0,56	1,39
SU	S	1,42	1,304	2,72	2	2	2	0,71	0,65	1,36
	F	0,17	0,13	0,3	2	2	2	0,08	0,06	0,15
A	S	1,63	0,97	2,59	2	2	2	0,81	0,48	1,3
	F	1,49	1,17	2,65	2	2	2	0,74	0,58	1,33
W	S	1,38	1,02	2,4	1,58	1,58	3,17	0,87	0,64	1,52
	F	0	0	0	0	0	0	0	0	0

SP: spring / SU: summer / A: autumn / W: winter / F: female / M: male

Table 13: Species- and sex-specific patterns of ecological indices in tick infesting cattle in Sidi Baizid locality

A									
Species		Spring		Summer		Autumn		Winter	
		ni	RA%	ni	RA%	ni	RA%	ni	RA%
<i>H. anatolicum</i>	F	33	34,4	96	32,11	18	12,77	22	36,1
	M	45	46,9	40	13,38	25	17,73	39	63,9
<i>H. excavatum</i>	F	63	65,6	56	18,73	27	19,15	6	9,84
	M	83	86,5	9	3,01	44	31,21	9	14,8
<i>H. impletatum</i>	F	10	10,4	14	4,68	4	2,84	14	23
	M	17	17,7	21	7,02	8	5,67	25	41
<i>H. marginatum</i>	F	19	19,8	26	8,7	4	2,84	1	1,64
	M	29	30,2	4	1,34	11	7,8	2	3,28
B									
<i>H'</i>	F	1,73	3,39	1,49	2,38	1,13	2,62	1,44	2,95
	M	1,66		0,89		1,49		1,51	
<i>H' max</i>	F	2	2	2	2	2	2	2	2
	M	2		2		2		2	
<i>E</i>	F	0,87	1,69	0,75	1,19	0,56	1,31	0,72	1,48
	M	0,83		0,45		0,74		0,75	



3.1.6. Statistical analysis of seasonal variation in tick infestation prevalence across localities

In comparing tick infestation prevalence across the two localities throughout the different seasons, significant variations were observed (Table 14). In the spring, the Chi-Square test revealed a significant difference between localities, with a p -value of 0.0137, indicating that the locality plays a role in the prevalence of tick infestations during this season. In the summer, however, Fisher's Exact Test yielded a p -value of 1.0000, suggesting no significant difference in prevalence between the two localities, indicating that locality does not significantly affect tick infestations during this season. The autumn season showed a highly significant difference in infestation prevalence, with a p -value of 1.11×10^{-5} from the Chi-Square test, highlighting a strong influence of locality on tick prevalence during this time. Similarly, the winter season displayed a highly significant difference, with a p -value of 0.00048 from Fisher's Exact Test, further confirming the influence of locality on tick infestations. In summary, while spring, autumn, and winter all showed significant to highly significant differences in tick prevalence across the localities, summer did not exhibit any significant variation. These results suggest that seasonal factors, alongside locality, likely affect tick infestation dynamics in the studied regions.

Table 14: Chi-square and Fisher's test results of seasonal variation in tick infestation prevalence across localities

Season	Test Used	Chi ² / Fisher's p -value	Significance
Spring	Chi-Square Test	0.0137	Significant ($p < 0.05$)
Summer	Fisher's Exact Test	1.0000	Not significant ($p > 0.05$)
Autumn	Chi-Square Test	1.11×10^{-5}	Highly significant ($p < 0.001$)
Winter	Fisher's Exact Test	0.00048	Highly significant ($p < 0.001$)



3.1.7. Statistical analysis of seasonal variation in tick infestation prevalence across hosts

The results of the statistical tests show that both seasonal variations and host factors influence tick infestation prevalence (Table 15). In spring, the Chi-Square Test revealed a significant difference in infestation prevalence (p -value = 0.032), indicating that seasonal factors, combined with host characteristics, contribute to changes in tick abundance. In summer, however, the Fisher's Exact Test yielded a non-significant result (p -value = 0.085), suggesting that tick infestation prevalence remains stable across hosts during this period, with no notable influence of seasonal changes. In autumn, the Chi-Square Test showed a highly significant difference (p -value = 5.060308e-18), suggesting that environmental factors, such as temperature and humidity, along with host-related variables, strongly impact tick dynamics. Similarly, in winter, a significant difference was observed (p -value = 0.0000767), emphasizing the role of colder temperatures and host behavior in influencing tick activity. Overall, the findings suggest that seasonal differences in tick infestation prevalence are influenced by both environmental conditions and the type of host, with notable effects seen in spring, autumn, and winter, while summer remains relatively stable across hosts.

Table 15: Chi-square and Fisher’s test results of seasonal variation in tick infestation prevalence across hosts

Season	Test Used	p -value	Significance
Spring	Chi-Square Test	3.231896e-02	Significant difference in infestation prevalence
Summer	Fisher's Exact Test	8.452387e-02	No significant difference in infestation prevalence
Autumn	Chi-Square Test	5.060308e-18	Significant difference in infestation prevalence
Winter	Chi-Square Test	7.669975e-05	Significant difference in infestation prevalence



3.2. Section 2: Pathogen interaction in *Hyalomma* ticks

This section presents the results of the analysis of pathogen–pathogen interactions identified in *Hyalomma* ticks collected from cattle. The detection of tick-borne pathogens (TBPs) was achieved using high-throughput real-time PCR and DNA sequencing. The analysis focuses on identifying both positive and negative associations between pathogens across seasonal networks, providing insights into potential synergistic or competitive relationships that may affect pathogen persistence and dissemination.

3.2.1. Tick morphological and genetic classification

The ticks were morphologically identified as *H. excavatum*. To confirm this identification with higher precision, advanced PCR techniques were applied. Subsequent sequencing of the 16S rRNA gene definitively confirmed the presence of *H. excavatum*. The phylogenetic relationships of the sequences obtained further supported this identification (Figure 15). The sequences were submitted to GenBank and assigned the following accession numbers: PP800859, PP800860, PP800863, PP800864, PP800865, and PP800866. This multitiered approach of morphological examination followed by genetic verification ensured a robust classification of the tick specimens.

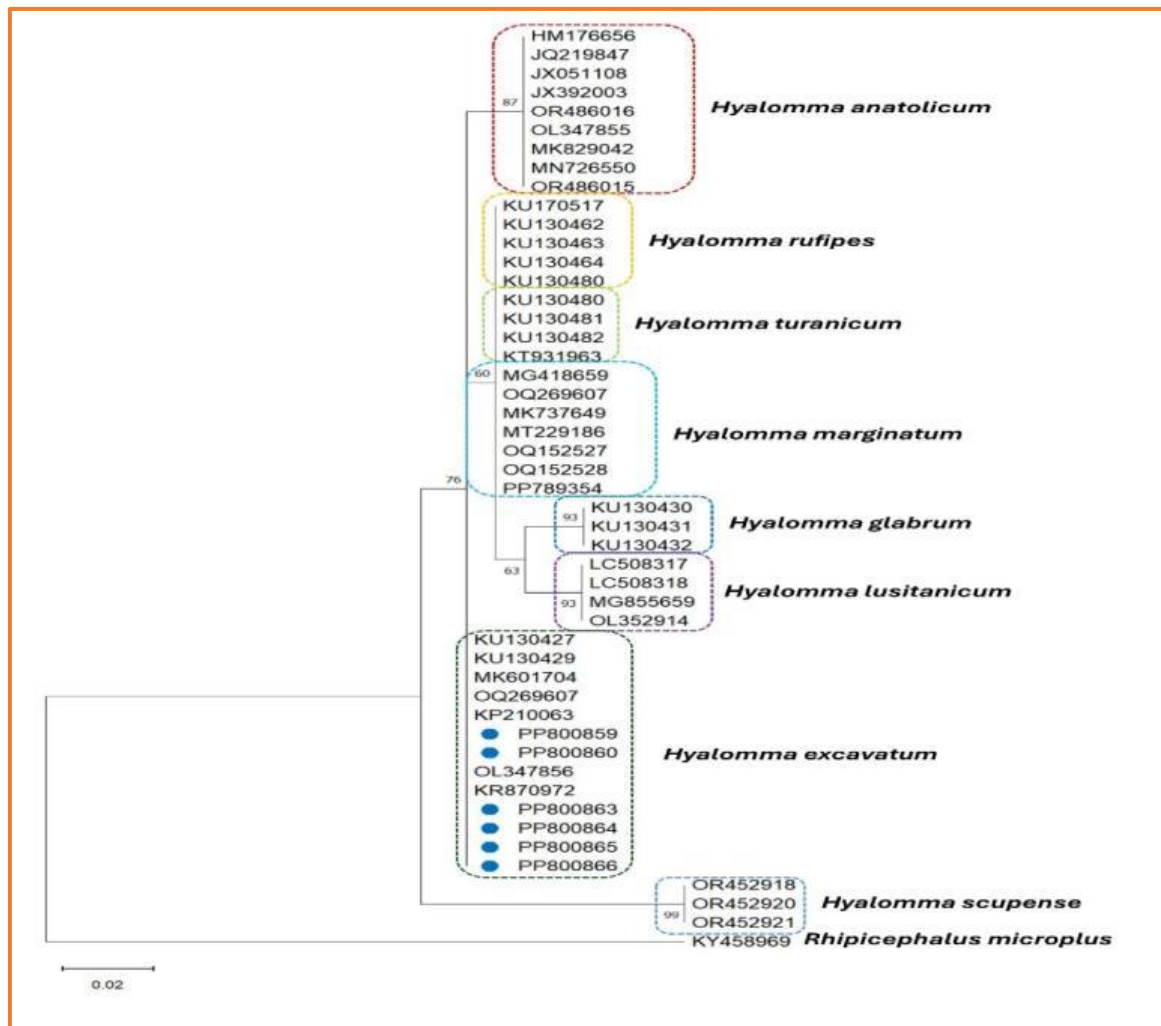


Figure 27 : Phylogeny of the genus *Hyalomma* based on 16S rRNA gene. The evolutionary history was inferred by using the maximum likelihood method and the Tamura 3-parameter model (T92). The analysis contains sequences identified in the current study (marked with blue dot) and retrieved from GenBank database. Accession numbers of sequences are given. Bootstrap values are represented as percentage of internal branches (1000 replicates), and values lower than 50 are hidden. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Rhipicephalus microplus* sequence KY458969 was used to root the tree.

3.2.2. Diversity of TBPs in ticks

The diversity of TBPs was analyzed in 166 *Hyalomma* ticks, consisting of 72 females and 94 males. Overall, 63.9% of female ticks (46/72; Table 16) and 56.4% of male ticks (53/94; Table 17) tested positive for at least one pathogen. Single infections were more common in males (45.8%, 43/94) than females (19.4%, 14/72), while coinfections were more frequent in females (44.4%, 32/72; Table 16) compared to males (10.6%, 10/94; Table 17). Across both sexes, *Rickettsia* spp. dominated the pathogen landscape, with *R. slovaca* most prevalent in females (26.4%) and *Rickettsia* spp. highest in males (31.1%). Other notable pathogens in females



included Apicomplexa (22.2%) and *Borrelia afzelii* (19.4%; Table 16), while males showed lower prevalence for Apicomplexa (5.3%) and *R. slovaca* (15.1%; Table 17).

A χ^2 test ($\chi^2 = 62.94$, $p < 0.001$) confirmed significant differences in TBP diversity between sexes, suggesting distinct transmission dynamics and ecological exposures for males and females. Pathogens such as *A. phagocytophilum*, *B. afzelii*, and *B. spielmanii* were detected only in females, while *Ehrlichia* was found exclusively in males, further highlighting sex-specific pathogen associations.

3.2.3. Coinfections between tick-borne microorganisms

Coinfections were more frequent in females (44.4%, 32/72; Table 16) compared to males (10.6%, 10/94; Table 17). In females, coinfections involving two pathogens occurred in 15.3%, while coinfections of three to eight pathogens were also observed, with *R. slovaca*, *R. conorii*, and Apicomplexa being the most frequent combination (4.2%, 3/72; Table 16). In males, coinfections typically involved two pathogens (9.6%), with the most common pairing being Apicomplexa and *Rickettsia* (3.2%, 3/94; Table 17).

Table 16 : Tick-borne pathogens detected in female ticks collected from cattle using microfluidic PCR.

Vector-borne pathogen(s)	Total	Prevalence rate (%)	95% CI
Total infected ticks (≥ 1 pathogen)	46	63.89	51.65 - 74.63
<i>R. slovaca</i>	19	26.39	17.01 - 38.31
Apicomplexa	16	22.22	13.61 - 33.85
<i>B. afzelii</i>	14	19.44	11.41 - 30.80
<i>Rickettsia</i> sp.	13	18.06	10.33 - 29.26
<i>R. conorii</i>	12	16.67	9.27 - 27.70
<i>N. mikurensis</i>	11	15.28	8.23 - 26.12
<i>B. spielmanii</i>	10	13.89	7.22 - 24.52
<i>Anaplasma</i> sp.	8	11.11	5.26 - 21.26
<i>Hepatozoon</i> sp.	6	8.33	3.43 - 17.88
<i>R. aeschlimannii</i>	5	6.94	2.58 - 16.14
<i>Mycoplasma</i> sp.	2	2.78	0.48 - 10.58
<i>Theileria</i> sp.	2	2.78	0.48 - 10.58
<i>A. phagocytophilum</i>	1	1.39	0.07 - 8.54
<i>Bartonella</i> sp.	1	1.39	0.07 - 8.54
<i>F. tularensis</i>	1	1.39	0.07 - 8.54
Single infections	14	19.44	11.41 - 30.80
<i>Rickettsia</i> sp.	5	6.94	2.58 - 16.14



Apicomplexa	2	2.78	0.48 - 10.58
<i>N. mikurensis</i>	2	2.78	0.48 - 10.58
<i>R. slovac</i>	2	2.78	0.48 - 10.58
<i>B. spielmanii</i>	1	1.39	0.07 - 8.54
<i>Mycoplasma sp.</i>	1	1.39	0.07 - 8.54
<i>Anaplasma sp.</i>	1	1.39	0.07 - 8.54
Mixed infections	32	44.44	32.90 - 56.59
Mixed infection with two pathogens	11	15.28	6.22 - 22.90
<i>R. slovac</i> + <i>R. aeschlimannii</i>	2	2.78	0.48 - 10.58
<i>R. slovac</i> + <i>R. conorii</i>	1	1.39	0.07 - 8.54
<i>R. slovac</i> + Apicomplexa	1	1.39	0.07 - 8.55
<i>Rickettsia sp.</i> + <i>B. spielmanii</i>	1	1.39	0.07 - 8.54
<i>B. afzelii</i> + <i>B. spielmanii</i>	1	1.39	0.07 - 8.55
<i>A. phagocytophilum</i> + <i>Rickettsia sp.</i>	1	1.39	0.07 - 8.56
<i>B. afzelii</i> + <i>Rickettsia sp.</i>	1	1.39	0.07 - 8.57
<i>N. mikurensis</i> + Apicomplexa	1	1.39	0.07 - 8.58
<i>R. slovac</i> + <i>F. tularensis</i>	1	1.39	0.07 - 8.59
<i>B. afzelii</i> + <i>Anaplasma sp.</i>	1	1.39	0.07 - 8.60
Mixed infection with three pathogens	9	12.5	6.22 - 22.90
<i>R. slovac</i> + <i>R. conorii</i> + Apicomplexa	3	4.17	1.08 - 12.50
<i>R. slovac</i> + <i>R. conorii</i> + <i>N. mikurensis</i>	1	1.39	0.07 - 8.54
<i>N. mikurensis</i> + Apicomplexa + <i>R. conorii</i>	1	1.39	0.07 - 8.55
<i>R. slovac</i> + <i>R. conorii</i> + <i>Anaplasma sp.</i>	1	1.39	0.07 - 8.56
<i>B. afzelii</i> + <i>B. spielmanii</i> + <i>R. slovac</i>	1	1.39	0.07 - 8.57
<i>N. mikurensis</i> + Apicomplexa + <i>Rickettsia sp.</i>	1	1.39	0.07 - 8.58
<i>R. slovac</i> + <i>R. conorii</i> + <i>B. afzelii</i>	1	1.39	0.07 - 8.54
Mixed infection with four pathogens	6	8.33	3.43 - 17.88
<i>Rickettsia sp.</i> + <i>Bartonella sp.</i> + <i>B. afzelii</i> + Apicomplexa	1	1.39	0.07 - 8.54
<i>R. slovac</i> + <i>R. conorii</i> + <i>R. aeschlimannii</i> + <i>Hepatozoon sp.</i>	1	1.39	0.07 - 8.55
<i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Rickettsia sp.</i> + <i>Anaplasma sp.</i>	1	1.39	0.07 - 8.56
<i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Anaplasma sp.</i> + Apicomplexa	1	1.39	0.07 - 8.57
Apicomplexa + <i>Mycoplasma sp.</i> + <i>Theileria sp.</i> + <i>Hepatozoon sp.</i>	1	1.39	0.07 - 8.58
<i>B. afzelii</i> + <i>N. mikurensis</i> + <i>Rickettsia sp.</i> + <i>Hepatozoon sp.</i>	1	1.39	0.07 - 8.59
Mixed infection with five pathogens	4	5.56	1.79 - 14.35
<i>B. afzelii</i> + <i>Anaplasma sp.</i> + <i>N. mikurensis</i> + <i>Rickettsia sp.</i> + Apicomplexa	1	1.39	0.07 - 8.54
<i>R. slovac</i> + <i>B. spielmanii</i> + <i>R. conorii</i> + Apicomplexa + <i>Hepatozoon sp.</i>	1	1.39	0.07 - 8.55
<i>B. afzelii</i> + <i>Anaplasma sp.</i> + <i>N. mikurensis</i> + <i>R. aeschlimannii</i> + <i>Hepatozoon sp.</i>	1	1.39	0.07 - 8.56
<i>R. slovac</i> + <i>R. conorii</i> + <i>B. afzelii</i> + <i>B. spielmanii</i> + <i>N. mikurensis</i>	1	1.39	0.07 - 8.57



Mixed infection with six pathogens	1	1.39	0.07 - 8.58
<i>R. slovaca</i> + <i>R. conorii</i> + <i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Theileria</i> sp. + Apicomplexa	1	1.39	0.07 - 8.59
Mixed infection with eight pathogens	1	1.39	0.07 - 8.60
<i>Hepatozoon</i> sp. + Apicomplexa + <i>R. slovaca</i> + <i>R. aeschlimannii</i> + <i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Anaplasma</i> sp. + <i>N. mikurensis</i>	1	1.39	0.07 - 8.61
Not detected	26	36.11	25.37 - 48.35

Table 17: Tick-borne pathogens detected in male ticks collected from cattle using microfluidic PCR.

Vector-borne pathogen(s)	Total	Prevalence rate (%)	95% CI
Total infected ticks (≥1 pathogen)	53	56.38	45.78 - 66.46
<i>Rickettsia</i> sp.	30	31.91	22.89 - 42.44
<i>R. slovaca</i>	15	15.96	9.5 - 25.27
<i>R. conorii</i>	6	6.38	2.62 - 13.91
Apicomplexa	5	5.32	1.97 - 12.55
<i>Anaplasma</i> sp.	1	1.06	0.06 - 6.62
<i>Bartonella</i> sp.	1	1.06	0.06 - 6.62
<i>Ehrlichia</i> sp.	1	1.06	0.06 - 6.62
<i>Francisella tularensis</i>	1	1.06	0.06 - 6.62
<i>N. mikurensis</i>	1	1.06	0.06 - 6.62
<i>R. Aeschlimannii</i>	1	1.06	0.06 - 6.62
<i>Theileria</i> sp.	1	1.06	0.06 - 6.62
Single infections	43	45.74	35.54 - 56.3
<i>Rickettsia</i> sp.	27	28.72	20.09 - 39.12
<i>R. slovaca</i>	11	11.7	6.27 - 20.38
<i>R. conorii</i>	2	2.13	0.37 - 8.21
<i>Ehrlichia</i> sp.	1	1.06	0.06 - 6.62
<i>N. mikurensis</i>	1	1.06	0.06 - 6.62
<i>R. aeschlimannii</i>	1	1.06	0.06 - 6.62
Mixed infections	10	10.64	5.5 - 19.12
Mixed infection with two pathogens	9	9.57	4.74 - 17.85
Apicomplexa + <i>Rickettsia</i> sp.	3	3.19	0.83 - 9.71
<i>R. slovaca</i> + <i>R. conorii</i>	3	3.19	0.83 - 9.71
Apicomplexa + <i>Theleiria</i>	1	1.06	0.06 - 6.62
<i>Anaplasma</i> sp. + <i>F. tularensis</i>	1	1.06	0.06 - 6.62



<i>Bartonella</i> sp. + <i>Rickettsia</i> sp.	1	1.06	0.06 - 6.62
Mixed infection with three pathogens	1	1.06	0.06 - 6.62
Apicomplexa + <i>R. slovaca</i> + <i>R. conorii</i>	1	1.06	0.06 - 6.62
Not detected	41	43.62	33.54 - 54.22

3.2.4. Influence of biotic and abiotic ecological determinants on microbe–microbe interactions

3.2.4.1. Tick sex as a biotic ecological determinant of microbe–microbe interactions

Network analysis of *Hyalomma* ticks revealed sex-specific pathogen interactions (Figure 16a,b). In females, negative associations between *A. phagocytophilum*, *B. afzelii*, and *F. tularensis* (Figure 16a) indicated competitive exclusion, where one pathogen’s presence inhibits others. In males, strong negative interactions were found between *Anaplasma*, *Ehrlichia*, *N. mikurensis*, and *Rickettsia* species (Figure 16b), suggesting competition for resources or immune evasion strategies. FLEs and CLEs played a central role in both sexes, showing positive associations with multiple pathogens, possibly facilitating their coexistence. Moderate positive associations, such as between *R. conorii* and *R. slovaca* in males and between *B. afzelii* and *B. spielmanii* in females, further suggest reduced competition in some coinfections.

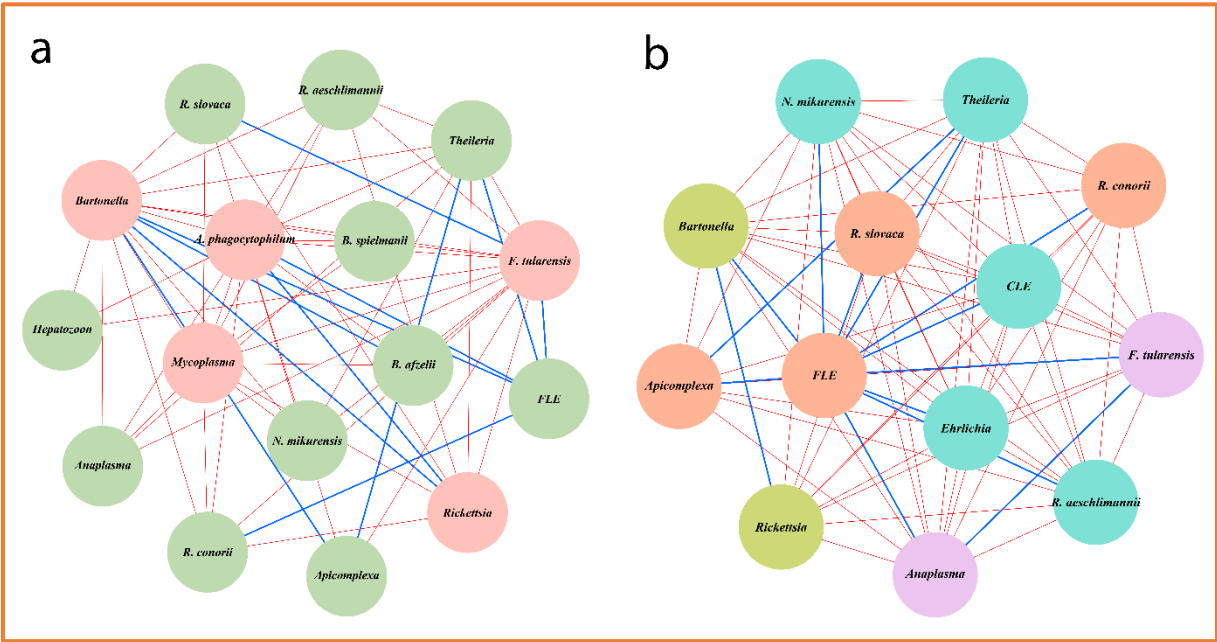




Figure 28 : Microbial co-occurrence networks. Meaningful connections between pairs of microbial species using Yule's Q statistic: (a) female network and (b) male network. Nodes represent distinct microbial taxa, including pathogens and symbionts, while edges signify statistically significant associations with weights between 1 and -1. The colors of nodes are based on modularity class metric, and the size is proportional to the eigenvector centrality value of each taxon. Blue edges denote positive connections, while red edges represent negative ones. CLE, Coxiella-like endosymbionts; FLE, Francisella-like endosymbiont.

3.2.4.2. Seasonal changes as an abiotic ecological determinants of the pathogen–pathogen interaction

The co-occurrence networks reveal clear seasonal differences in pathogen interactions between female and male *Hyalomma* ticks (Figure 17a–h). In winter, female networks show balanced interactions between *Rickettsia* species and FLE (Figure 17a), while male networks exhibit more competitive dynamics, such as negative interactions between *R. slovacica* and Apicomplexa (Figure 17b). In spring, female networks are more complex, dominated by positive interactions suggesting cooperation (Figure 17c), while male networks are simpler and more competitive, with taxa like *Bartonella* absent from females but present in males (Figure 17d). In summer, females show a more diverse and complex network, with largely positive interactions and the presence of *F. tularensis* (Figure 17e), while males display stronger negative interactions, particularly between *Rickettsia* and *Theileria* (Figure 17f). Autumn networks reflect similar patterns, with females showing more balanced interactions (Figure 17g), while males demonstrate stronger competitive pressures, particularly between species like *Anaplasma* and *R. conorii* (Figure 17h). In pathogen–pathogen co-occurrence network of the same guild, the nodes all maintained the same value of degree centrality, suggesting the same numbers of connections for each node within the network regardless of the differences in the nature and preference of interaction (Appendix 5). On the other hand, the degree centrality values of the shared nodes varied between the TBPGs networks for the same node, demonstrating that tick sex and seasonal changes influence not only the nature of interaction but also the number of associations that a taxon can establish within one condition (Appendix 6). Overall, while both female and male networks display seasonal variations in species composition and interaction patterns, males tend to exhibit more pronounced competitive interactions, particularly in summer and autumn. Females show a similarly dynamic but slightly less competitive network structure, indicating subtle differences in ecological strategies and adaptations between the sexes throughout the year.

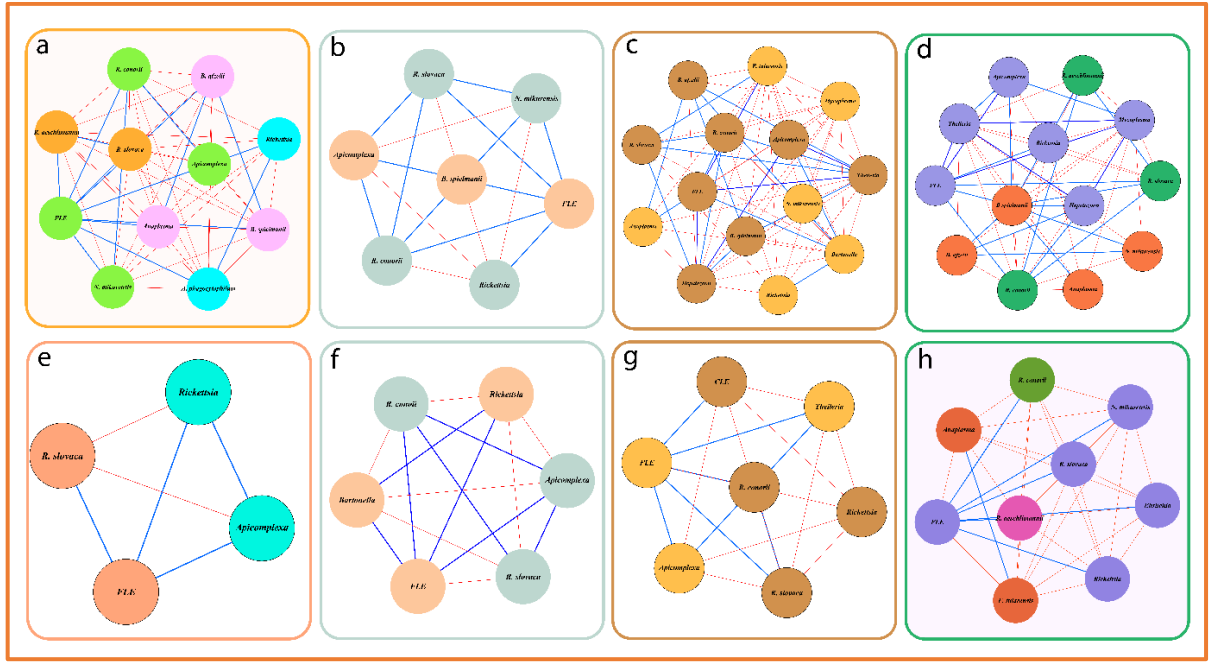


Figure 29 : Microbial co-occurrence networks across seasons and sexes. Using co-occurrence networks, we analyzed the complex dynamics of microbe-microbe interactions in male and female *Hyalomma* ticks across different seasons. The figure includes separate networks for each season, presented as follows: winter networks in (a) for females and (b) for males; spring networks in (c) for females and (d) for males; summer networks in (e) for females and (f) for males; and autumn networks in (g) for females and (h) for males. The visualization showcases significant connections between pairs of microbes using Yule's Q statistic. Each node symbolizes a unique microbe, with edges indicating statistically significant associations with weights between 1 and -1 . Blue edges denote positive connections, while red edges represent negative ones. The color and size of nodes reflect modularity class and eigenvector centrality, respectively. CLE, *Coxiella*-like endosymbiont; FLE, *Francisella*-like endosymbiont.



3.2.5. Genetic diversity and variation in pathogen guilds

Significant genetic diversity was observed among sequences within guilds composed of bacterial pathogens (16S rRNA), surpassing that found within guilds grouping eukaryotic microorganisms (18S rRNA; Figures 18 and 19). *Rickettsia slovaca* was the only pathogen identified in all TBPGs, while *Ehrlichia* sp. was only identified in M and MA guilds (Figure 18a). The rest of the bacterial pathogens were identified in both F and M guilds and in at least one corresponding to a seasonal change guild (Figure 18a). Protozoan pathogens presented lower genetic diversity, Apicomplexa (other) was identified in a greater number and variety of guilds followed by *Hepatozoon* sp., while *Theileria* sp. was only identified in F, FSU, and FA guilds (Figure 18b). Analysis revealed that the majority of studied guilds, with the exception of MSP 16S rRNA, displayed statistically significant variations ($p < 0.05$) in genetic distances among their constituent sequences (Figure 19, Tables 18 and 19). This trend was consistently observed across comparisons between different guilds (Figures 18 and 19 and Tables 18 and 19).

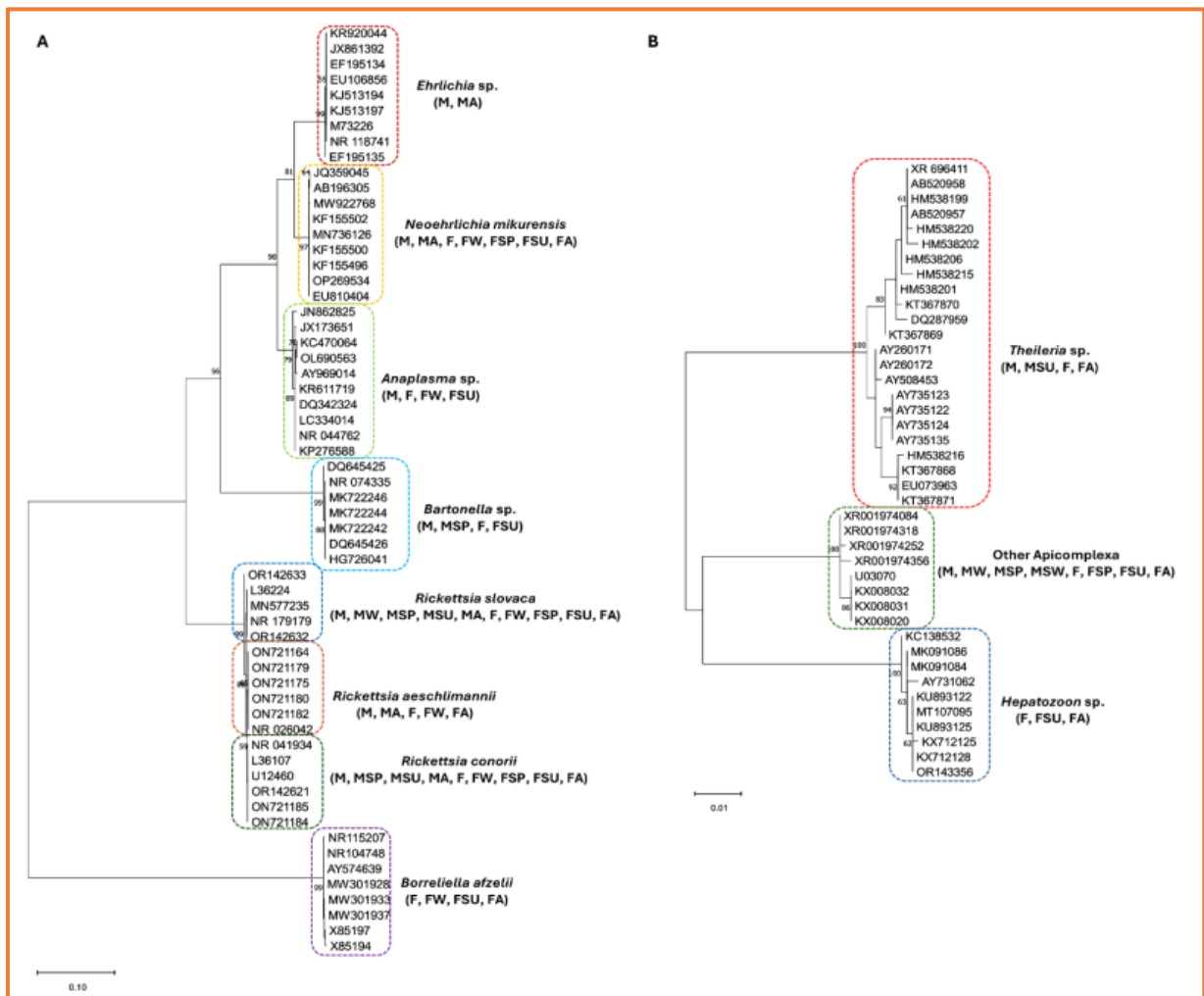




Figure 30 : Distribution of guilds across the phylogenetic trees of tick-borne pathogens (TBPs) linked with *Hyalomma excavatum*. (a) Tickborne bacteria associated with *H. excavatum*. The phylogram was constructed from the 16S rRNA gene, and the evolutionary history was inferred using the maximum likelihood method with the Tamura–Nei model and Gamma distribution (TN93+G). (b) Tick-borne protozoa associated with *H. excavatum*. The phylogram was constructed from the 18S rRNA gene, and the evolutionary history was inferred using the maximum likelihood method with the Tamura 3-parameter model (T92). For both trees, accession numbers of sequences are given. Bootstrap values are represented as percentages of internal branches (1000 replicates), with values lower than 50 hidden. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Letters represent different guilds: F, females; FA, females collected in autumn; FSP, females collected in spring; FSU, females collected in summer; FW, females collected in winter; M, males; MA, males collected in autumn; MSP, males collected in spring; MSU, males collected in summer; MW, males collected in winter.

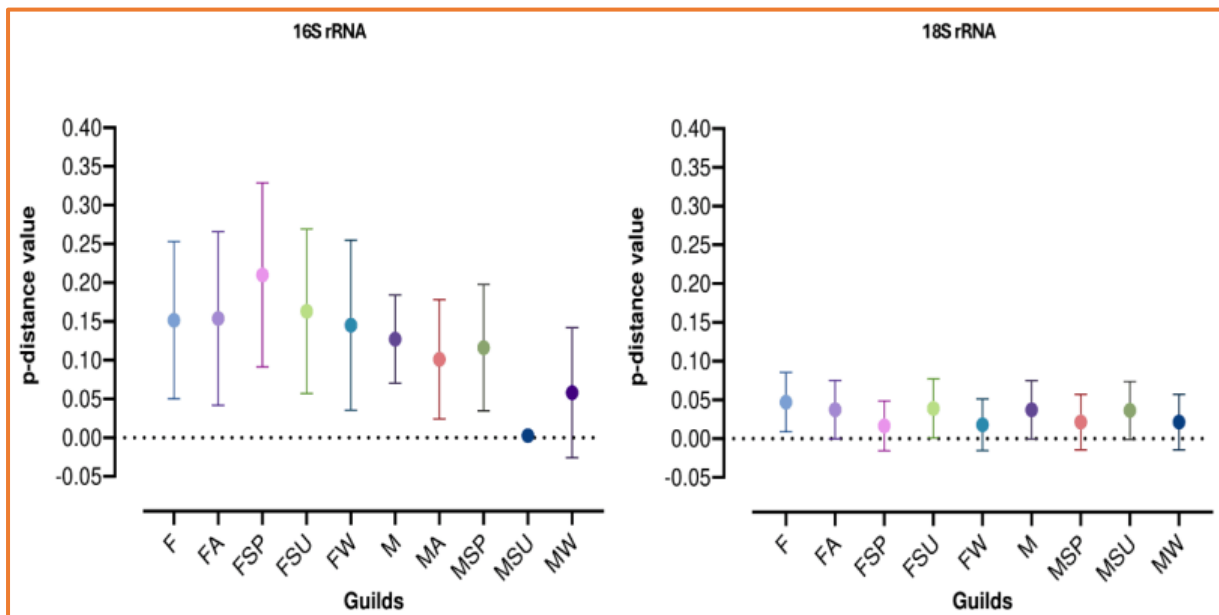


Figure 31 : Genetic distances between sequences grouped into different guilds. a) Genetic distances between 16S rRNA sequences grouped into different guilds. (b) Genetic distances between 18S rRNA sequences grouped into different guilds. The genetic distances were calculated as pairwise distances. The diagram shows the mean p-distance values and standard deviation ranges within each studied guild. The guilds are represented by the following abbreviations: F (females); FA (females collected in autumn); FSP (females collected in spring); FSU (females collected in summer); FW (females collected in winter); M (males); MA (males collected in autumn); MSP (males collected in spring); MSU (males collected in summer); MW (males collected in winter).



Table 18 : Statistical significance of genetic distances calculated as pairwise distance between particular 16 rRNA sequences grouped into guilds

Guilds	Guilds and p-values								
	F	FA	FSP	FSU	FW	M	MA	MSP	MSU
FA	0.862								
FSP	<0.001*	<0.001*							
FSU	0.154	0.185	<0.001*						
FW	0.131	0.124	<0.001*	0.004*					
M	0.009*	0.007*	<0.001*	<0.001*	0.525				
MA	0.003*	<0.001*	<0.001*	<0.001*	0.036*	0.211			
MSP	0.049*	0.041*	<0.001*	0.814	0.099	<0.001*	0.3811		
MSU	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	
MW	0.008*	0.005*	<0.001*	0.002*	0.009*	0.012*	0.001*	0.002*	0.189

p - level of statistical significance, * - statistically significant

Table 19 : Statistical significance of genetic distances calculated as pairwise distance between particular 18S rRNA sequences grouped into guilds

Guilds	Guilds and p-values							
	F	FA	FSP	FSU	FW	M	MSP	MSU
FA	0.006*							
FSP	<0.001*	<0.001*						
FSU	0.010*	0.753	<0.001*					
FW	<0.001*	<0.001*	0.887	<0.001*				
M	0.005*	0.904	<0.001*	0.665	<0.001*			
MSP	<0.001*	<0.001*	0.534	<0.001*	0.627	<0.001*		
MSU	0.003*	0.816	<0.001*	0.591	<0.001*	0.913	<0.001*	
MW	<0.001*	<0.001*	0.5344	<0.001*	0.6275	<0.001*	0.999	<0.001*



3.2.6. Confirmation of pathogen presence using conventional PCR.

Utilizing conventional PCR techniques, specific genetic targets were amplified to confirm the presence of selected pathogenic species. Amplification of the 18S rRNA gene generated fragments of 1258 and 1373 bp, indicating the presence of *Babesia occultans* (accession numbers: P809771 and PP809772) in two out of nine samples tested. For *Rickettsia* species, PCR assays targeting the *gltA* and *ompB* genes produced amplicons of 282, 380, 173, and 169 bp, respectively. These results confirmed two distinct *Rickettsia* sequences in 2 out of 15 samples tested (PP828624 (282 bp) and PP828625 (380 bp)). Further analysis specifically identified *Rickettsia sibirica* (PP828626 (173 bp)) and *Rickettsia africae* (PP828627 (169 bp)) in 2 out of 18 samples tested. Additionally, two samples tested positive for *F. tularensis subsp. holarctica* via PCR in two out of two samples tested. However, sequencing of these PCR products was not attempted. The utilization of species-specific primers in these PCR assays ensured accurate identification of the target pathogens, thereby, enhancing the reliability and robustness of the study's findings.

3.3. Section 3: Microbiome dynamics in *Hyalomma* ticks

This section presents the results of the microbiome analysis of *Hyalomma excavatum* ticks collected across three seasons: autumn, spring, and summer. The characterization of the microbial communities was based on 16S rRNA sequencing, identifying 640 taxa across 21 samples. The analysis focuses on alpha and beta diversity to assess the richness, evenness, and compositional differences of the microbiome across seasons.

Additionally, network and sub-network analyses were performed to explore microbial co-occurrence patterns and identify key taxa driving microbiome structure. The robustness of these networks was assessed to evaluate the stability and resilience of microbial associations under potential ecological disturbances.

Seasonal variations in microbial composition, as well as their implications for tick biology and pathogen transmission, are highlighted throughout this section



3.3.1. Seasonal variations in diversity and taxonomic composition of *H. excavatum* microbiome.

Distinct patterns were observed in the diversity, and composition, of microbial communities within *H. excavatum* ticks collected in spring, summer, and autumn. Alpha diversity metrics, including richness and evenness (Figure 20a), showed no significant differences across the three seasons (Kruskal–Wallis test, $p > .05$). Beta diversity analysis, based on Bray–Curtis dissimilarity, revealed significant seasonal clustering of microbial communities (PERMANOVA: $R^2 = 0.23$, $p = 0.001$; Figure 20b). Pairwise comparisons showed that microbial compositions in spring differed significantly from both summer ($p_{\text{adj}} = 0.0285$) and autumn ($p_{\text{adj}} = 0.0030$), while summer and autumn communities were more similar ($p_{\text{adj}} = 0.102$). These findings suggest that while overall microbial richness remained stable, community composition was significantly shaped by seasonal variation.

The analysis of taxonomic composition (Figure 20c) highlighted a group of microbial taxa shared across all seasons (Supplementary Table S1), accounting for largest part of the microbial community. However, unique taxa were predominantly detected in autumn, reflecting potential environmental influences during this period. The Jaccard clustering (Figure 20d) provided additional support for seasonal differentiation, with samples clustering tightly within their respective seasons, underscoring the distinct microbial profiles associated with each sampling period. Furthermore, differential abundance analyses detected eight specific bacterial taxa with significant differences between seasons (Figure 20e; Supplementary Table S2). For instance, *Francisella* and *Candidatus* Midichloria exhibited higher relative abundances in autumn compared to spring and summer. Yersiniaceae and Enterobacteriaceae were predominantly found in spring, while *Staphylococcus* was dominant in autumn and spring but not in summer. Conversely, *Acinetobacter*, *Pseudomonas*, and *Veillonella* were less abundant in spring.

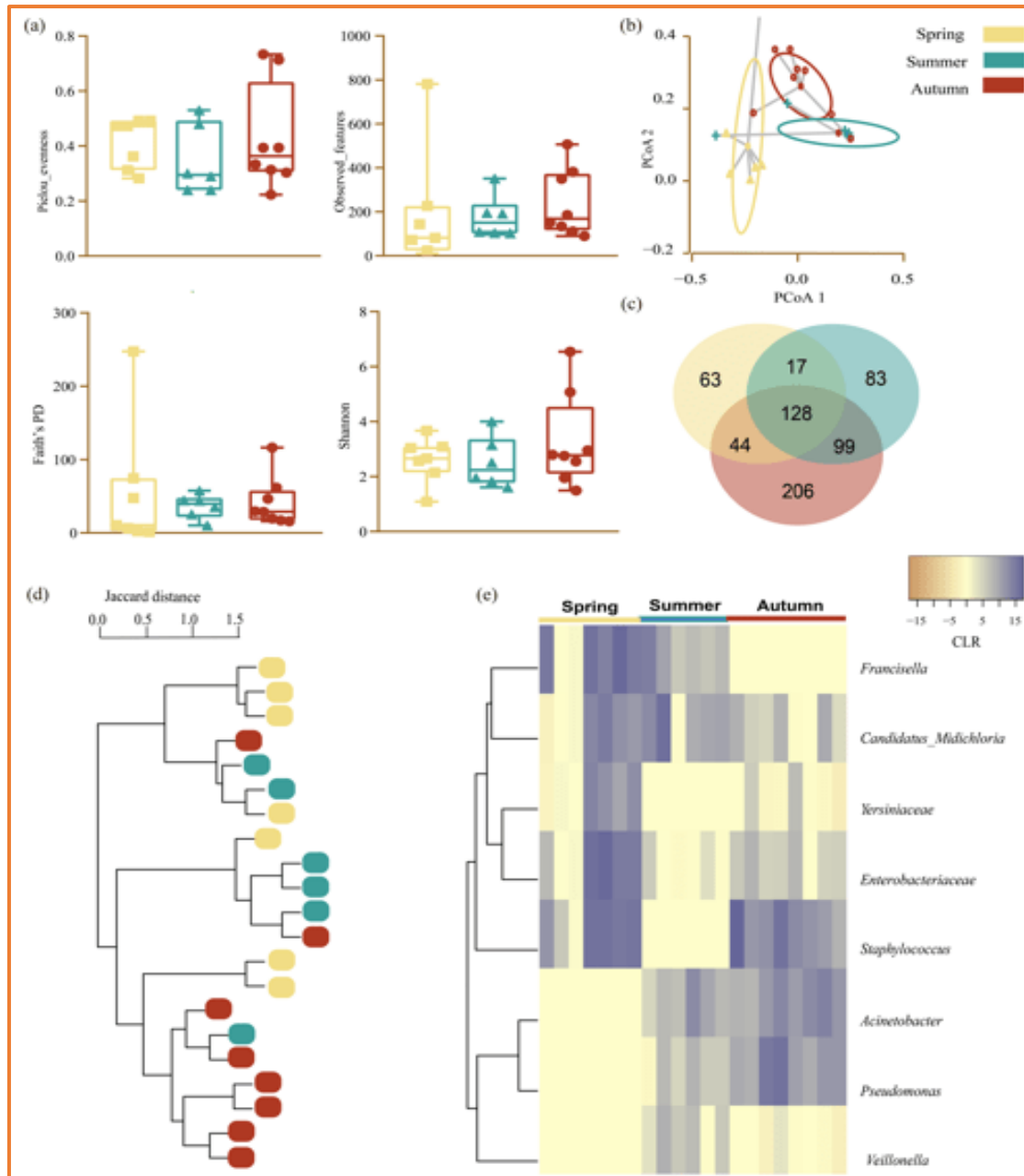


Figure 32 : Comparison of diversity of complex microbial communities within *Hyalomma excavatum* over seasons. (a) Comparison of alpha diversity between A, SP and SU (Kruskal–Wallis test, no significant differences for $p > 0.05$), (b) Comparison of beta – diversity with Bray Curtis dissimilarity index between A, SP, and SU. Beta dispersion of three sets of samples (global comparison). Small circles, crosses and triangles represent samples, and ellipses represent centroid position for each group. This test use principal coordinate analysis (PCoA), it is used to explore and to visualize variability in a microbial community. ANOVA test was performed and showed that beta dispersion of the three sets of samples (three conditions) is significantly different ($p \leq 0.05$). (c) Venn Diagram displaying the comparison of taxa composition in ticks collected at the three sampling times. Common and unique taxa between the conditions are represented. (d) Jaccard clusterisation of the tick samples collected in A, SP and SU. The samples are represented by circles and the groups by colors (legend). (e) Comparison of relative abundance of complex microbial communities within *H. excavatum* over seasons. The taxa were clustered based on relative abundance (calculated as clr transformed values). Each column represents the clr values for bacterial taxa per sample and per group. Each line represents bacterial taxa with significant changes between the datasets. Color represent the clr value (range from -15 to 15)



3.3.2. Seasonal variation in *H. excavatum* microbial community networks

The seasonal variations of microbial communities assembly in *H. excavatum* ticks were explored using co-occurrence networks constructed separately for autumn, spring, and summer, as illustrated in Figure 21a–c. The autumn network showed intermediate complexity with 144 nodes and 538 edges (Figure 21a, Table 20). The spring network was the simplest, with only 35 nodes and 51 edges (Figure 21b, Table 20). The summer network was the most complex, with 169 nodes and 404 edges (Figure 21c, Table 20), reflecting high microbial diversity and interactions. These networks provide insight into microbial interactions and community structures across seasons. Modularity varied across seasons, being highest in summer (0.77) and lowest in autumn (0.45) (Table 20), indicating more structured communities in warmer conditions. Other metrics, such as clustering coefficient and average degree, also highlighted distinct seasonal dynamics (Table 20). Seasonal differences significantly influenced the microbial networks in *H. excavatum*.

Table 20 : Topological features of microbial networks with *Francisella* and *Rickettsia* presence in each season.

Network features	Autumn	Spring	Summer
No. of nodes	144	35	169
No. of edges	538	51	404
Positive interaction	538	51	404
Negative interaction	0	0	0
Modularity	0,45	0,7	0,77
Network diameter	9	5	8
Average degree	7,47	2,91	4,78
Weighted degree	6,01	2,52	3,83
Clustering coefficient	0,54	0,57	0,57
Connectivity	13	7	15

3.3.3. Local connectivity of *Francisella* and *Rickettsia*

Analyzing the local connectivity of *Francisella* (Figure 21 d-f) and *Rickettsia* (Figure 21 g-h) across seasons revealed notable differences in their associations and potential roles in microbial networks. In autumn, *Francisella* exhibited a specific relationship with *Deinococcus* (Figure 2



d), while *Rickettsia* exhibited multiple associations -9 taxa- (Figure 21 g), highlighting distinct ecological roles for these taxa during this season. In spring, *Francisella* maintained its connection with *Deinococcus* (Figure 21e), whereas *Rickettsia* was not detected in any sample, as confirmed by 16S rRNA sequencing data, emphasizing a seasonal absence of *Rickettsia* and the persistent influence of *Francisella*. In summer, *Francisella* displayed extensive connectivity, interacting with 14 taxa (Figure 21 f), compared to *Rickettsia*, which was related to 2 taxa (Figure 21 h). This broader connectivity of *Francisella* suggests a more dominant role in shaping microbial interactions during summer, potentially reflecting its ecological importance and adaptability across varying seasonal conditions.

Table 21 : Topological features of microbial networks without *Francisella* taxon in each season.

Network features	Autumn	Spring	Summer
No. of nodes	151	31	173
No. of edges	533	49	379
Positive interaction	533	49	379
Negative interaction	0	0	0
Modularity	0.45	0.69	0.75
Network diameter	8	5	9
Average degree	7.06	3.16	4.38
Weighted degree	5.69	2.74	3.52
Clustering coefficient	0.53	0.66	0.54
Connectivity	19	6	18

Table 22 : Topological features of microbial networks without *Rickettsia* taxon in each season.

Network features	Autumn	Summer
No. of nodes	156	171
No. of edges	508	396
Positive interaction	508	396
Negative interaction	0	0
Modularity	0.48	0.75
Network diameter	8	9
Average degree	6.51	4.36
Weighted degree	5.24	3.71



Clustering coefficient	0.58	0.56
Connectivity	21	14

3.3.4. Impact of *Francisella* and *Rickettsia* on microbial community assembly

To evaluate the impact of *Francisella* and *Rickettsia* on the microbial community structure of *H. excavatum*, seasonal sub-networks were constructed excluding these taxa—without *Francisella* and without *Rickettsia* (Figure 22a-e). When *Francisella* was removed, the number of nodes increased slightly across all seasons compared to the complete networks, with 151, 31, and 173 nodes in autumn, spring, and summer (Figure 22 a-c, Table 21), respectively. This suggests that *Francisella*'s dominance might influence the presence of other microbial taxa. The number of edges decreased marginally, with 533, 49, and 379 edges observed in autumn, spring, and summer, respectively (Table 21), when compared with the network with *Francisella* (Table 20). Positive interactions remained the sole type of correlation, while modularity values were consistent with the complete networks, showing a slightly structured microbial community, particularly in summer (0.75) (Table 20-21).

Topological metrics such as network diameter and clustering coefficient showed nuanced differences. The clustering coefficient decreased in summer but increased in spring when *Francisella* was removed (Table 21), indicating changes in localized interaction density. Similarly, the network diameter expanded in autumn and summer), reflecting longer paths between taxa in the absence of *Francisella* (Table 21). These results suggest that *Francisella* plays a central role in shaping microbial network structure, particularly by maintaining connectivity and localized clustering in summer.

Excluding *Rickettsia* had a distinct impact on the microbial networks in autumn and summer - As it was absent from all samples in spring - (Figure 22d-e, Table 22). The number of nodes increased slightly to 156 and 171 in autumn and summer, respectively, while the number of edges decreased to 508 and 396, respectively (Table 22). Modularity values were similar to the networks without *Francisella*, with a structured and compartmentalized network observed in summer (Table 22). The clustering coefficient was highest in autumn, indicating tight interactions among remaining taxa, while the connectivity values dropped slightly compared to the networks with *Rickettsia* (Table 22).



Comparing the sub-networks without *Francisella* or *Rickettsia* highlights their differential roles in the microbial ecology of *H. excavatum*. While both taxa contribute significantly to network structure and interactions, *Francisella* appears to have a stronger effect on network density and clustering, particularly in summer. *Rickettsia*, on the other hand, influences connectivity and overall interaction dynamics.

3.3.5. Comparison of community compositions across seasons with and without *Francisella* and *Rickettsia*

The microbial communities in *H. excavatum* ticks were analyzed using Venn diagrams to identify taxa shared across and exclusively present in one of the three networks—network with *Francisella* and *Rickettsia* (N), network without *Francisella* (WoF), and network without *Rickettsia* (WoR)—across autumn, spring, and summer (Figure 22f–h; Appendix 9). Here, “unique” refers to taxa detected only in a given network and absent in the others. In autumn (Figure 22 f), 126 taxa formed a stable community shared across all networks, while the removal of *Francisella* or *Rickettsia* revealed distinct patterns. WoF included 16 unique taxa, and WoR revealed nine, demonstrating their individual roles in the microbial network (Figure 22 f). In spring (Figure 22 g), *Francisella*'s dominance profoundly influenced the community. Removing *Francisella* exposed 21 unique taxa in WoF, previously overshadowed, while only 10 taxa were shared between N and WoF. In summer (Figure 22 h), 142 taxa were consistently shared across all networks, indicating a resilient core. However, removing *Francisella* or *Rickettsia* revealed 7 and 13 unique taxa in WoF and WoR (Figure 22 h), respectively, emphasizing their contributions to the microbial structure. These findings reveal a dynamic interplay between core and unique taxa, underscoring the significant roles of *Francisella* and *Rickettsia* in shaping the tick microbiome across seasons.

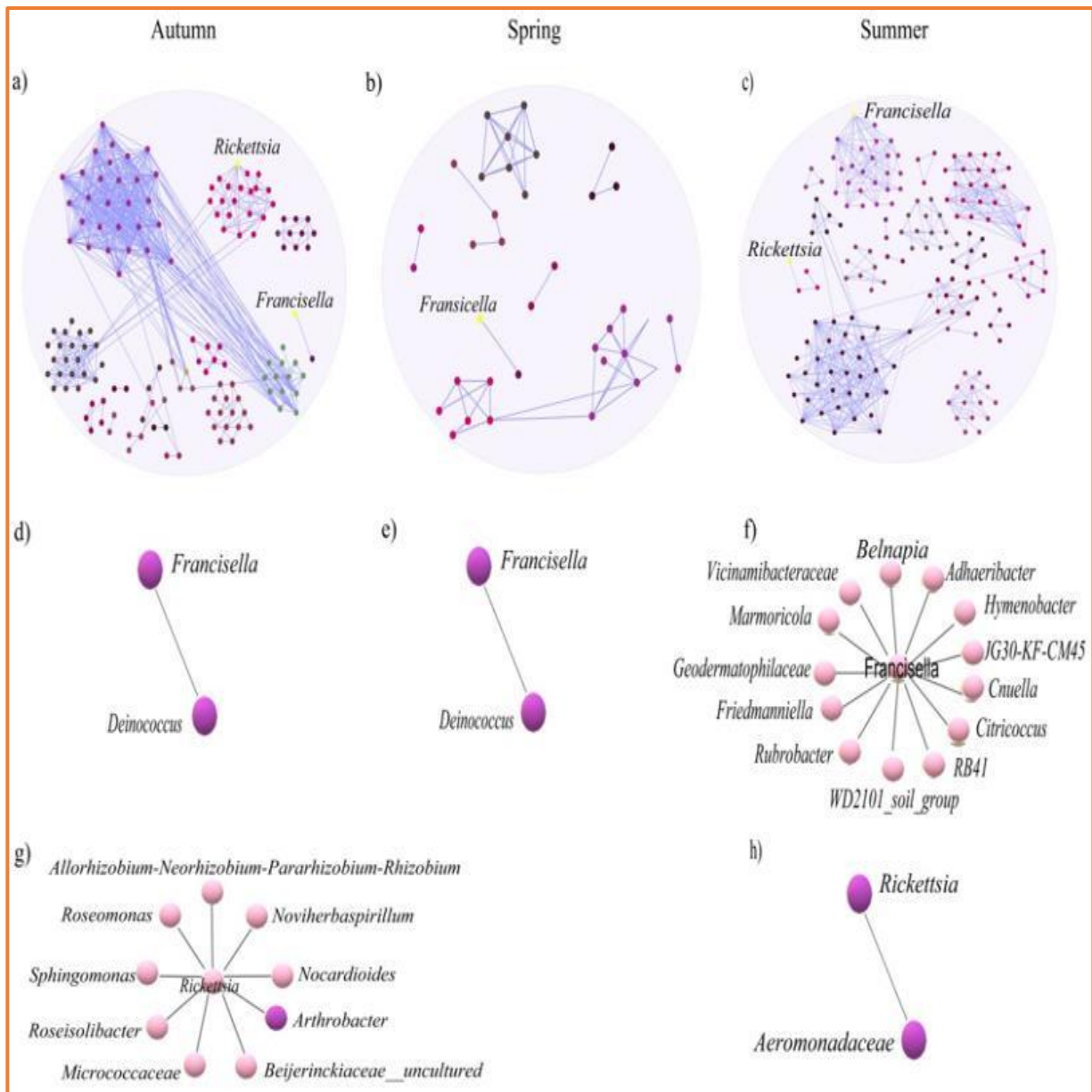


Figure 33 : Seasonal variation and Connectivity, of *Francisella* and *Rickettsia*. (a–c) Co-occurrence networks of tick microbiota for (a) autumn, (b) spring, and (c) summer, where node colors indicate modularity classes (modules of co-occurring taxa), node size represents eigenvector centrality, and edge colors indicate strong positive correlations (blue); (d–h) local connectivity and module composition of *Francisella* during (d) autumn, (e) spring, and (f) summer, and of *Rickettsia* during (g) autumn and (h) summer.

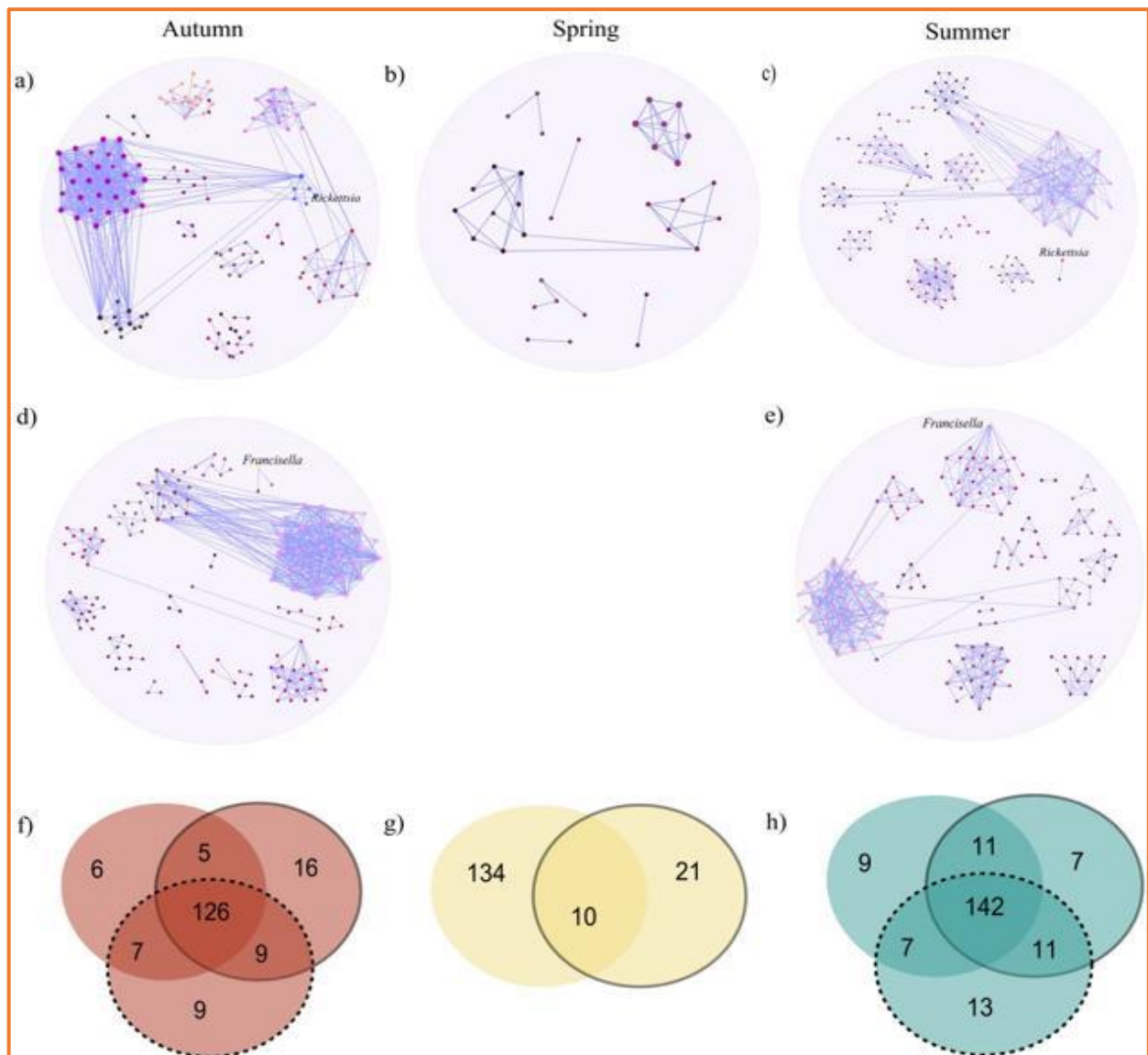


Figure 34 : Impact of *Francisella* and *Rickettsia* Removal on Seasonal Pathogen and Module Composition Comparisons. (a-e) sub-networks without *Francisella* in (a) autumn, (b) spring, and (c) summer, and without *Rickettsia* in (d) autumn and (e) summer. Venn diagrams (f-h) compare module compositions across seasons (autumn, spring, and summer) with and without the presence of *Francisella* (solid contour) and *Rickettsia* (dotted contour).

3.3.6. The robustness comparison of microbial networks under various node addition and removal scenarios across seasons

Network robustness varies across seasons and is influenced by the presence of *Francisella* and *Rickettsia*. Cascading failures (green) and betweenness-based attacks (red) emerge as the most disruptive, rapidly reducing connectivity. In the autumn network, where both *Francisella* and *Rickettsia* are present (Figure 23a), connectivity drops by nearly 80% when



only 20% of nodes are removed under cascading failure, whereas random attacks (blue) lead to a more gradual decline, with less than 40% connectivity loss at the same removal fraction. A similar pattern is observed in (Figure 23b), though the gap between cascading and other targeted attacks is smaller, indicating variations in network resilience. Seasonal differences further influence robustness, with the summer network (Figure 23c) exhibiting greater stability, as connectivity remains higher across all attack strategies compared to autumn and spring. The absence of *Francisella* (Figures 23d–f) increases network vulnerability, particularly under cascading and degree-based attacks, while the removal of *Rickettsia* (Figures 23g–h) similarly reduces structural stability. For example, in the autumn network without *Rickettsia* (Figure 23g), betweenness and cascading attacks result in over 90% connectivity loss when 30% of nodes are removed. The network robustness varied across seasons and was influenced by the presence of *Francisella* and *Rickettsia*. The results indicate that cascading failures (green) and betweenness-based attacks (red) were the most disruptive, causing rapid connectivity loss.

The comparison of predicted APL values across autumn, spring, and summer microbial networks (Figure 24a) demonstrates clear seasonal variations in response to node addition. In all three seasons, APL gradually increases as nodes are added, indicating a consistent structural trend. The autumn network (3.72 to 4.62) closely parallels the spring network (3.73 to 4.57), while the summer network starts at a higher APL (4.17) and increases modestly to 4.29, suggesting a more compact baseline structure. Despite overall similarity, autumn and spring diverge in later stages, with autumn reaching a higher final APL, indicating slightly more fragmentation. The summer network maintains the lowest range of APL values, highlighting greater inherent connectivity and robustness under node addition (Figure 24i).

The removal of *Francisella* (Figure 24b) revealed notable changes in network robustness, particularly in spring, where its absence significantly increased APL. In autumn, *Francisella* removal increased APL from 3.89 to 4.44, though the effect was weaker than that of *Rickettsia* removal. In summer, *Francisella* removal caused minimal changes (4.18 to 4.25), indicating limited impact compared to autumn.

The removal of *Rickettsia* (Figure 24c) had varying effects across seasons. In autumn, APL increased more significantly than with *Francisella* removal, suggesting a greater impact on network fragmentation. In spring, *Rickettsia* removal resulted in minimal changes compared



to *Francisella* removal, highlighting differences in their roles across seasons. The summer network exhibited a slight increase in APL, reinforcing the idea that *Rickettsia* plays a more limited stabilizing role in this season.

The LCC analysis (Figure 24d) further highlights the season-specific roles of *Francisella* and *Rickettsia*. In autumn, both bacteria maintained LCC values between 138.42 and 157.27. When *Francisella* was removed (Figure 24e), LCC values increased (141.61 to 165.84), indicating a destabilizing effect, while *Rickettsia* removal (Figure 5f) led to even higher LCC values (144.30 to 171.62), suggesting it also weakens network robustness. In spring, *Francisella* removal caused a significant drop in LCC (26.22 to 91.90), underscoring its essential role in maintaining network connectivity, whereas in summer, *Francisella* removal slightly increased LCC (162.61 to 187.45), suggesting a minor destabilizing effect, while *Rickettsia* removal further raised LCC (164.03 to 183.52), indicating a limited stabilizing role in this season.

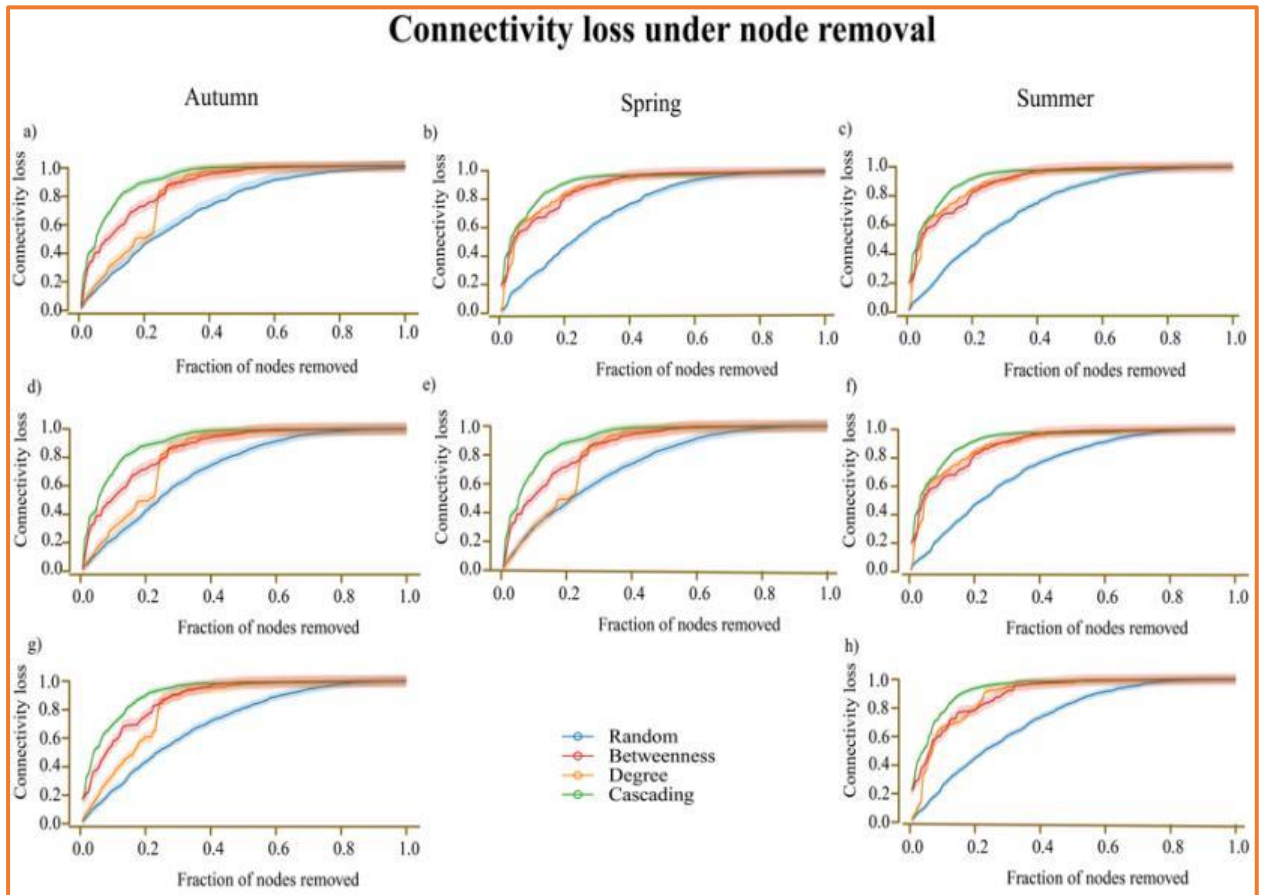


Figure 35 : Seasonal effects of *Francisella* and *Rickettsia* on network robustness. Connectivity loss under node addition and removal scenarios is depicted for various attack strategies - betweenness (red), cascading (green), degree (orange), and random (blue)- in networks with *Francisella* and *Rickettsia* presence in (a) autumn, (b) spring, and (c) summer; *Francisella*



absence in (d) autumn, (e) spring, and (f) summer; and *Rickettsia* absence in (g) autumn and (h) summer.

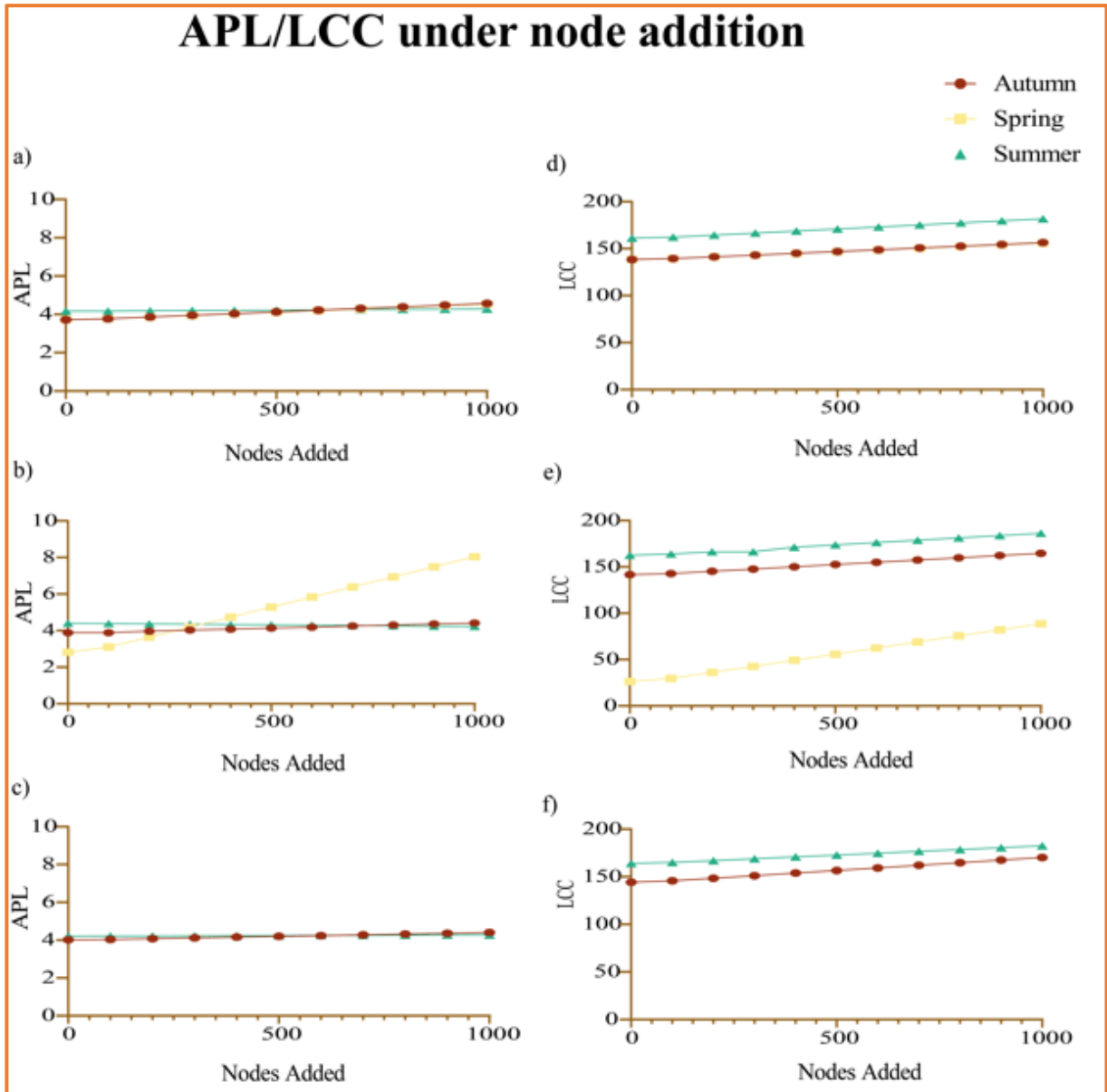


Figure 36 : Seasonal effects of *Francisella* and *Rickettsia* on network structure. Average path length (APL) values are shown for networks with *Francisella* and *Rickettsia* presence (a), *Francisella* absence (b), and *Rickettsia* absence (c) across seasons. Largest connected component (LCC) values are compared across seasons for networks with *Francisella* and *Rickettsia* presence (d), *Francisella* absence (e), and *Rickettsia* absence (f).



3.4. Section 4: Botanical control of *Rhipicephalus sanguineus*

This section presents the results of evaluating the acaricidal efficacy of three essential oils against *Rhipicephalus sanguineus* larvae and engorged females. The study involved two distinct immersion tests: one targeting larval stages and the other focusing on the reproductive aspects of engorged females.

Initially, the yield and chemical composition of the essential oils were analyzed to determine the major bioactive compounds responsible for their acaricidal properties. This chemical profiling provides insights into the potential mechanisms underlying the observed effects.

For larvae, the analysis includes mortality rates at various concentrations and exposure times, alongside the estimation of lethal doses (LD_{50} and LD_{90}) and lethal times (LT_{50} and LT_{90}). In addition, pairwise comparisons were conducted using the Tukey test to identify significant differences between essential oil doses.

For engorged females, the results cover the impact on reproductive parameters, including egg mass reduction, reproductive efficiency index, and egg hatching inhibition rates. The study highlights dose-dependent variations and the potential of these botanical extracts as alternatives for tick management.

3.4.1. Yield and chemical composition of the essential oils

The extraction yield of essential oils from each plant is presented in the table below, offering insights into the efficiency of the extraction process. Additionally, the chemical composition is detailed, highlighting key bioactive compounds that may contribute to their acaricidal properties.

**Table 23 :** The extraction yield of essential oils from each plant.

Plant species	Extracted weight (g)	essential oil	Treated weight (g)	plant	Yield %
<i>Artemisia herba alba</i>	3		200		1.5
<i>Rosmarinus officinalis</i>	2.97		200		1.49
<i>Thymus vulgaris</i>	2.15		50		4.3

Table 24 : Percentages of main chemical compound found in the Essential Oils

Plant	Molecule	Percentage %
<i>Artemisia herba alba</i>	Davanone	48.84
<i>Rosmarinus officinalis</i>	Camphor	43.52
<i>Thymus vulgaris</i>	Carvacrol	18.3

The essential oil yield of *Artemisia herba-alba* obtained in this study was 1.5%. Within forty minutes of extraction, twenty-nine major compounds were identified. Davanone dominated the composition, representing nearly half of the total content (48.84%), followed by chrysanthenone (15.97%) and camphor (14.84%), with the remaining compounds ranging from 0.04% to 5.69%. For *Rosmarinus officinalis*, the essential oil yield was 1.49%. Forty-nine main compounds were extracted within forty minutes. Camphor accounted for the largest proportion (43.52%), representing almost half of the total composition. This was followed by γ -terpinene (13.66%), camphene (13.20%), and α -pinene (8.9%), with the remaining compounds ranging from 0.01% to 4.32%.



The essential oil yield of *Thymus vulgaris* was notably higher than the other plants, reaching 4.3%. Gas chromatography-mass spectrometry (GC-MS) analysis (Table 24) identified twelve compounds, accounting for 99.98% of the total composition. Six major constituents were detected: carvacrol (18.3%), spathulenol (13.78%), borneol (13.39%), camphor (12.59%), linalool (10.05%), and eucalyptol (9.18%), with the remaining compounds ranging from 2.98% to 4.54%. Full chromatograms and detailed compositions are available in Appendixes A and B.”

3.4.2. The mortality rate of larvae treated with essential oils

The table below presents the mortality rates of *Rhipicephalus sanguineus* larvae following treatment with three essential oils. It includes data collected after specified exposure times and concentrations for each oil, providing an overview of larval responses under controlled conditions.

Table 25 : Toxicity of essential oils on *Rhipicephalus sanguineus* larvae

Essential oil	Concentration (µl/ml)	Mortality of treated larvae (%)			Mortality of control larvae %
		24h	48h	72h	24h, 48h, 72h
<i>Artemisia herba alba</i>	0.5	27	29	32	0
	1	40	46	51	0
	2	65	71	76	0
	3	80	84	87	0
	5	93	96	100	0
	0.5	20	23	26	0



<i>Rosmarinus officinalis</i>	1	31	34	38	0
	2	46	49	57	0
	3	65	70	74	0
	5	85	89	100	0
<i>Thymus vulgaris</i>	0.5	31	34	40	0
	1	48	57	60	0
	2	74	80	84	0
	3	85	88	90	0
	5	97	98	100	0

The mortality rate of *Rhipicephalus sanguineus* larvae treated with essential oils ranged from 20% to 100%, depending on the type of oil, exposure time, and concentration used. Mortality assessments were conducted after 24, 48, and 72 hours of treatment using two concentrations: a minimum dose of 0.5 µl/ml and a maximum dose of 5 µl/ml. Among the three essential oils tested, *Thymus vulgaris* exhibited the most potent larvicidal activity. At the minimum dose of 0.5 µl/ml, it achieved mortality rates of 31%, 34%, and 40% after 24, 48, and 72 hours, respectively. When the concentration was increased to 5 µl/ml, the mortality rates rose significantly to 97% after 24 hours, 98% after 48 hours, and reached complete mortality (100%) after 72 hours of exposure.

In contrast, *Rosmarinus officinalis* showed the lowest larvicidal effectiveness. At the minimum concentration of 0.5 µl/ml, mortality rates were comparatively lower, with 20% after 24 hours, 23% after 48 hours, and 26% after 72 hours. Even at the highest dose of 5 µl/ml, *R. officinalis* resulted in 85% mortality after 24 hours and 89% after 48 hours. However, like the other



extracts, it induced complete mortality (100%) after 72 hours of treatment at the maximum dose.

Despite the differences observed in larvicidal activity at lower concentrations and shorter exposure times, all three essential oils demonstrated complete efficacy at the highest concentration after 72 hours of treatment (Figure 23).

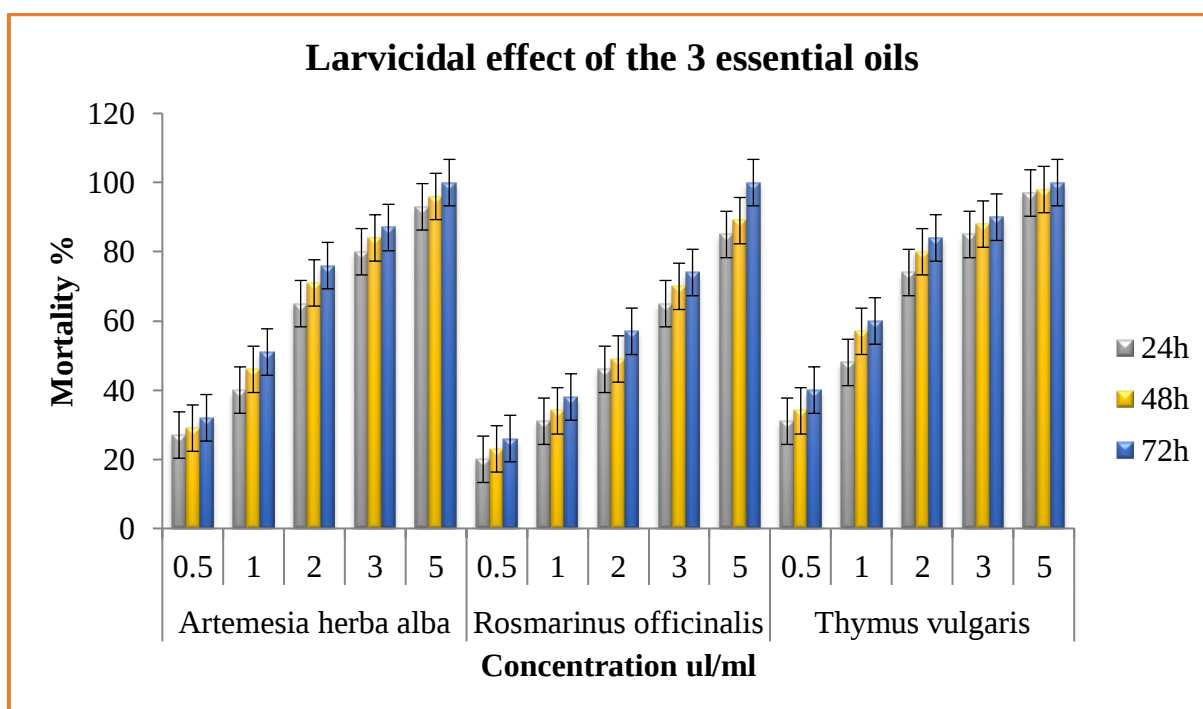


Figure 37 : Evolution of mortality in *Rhipicephalus sanguineus* larvae treated with different doses of essential oils from 3 plants

3.4.3. Estimated LD₅₀ and LD₉₀ obtained after treatment of larvae with essential oils

Table 25 presents the estimated LD₅₀ and LD₉₀ values for the three essential oils tested against *Rhipicephalus sanguineus* larvae after 24, 48, and 72 hours of exposure. These toxicological parameters represent the doses required to achieve 50% (LD₅₀) and 90% (LD₉₀) larval mortality, providing essential information on the potency of each essential oil.

The data illustrate how the required lethal doses vary depending on the exposure time, with longer durations generally reducing the concentrations needed to achieve the same mortality



rates. This comparison allows for a better understanding of the relative efficacy of each essential oil and offers insights into the optimal dosage and application timing for effective tick control.

Table 26 : Lethal doses 50 and 90 of various essential oils (probits as a function of dose)

Plants	Time (hour)	Regression equation	LD50	LD90	R ²
<i>Artemisia herba alba</i>	24h	$Y = -0.69 + 0.46x$	1.493	4,127	0.968
	48h	$Y = -0.62 + 0.5x$	1.249	3.632	0.971
	72h	$Y = -0.68 + 0.63x$	1.077	2.990	0.974
<i>Rosmarinus officinalis</i>	24h	$Y = -0.95 + 0.41x$	2.286	5.380	0.988
	48h	$Y = -0.88 + 0.43x$	2.021	4.935	0.991
	72h	$Y = -0.85 + 0.51x$	1.635	3.783	0.995
<i>Thymus vulgaris</i>	24h	$Y = -0.57 + 0.51x$	1.133	3.413	0.971
	48h	$Y = -0.42 + 0.52x$	0.879	3.102	0.962
	72h	$Y = -0.43 + 0.61x$	0.734	2.677	0.947

Rhipicephalus sanguineus larvae exposed to the three essential oils for 24, 48, and 72 hours exhibited average mortality rates that correlated with the doses applied. The coefficient of determination (R²) values exceeded 0.90 for all tests, indicating a strong relationship between dose and mortality. After 72 hours of exposure, *Artemisia herba-alba* essential oil showed LD₅₀ and LD₉₀ values of 1.077 µl/ml and 2.990 µl/ml, respectively. For *Rosmarinus officinalis*, the corresponding LD₅₀ and LD₉₀ values were 1.635 µl/ml and 3.783 µl/ml. In contrast, *Thymus vulgaris* essential oil demonstrated the lowest LD₅₀ and LD₉₀ values, at 0.734 µl/ml and 2.677 µl/ml, respectively, after 72 hours of exposure.



3.4.4. Estimation of LT 50 and LT 90 after treatment of larvae with essential oils

Table 27 presents the toxicological parameters (LT₅₀ and LT₉₀) for the three essential oils tested against *Rhipicephalus sanguineus* larvae. These parameters indicate the time required to achieve 50% (LT₅₀) and 90% (LT₉₀) mortality, providing essential information on the speed and efficacy of each essential oil.

The LT₅₀ and LT₉₀ values reflect the rapidity with which each essential oil acts on the larvae, with variations linked to their chemical compositions and bioactive compounds. Understanding these differences is critical for selecting suitable essential oils for practical applications.

Table 27 : Lethal times 50 and 90 for various essential oils (probits as a function of time)

Plants	Dose ($\mu\text{l/ml}$)	Regression equation	LT50 (h)	LT90 (h)	R ²
<i>Artemisia herba alba</i>	0.5	$Y = -0.69 + 3.02E-3x$	227.835	650.998	0.989
	1	$Y = -0.39 + 5.8E-3x$	66.893	287.921	0.997
	2	$Y = 0.23 + 6.69E-3x$	*	157.531	0.999
	3	$Y = 0.7 + 5.93E-3x$	*	97.433	0.998
	5	$Y = 1.2 + 0.01x$	*	18.158	1
<i>Rosmarinus officinalis</i>	0.5	$Y = -0.94 + 413E-3x$	227.572	538.028	1
	1	$Y = -0.59 + 3.97E-3x$	149.898	472.656	0.995
	2	$Y = -0.26 + 5.77E-3x$	45.076	267.411	0.935



<i>Thymus vulgaris</i>	3	$Y = 0.26 + 5.38E-3x$	*	189.917	0.998
	5	$Y = 0.85 + 7.92E-3x$	*	38.561	1
	0.5	$Y = -0.63 + 5.05E-3x$	124.375	377.154	0.969
	1	$Y = -5.53 + 0.1x$	55.66	234.389	0.929
	2	$Y = 0.5 + 6.86E-3x$	*	113.096	0.998
	3	$Y = 0.96 + 4.47E-3x$	*	71.939	1
	5	$Y = Y = 1.71 + 7.21E-3x$	*	*	1

* Calcul was not possible

Rhipicephalus sanguineus larvae exposed to varying doses of the three essential oils exhibited dose- and time-dependent mortality rates. The coefficient of determination values exceeded 0.9 for all tests, indicating a strong correlation between exposure time and larval mortality. The lethal times (LT₅₀ and LT₉₀) varied depending on the concentration and type of plant extract used. Notably, essential oils from all three plants achieved 100% mortality in the shortest recorded time when applied at a dose of 5 µl/ml.

3.4.5. Comparative study of doses of essential oils used against *Rhipicephalus sanguineus* Larvae

Table 28 presents the results of the analysis of variance (ANOVA) performed on arcsine-transformed larval mortality rates, highlighting the variations in larval mortality rates based on the different doses of essential oils tested. This comparative study aims to evaluate how increasing concentrations of essential oils influence the mortality of *Rhipicephalus sanguineus* larvae, thereby determining the most effective dosage for larvicidal activity.

The analysis provides a clear understanding of the dose-response relationship, where higher doses are expected to result in greater mortality rates. Such findings are essential for identifying the optimal concentration that achieves high mortality with minimal use of plant material,



ensuring both cost-effectiveness and sustainability. Additionally, the comparison of doses sheds light on the potency of each essential oil, revealing differences in their acaricidal activities.

Table 28 : Analysis of variance applied to variations in larval mortality rates according to the doses of essential oils used

	Sum of Squares	df	Mean Square	F	p-value
Between Groups	1.270	4	0.317	82.451	.000
Within Groups	0.039	10	0.004		
Total	1.308	14			

Table 28 presents the results of the one-way analysis of variance (ANOVA) performed on arcsine-transformed larval mortality rates, revealing highly significant differences between the doses of essential oils tested ($F = 82.45$; $p < 0.001$). This analysis confirms the existence of a strong dose-response relationship, with higher doses resulting in significantly greater larval mortality.

The pairwise comparison was performed using the Tukey test to identify significant differences between the mortality rates associated with each essential oil dose. This post-hoc analysis helps determine which specific doses differ significantly from one another, providing a clearer understanding of the dose-response relationship. The results highlight the most effective concentrations, allowing for precise recommendations on optimal doses for controlling *Rhipicephalus sanguineus* larvae while ensuring efficient use of essential oils.

Table 29 : Pairwise Comparison Between Doses Using the Tukey Test for larvicidal effect

(I) Dose	(J) Dose	Mean Difference (I-J)	Standard Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
0.500	1	-,20312*	.05067	.016	-.3699	-.0364



	2	-.47081*	.05067	.000	-.6375	-.3041
	3	-.58378*	.05067	.000	-.7505	-.4170
	5	-.83289*	.05067	.000	-.9996	-.6661
1	0.5	.20312*	.05067	.016	.0364	.3699
	2	-.26769*	.05067	.003	-.4344	-.1009
	3	-.38067*	.05067	.000	-.5474	-.2139
	5	-.62977*	.05067	.000	-.7965	-.4630
2	0.5	.47081*	.05067	.000	.3041	.6375
	1	.26769*	.05067	.003	.1009	.4344
	3	-.11298	.05067	.244	-.2797	.0538
	5	-.36208*	.05067	.000	-.5288	-.1953
3	0.5	.58378*	.05067	.000	.4170	.7505
	1	.38067*	.05067	.000	.2139	.5474
	2	.11298	.05067	.244	-.0538	.2797
	5	-.24910*	.05067	.004	-.4158	-.0824
5	0.5	.83289*	.05067	.000	.6661	.9996
	1	.62977*	.05067	.000	.4630	.7965
	2	.36208*	.05067	.000	.1953	.5288
	3	.24910*	.05067	.004	.0824	.4158



Table 26 presents the results of the Tukey post-hoc test, revealing distinct groupings among the tested doses. Nearly all dose comparisons showed statistically significant differences in larval mortality, except between 2 and 3 $\mu\text{l/ml}$, which did not differ significantly ($p = 0.244$). This suggests that these two intermediate concentrations produced comparable effects on larval mortality. In contrast, the remaining doses showed progressively greater differences, reflecting a clear dose-response pattern. Lower doses (0.5 and 1 $\mu\text{l/ml}$) were significantly less effective than higher doses (3 and 5 $\mu\text{l/ml}$), with the highest mortality observed at 5 $\mu\text{l/ml}$. These results confirm that increasing the concentration of essential oils significantly enhances their acaricidal activity against *Rhipicephalus sanguineus* larvae, with each dose increment—except between 2 and 3 $\mu\text{l/ml}$ —leading to a notable rise in efficacy.

3.4.6. The reproductive aspects of the *R.sanguineus* females treated with essential oils

The table below provides a comprehensive overview of the impact of the three essential oils on the reproductive parameters of *Rhipicephalus sanguineus* engorged females. It details key reproductive aspects such as the oviposition rate, egg mass weight, and reproductive index following treatment with different essential oils. These parameters are crucial in evaluating the sublethal effects of essential oils, as they directly influence the reproductive success and population dynamics of the tick species.

By comparing the results obtained for each essential oil, the table highlights variations in reproductive performance, shedding light on how certain oils may inhibit or reduce the reproductive potential of *R. sanguineus* females.

Table 30 : Impact of Essential Oils on Reproductive Parameters of *Rhipicephalus sanguineus* Females

Essential oil	Dose ($\mu\text{l/ml}$)	EPI (%)	RO (%)	REI	EP (%)	Hatching (%)	HR (%)
<i>Artemisia herba alba</i>	1	26.04	27.42	442745.62	38.31	85	15
	2	13.44	57.61	201617.65	68.21	75	25
	10	11.79	61.50	129691.36	78.83	55	45



	30	6.66	78.44	39930.07	93.53	30	70
<i>Rosmarinus officinalis</i>	1	33.82	5.75	642579.37	10.46	95	5
	2	25.15	20.68	427529.76	32.58	85	15
	10	20.44	33.27	265659.4	56.63	65	35
	30	16.41	46.84	131256.16	78.74	40	60
<i>Thymus vulgaris</i>	1	20.60	35.03	299679.94	58.24	75	25
	2	19.98	44.32	247187.74	61.02	60	40
	10	9.65	68.48	48271.6	92.12	25	75
	30	5	83.80	10000	98.38	10	90
Control		32.27	0	645394.34	0	100	0

The three essential oils tested demonstrated varying degrees of efficacy. The lowest efficacy was recorded for *Rosmarinus officinalis* at the minimum dose, with a reduction of 10.46%, while the highest was observed for *Thymus vulgaris* at the maximum dose, reaching 98.38%. This variation in efficacy was reflected in the reduction of egg mass produced by engorged females, ranging from 33.82% for the minimum dose of *Rosmarinus officinalis* to 5% for the maximum dose of *Thymus vulgaris*.

These reductions were accompanied by a significant decrease in the reproductive efficacy index compared to the control group. Consequently, the overall reduction in egg production by *R. sanguineus* females ranged from 5.75% at the lowest concentration of *Rosmarinus officinalis* to 83.8% at the highest concentration of *Thymus vulgaris*.



Moreover, *Thymus vulgaris* essential oil exhibited a strong inhibitory effect on egg hatching, with up to 90% inhibition observed at the maximum concentration. Notably, the few larvae that did hatch failed to survive, dying within a few hours post-hatching.

3.4.7. Comparative study of the doses of essential oils used against the hatching of *Rhipicephalus sanguineus* eggs

The table below presents the results of the analysis of variance (ANOVA) applied to assess variations in the hatching rates of *Rhipicephalus sanguineus* eggs based on different doses of the three essential oils. The analysis was conducted using arcsine-transformed values to ensure the normality of percentage data and to evaluate the effect of increasing concentrations on egg hatching over a defined period.

Table 31 : Analysis of variance applied to variations in egg hatching rates as a function of essential oil doses used

	Sum of Squares	Df	Mean Square	F	<i>p</i> -value
Between groupes	,768	3	,256	8,219	,008
Within groupes	,249	8	,031		
Total	1,017	11			

This table shows a statistically significant difference in the egg-hatching rates of *Rhipicephalus sanguineus* based on the doses of essential oils applied ($F = 8.219$; $df = 3$; $p = 0.008$). The dose factor appears to be a major source of variability, highlighting its influence on the ovicidal activity of the oils.

Additionally, Table 32 compares the different doses in pairs. This two-by-two comparison, conducted using the Tukey test, provides a detailed understanding of how each dose level differs from the others in terms of its impact on egg-hatching rates.

**Table 32 :** Pairwise comparison between Essential Oil doses using the Tukey Test for egg hatching effect

(I) Dose	(J) Dose	Mean Difference (I-J)	Standard Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
1.000	2.000	.15307	.14406	.720	-.3083	.6144
	10.000	.42292	.14406	.073	-.0384	.8842
	30.000	.65982*	.14406	.008	.1985	1.1211
2.000	1.000	-.15307	.14406	.720	-.6144	.3083
	10.000	.26985	.14406	.310	-.1915	.7312
	30.000	.50675*	.14406	.032	.0454	.9681
10.000	1.000	-.42292	.14406	.073	-.8842	.0384
	2.000	-.26985	.14406	.310	-.7312	.1915
	30.000	.23691	.14406	.409	-.2244	.6982
30.000	1.000	-.65982*	.14406	.008	-1.1211	-.1985
	2.000	-.50675*	.14406	.032	-.9681	-.0454
	10.000	-.23691	.14406	.409	-.6982	.2244



Table 32 presents a detailed comparison of the different essential oil doses, revealing a significant separation between lower and higher concentration groups. The first group comprises doses of 1 $\mu\text{l/ml}$ and 2 $\mu\text{l/ml}$, which showed comparable hatching rates. In contrast, the second group, including the 30 $\mu\text{l/ml}$ dose, exhibited a more substantial reduction in egg hatching. This classification highlights the increasing ovicidal efficacy with higher concentrations, indicating a clear dose-dependent pattern in the inhibition of *Rhipicephalus sanguineus* egg hatching, as supported by statistical analysis.



4. CHAPTER IV: DISCUSSION

4.1. Section 1: Tick inventory and sampling

This section discusses the patterns observed in the tick inventory and sampling across the studied localities, highlighting the seasonal variations, host preferences, and species diversity of hard ticks. The results are compared with previous findings to elucidate regional differences and ecological factors influencing tick distribution. The discussion further explores the implications of parasitic and ecological indices and how statistical analyses across localities provide insights into infestation dynamics and potential risk factors for tick-borne diseases

Eight tick species were identified in this study, with *Rhipicephalus sanguineus* being the most prevalent and infestations peaking in summer. These findings align with previous research in Algeria and other regions, although tick species richness and composition vary considerably across studies. Tick diversity is influenced by geography, climate, and host availability. While this study identified eight species, other studies in Algeria have reported different levels of richness. For instance, Boulkaboul (2003) recorded 13 species across six genera in Tiaret, whereas Abdul hussain et al. (2004) found nine species in Tizi Ouzou, with *Hyalomma marginatum* being the most abundant. Similar trends are observed in neighboring Morocco (Laamari et al., 2012) and internationally, where species counts vary based on environmental and methodological factors (Bryson et al., 2000 ; De Mantos et al., 2008 ; Kumsa and Mekonnen, 2011). Such variations are often attributed to climate, host management, and habitat characteristics (Krčmar et al., 2014). Additionally, climate change, vegetation shifts, and habitat alterations have been suggested as key drivers of tick population dynamics (Gray et al., 2009).

The detection of *Rhipicephalus camicasi* in the Algerian steppe is noteworthy, as it has not been previously documented in national tick inventories and is absent from Walker et al.'s key for North Africa. Its presence raises several questions. One possibility is that previous surveys overlooked this species due to limited geographic coverage or reliance on morphological identification, which can lead to misclassification. Environmental changes and host movements may have also contributed to its establishment. Given the potential epidemiological implications, further molecular investigations are needed to confirm its identity, assess its genetic lineage, and determine its role in pathogen transmission. This finding underscores the importance of ongoing tick surveillance and taxonomic revisions.



Seasonal dynamics were evident, with *Rhipicephalus bursa* and *R. turanicus* showing the highest infestation rates, particularly in summer, when all examined dogs in Faidh El Botma were infested. In contrast, *R. camicasi* and *R. sanguineus* were less abundant, declining sharply in autumn and nearly absent in winter. These findings align with previous studies. *R. sanguineus* has been reported as dominant in some studies, likely due to its primary association with dogs (Matallah et al., 2013). *R. bursa* is among the most prevalent species in Sétif (Bouchama, 2020), Mila and El Tarf (Benchikh-Elfeghoun et al. 2013), and Tiaret (Boulkaboul, 2003). This species is a key vector of *Babesia bovis*, *B. bigemina*, and *Anaplasma marginale* in cattle (Sahibi and Rhalem, 2007).

Among cattle, *Hyalomma anatolicum* and *H. excavatum* were the most prevalent, particularly in summer, with *H. anatolicum* showing the highest infestation rates. *H. impletatum* and *H. marginatum* were less abundant but exhibited increased infestation intensity during this season. In Mila, a semi-arid region, low abundances of *H. marginatum* (Mokhtaria et al., 2018), *H. excavatum*, and *H. anatolicum* (Benchikh-Elfeghoun et al. 2013) have been reported. In contrast, the considerable prevalence observed in this study suggests that local environmental conditions play a key role in infestation dynamics. *H. impletatum* has been identified as a vector of *Rickettsia aeschlimannii* in Algeria (Sadeddine et al., 2020), while *H. marginatum* and *H. excavatum* also harbor *R. aeschlimannii*, *Candidatus Rickettsia barbariae*, and *Coxiella burnetii* (Abdelkadir et al., 2019). Additionally, *H. excavatum* has been implicated in the transmission of *Theileria lestoquardi* in sheep in Tunisia (Rjeibi et al., 2018). Variations in tick abundance and distribution across studies may be influenced by climatic conditions, ecological interactions, and livestock management practices.

Sex ratio differences observed in *Rhipicephalus* and *Hyalomma* species suggest that population structures are influenced by ecological and biological factors. In this study, *Rhipicephalus* species exhibited a female-biased population, whereas *Hyalomma* species showed a male-biased trend. Similar patterns have been reported by Gharbi et al., (2013) and Little et al. (2007), suggesting that male *Hyalomma* ticks' higher mobility and prolonged attachment contribute to this pattern. However, contrasting results exist, with some studies finding no significant sex-based differences (Ebrahimzade et al., 2016) or even opposite trends (Mosallanejad et al., 2012). Statistical analyses, such as chi-square tests (Wasihun and Doda, 2013), highlight the importance of quantitative validation in sex ratio assessments. The observed female bias in



Rhipicephalus may be linked to their prolonged blood-feeding for egg production, while *Hyalomma* males' higher mobility could explain their prevalence in collections.

Infestation differences between localities (S: 840 m, F: 1250 m) highlight altitude-driven environmental influences. In spring, infestation was significantly higher in F, suggesting prolonged tick activity at higher altitudes. By summer, infestation reached 100% in both localities, indicating optimal conditions regardless of altitude. In autumn, infestation declined faster in S due to earlier seasonal cooling, while F remained favorable for a longer period. In winter, infestation disappeared in F due to harsher conditions, whereas some ticks persisted in S, likely benefiting from milder temperatures. Previous studies indicate that higher elevations reduce tick activity and pathogen prevalence (reference needed), with altitude impacting tick survival, distribution, and host interactions. Additionally, high rainfall at elevated sites can lower ambient temperatures, negatively affecting tick density. These findings reinforce the role of altitude in shaping seasonal tick infestation patterns.

Differences in infestation between dogs and cattle further emphasize host specificity among tick species. *Hyalomma* ticks, known for their preference for large mammals, were predominantly found on cattle, whereas *Rhipicephalus* species were more prevalent on dogs. These patterns align with studies from Tunisia (M'ghirbi and Bouattour, 2008) and Morocco (Sarih et al., 2008), which reported similar host associations in North Africa.

These observations fit into a broader framework where environmental factors and host characteristics shape tick dynamics. For instance, a study on cattle in the Kassena-Nankana District found higher tick infestations during the wet season, highlighting the impact of favorable environmental conditions and host availability (Offei Addo et al., 2024). Similarly, research on *Amblyomma cohaerens* ticks demonstrated that rainfall significantly affects seasonal infestations, with higher prevalence during wetter periods (Gashaw, 2005).

This study highlights the intricate relationship between environmental factors, host specificity, and seasonal variations in tick infestations. Future research integrating molecular tools and ecological modeling will be essential for a deeper understanding of tick-host-pathogen interactions and vector ecology.



4.2.Section 2: Pathogen interactions in *Hyalomma* ticks

This section explores the complex interactions among tick-borne pathogens detected in *Hyalomma* ticks. The discussion focuses on the observed positive and negative associations among pathogens, examining potential mechanisms behind these interactions. By comparing the findings with existing literature, this section highlights the ecological significance of pathogen coexistence and competition and their implications for vector competence and disease transmission dynamics. The role of biotic and abiotic factors in shaping these interactions is also addressed.

Traditional TBP detection methods in North Africa, like PCR and real-time PCR, are limited to identifying single pathogens. Recent studies emphasize the importance of co-infections in pathogen transmission and disease severity (Said et al., 2021; Borşan et al., 2021; Moutailler et al., 2016). This study utilizes microfluidic PCR and network analysis to examine interactions among 43 microorganisms in *Hyalomma* ticks infesting cattle in Algeria's steppe region. This innovative approach reveals the prevalence and diversity of pathogens while highlighting the complex dynamics of co-infections, providing crucial insights into pathogen community structures and their influence on disease transmission in North Africa.

One of the key findings of this study is the significant difference in pathogen prevalence and co-infection patterns between male and female ticks. These variations are likely influenced by several factors. Female ticks, which typically have longer feeding periods and consume larger blood meals compared to males, face increased exposure to pathogens (Sonenshine and Roe, 2013). Krawczyk et al. (2022) suggest that this extended feeding duration, coupled with physiological differences like hormonal variations, enhances females' susceptibility to infections, such as *Borrelia burgdorferi*, and increases their likelihood of harboring multiple pathogens. Hormones like ecdysteroids and juvenile hormones, which vary between sexes, are believed to modulate immune responses and pathogen susceptibility in arthropods (Liu et al., 2023; Adegoke et al., 2022). Additionally, these physiological differences may alter the tick microbiome, potentially impacting pathogen colonization and persistence (Adegoke et al., 2022).

The presence of unique pathogens in female (e.g., *B. afzelii*, *B. spielmanii*, *Hepatozoon*, *Mycoplasma*) and male (e.g., *Ehrlichia*) ticks suggests sex-specific ecological niches and behaviors that influence pathogen acquisition and transmission. These observations are



consistent with findings from studies by Van Treuren et al., (2015) and Benyedem et al. (2022), which highlighted sex-specific differences in bacterial communities within ticks.

Ecological factors are crucial in the epidemiology of zoonotic diseases (Patricia, 2021). Climate change, marked by increased heat waves, heavy rains, and droughts, alters environmental conditions (Allen et al., 2018), affecting animal distribution and, in turn, the biology and redistribution of ticks (Boulanger et al., 2019). As ticks expand, the pathogens they carry follow (Wikel, 2018). Tick life cycles, primarily driven by heat, rely on favorable conditions like humidity and host availability to support egg development and larval metamorphosis (Estrada-Peña et al., 2021). High temperatures can also accelerate pathogen replication, as seen with *Theileria parva*, which causes East Coast fever in cattle, while reducing transmission time in infected ticks (Ochanda et al., 1988). In North Africa, *Hyalomma excavatum* is active year-round, with developmental rates peaking during warmer months (Elati et al., 2024). This tick follows either a two- or three-host life cycle depending on host availability, adding complexity to its seasonal development (Mechouk et al., 2022). Larvae and nymphs may feed on different hosts or the same one before molting into adults, creating a fluctuating landscape for pathogen transmission (Walker et al., 2003). Seasonal peaks in tick activity often coincide with higher pathogen presence in large mammals, particularly in summer when adult ticks are most active (Bouattour, 2002).

Moreover, this study underscores the crucial role of *Francisella*-like endosymbionts (FLE) in supporting pathogen coexistence in *Hyalomma* ticks, particularly with *Rickettsia*. FLE enhance the stability of tick microbial communities, promoting co-infections and pathogen persistence. This aligns with findings from Kumar et al. (2022), who highlighted the competitive advantage of FLE over ancient endosymbionts in *Amblyomma americanum*, suggesting their ecological dominance. Azagi et al. (2017) also found that imported *Hyalomma* ticks may exhibit different endosymbiont-pathogen relationships, indicating that geographical factors influence disease transmission dynamics. The evolutionary link between FLE and pathogens is further supported by Gerhart *et al.* (Gerhart et al., 2016), who showed that a FLE evolved from a mammalian pathogen, emphasizing its role in pathogen interactions. Additionally, Sesmero-García et al. (2023) discuss how climate change could enhance FLE's role in disease transmission, as they may help *Hyalomma* ticks adapt to changing environments.



Hussain et al. (2022) propose that targeting FLE could serve as an effective tick management strategy by disrupting their symbiotic relationships, thereby reducing tick fitness and pathogen transmission. Developing anti-microbiota vaccines to target FLE presents a promising strategy to influence tick microbiota and reduce pathogen transmission. For example, vaccination of mice against a commensal *Escherichia* in *Ixodes ricinus* altered the tick microbiota (Mateos-Hernández et al., 2020), leading to decreased levels of *Borrelia afzelii* (Wu-Chuang et al., 2023). Similarly, vaccination of alpha-gal knockout mice with the same commensal decreased tick survival (Mateos-Hernández et al., 2021). Additionally, microbiota-driven vaccination in soft ticks, such as *Ornithodoros moubata*, has demonstrated implications for survival, fitness, and reproductive capabilities (Cano-Argüelles et al., 2024). In another study, vaccination of birds against a commensal in *Culex quinquefasciatus* effectively reduced *Plasmodium* colonization in the mosquito (Aželytė et al., 2022). These results support the concept that vector microbiota manipulation by host antibodies can be utilized as a strategy to develop transmission-blocking vaccines (Maitre et al., 2022).

The observed co-infections reveal important insights into disease dynamics, particularly the positive associations between pathogens like *Rickettsia conorii* and *Rickettsia slovaca* in male ticks, and *Borrelia afzelii* and *Borrelia spielmanii* in female ticks, indicating a lack of competition. Moutailler et al. (2016) found a strong association between *Borrelia garinii* and *Borrelia afzelii*, suggesting that biological interactions may promote their co-infection. Similarly, *R. conorii* and *R. slovaca* have been found to coexist without competition, as noted by Torina et al. (2012). These interactions may contribute to more complex infection patterns, influencing the epidemiology of tick-borne diseases.

Pathogens can cooperate by producing shared resources, or "common goods," essential for their collective growth and survival. In bacterial communities, for instance, siderophores are produced to capture iron from the environment, a critical element for bacterial growth. These siderophores benefit multiple strains within the population, enhancing the overall fitness and survival of the community (Griffin et al., 2004 ; Buckling et al., 2007). Additionally, such cooperative behaviors are often regulated by quorum sensing, where bacteria use chemical signals to coordinate the production of these shared resources, further demonstrating the intricate cooperation among pathogens (Kümmerli et al., 2015).



In contrast, strong negative associations between pathogens like *Anaplasma phagocytophilum* and *Francisella tularensis* suggest mutual exclusion. Competition among parasites within a host can lead to varied evolutionary outcomes, driven by different mechanisms (Susi et al., 2015). Exploitation competition occurs when parasites compete for the host's limited resources by occupying overlapping ecological niches, intensifying during co-infections (Read and Taylor, 2001; Izhar et al., 2015). Apparent competition, on the other hand, arises from cross-reactive immune responses, where the host's nonspecific defenses affect the abundance and success of different parasites (Mideo, 2009; Smith and Holt, 1996). Lastly, interference competition involves direct suppression, where parasites actively inhibit their rivals through chemical or mechanical means (Balmer and Tanner, 2011 ; Dallas et al., 2019). These competitive interactions may limit the co-occurrence of certain pathogens, impacting disease prevalence and influencing control strategies.

Interactions between pathogens in multi-infections significantly influence the evolution of virulence. Pathogens may compete for resources or cooperate to enhance survival and share resources. The observed sex-specific and seasonal variations in these interactions provide important insights into tick-borne disease dynamics. These findings highlight the need to consider both biotic and abiotic factors when developing control strategies. By combining molecular techniques with ecological and epidemiological approaches, this study enhances the understanding of TBPs and improves predictions and management strategies for their spread, leading to more effective public health interventions. While the study provides valuable insights, its findings may be constrained by the limited sample size, focus on specific tick species, and potential geographical biases. These limitations should be considered when interpreting the results and applying them to broader ecological or epidemiological contexts.

4.3.Section 3: Microbiome dynamics in *Hyalomma* ticks

This section discusses the microbial composition and dynamics within the *Hyalomma* tick microbiome. Emphasis is placed on the patterns revealed by alpha and beta diversity analyses, as well as the network and sub-network structures that outline microbial interactions. The robustness of the microbial network is examined to understand the stability of the tick microbiome under various seasonal and environmental conditions. The discussion also compares these findings with previous studies, highlighting how microbial communities may influence pathogen persistence, vector competence, and tick fitness.



The microbiome of *Hyalomma excavatum* ticks is subject to seasonal variation, influencing microbial diversity, pathogen prevalence, and interspecies interactions. This study highlights how key microbial taxa such as *Francisella* and *Rickettsia* play significant roles in shaping the tick microbiome, with their relative abundance and microbial network connectivity fluctuating across different seasons. These findings align with previous research on other tick species, suggesting that microbiome dynamics are driven by environmental factors, including temperature and humidity (Piloto-Sardiñas et al., 2024; Thapa et al., 2019; Lejal et al., 2021).

However, by focusing on *Hyalomma excavatum*, a relatively underexplored vector compared to well-studied ticks like *Ixodes*, this study provides novel insights into how seasonal shifts shape its microbial community and pathogen associations. These findings contribute to a better understanding of *H. excavatum*'s potential role in pathogen transmission under changing ecological conditions.

Environmental factors such as temperature are known to be major drivers of tick microbiome structure (Thapa et al., 2019). Our results show distinct seasonal shifts in microbial communities, with *Francisella* and *Candidatus* Midichloria being more abundant in autumn, whereas Yersiniaceae and Enterobacteriaceae were predominant in spring. These seasonal fluctuations are consistent with findings in *Ixodes scapularis*, where bacterial diversity was significantly altered by temperature (Thapa et al., 2019). However, there are discrepancies across studies regarding the impact of high temperatures on bacterial diversity in ticks.

In *I. scapularis*, high temperatures reduced microbial diversity and weakened microbial network connectivity (Wu-Chuang et al., 2022). In contrast, in *Ixodes ricinus*, microbial interactions became more complex in warmer conditions, suggesting that higher temperatures might facilitate co-occurrence of microbial taxa rather than causing diversity loss (Lejal et al., 2021). On the other hand, the *H. dromedarii* study in the UAE reported that microbial diversity remained stable despite seasonal variations, indicating a more resilient microbiome (Perveen et al., 2022). Our study on *H. excavatum* aligns more closely with *I. ricinus*, as we observed higher microbial network complexity in summer, indicating that warmer conditions may enhance microbial interactions rather than reduce diversity.

Several factors could explain these discrepancies. Species-specific adaptations may play a critical role, as *H. dromedarii* is adapted to desert conditions, potentially harboring a microbiome that is more resistant to extreme temperature shifts. Conversely, *I. scapularis*,



which inhabits temperate regions, may experience a greater microbiome disruption under heat stress. Another possible explanation is differences in experimental conditions. The study on *I. scapularis* was conducted under controlled laboratory settings (Thapa et al., 2019), where heat stress was imposed artificially, whereas the studies on *Hyalomma* (Perveen et al., 2022) species and *I. ricinus* (Lejal et al., 2021) used field-collected ticks, allowing for natural environmental buffering, including potential microbial acquisition from the habitat.

Recent research by Abdelali et al. (2024), using the same *Hyalomma excavatum* tick samples analyzed here, provides valuable insight into pathogen–pathogen interactions. Their study revealed that certain pathogen guilds exhibit cooperative associations, such as *Rickettsia slovaca* and *Rickettsia conorii*, while others, such as *Anaplasma phagocytophilum* and *Francisella tularensis*, may engage in competitive exclusion. In the present network analysis, this specific negative association was not observed, likely due to differences in detection thresholds or seasonal filtering. It is important to note that the current study was based on presence/absence data derived from 16S rRNA gene sequencing, not on quantitative pathogen load, which may influence the resolution of inferred interactions. These findings suggest that tick microbiomes may influence pathogen persistence and co-occurrence patterns, a hypothesis supported by our results showing the pivotal role of *Francisella* and *Rickettsia* in microbial networks.

Abdelali et al. (2024) also observed seasonal differences in pathogen prevalence, with female ticks exhibiting higher infection rates than males. This aligns with our microbiome findings, where seasonality influenced microbial community structure and complexity. The seasonal co-occurrence of certain bacteria and pathogens suggests that environmental factors regulate both microbiota and pathogen load, shaping tick-borne disease transmission dynamics.

The positive and negative pathogen–pathogen interactions reported by Abdelali et al. (2024) could be mediated by microbiome composition. For instance, the negative association between *A. phagocytophilum* and *F. tularensis* suggests that *Francisella* may play a role in pathogen exclusion. Our study found that *Francisella* is a highly connected keystone taxon, which could be influencing the tick's pathogen load by either outcompeting certain pathogens or indirectly altering microbial community dynamics. These results reinforce the hypothesis that the tick microbiome may act as a selective filter for pathogen colonization (Narasimhan et al., 2014) (Wu-Chuang et al., 2023).



The stability of microbial networks may be influenced by taxa that occupy central or structurally important positions within the community. In *Hyalomma excavatum*, *Francisella* and *Rickettsia* consistently maintained microbial balance across seasons, acting as potential stabilizers within the network due to their strong connectivity and persistence. Similarly, in *Ixodes scapularis*, certain thermostable bacteria such as *Pseudomonas*, *Ralstonia*, *Acinetobacter*, and *Bradyrhizobium* were identified as key contributors to microbiome stability under heat stress (Wu-Chuang et al., 2022). However, while these bacteria were lost at higher temperatures in *I. scapularis*, microbial interactions in *H. excavatum* appeared to be enhanced rather than diminished, as evidenced by *Francisella* being connected to 13 taxa in the summer network, compared to only one taxon in both spring and autumn. This contrast suggests that functional redundancy, where different microbial taxa can fulfill similar ecological roles, may buffer some tick species against environmental fluctuations, whereas others remain more sensitive to thermal stress.

The seasonal modulation of microbial networks has direct implications for tick-borne disease transmission. Increased *Rickettsia* abundance in specific seasons may enhance pathogen transmission, similar to findings in *I. ricinus*, where *Borrelia* and *Anaplasma* disrupted microbial interactions and increased disease risk (Lejal et al., 2021). Additionally, the study on *I. scapularis* (Thapa et al., 2019) highlights that heat stress reduces microbial diversity but preserves key metabolic pathways (Wu-Chuang et al., 2022), suggesting that keystone bacteria buffer environmental fluctuations. This functional stability may contribute to the persistence of tick-borne pathogens across different seasons, reinforcing the importance of microbiome monitoring in tick control strategies.

A crucial aspect of microbial ecology is whether observations in controlled environments translate to natural conditions. The *I. scapularis* study found that lab-reared and field-collected ticks harbored similar keystone bacteria (Wu-Chuang et al., 2022), suggesting that laboratory experiments can accurately reflect microbial dynamics in natural tick populations. Our results align with this, indicating that seasonal shifts in *H. excavatum* microbiomes observed in the field are not random but rather follow predictable environmental patterns. Future research should explore how microbiome stability varies across tick species under controlled and field conditions, focusing on the functional implications of microbial shifts for vector competence and pathogen transmission.



Despite the valuable insights provided by this study, several limitations should be acknowledged. The sample size was limited to 21 engorged female *Hyalomma excavatum* ticks, which may affect the statistical power and generalizability of the findings. Although all ticks were collected during the early stage of engorgement and no host DNA contamination was detected using the Decontam tool, individual variation in host immune status and feeding duration could still influence the microbiome profiles. Furthermore, the absence of unfed or male ticks limits our ability to assess sex- or feeding-related microbial differences. These constraints highlight the need for future studies with larger and more diverse sample sets under controlled experimental conditions.

4.4. Section 4: Botanical control of *Rhipicephalus sanguineus*

This section discusses the acaricidal efficacy of the tested essential oils on both larvae and engorged females of *Rhipicephalus sanguineus*. The impact of chemical composition and yield of each essential oil on their bioactivity is analyzed. The discussion examines the dose-dependent larvicidal effects, as indicated by LD₅₀, LD₉₀, LT₅₀, and LT₉₀ values, and the reproductive inhibition observed in engorged females. Additionally, the potential of these essential oils as botanical alternatives for tick control is considered, along with their broader implications for sustainable tick management strategies.

4.4.1. The yield of the essential oils and their chemical characterization

The oil yield of *Artemisia herba alba* recorded in the present study was relatively higher than those extracted from the same species collected in Spain with 0.8% (Salido et al. 2001) and Tunisia 0.7% (Haouari and Ferchichi, 2009). While it is equal to those extracted in Tunisia by Zouari et al. (2010) and by Boutemak et al. (2009) in Algeria. Also, it is lower than the one extracted in Morocco: 3.3% by Paolini et al. (2010). Regarding the chemical composition of this oil, a variability of volatile constituents was observed in many countries from previous studies as such in Morocco [Camphor (40–70%), α - or β -Thujone (32–82% and 43–93%, respectively), Chrysanthenone (51.4%), Chrysanthenyl acetate (32–71 %), or Davanone (20–70%)] were the major components from that of Paolini et al. (2010). On the other hand, with the exception of Davanone, which is the main compound of the present work, it was not detected in the study of Abu-Darwish et al. (2015) in Jordan [β -Thujones (25.1%), α -Thujones (22.9%), Eucalyptol (20.1%) and Camphre (10%)] neither in that of Abou El-Hamd et al. (2010) in Egypt [1,8-Cineole (50%), Thujone (27%), Terpinen-4-ol (3.3%), Camphor (3%) and Borneol



(3%)]. In addition, even within Algeria, different chemical compositions of the essential oil of *A. herba-alba* have been recorded. For example, in the region of Djelfa, Touil and Benrebiha (2014) found Davanone (62.20%), Carvacrol (4.88%), Davana ether (3.62%), Camphor (3.48%) as major components.

The essential oil yield of *Rosmarinus officinalis* was 1.49%, it is higher than many other works done, noting that the yield of that collected in Kenya was 0.59% (Mwithiga et al. 2022), in Portugal was 0.3–0.7% (Serrano et al. 2002), also in Turkey was 0.71–0.94% (Gurbuz et al. 2016). However, it is lower if compared to those collected in Algeria, the yield of essential oil in Tbessa was 1.85–2.29% (Boutabia et al. 2016). Regarding the chemical composition of this oil, it differs from those obtained by Bakkali et al. (2018) in Morocco, in which 17 compounds account for around 75.6% of the total. The main constituents are: α pinene (32.64%), β humulene (8.71%), and Camphene (5.95%). Also for the Indian *rosmarinus* essential oil the most important constituents were alpha -pinene (31.91%) and 1, 8 - cineole (14.66%). However, in France, Kaloustian et al. (2002) recorded a camphor chemotype with a high level (30–45%). In addition, in Algeria, Boutabia et al. (2016) showed that 1,8-cineole is the predominant chemotype of *Rosmarinus officinalis* essential oil. But those of Lograda et al. (2013) noted that the chemical composition of essential oils of rosemary collected from five regions of eastern Algeria is dominated by camphor (42.7%).

The essential oil yield of *Thymus vulgaris* calculated on the dry weight of the aerial part of the plant was 4.3%, it is higher than many other works done regarding the same plant species, noting that the yield of that collected in Romania was 1.25% (Borugă et al. 2014), in Bosnia and Herzegovina was 1.5% (Niksic et al. 2021), Imelouane et al. (2009) recorded 1% in Morocco, also in Iran was 0.83%–1.39% (Nezhadali et al. 2014) and 0.47%–2.8% in Italy by Najar et al. (2021). It is even higher if compared to those collected in Algeria, the yield of essential oil in El Tarf was 1.58% (Benaliouche et al. 2021), in Blida was 2% (Bouguerra et al. 2017), in Batna Yakhlef (2010) noted a yield of 1.94%, also in Mostaghanim the yield was 2.2%, however, it is almost equal to that obtained in Telemcen 4.2% (Abdelli et al. 2017). Regarding the chemical composition of this oil, it differs from those obtained by Galovičová et al. (2021) who studied the oil composition of thyme of the same specie sample in Slovakia, in which thymol (48.1%), p-cymene (11.7%), 1,8-cineole (6.7) were revealed to be dominant. For the Moroccan thyme essential oil, it has been reported a camphor-type, the major components quantified were camphor (38.54%), camphene (17.19%), α -pinene (9.35%) (Imelouane et al.,



2009). On the other hand, except for carvacrol, which is the main compound of the present work, it was not detected in the study of Borugă et al. (2014) in Romania, nor in those collected from Serbia which were geraniol chemotype and a sabinene hydrate chemotype. In addition, even within Algeria, it was recorded the total absence of carvacrol in two essential oils obtained at Mostaghanim and Telemcen (Abdelli et al. 2017).

4.4.2. Acaricidal effect of essential oils

For a long time, resource-poor farmers in Africa and Asia have practiced traditional medicine based on the use of plant materials to treat endo- and ectoparasites of livestock including ticks (Mondal et al. 2013), the first intensive trials on the acaricidal activity were launched by Khaidarov (1971) evaluating 84 plants, currently at the global level 200 plant species have been registered for their repellent or acaricidal properties (Adenubi et al. 2016). The orientation towards biocides is due to the abundance of plant secondary metabolites with toxicological activity, their low cost, and relatively lower toxicity to the environment and the hosts (Borges et al. 2011) in addition to the slow development of resistance due to the variability of active agents with different mechanisms of action (Balandrin et al., 1985; Chagas et al., 2002; Olivo et al., 2009) make plant extracts a better alternative to control tick populations (Oliveira et al. 2016).

Toxicological tests of the present study reveal a considerable and variable sensitivity of *R. sanguineus* using *Artemisia herba halba*, *Rosmarinus officinalis*, and *Thymus vulgaris* essential oil, translated by a significant reduction of the egg mass of the engorged females from 20.6% to 5% with a significant reduction of the reproductive efficiency index compared to the control group. Moreover, the essential oil was also toxic to larvae expressed by rates of low to very high mortality, which correlates with the extension of time from one concentration to the other, with an LC₅₀ of 1.133 µl/ml for 24 hours, 0.879 µl/ml for 48 hours, and 0.734 µl/ml for 72 hours.

Compared to the tick species chosen for this work, Daemon et al. (2009) and Monteiro et al. (2009) showed the efficacy of thymol on *R. sanguineus* larvae and pupae by a mortality rate that reached 100% at the concentration of 2 and 0.5%, respectively, However, in the case of non-engorged *R. sanguineus* larvae, only 37.7% mortality was recorded at the 2% concentration of thymol (Daemon et al. 2009), besides Godara et al. (2013) showed the in vitro efficacy of chloroform extract obtained from *Artemisia absinthium* on adults, eggs and larvae using the



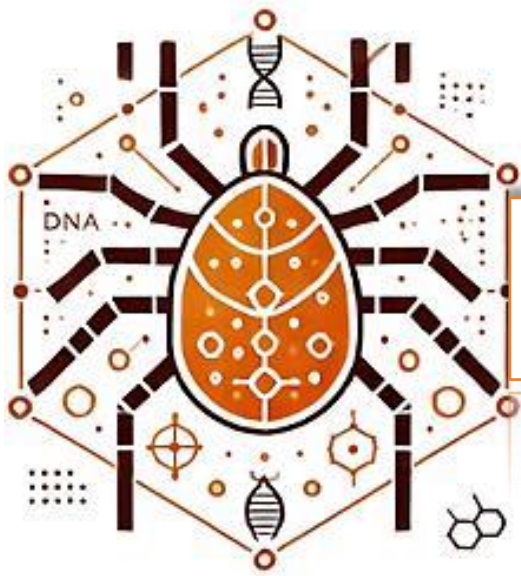
adult immersion test (AIT) which cause a mortality rate reached 93.3% with LC50 and LC95 values of 8.793% and 34.59%, the egg hatch test (EHT) reducing egg production to 85.1% with complete inhibition of hatching, and the larval package test (LPT) provokes 100% mortality of larvae with LC50 and LC95 values of 1.11% and 2.37%.

In Algeria, few works have been carried out on the fight against ticks by plant extracts, these are two studies on the same species *Hyalomma scupense* revealing considerable toxic activity, the first by Djebir et al. (2019) evaluating the acaricidal activity of six aromatic plants belonging to the Lamiaceae and Myrtaceae families by an adult immersion test (AIT) and a larval immersion test (LIT) and the second by Alimi et al., (2022) evaluating the acaricidal activity of *Ocimum basilicum* essential oil and its main constituents by adult immersion test (AIT) and larval packet test (LPT).

However, the variations between the methods used and the conditions for testing the repellent and acaricidal effects of certain plant extracts, such as the choice of test type, the duration of the test, the presence or absence of index host, the species and stage of ticks, also the plant, the extraction, type and the solvent, have made it difficult to compare studies and select the best plant species.

Noting that the toxicity of different extracts of some plants is not only limited to mortality, but it can also affect the fecundity and hatching rate of female eggs (Ellse and Wall 2014) while altering the morphophysiology of some important organs (Camargo-Mathias 2018), such as ovaries (Konig et al. 2020), salivary glands (Remedio et al. 2016), the synganglion (Roma et al. 2013). The mechanism of action of the essential oil on arthropods is mainly due to neurotoxic effects involving several modes of action, including acetylcholinesterase (AChE) inhibition (Mills et al. 2004), disruption of gamma-aminobutyric acid (GABA) receptor functionality (Priestley et al. 2003) and agonist of the octopamine system (Enan 2005). In the same context, Jun-Hyung and Murray (2015) note that the arthropocidal activity results from a series of complex actions and contractions between a toxic tissue and an arthropod tissue. This mechanism of toxicity can be expressed in three steps: penetration, activation (target site interaction), and detoxification. Plant extracts act in two possible ways: a larvicidal action that can cause appreciable mortality of larvae in 1 to 12 days, or a juvenile hormone mimetic action, with an extension of the larval life span that can inhibit pupation (Rageau and Delaveau, 1979)





CONCLUSION

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Ticks are significant vectors of pathogens affecting both animal and human health, requiring a comprehensive understanding of their distribution, associated microorganisms, and potential control strategies. This study aimed to investigate the tick fauna in the Djelfa region, explore the molecular diversity of *Hyalomma excavatum* from cattle with a focus on pathogen interactions and microbiome dynamics, and assess the acaricidal potential of essential oils against *Rhipicephalus sanguineus* from dogs.

The tick inventory provided a detailed overview of tick species infesting domestic animals in the Djelfa region, revealing clear seasonal and host-related patterns. A total of 8,405 ticks were collected from cattle and dogs, with *Hyalomma excavatum* dominating in cattle and *Rhipicephalus turanicus* being the most frequent species on dogs. Infestation rates varied significantly across seasons, with the highest prevalence observed in summer and the lowest in winter. Statistical analysis confirmed locality-based differences, highlighting the influence of environmental and ecological factors on tick distribution. This baseline knowledge is crucial for understanding tick population dynamics and guiding targeted control measures.

In order to deepen our understanding of *H. excavatum* as a vector, molecular analyses were conducted to assess both pathogen interactions and microbiome dynamics. Molecular screening of *H. excavatum* ticks collected from cattle confirmed their identity through 16S rRNA sequencing and revealed a high prevalence of tick-borne pathogens (TBPs). Infection rates differed between sexes, with females displaying higher co-infection rates (44.4%) than males (10.6%). *Rickettsia slovaca*, *Borrelia afzelii*, and Apicomplexa were the most frequently detected pathogens, while *Anaplasma phagocytophilum* and *Ehrlichia* species exhibited sex-specific associations.

Network analysis revealed that tick sex and seasonal changes influenced pathogen co-occurrence patterns. Female ticks exhibited more balanced and cooperative pathogen interactions, whereas males showed stronger negative associations, suggesting competitive exclusion dynamics. *Francisella-like endosymbionts* played a key role in pathogen coexistence, further emphasizing the complex interactions shaping TBP transmission. These findings provide new insights into the ecological factors influencing pathogen persistence and co-infections in *H. excavatum*.

The microbiome analysis of *H. excavatum* revealed significant seasonal shifts in microbial composition, despite stable overall diversity. Core microbial taxa were present throughout the year, but specific bacterial groups exhibited seasonal variations. *Francisella* and *Candidatus Midichloria* were dominant in autumn, while *Yersiniaceae* and *Enterobacteriaceae* were more abundant in spring.

Microbial co-occurrence networks varied across seasons, with summer displaying the highest complexity. *Francisella* played a dominant role in shaping microbial interactions, maintaining network cohesion and stability, while *Rickettsia* influenced connectivity patterns. The removal of these taxa from network analyses disrupted microbial associations, underscoring their ecological significance. These results highlight the importance of microbiome dynamics in vector competence and pathogen transmission, suggesting potential microbiome-based strategies for tick control.

To explore environmentally sustainable tick control alternatives, the acaricidal effects of essential oils from *Artemisia herba-alba*, *Rosmarinus officinalis*, and *Thymus vulgaris* were evaluated against *Rhipicephalus sanguineus* larvae and engorged females. *T. vulgaris* exhibited the highest acaricidal activity, achieving 100% larval mortality at 5 µl/ml within 72 hours, while *R. officinalis* was the least effective.

Reproductive inhibition assays showed that *T. vulgaris* significantly reduced egg production and hatching, with a 98.38% reduction at the highest concentration. The statistical analyses confirmed significant differences between essential oil doses, reinforcing the potential of plant-derived acaricides as viable alternatives to synthetic chemical treatments.

This study provides an integrated approach to tick ecology, vector-pathogen interactions, microbiome dynamics, and botanical control strategies. The findings emphasize the importance of seasonality and host interactions in shaping tick populations and microbial communities. The demonstrated efficacy of essential oils suggests a promising avenue for sustainable tick management.

Future research should focus on field-based testing of botanical acaricides, long-term monitoring of TBP dynamics, and functional studies on microbiome-pathogen interactions. Additionally, given the central role of *Francisella* and *Rickettsia* in microbial network stability, a crucial next step is to investigate the impact of essential oils on the tick microbiome. Assessing

microbiome stability after botanical treatments will provide insights into potential disruptions or resilience within microbial communities, helping to refine vector control strategies with minimal ecological disturbance.

A deeper understanding of these interconnected factors will contribute to the development of more effective and ecologically sound tick control strategies, ultimately reducing the risk of tick-borne diseases.

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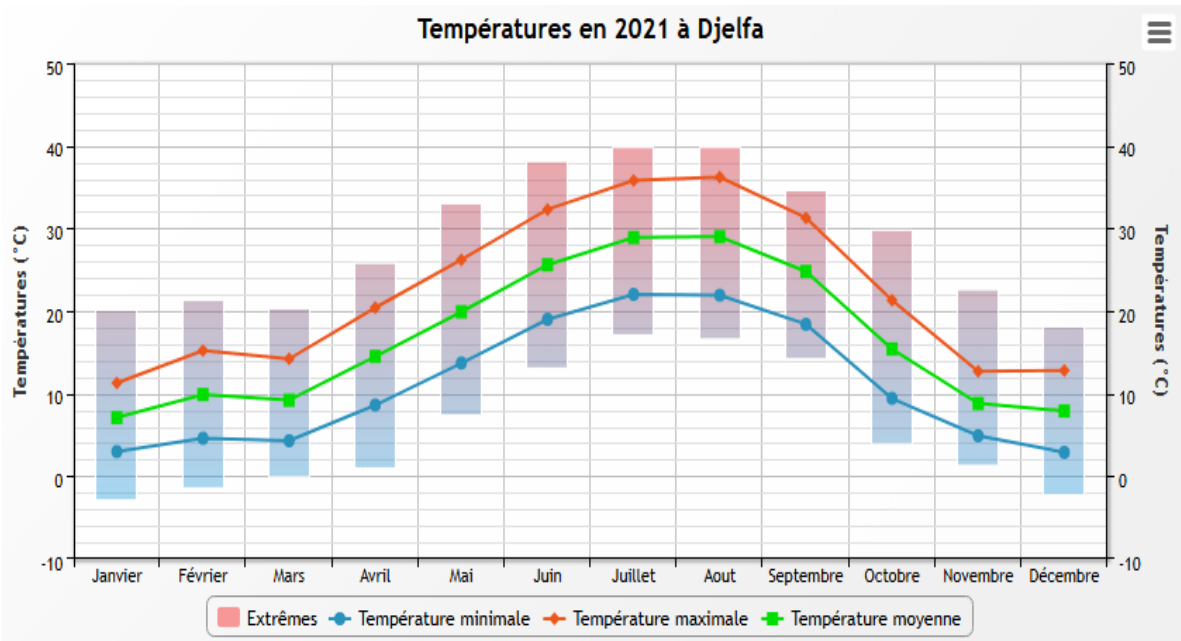
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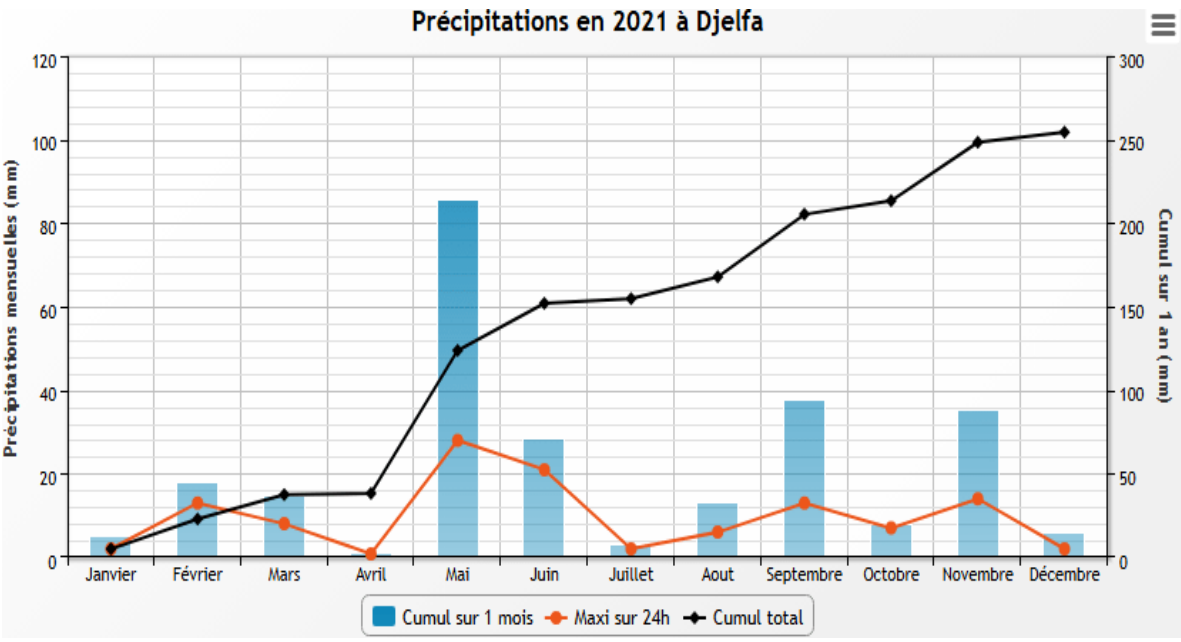
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APPENDIX 1

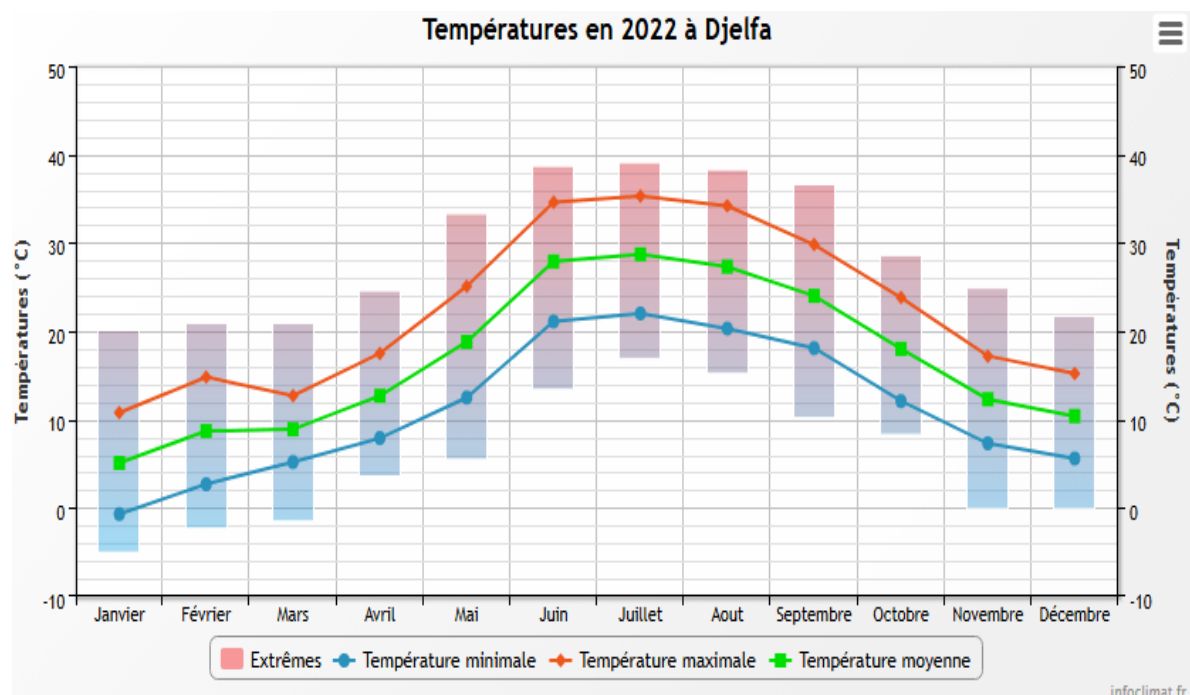
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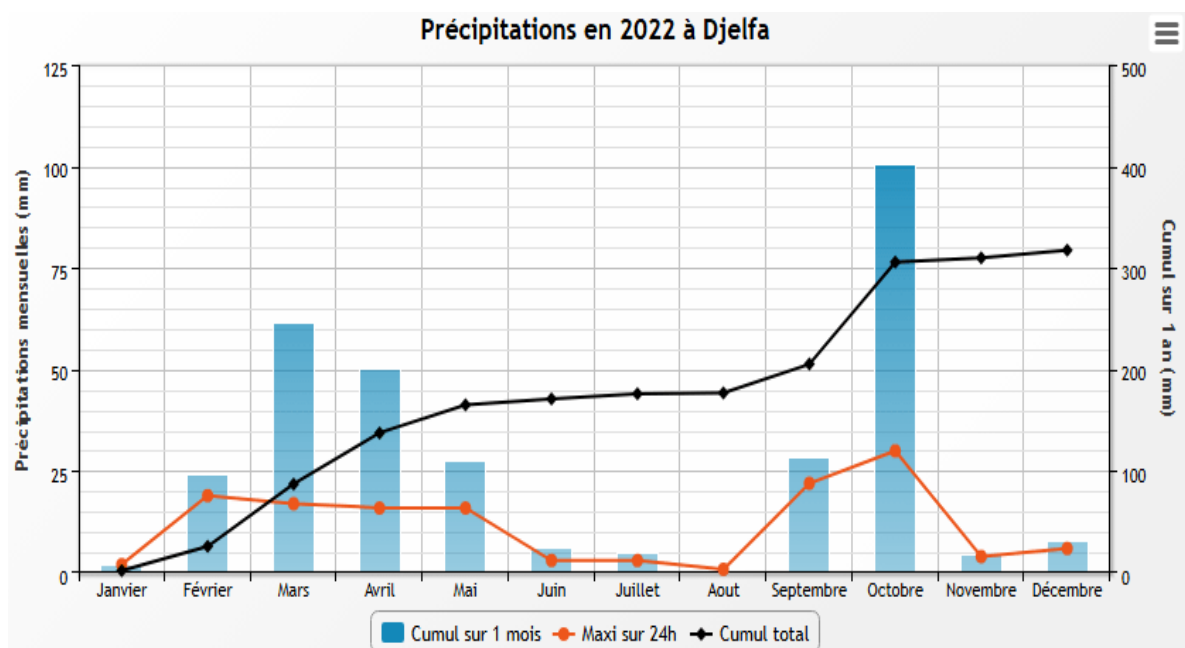
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APPENDIX 2

Supplementary Table 1. List of primer/probe sets used in the BioMark™ real-time PCR system.

Organism	Targeted gene	Primers (F and R - 5'-3') and Probes (P)	Product length (bp)	Reference
<i>Borrelia burgdorferi sensu stricto</i>	<i>rpoB</i>	F-GCTTACTCACAAAAGGCGTCTT R-GCACATCTCTTACTTCAAATCCT P-AATGCTCTTGGACCAGGAGGACTTTCA	83	Michelet et al. 2014
<i>Borrelia garinii</i>	<i>rpoB</i>	F-TGGCCGAAGTTACCCACAAAA R-ACATCTCTTACTTCAAATCCTGC P-TCTATCTCTTGAAAGTCCCCCTGGTCC	88	Michelet et al. 2014
<i>Borrelia afzelii</i>	<i>fla</i>	F-GGAGCAAATCAAGATGAAGCAAT R-TGAGCACCTCTTGAACAGG P-TGCAGCCTGAGCAGCTTGAGCTCC	116	Michelet et al. 2014
<i>Borrelia valaisiana</i>	<i>ospA</i>	F-ACTCACAAATGACAGATGCTGAA R-GCTTGCTTAAAGTAACAGTACCT P-TCCGCCTACAAGATTTCTGGAAGCTT	135	Michelet et al. 2014
<i>Borrelia lusitaniae</i>	<i>rpoB</i>	F-CGAACTTACTCATAAAAAGGCGTC R-TGGACGTCTCTTACTTCAAATCC P-TTAATGCTCTCGGGCCTGGGGGACT	87	Michelet et al. 2014
<i>Borrelia spielmanii</i>	<i>fla</i>	F-ATCTATTTTCTGGTGAGGGAGC R-TCCTTCTTGTTGAGCACCTTC P-TTGAACAGGCGCAGTCTGAGCAGCTT	71	Michelet et al. 2014

<i>Borrelia bissetti</i>	<i>rpoB</i>	F-GCAACCAGTCAGCTTTCACAG R-CAAATCCTGCCCTATCCCTTG P-AAAGTCCTCCCGGCCCAAGAGCATTAA	118	Michelet et al. 2014
<i>Borrelia myamotoi</i>	<i>glpQ</i>	F-CACGACCCAGAAATTGACACA R-GTGTGAAGTCAGTGGCGTAAT P-TCGTCCGTTTTCTCTAGCTCGATTGGG	94	Michelet et al. 2014
<i>Borrelia</i> spp.	23S <i>rRNA</i>	F-GAGTCTTAAAAGGGCGATTTAGT R-CTTCAGCCTGGCCATAAATAG P-AGATGTGGTAGACCCGAAGCCGAGT	73	Michelet et al. 2014
<i>Anaplasma marginale</i>	<i>msp1</i>	F-CAGGCTTCAAGCGTACAGTG R-GATATCTGTGCCTGGCCTTC P-ATGAAAGCCTGGAGATGTTAGACCGAG	85	Michelet et al. 2014
<i>Anaplasma platys</i>	<i>groEL</i>	F-TTCTGCCGATCCTTGAAAACG R-CTTCTCCTTCTACATCCTCAG P-TTGCTAGATCCGGCAGGCCTCTGC	75	Michelet et al. 2014
<i>Anaplasma phagocytophilum</i>	<i>msp2</i>	F-GCTATGGAAGGCAGTGTTGG R-GTCTTGAAGCGCTCGTAACC P-AATCTCAAGCTCAACCCTGGCACCAC	77	Michelet et al. 2014
<i>Anaplasma ovis</i>	<i>msp4</i>	F-TCATTGACATGCGTGAGTCA R-TTTGCTGGCGCACTCACATC P-AGCAGAGAGACCTCGTATGTTAGAGGC	92	Michelet et al. 2014
<i>Anaplasma centrale</i>	<i>groEL</i>	F-AGCTGCCCTGCTATACACG	79	

		R-GATGTTGATGCCCAATTGCTC		Michelet et al. 2014
		P-CTTGCATCTCTAGACGAGGTAAAGGGG		
<i>Anaplasma bovis</i>	<i>groEL</i>	F-GGGAGATAGTACACATCCTTG	73	Sprong et al. 2019
		R-CTGATAGCTACAGTTAAGCCC		
		P-AGGTGCTGTTGGATGTACTGCTGGACC		
<i>Anaplasma spp.</i>	16S rRNA	F-CTTAGGGTTGTAAACTCTTTCAG	160	Gondard et al. 2020
		R-CTTTAACTTACCAAACCGCCTAC		
		P-ATGCCCTTTACGCCCAATAATTCCGAACA		
<i>Ehrlichia canis</i>	<i>gltA</i>	F-GACCAAGCAGTTGATAAAGATGG	136	Gondard et al. 2020
		R-CACTATAAGACAATCCATGATTAGG		
		P-ATTAAAACATCCTAAGATAGCAGTGGCTAAGG		
<i>Ehrlichia/Neoehrlichia spp.</i>	16S rRNA	F-GCAACGCGAAAAACCTTACCA	98	Gondard et al. 2020
		R-AGCCATGCAGCACCTGTGT		
		P-AAGGTCCAGCCAAACTGACTCTTCCG		
<i>Candidatus Neoehrlichia mikurensis</i>	<i>groEL</i>	F-AGAGACATCATTCGCATTTTGGA	96	Michelet et al. 2014
		R-TTCCGGTGTACCATAAGGCTT		
		P-AGATGCTGTTGGATGTACTGCTGGACC		
<i>Rickettsia conorii</i>	23S-5S ITS	F-CTCACAAAGTTATCAGGTAAATAG	118	Michelet et al. 2014
		R-CGATACTCAGCAAAATAATTCTCG		
		P-CTGGATATCGTGGCAGGGCTACAGTAT		
<i>Rickettsia slovaca</i>		F-GTATCTACTCACAAAGTTATCAGG	138	

	23S-5S ITS	R-CTTAACTTTTACTACAATACTCAGC P-TAATTTTCGCTGGATATCGTGGCAGGG		Michelet et al. 2014
<i>Rickettsia massiliae</i>	23S-5S ITS	F-GTTATTGCATCACTAATGTTATACTG R-GTTAATGTTGTTGCACGACTCAA P-TAGCCCCGCCACGATATCTAGCAAAAA	128	Michelet et al. 2014
<i>Rickettsia Helvetica</i>	23S-5S ITS	F-AGAACCGTAGCGTACACTTAG R-GAAAACCCTACTTCTAGGGGT P-TACGTGAGGATTTGAGTACCGGATCGA	79	Michelet et al. 2014
<i>Rickettsia aeshlimannii</i>	23S-5S ITS	F-CTCACAAAGTTATCAGGTTAAATAG R-CTTAACTTTTACTACGATACTTAGCA P-TAATTTTTCGCTGGATATCGTGGCGGGG	134	Sprong et al.2019
<i>Rickettsia felis</i>	orfB	F-ACCCTTTTCGTAACGCTTTGC R-TATACTTAATGCTGGGCTAAACC P- AGGGAAACCTGGACTCCATATTCAAAAGA G	163	Gondard et al. 2020
<i>Rickettsia spp.</i>	gltA	F-GTCGCAAATGTTACGGTACTT R-TCTTCGTGCATTTCTTCCATTG P-TGCAATAGCAAGAACCGTAGGCTGGATG	145	Michelet et al. 2014
<i>Bartonella henselae</i>	pap31	F-CCGCTGATCGCATTATGCCT R-AGCGATTCTGCATCATCTGCT P-ATGTTGCTGGTGGTGTTTCCTATGCAC	107	Michelet et al. 2014
<i>Bartonella spp.</i>	ssrA	F-CGTTATCGGGCTAAATGAGTAG	118	

		R-ACCCCGCTTAAACCTGCGA		Gondard et al. 2020
		P-TTGCAAATGACAACTATGCGGAAGCACGT C		
<i>Francisella tularensis</i>	<i>tul4</i>	F-ACCCACAAGGAAGTGTAAGATTA	76	Michelet et al. 2014
		R-GTAATTGGGAAGCTTGTATCATG		
		P-AATGGCAGGCTCCAGAAGGTTCTAAGT		
<i>Francisella-like endosymbiont</i>	<i>fop4</i>	F-GGCAAATCTAGCAGGTCAAGC	91	Michelet et al. 2014
		R-CAACACTTGCTTGAACATTTCTAG		
		P-AACAGGTGCTTGGGATGTGGGTGGTG		
<i>Coxiella burnetii</i>	<i>IS1111</i>	F-TGGAGGAGCGAACCATTGGT	86	Michelet et al. 2014
		R-CATACGGTTTGACGTGCTGC		
		P-ATCGGACGTTTATGGGGATGGGTATCC		
<i>Coxiella-like</i>	<i>idc</i>	F-AGGCCCGTCCGTTATTTTACG	74	Michelet et al. 2014
		R-CGGAAAATCACCATATTCACCTT		
		P-TTCAGGCGTTTTGACCGGGCTTGGC		
<i>Apicomplexa</i>	<i>18S</i>	F-TGAACGAGGAATGCCTAGTATG	104	Gondard et al. 2020
		R-CACCGGATCACTCGATCGG		
		P-TAGGAGCGACGGGCGGTGTGTAC		
<i>Babesia microti</i>	<i>CCTeta</i>	F-ACAATGGATTTTCCCCAGCAAAA	145	Michelet et al. 2014
		R-GCGACATTTGCGCAACTTATATA		
		P-TACTCTGGTGCAATGAGCGTATGGGTA		
	<i>18 rRNA</i>	F-TGGCCGTTCTTAGTTGGTGG	104	

<i>Babesia canis</i> (3 subspecies)		R-AGAAGCAACCGGAAACTCAAATA P-ACCGGCACTAGTTAGCAGGTTAAGGTC		Michelet et al. 2014
<i>Babesia ovis</i>	18S rRNA	F-TCTGTGATGCCCTTAGATGTC R-GCTGGTTACCCGCGCCTT P-TCGGAGCGGGGTCAACTCGATGCAT	92	Michelet et al. 2014
<i>Babesia bovis</i>	CCTeta	F-GCCAAGTAGTGGTAGACTGTA R-GCTCCGTCATTGGTTATGGTA P-TAAAGACAACACTGGGTCCGCGTGG	100	Michelet et al. 2014
<i>Babesia caballi</i>	Rap1	F-GTTGTTCGGCTGGGGCATC R-CAGGCGACTGACGCTGTGT P-TCTGTCCCGATGTCAAGGGGCAGGT	94	Michelet et al. 2014
<i>Babesia venatorum</i> (sp. EU1)	18S rRNA	F-GCGCGCTACACTGATGCATT R-CAAAAATCAATCCCCGTCACG P-CATCGAGTTTAATCCTGTCCCGAAAGG	91	Michelet et al. 2014
<i>Babesia divergens</i>	hsp70	CTCATTGGTGACGCCGCTA R-CTCCTCCCGATAAGCCTCTT P-AGAACCAGGAGGCCCGTAACCCAGA	83	Michelet et al. 2014
<i>Babesia vogeli</i>	hsp70	F-TCACTGTGCCTGCGTACTTC R-TGATACGCATGACGTTGAGAC P-AACGACTCCCAGCGCCAGGCCAC	87	Michelet et al. 2014
<i>Theileria</i> spp	18S rRNA	GTCAGTTTTTACGACTCCTTCAG CCAAAGAATCAAGAAAGAGCTATC	213	Michelet et al. 2014

		AATCTGTCAATCCTTCCTTTGTCTGGACC		
<i>Hepatozoon</i> spp.	18S rRNA	F-ATTGGCTTACCGTGGCAGTG R-AAAGCATTTTAACTGCCTTGTATTG P-ACGGTTAACGGGGGATTAGGGTTCGAT R-CCGCTCCGCGCAAGAATCT P-TTCGGAGTACGTCGAGCTCTAGCAGA	175	Gondard et al. 2020
Tick spp.	16S rRNA	F-AAATACTCTAGGGATAACAGCGT R-TCTTCATCAAACAAGTATCCTAATC P- CAACATCGAGGTCGCAAACCATTTTGTCT A	99	Gondard et al. 2020
<i>Escherichia coli</i>	eae	F-CATTGATCAGGATTTTCTGGTGATA R-CTCATGCGGAAATAGCCGTTA P- ATAGTCTCGCCAGTATTCGCCACCAATACC	102	Michelet et al. 2014

APPENDIX 3

Supplementary Table 2. Primer pairs and PCR conditions used for validation of microfluidic real-time PCR results.

Pathogens	Primers sequences (5' - 3')	Target gene	Amplicon size	PCR conditions	References
<i>Hyalomma excavatum</i>	TTT GAC TAT ACA AAG GTA TTG CGG TCT GAA CTC AGA TCA AGT AGG	16S rRNA	-	35 cycles: 10 sec 98°C; 30 sec 51.4°C; 30 sec 72°C	Roth et al. (2019)
<i>Babesia</i> spp./ <i>Hepatozoon</i> spp./ <i>Theileria</i> spp.	PCR 1 GTGAAACTGCGAATGGCTCA TTAC AAGTGATAAGGTTACAAAA ACTTCCC	18S rRNA	1500 bp	35 cycles: 10 sec 98°C; 30 sec 58°C; 45 sec 72°C	Masatani et al. (2017)
	PCR 2 GGCTCATTACAACAGTTATA GTTTATTTG CGGTCCGAATAATTCACCGG AT		1500 bp	35 cycles: 10 sec 98°C; 30 sec 58°C; 30 sec 72°C	
<i>Anaplasma</i> spp./ <i>Ehrlichia</i> spp.	PCR 1 GAACGAACGCTGGCGGCAA GC AGTAYCGRACCAGATAGCC GC	16S rRNA	693 bp	35 cycles: 10 sec 98°C; 30 sec 60°C; 30 sec 72°C	Rar et al. (2005)
	PCR 2 TGCATAGGAATCTACCTAGT AG AGTAYCGRACCAGATAGCC GC		629 bp	35 cycles: 10 sec 98°C; 30 sec 55°C; 30 sec 72°C	
<i>Rickettsia sibirica</i> / <i>R. africae</i>	PCR 1 GTCAGCGTTACTTCTTCGAT GC CCGTACTCCATCTTAGCATC AG	ompB	475 bp	35 cycles: 10 sec 98°C; 30 sec 57°C; 30 sec 72°C	Choi et al. (2005)

<i>Rickettsia</i> spp.	PCR 2			267 bp	35 cycles:	Regnery et al. (1991)
	CCAATGGCAGGACTTAGCTA				10 sec	
	CT				98°C;	
	AGGCTGGCTGATACACGGA				30 sec	
	GTAA				58°C;	
					30 sec	
	PCR 1			72°C		
				35 cycles:		
	GGG GGC CTG CTC ACG GCG			10 sec		
	G			98°C;		
ATT GCA AAA AGT ACA GTG			30 sec			
AAC A			56°C;			
			30 sec			
			72°C			

APPENDIX 4

Supplementary Table 3: Tick-borne microorganisms identified in TBPGs associated with *Hyalomma excavatum*.

TBPG Identifier	Pathogen (TBP)
M	<i>Anaplasma</i>
	<i>Apicomplexa</i>
	<i>Bartonella</i>
	<i>Coxiella</i> -like endosymbiont
	<i>Ehrlichia</i>
	<i>Francisella</i> -like endosymbiont
	<i>Francisella tularensis</i>
	<i>Neoehrlichia mikurensis</i>
	<i>Rickettsia aeschlimannii</i>
	<i>Rickettsia conorii</i>
	<i>Rickettsia slovaca</i>
	<i>Rickettsia</i>
	<i>Theleiria</i>
MW	<i>Apicomplexa</i>
	<i>Francisella</i> -like endosymbiont
	<i>Rickettsia slovaca</i>
	<i>Rickettsia</i>
MSP	<i>Apicomplexa</i>
	<i>Bartonella</i>
	<i>Francisella</i> -like endosymbiont
	<i>Rickettsia conorii</i>
	<i>Rickettsia slovaca</i>
MSU	<i>Rickettsia</i>
	<i>Apicomplexa</i>
	<i>Coxiella</i> like endosymbiont
	<i>Rickettsia conorii</i>
	<i>Francisella</i> -like endosymbiont
	<i>Rickettsia slovaca</i>
	<i>Rickettsia</i>
MA	<i>Theleiria</i>
	<i>Apicomplexa</i>
	<i>Ehrlichia</i>
	<i>Francisella</i> -like endosymbiont
	<i>Francisella tularensis</i>
	<i>Neoehrlichia mikurensis</i>
	<i>Rickettsia aeschlimannii</i>
	<i>Rickettsia conorii</i>
	<i>Rickettsia slovaca</i>
F	<i>Rickettsia</i>
	<i>Anaplasma phagocytophilum</i>
	<i>Anaplasma</i>
	<i>Apicomplexa</i>
	<i>Bartonella</i>
	<i>Borrelia afzelii</i>

	<i>Borrelia spielmanii</i> <i>Francisella-like endosymbiont</i> <i>Francisella tularensis</i> <i>Hepatozoon</i> <i>Mycoplasma</i> <i>Neoehrlichia mikurensis</i> <i>Rickettsia aeschlimannii</i> , <i>Rickettsia conorii</i> <i>Rickettsia slovaca</i> <i>Rickettsia</i> <i>Theleiria</i>
FW	<i>Anaplasma phagocytophilum</i> <i>Anaplasma</i> <i>Apicomplexa</i> <i>Borrelia afzelii</i> <i>Borrelia spielmanii</i> <i>Francisella-like endosymbiont</i> <i>Neoehrlichia mikurensis</i> <i>Rickettsia aeschlimannii</i> , <i>Rickettsia conorii</i> <i>Rickettsia slovaca</i> <i>Rickettsia</i>
FSP	<i>Apicomplexa</i> <i>Borrelia spielmanii</i> <i>Francisella-like endosymbiont</i> <i>Neoehrlichia mikurensis</i> <i>Rickettsia conorii</i> <i>Rickettsia slovaca</i> <i>Rickettsia</i>
FSU	<i>Anaplasma</i> <i>Apicomplexa</i> <i>Bartonella</i> <i>Borrelia afzelii</i> <i>Borrelia spielmanii</i> <i>Francisella-like endosymbiont</i> <i>Francisella tularensis</i> <i>Hepatozoon</i> <i>Mycoplasma</i> <i>Neoehrlichia mikurensis</i> <i>Rickettsia conorii</i> <i>Rickettsia slovaca</i> <i>Rickettsia</i>
FA	<i>Theleiria</i> <i>Anaplasma</i> <i>Apicomplexa</i> <i>Borrelia afzelii</i> <i>Borrelia spielmanii</i> <i>Francisella-like endosymbiont</i> <i>Hepatozoon</i> <i>Mycoplasma</i>

Neoehrlichia mikurensis

Rickettsia aeschlimannii

Rickettsia conorii

Rickettsia slovaca

Rickettsia

Theleiria

APPENDIX 5

Supplementary Table 4: Centrality Measures of Pathogens in Tick-Borne Pathogen Guilds (TBPGs) Networks.

TBPG networks	Pathogen (TBP)	Degree Centrality	Betweenness Centrality	Eigenvector Centrality	Closeness Centrality
M	<i>Anaplasma</i>	11	0	1	1
	<i>Apicomplexa</i>	11	0	1	1
	<i>Bartonella</i>	11	0	1	1
	<i>Ehrlichia</i>	11	0	1	1
	<i>Francisella like endosymbiont</i>	11	0	1	1
	<i>Francisella tularensis</i>	11	0	1	1
	<i>Neoehrlichia mikurensis</i>	11	0	1	1
	<i>Rickettsia aeschlimannii</i>	11	0	1	1
	<i>Rickettsia conorii</i>	11	0	1	1
	<i>Rickettsia slovaca</i>	11	0	1	1
	<i>Rickettsia</i>	11	0	1	1
	<i>Theleiria</i>	11	0	1	1
MW	<i>Apicomplexa</i>	3	0	1	1
	<i>Francisella like endosymbiont</i>	3	0	1	1
	<i>Rickettsia slovaca</i>	3	0	1	1
	<i>Rickettsia</i>	3	0	1	1
MSP	<i>Apicomplexa</i>	5	0	1	1
	<i>Bartonella</i>	5	0	1	1
	<i>Francisella like endosymbiont</i>	5	0	1	1
	<i>Rickettsia conorii</i>	5	0	1	1
	<i>Rickettsia slovaca</i>	5	0	1	1
	<i>Rickettsia</i>	5	0	1	1
MSU	<i>Apicomplexa</i>	6	0	1	1
	<i>Coxiella like endosymbiont</i>	6	0	1	1
	<i>Francisella like endosymbiont</i>	6	0	1	1
	<i>Rickettsia conorii</i>	6	0	1	1
	<i>Rickettsia slovaca</i>	6	0	1	1
	<i>Rickettsia</i>	6	0	1	1
	<i>Theleiria</i>	6	0	1	1
MA	<i>Anaplasma</i>	8	0	1	1
	<i>Ehrlichia</i>	8	0	1	1

F	<i>Francisella like endosymbiont</i>	8	0	1	1
	<i>Francisella tularensis</i>	8	0	1	1
	<i>Neoehrlichia mikurensis</i>	8	0	1	1
	<i>Rickettsia aeschlimannii</i>	8	0	1	1
	<i>Rickettsia conorii</i>	8	0	1	1
	<i>Rickettsia slovaca</i>	8	0	1	1
	<i>Rickettsia</i>	8	0	1	1
	<i>Anaplasma phagocytophilum</i>	15	0	1	1
	<i>Anaplasma</i>	15	0	1	1
	<i>Apicomplexa</i>	15	0	1	1
	<i>Bartonella</i>	15	0	1	1
	<i>Borrelia afzelii</i>	15	0	1	1
	<i>Borrelia spielmanii</i>	15	0	1	1
	<i>Francisella like endosymbiont</i>	15	0	1	1
	<i>Francisella tularensis</i>	15	0	1	1
	<i>Hepatozoon</i>	15	0	1	1
	<i>Mycoplasma</i>	15	0	1	1
	<i>Neoehrlichia mikurensis</i>	15	0	1	1
	<i>Rickettsia aeschlimannii</i>	15	0	1	1
	<i>Rickettsia conorii</i>	15	0	1	1
FW	<i>Rickettsia slovaca</i>	15	0	1	1
	<i>Rickettsia</i>	15	0	1	1
	<i>Theleiria</i>	15	0	1	1
	<i>Anaplasma phagocytophilum</i>	11	0	1	1
	<i>Anaplasma</i>	11	0	1	1
	<i>Apicomplexa</i>	11	0	1	1
	<i>Borrelia afzelii</i>	11	0	1	1
	<i>Borrelia spielmanii</i>	11	0	1	1
	<i>Francisella like endosymbiont</i>	11	0	1	1
	<i>Neoehrlichia mikurensis</i>	11	0	1	1
	<i>Rickettsia aeschlimannii</i>	11	0	1	1
	<i>Rickettsia conorii</i>	11	0	1	1
	<i>Rickettsia slovaca</i>	11	0	1	1
	<i>Rickettsia</i>	11	0	1	1
FSP	<i>Apicomplexa</i>	6	0	1	1
	<i>Borrelia spielmanii</i>	6	0	1	1
	<i>Francisella like endosymbiont</i>	6	0	1	1

FSU	<i>Neoehrlichia mikurensis</i>	6	0	1	1
	<i>Rickettsia conorii</i>	6	0	1	1
	<i>Rickettsia slovaca</i>	6	0	1	1
	<i>Rickettsia</i>	6	0	1	1
	<i>Anaplasma</i>	13	0	1	1
	<i>Apicomplexa</i>	13	0	1	1
	<i>Bartonella</i>	13	0	1	1
	<i>Borrelia afzelii</i>	13	0	1	1
	<i>Borrelia spielmanii</i>	13	0	1	1
	<i>Francisella like endosymbiont</i>	13	0	1	1
	<i>Francisella tularensis</i>	13	0	1	1
	<i>Hepatozoon</i>	13	0	1	1
	<i>Mycoplasma</i>	13	0	1	1
	<i>Neoehrlichia mikurensis</i>	13	0	1	1
FA	<i>Rickettsia conorii</i>	13	0	1	1
	<i>Rickettsia slovaca</i>	13	0	1	1
	<i>Rickettsia</i>	13	0	1	1
	<i>Theleiria</i>	13	0	1	1
	<i>Anaplasma</i>	12	0	1	1
	<i>Apicomplexa</i>	12	0	1	1
	<i>Borrelia afzelii</i>	12	0	1	1
	<i>Borrelia spielmanii</i>	12	0	1	1
	<i>Francisella like endosymbiont</i>	12	0	1	1
	<i>Hepatozoon</i>	12	0	1	1
	<i>Mycoplasma</i>	12	0	1	1
	<i>Neoehrlichia mikurensis</i>	12	0	1	1
	<i>Rickettsia aeschlimannii</i>	12	0	1	1
	<i>Rickettsia conorii</i>	12	0	1	1
	<i>Rickettsia slovaca</i>	12	0	1	1
	<i>Rickettsia</i>	12	0	1	1
	<i>Theleiria</i>	12	0	1	1

APPENDIX 6

Supplementary Table 5: Centrality Measures of Shared Pathogens Across TBPGs networks

Pathogen (TBP)	TBPG networks	Degree Centrality	Betweenness Centrality	Eigenvector Centrality	Closeness Centrality
<i>Anaplasma phagocytophilum</i>	F	15	0	1	1
<i>Anaplasma</i>	FW	11	0	1	1
	M	11	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Apicomplexa</i>	M	11	0	1	1
	MW	3	0	1	1
	MSP	5	0	1	1
	MSU	6	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
<i>Coxiella like endosymbiont</i>	M	11	0	1	1
	MSP	5	0	1	1
<i>Bartonella</i>	M	11	0	1	1
	MSP	5	0	1	1
	F	15	0	1	1
	FSU	13	0	1	1
<i>Borrelia afzelii</i>	F	15	0	1	1
	FW	11	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Borrelia spielmanii</i>	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Ehrlichia</i>	M	11	0	1	1
	MA	8	0	1	1
<i>Francisella like endosymbiont</i>	M	11	0	1	1
	MW	3	0	1	1
	MSP	5	0	1	1
	MSU	6	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Francisella tularensis</i>	M	11	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FSU	13	0	1	1

<i>Hepatozoon</i>	F	15	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Mycoplasma</i>	F	15	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Neoehrlichia mikurensis</i>	M	11	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Rickettsia aeschlimannii</i>	M	11	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FA	12	0	1	1
<i>Rickettsia conorii</i>	M	11	0	1	1
	MSP	5	0	1	1
	MSU	6	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Rickettsia slovaca</i>	M	11	0	1	1
	MW	3	0	1	1
	MSP	5	0	1	1
	MSU	6	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Rickettsia</i>	M	11	0	1	1
	MW	3	0	1	1
	MSP	5	0	1	1
	MSU	6	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FW	11	0	1	1
	FSP	6	0	1	1
	FSU	13	0	1	1
	FA	12	0	1	1
<i>Theleiria</i>	M	11	0	1	1
	MA	8	0	1	1
	F	15	0	1	1
	FA	12	0	1	1

APPENDIX 7

Supplementary Table 6: Microbial taxa shared across seasons

Names			Total	Elements
Autumn	Spring	Summer	128	g__Segetibacter g__Mannheimia f__Gemmatimonadaceae__uncultured g__Moraxella g__Aeromonas f__Geodermatophilaceae f__Pasteurellaceae g__Francisella f__Beijerinckiaceae__uncultured g__Geminicoccus o__Bacillales g__Methanobrevibacter g__Bibersteinia g__Selenomonas g__Aquipuribacter g__UCG-005 g__Rubrobacter g__Christensenellaceae_R-7_group

g__Brachybacterium

g__Blastococcus

g__PMMR1

g__Deinococcus

g__Haemophilus

g__Sphingobacterium

g__Pontibacter

g__Corynebacterium

g__Iamia

g__Clostridium_sensu_stricto_1

f__Microbacteriaceae

f__Micrococcaceae

g__Kocuria

g__Streptomyces

f__Carnobacteriaceae

g__Cnuella

f__Comamonadaceae

g__Arthrobacter

g__JG30-KF-CM66

g__Pedobacter

f__Planococcaceae

g__Paracoccus

g__Turicibacter

f__Kineosporiaceae

g__Noviherbaspirillum

g__Lysinibacillus

g__Actinomadura

f__Enterobacteriaceae

g__Atopostipes

g__Solirubrobacter

g__Skermanella

g__Actinomycespora

g__Cutibacterium

g__Candidatus_Alysiosphaera

g__Sphingomonas

g__Rickettsia

g__Truepera

f__Intrasporangiaceae

g__WD2101_soil_group

g__Bryobacter

g__JG30-KF-CM45

g__Massilia

g__Bacillus

g__Candidatus_Midichloria

g__Stenotrophomonas

g__Pseudonocardia

g__Lachnospiraceae_NK3A20_group

g__Pseudomonas

g__Geodermatophilus

g__Bergeyella

g__Exiguobacterium

g__Mycobacterium

f__Beijerinckiaceae

g__Hymenobacter

o__Vicinamibacterales__uncultured

g__Brevibacterium

g__TM7a

c__Acidimicrobiia__uncultured

g__Microvirga

g__Adhaeribacter

g__Qipengyuania

g__Saccharimonadales

g__Streptococcus

g__Devosia

g__Chryseobacterium

g__Escherichia-Shigella

g__Mitochondria

g__Citricoccus

f__Acetobacteraceae

f__Neisseriaceae__uncultured

g__Brevundimonas

g__Alloprevotella

o__Frankiales__uncultured

f__Isosphaeraceae__uncultured

g__Alysiella

g__Lactobacillus

g__Friedmanniella

g__Allorhizobium-Neorhizobium-Pararhizobium-
Rhizobium

g__[Eubacterium]_coprostanoligenes_group

g__Modestobacter

f__Rhodobacteraceae

d__Bacteria

g__Staphylococcus

g__Mesorhizobium

f__Neisseriaceae

g__Nocardioides

g__Gemmatimonas

g__Cellulomonas

g__Mogibacterium

g__Georgenia

g__Methylobacterium-Methylobacterium

g__Ferruginibacter

g__Roseisolibacter

g__RB41

f__Sphingomonadaceae

g__Kineococcus

g__Flavobacterium

g__Paenibacillus

g__Dietzia

g__Romboutsia

g__Rubellimicrobium

g__Craurococcus-Caldovatus

g__Oceanobacillus

g__Sporosarcina

g__Ornithinimicrobium

g__Prevotella

g__Acinetobacter

g__Aerococcus

g__Longimicrobium

Autumn Spring

45

g__Lawsonella

g__Flaviaesturariibacter

o__Enterobacterales

g__Pantoea

g__Trichococcus

g__Luteimonas

g__Agrococcus

g__Aeromicrobium

g__Edaphobaculum

g__Paenarthrobacter

f__Rhizobiaceae

o__Rhizobiales__uncultured

g__Shewanella

g__Clostridioides

g__Lactococcus

f__Caldilineaceae__uncultured

g__Altererythrobacter

g__Olsenella

f__Myxococcaceae__uncultured

g__Limnobacter

f__Caulobacteraceae__uncultured

g__Glutamicibacter

g__Longimicrobiaceae

g__Bdellovibrio

g__Methanosphaera

o__Gaiellales

g__Muribaculaceae

g__Enteractinococcus

g__Paeniclostridium

g__Lysobacter

g__Antricoccus

g__Rikenellaceae_RC9_gut_group

g__Oligoflexus

f__Cyclobacteriaceae

g__EPR3968-O8a-Bc78

g__WPS-2

g__Macrococcus

g__Jeotgalicoccus

g__F082

f__Yersiniaceae

g__Alistipes

g__Anaerosporobacter

g__Mailhella

Autumn Summer

99

o__Coriobacteriales

o__Lactobacillales

g__Subgroup_7

g__Vulcaniibacterium

g__Cloacibacterium

g__Veillonella

g__Candidatus_Saccharimonas

g__67-14

f__Ruminococcaceae__uncultured

f__Isosphaeraceae

g__Rheinheimera

g__Anaerococcus

g__Cardiobacterium

g__Quadrisphaera

g__Absconditabacteriales_(SR1)

g__Novosphingobium

g__Fusobacterium

f__Bacillaceae

g__Enhydrobacter

g__Nitrospira

g__RF39

f__Gemmataceae__uncultured

g__Vogesella

g__Gracilibacteria

g__TK10

g__Family_XIII_AD3011_group

g__Rhodococcus

f__Sporichthyaceae__uncultured

f__Dermabacteraceae

g__Syntrophococcus

g__Bifidobacterium

o__Gaiellales__uncultured

g__Marmoricola

f__Microtrichaceae__uncultured

g__Saccharomonospora

g__Aquabacterium

g__KD4-96

g__Riemerella

g__S0134_terrestrial_group

g__Xanthomonas

f__Micromonosporaceae

g__YC-ZSS-LKJ147

p__Armatimonadota__uncultured

g__Lautropia

g__S085

g__Vicinamibacteraceae

g__Conexibacter

g__Helcococcus

g__Woeseia

g__Rothia

g__Clostridia_UCG-014

g__Bradyrhizobium

g__Aureimonas

g__Gemella

f__Carnobacteriaceae__uncultured

g__Rubritepida

f__Lachnospiraceae

g__MB-A2-108

g__Schlegelella

g__Parviterribacter

o__Planctomycetales__uncultured

g__Coproccoccus

f__Blastocatellaceae__uncultured

o__Microtrichales__uncultured

g__Roseomonas

f__Lachnospiraceae__uncultured

g__NK4A214_group
f__Ilumatobacteraceae_uncultured
f__Blastocatellaceae
g__Nocardiopsis
g__Bosea
g__Candidatus_Nitrososphaera
g__Actinomyces
g__Pir4_lineage
o__Frankiales
g__Anoxybacillus
g__AKIW781
g__Patulibacter
g__Candidatus_Proteochlamydia
f__Thermomonosporaceae
g__Flavisolibacter
g__Hydrogenophilus
f__Rhodobacteraceae_uncultured
g__Neisseria
g__Micrococcus
c__Acidimicrobiia
f__Ruminococcaceae
g__Leptotrichia

		g__Gaiella
		f__Aerococcaceae__uncultured
		c__Clostridia
		g__Chthoniobacter
		g__Belnapia
		f__Erysipelotrichaceae__uncultured
		g__Hydrogenophaga
		g__Psychroglaciecola
		g__Porphyromonas
		g__Geobacillus
		g__Rhodocytophaga
		g__Trueperella
		g__Enterococcus
Spring Summer	17	f__Microscillaceae__uncultured
		g__Steroidobacter
		g__Psychrobacter
		g__Acidiphilium
		g__Empedobacter
		g__Amaricoccus
		g__Leuconostoc
		g__Actinophytocola
		f__Aerococcaceae

		g__Nitrososphaeraceae
		g__Spirosoma
		g__Thermomonas
		g__Candidatus_Nitrocosmicus
		g__Virgibacillus
		g__Rubrivirga
		g__Tundrisphaera
		g__Chloroplast
Autumn	206	g__Hyphomicrobium
		g__Klenkia
		g__Saccharimonadaceae
		g__Pseudolabrys
		g__Haliangium
		g__Blfdi19
		g__Sphingopyxis
		g__Devosiaceae
		g__Candidatus_Peribacteria
		g__P2-11E
		f__Geobacteraceae
		g__Stenotrophobacter
		f__Caloramatoraceae
		g__Gastranaerophilales

g__Asanoa

g__Alkanindiges

g__Monoglobus

g__Curvibacter

g__Caldicoprobacter

g__Anaeromyxobacter

g__MVP-15

g__Sericytochromatia

g__[Ruminococcus]_gnavus_group

g__Peptoniphilus

g__Suttonella

g__Phyllobacterium

g__Negativicoccus

f__Cellulomonadaceae

g__CL500-29_marine_group

g__Flavitalea

g__Cryptosporangium

g__Rhodopseudomonas

g__Defluviicoccus

f__Geminicoccaceae__uncultured

o__Oscillospirales

g__Phreatobacter

g__Slackia

f__Rhodocyclaceae

g__Desemzia

f__Anaerolineaceae__uncultured

g__Alkaliphilus

g__Parasutterella

o__Azospirillales__uncultured

g__Caldibacillus

f__Clostridiaceae

g__Phenylobacterium

f__Solirubrobacteraceae

g__Neochlamydia

g__Terrisporobacter

g__[Eubacterium]_ventriosum_group

g__Peredibacter

o__Rhizobiales

f__Ilumatobacteraceae

g__Dactylosporangium

g__Aequorivita

g__Candidatus_Soleaferrea

f__Peptostreptococcaceae

o__Acidobacteriales

g__0319-6G20

g__TRA3-20

g__Tolumonas

g__BD2-11_terrestrial_group

g__Proteocatella

f__Solirubrobacteraceae__uncultured

g__Vermiphilaceae

f__Nocardiodaceae

g__Thermonema

g__Glycomyces

f__Pseudonocardiaceae

g__UCG-010

f__Prolixibacteraceae__uncultured

f__Weeksellaceae

g__DNF00809

g__Neo-b11

g__Halomonas

g__Planifilum

g__Promicromonospora

f__Oxalobacteraceae

o__Xanthomonadales__uncultured

g__Muribaculum

g__Xanthobacter

g__Microbacterium

g__Caenimonas

g__Rufibacter

o__Solirubrobacterales

g__Fervidobacterium

g__Ochrobactrum

g__Facklamia

g__Marisediminicola

p__Patescibacteria__uncultured

f__Rikenellaceae

g__Abditibacterium

g__Thermoactinomyces

g__Amb-16S-1323

g__Ellin6067

g__Duganella

g__Flexilinea

f__Cellvibrionaceae__uncultured

f__Hyphomicrobiaceae

g__Asticcacaulis

g__[Ruminococcus]_gauvreauii_group

g__Dielma

g__P3OB-42

g__Sphaerisporangium

g__Bordetella

f__Pseudomonadaceae

f__Hungateiclostridiaceae__uncultured

o__Elsterales__uncultured

f__Moraxellaceae__uncultured

g__Treponema

g__Sphingobium

f__Selenomonadaceae

g__Legionella

g__Elev-16S-1166

g__Solibacillus

f__Xanthomonadaceae

g__Haloactinopolyspora

f__Acetobacteraceae__uncultured

g__Tepidimonas

g__Ramlibacter

g__Opitutus

f__Rubinisphaeraceae

g__SC-I-84

g__Caulobacter

g__Bacteroides

g__Gemmata

f__Oscillospiraceae

g__Propionibacterium

g__Erysipelotrichaceae_UCG-009

g__Fonticella

g__Candidatus_Entotheonella

g__BIRii41

g__Armatimonadales

o__Saccharimonadales

g__Niveibacterium

g__Porphyrobacter

f__Nannocystaceae__uncultured

g__Variovorax

g__Caloramator

g__Thermoanaerobacterium

g__Methyloversatilis

g__mle1-27

g__Microlunatus

g__Kribbella

g__Agromyces

g__Perlucidibaca

g__LiUU-11-161

g__Candidatus_Solibacter

g__Jeotgalibaca

g__Blastomonas

g__Bacteroidales_RF16_group

g__Clostridia_vadinBB60_group

g__Pedomicrobium

g__Moryella

g__Rhodobacter

g__Hungateiclostridium

f__Sphingobacteriaceae

g__Burkholderia-Caballeronia-Paraburkholderia

g__Kibdelosporangium

g__IheB3-7

p__Proteobacteria

g__1-20

g__Thermicanus

g__Dechloromonas

g__WCHB1-41

g__KF-JG30-B3

g__Pseudorhodoferax

g__Fimbriimonadaceae

g__Nibribacter

g__Tissierella

g__Sumerlaea

g__Lechevalieria

g__D05-2

g__Catellatospora

g__type_III

g__Rickettsiella

g__Arcanobacterium

g__env.OPS_17

g__Planctomicrobium

g__Alloiococcus

o__Babeliales

g__Sphingaurantiacus

f__Methylophilaceae

f__Pirellulaceae__uncultured

g__Blastocatella

g__Rhodoplanes

g__Thermosinus

f__Eggerthellaceae

g__Azospira

g__Novibacillus

		g__Tuwongella
		g__IMCC26256
		o__Erysipelotrichales__uncultured
		g__Thermus
		g__Acetitomaculum
		g__Elusimicrobium
		g__Kineosporia
		g__Paludibacter
		g__Phaselicystis
		g__dgA-11_gut_group
		g__Lachnospiraceae_UCG-010
		g__Sorangium
		g__SZB85
		c__Clostridia__uncultured
		g__CAP-aah99b04
		f__Xanthobacteraceae
Spring	64	g__Williamsia
		g__Sphingorhabdus
		g__Aestuariicella
		g__Bacteroidales_UCG-001
		g__Confluentibacter
		g__Algoriphagus

g__A4b

g__Blautia

g__Pontibacillus

g__Prevotellaceae_UCG-004

g__Micromonospora

g__LWQ8

Spring

g__Vitellibacter

g__Phascolarctobacterium

g__Brevibacillus

o__Microtrichales

g__Actinotalea

g__Kandleria

g__Halobacillus

g__Cellvibrio

g__Serratia

g__Kurthia

g__Gracilibacillus

f__Sandaracinaceae_uncultured

g__MWH-CFBk5

g__R7C24

f__Methylophilaceae

g__[Eubacterium]_xylanophilum_group

g__Clostridium_sensu_stricto_3

f__Dermatophilaceae

g__Leucobacter

g__SWB02

g__Tumebacillus

g__Carnobacterium

g__Myxococcus

g__Luteitalea

f__Chroococcidiopsaceae__uncultured

g__Marinicella

g__Ornithinicoccus

g__Ruminococcus

g__UCG-009

g__Ammoniphilus

g__Pirellula

g__Tessaracoccus

g__Ornithobacterium

g__Myroides

g__Salinimicrobium

g__Brochothrix

g__Fibrella

		g__HAW-RM37-2
		g__Demequina
		g__Weissella
		g__Psychrobacillus
		g__Mobilicoccus
		g__Succiniclasicum
		f__Ardenticatenaceae__uncultured
		g__Tomitella
		f__Erwiniaceae
		g__Crossiella
		o__Micrococcales
		g__Cereibacter
		g__Reyranella
		g__Raineiyella
Summer	83	f__Comamonadaceae__uncultured
		g__Paludisphaera
		f__Myxococcaceae
		c__Actinobacteria
		g__Atopobium
		g__Bauldia
		f__Sphingomonadaceae__uncultured
		f__Methanobacteriaceae

g__Lineage_IIb

g__[Anaerorhabdus]_furcosa_group

g__Prauserella

g__Fenollaria

g__Granulicatella

g__Actinocorallia

g__Abiotrophia

g__F0332

g__Proteiniclasticum

g__Nakamurella

g__Pseudoalteromonas

g__p-251-o5

g__Jatrophihabitans

g__Aridibacter

f__Eggerthellaceae__uncultured

f__Polyangiaceae

g__Marvinbryantia

g__Phocaeicola

g__Oribacterium

g__GCA-900066575

g__Tepidisphaera

g__Akkermansia

g__JCM_18997

g__Tychonema_CCAP_1459-11B

g__Domibacillus

g__Tepidisphaeraceae

g__Peptostreptococcus

f__Oscillospiraceae__uncultured

g__Dysgonomonas

g__Dermacoccus

g__Bergeriella

f__Xanthobacteraceae__uncultured

g__Motilibacter

f__Aeromonadaceae

g__Saccharothrix

g__Constrictibacter

g__Aerosphaera

g__Ohtaekwangia

g__CAG-352

f__Verrucomicrobiaceae__uncultured

g__SH-PL14

g__Capnocytophaga

g__IMCC26207

p__Chloroflexi

g__Sulfurimonas

g__Comamonas

o__Thermomicrobiales

g__Anaerofustis

g__bacteriap25

g__C0119

g__Enterorhabdus

g__0319-7L14

g__Taibaiella

f__Gemmatimonadaceae

f__Peptococcaceae__uncultured

f__Longimicrobiaceae

g__Alishewanella

f__Nitrososphaeraceae

c__Cyanobacteriia

g__Gitt-GS-136

g__Campylobacter

g__Lachnospiraceae_XPB1014_group

g__TM7x

g__Family_XIII_UCG-001

g__Tepidiphilus

g__11-24

g__Azospirillum

g__Subdoligranulum

g__Dyadobacter

c__Alphaproteobacteria__uncultured

g__JGI_0000069-P22

g__Antarcticibacterium

g__Lachnospiraceae_UCG-008

g__Simonsiella

g__wb1-P19

APPENDIX 8

Supplementary Table 7: Shared and unique microbial taxa across seasonal networks

Names	Total	Elements
Autumn	126	g__Streptomyces
AutumnWithoutFrancisella		
AutumnWithoutRickettsia		g__Massilia
		g__Segetibacter
		g__Burkholderia-Caballeronia-Paraburkholderia
		f__Comamonadaceae
		f__Lachnospiraceae
		g__IheB3-7
		g__Vulcaniibacterium
		g__Arthrobacter
		g__Nocardioides
		g__Pseudonocardia
		g__Lachnospiraceae_NK3A20_group
		g__Pseudomonas

g__Geodermatophilus

g__Bergeyella

f__Pseudomonadaceae

f__Planococcaceae

g__Paracoccus

g__Thermicanus

g__Hydrogenophilus

g__Moraxella

g__Mycobacterium

g__Aeromonas

f__Caloramatoraceae

g__Cellulomonas

g__Candidatus_Saccharimonas

g__Micrococcus

f__Beijerinckiaceae

g__Treponema

g__Pseudorhodofex

g__Hymenobacter

g__Sphingobium

f__Beijerinckiaceae__uncultured

o__Enterobacterales

f__Selenomonadaceae

g__Thermonema

f__Kineosporiaceae

g__67-14

g__Paenarthrobacter

g__Legionella

o__Bacillales

f__Ruminococcaceae

g__Pantoea

f__Xanthomonadaceae

o__Planctomycetales__uncultured

g__Noviherbaspirillum

g__Lysinibacillus

g__Tepidimonas

g__Aquabacterium

g__Methylobacterium-Methylorubrum

g__Roseisolibacter

f__Isosphaeraceae

g__Rubrobacter

g__Microvirga

g__Adhaeribacter

g__Xanthomonas

g__Phyllobacterium

g__Negativicoccus

g__Roseomonas

g__Rheinheimera

g__Flavobacterium

g__Arcanobacterium

f__Yersiniaceae

g__Solirubrobacter

g__Chryseobacterium

g__Muribaculum

f__Aerococcaceae__uncultured

c__Clostridia

g__Actinomycetospora

g__Bacteroides

g__Clostridioides

g__Christensenellaceae_R-7_group

g__Cutibacterium

g__Cardiobacterium

g__Microbacterium

g__Romboutsia

g__Fonticella

f__Ilumatobacteraceae__uncultured

f__Rhodocyclaceae

g__Luteimonas

g__Fervidobacterium

g__Thermosinus

f__Neisseriaceae__uncultured

f__Eggerthellaceae

g__Brevundimonas

g__Blastococcus

o__Frankiales__uncultured

g__Nocardiopsis

g__Rubellimicrobium

g__Candidatus_Alysiosphaera

g__Craurococcus-Caldovatus

g__Sphingomonas

g__Bosea

g__Actinomyces

f__Isosphaeraceae__uncultured

g__Fusobacterium

g__Sporosarcina

g__Caloramator

g__Haemophilus

g__Thermoanaerobacterium

g__Caldibacillus

g__Methyloversatilis

g__Thermus

g__Enhydrobacter

g__Altererythrobacter

g__Geobacillus

f__Gemmataceae__uncultured

g__Vogesella

g__Allorhizobium-Neorhizobium-
Pararhizobium-Rhizobium

g__Prevotella

g__Corynebacterium

g__Anoxybacillus

g__[Eubacterium]_coprostanoligenes_group

g__Lawsonella

		g__Helcococcus
		g__Family_XIII_AD3011_group
		g__Rhodococcus
		g__Modestobacter
		g__Bradyrhizobium
		g__JG30-KF-CM45
		d__Bacteria
		g__Staphylococcus
		f__Microbacteriaceae
		f__Micrococcaceae
		g__Rhodobacter
		g__Kocuria
Autumn	7	g__0319-6G20
AutumnWithoutFrancisella		g__Mogibacterium
		g__Sumerlaea
		g__Paenibacillus
		g__Rickettsia

		g__Trueperella
		g__Gemella
Autumn	5	g__Francisella
AutumnWithoutRickettsia		g__RB41
		g__Erysipelotrichaceae_UCG-009
		f__Erysipelotrichaceae__uncultured
		g__Deinococcus
AutumnWithoutFrancisella	9	g__Marmoricola
AutumnWithoutRickettsia		g__Riemerella
		g__Saccharimonadales
		f__Oscillospiraceae
		g__Marisediminicola
		p__Armatimonadota__uncultured
		g__Microlunatus
		f__Solirubrobacteraceae
		g__WD2101_soil_group
Autumn	6	g__JG30-KF-CM66

		g__Candidatus_Peribacteria
		g__Opitutus
		g__Pir4_lineage
		g__Flexilinea
		g__Moryella
AutumnWithoutFrancisella	9	g__Subgroup_7
		g__Leptotrichia
		g__Kineococcus
		o__Solirubrobacterales
		g__Porphyromonas
		g__Agromyces
		g__Friedmanniella
		g__Longimicrobium
		f__Hyphomicrobiaceae
AutumnWithoutRickettsia	16	g__Saccharimonadaceae
		g__Sphingopyxis
		g__Candidatus_Proteochlamydia

g__Exiguobacterium

o__Vicinamibacterales__uncultured

g__Bibersteinia

g__Methanosphaera

c__Acidimicrobiia__uncultured

g__Promicromonospora

g__Cryptosporangium

g__Chthoniobacter

g__Acetitomaculum

g__Acinetobacter

g__Olsenella

g__Rothia

f__Sporichthyaceae__uncultured

Names	total	elements
Spring	10	g__Pseudomonas
SpringWithoutFrancisella		f__Planococcaceae
		g__Aeromonas

		g__Pantoea
		g__Lysinibacillus
		g__Paenibacillus
		g__Sporosarcina
		g__Staphylococcus
		d__Bacteria
		g__Kocuria
Spring	134	g__Streptomyces
		g__Massilia
		g__Segetibacter
		g__Burkholderia-Caballeronia-Paraburkholderia
		f__Comamonadaceae
		g__0319-6G20
		f__Lachnospiraceae
		g__IheB3-7
		g__Vulcaniibacterium
		g__Arthrobacter

g__Nocardioides

g__JG30-KF-CM66

g__Pseudonocardia

g__Lachnospiraceae_NK3A20_group

g__Bergeyella

g__Geodermatophilus

f__Pseudomonadaceae

g__Candidatus_Peribacteria

g__Paracoccus

g__Thermicanus

g__Hydrogenophilus

g__Moraxella

g__Mycobacterium

f__Caloramatoraceae

g__Cellulomonas

g__Candidatus_Saccharimonas

g__Micrococcus

f__Beijerinckiaceae

g__Pseudorhodofex

g__Treponema

g__Hymenobacter

g__Sphingobium

g__Francisella

f__Beijerinckiaceae__uncultured

o__Enterobacterales

f__Selenomonadaceae

g__Thermonema

f__Kineosporiaceae

g__67-14

g__Paenarthrobacter

g__Legionella

o__Bacillales

f__Ruminococcaceae

g__Mogibacterium

f__Xanthomonadaceae

o__Planctomycetales__uncultured

g__Noviherbaspirillum

g__Sumerlaea

g__Tepidimonas

g__Aquabacterium

g__Methylobacterium-Methylorubrum

f__Isosphaeraceae

g__Roseisolibacter

g__Rubrobacter

g__Adhaeribacter

g__Microvirga

g__Xanthomonas

g__RB41

g__Phyllobacterium

g__Negativicoccus

g__Roseomonas

g__Rheinheimera

g__Flavobacterium

g__Arcanobacterium

f__Yersiniaceae

g__Opitutus

g__Solirubrobacter

g__Chryseobacterium

g__Muribaculum

f__Aerococcaceae__uncultured

c__Clostridia

g__Actinomycetospora

g__Bacteroides

g__Clostridioides

g__Christensenellaceae_R-7_group

g__Cutibacterium

g__Microbacterium

g__Erysipelotrichaceae_UCG-009

g__Cardiobacterium

g__Romboutsia

g__Fonticella

f__Ilumatobacteraceae__uncultured

f__Rhodocyclaceae

g__Luteimonas

g__Fervidobacterium

f__Erysipelotrichaceae__uncultured

g__Thermosinus

f__Neisseriaceae__uncultured

f__Eggerthellaceae

g__Brevundimonas

g__Blastococcus

o__Frankiales__uncultured

g__Nocardiopsis

g__Rubellimicrobium

g__Candidatus_Alysiosphaera

g__Deinococcus

g__Sphingomonas

g__Craurococcus-Caldovatus

g__Bosea

f__Isosphaeraceae__uncultured

g__Actinomyces

g__Fusobacterium

g__Rickettsia

g__Caloramator

g__Haemophilus

g__Thermoanaerobacterium

g__Caldibacillus

g__Methyloversatilis

g__Thermus

g__Enhydrobacter

g__Altererythrobacter

g__Geobacillus

f__Gemmataceae__uncultured

g__Vogesella

g__Prevotella

g__Allorhizobium-Neorhizobium-Pararhizobium-
Rhizobium

g__Corynebacterium

g__Pir4_lineage

g__Flexilinea

g__Anoxybacillus

g__Lawsonella

g__[Eubacterium]_coprostanoligenes_group

g__Helcococcus

g__Rhodococcus

g__Family_XIII_AD3011_group

g__Modestobacter

g__Bradyrhizobium

g__Trueperella

g__JG30-KF-CM45

		f__Microbacteriaceae
		f__Micrococcaceae
		g__Moryella
		g__Gemella
		g__Rhodobacter
SpringWithoutFrancisella	21	g__Bacillus
		g__Candidatus_Midichloria
		g__Serratia
		g__Kurthia
		g__Psychrobacillus
		f__Pasteurellaceae
		g__Virgibacillus
		g__Bibersteinia
		f__Enterobacteriaceae
		g__Leuconostoc
		g__Shewanella
		g__Psychrobacter

g__Mitochondria

g__Lactococcus

g__Myroides

g__Brochothrix

f__Erwiniaceae

g__Chloroplast

g__Acinetobacter

g__Weissella

o__Lactobacillales

Names

total

elements

Summer

129

g__Massilia

SummerWithoutFrancisella

SummerWithoutRickettsia

f__Carnobacteriaceae

f__Carnobacteriaceae__uncultured

g__Bacillus

g__Segetibacter

f__Neisseriaceae

g__Mannheimia

g__Patulibacter

g__Cnuella

f__Comamonadaceae

g__Aridibacter

g__Vulcaniibacterium

g__Cloacibacterium

g__Arthrobacter

g__Stenotrophomonas

g__Nocardioides

g__Pedobacter

g__Pseudonocardia

g__Bergeyella

g__Geodermatophilus

g__Bifidobacterium

f__Planococcaceae

g__Veillonella

g__Exiguobacterium

g__Hydrogenophilus

g__Moraxella

g__Mycobacterium

g__Thermomonas

g__Schlegelella

g__Neisseria

g__Aeromonas

f__Aeromonadaceae

f__Geodermatophilaceae

g__Cellulomonas

g__Candidatus_Saccharimonas

f__Pasteurellaceae

g__Micrococcus

g__Bauldia

g__Hymenobacter

f__Methanobacteriaceae

f__Kineosporiaceae

g__67-14

g__Methanobrevibacter

f__Ruminococcaceae

g__Mogibacterium

g__Virgibacillus

g__Brevibacterium

g__Marmoricola

g__Bibersteinia

g__Lysinibacillus

g__Coprococcus

g__Riemerella

f__Ruminococcaceae__uncultured

f__Eggerthellaceae__uncultured

g__Ferruginibacter

g__Rubrobacter

g__Adhaeribacter

g__Xanthomonas

g__Atopostipes

g__Rheinheimera

f__Lachnospiraceae__uncultured

g__Streptococcus

f__Aerococcaceae

g__Leuconostoc

f__Longimicrobiaceae

g__Phocaeicola

g__Escherichia-Shigella

g__Anaerococcus

g__Dietzia

g__Christensenellaceae_R-7_group

g__Cutibacterium

g__NK4A214_group

g__Oribacterium

g__Romboutsia

g__Campylobacter

g__Citricoccus

g__CAG-352

f__Acetobacteraceae

g__Belnapia

g__GCA-900066575

f__Neisseriaceae__uncultured

g__Brachybacterium

g__Fenollaria

g__Blastococcus

g__Actinocorallia

g__Alloprevotella

g__Quadrisphaera

g__Nocardiopsis

g__Rubellimicrobium

g__PMMR1

g__Abiotrophia

g__Sphingomonas

g__Actinomyces

g__Fusobacterium

g__Porphyromonas

g__Alysiella

g__Lautropia

g__F0332

g__Enhydrobacter

g__Lactobacillus

g__Subdoligranulum

g__Geobacillus

g__Sphingobacterium

g__Peptostreptococcus

g__Friedmanniella

g__Vogesella

g__Prevotella

g__Allorhizobium-Neorhizobium-
Pararhizobium-Rhizobium

f__Oscillospiraceae__uncultured

g__Acinetobacter

g__Gracilibacteria

g__Aerococcus

g__Lawsonella

g__Helcococcus

g__Rothia

g__Antarcticibacterium

g__Modestobacter

g__Dermacoccus

g__Trueperella

g__JG30-KF-CM45

g__AKIW781

g__Mesorhizobium

g__Simonsiella

g__Enterococcus

g__Pseudoalteromonas

g__p-251-o5

		f__Micrococcaceae
		g__Jatrophihabitans
		g__Gemella
Summer	20	o__Bacillales
SummerWithoutFrancisella		f__Blastocatellaceae__uncultured
		g__Methylobacterium-Methylorubrum
		g__Roseisolibacter
		o__Microtrichales__uncultured
		c__Clostridia
		g__Prauserella
		o__Frankiales__uncultured
		g__Lachnospiraceae_XPB1014_group
		g__Rickettsia
		g__Family_XIII_UCG-001
		g__RF39
		o__Frankiales
		g__WD2101_soil_group

		c__Alphaproteobacteria__uncultured
		g__Vicinamibacteraceae
		g__Longimicrobium
		g__Family_XIII_AD3011_group
		g__Clostridia_UCG-014
		g__Aureimonas
Summer	10	g__Taibaiella
SummerWithoutRickettsia		g__Francisella
		f__Isosphaeraceae
		g__RB41
		g__Devosia
		g__Paenibacillus
		g__Craurococcus-Caldovatus
		g__Truepera
		g__Clostridium_sensu_stricto_1
		f__Rhodobacteraceae
	11	g__Amaricoccus

SummerWithoutFrancisella SummerWithoutRickettsia			f__Thermomonosporaceae
			g__Turicibacter
			g__Noviherbaspirillum
			g__Leptotrichia
			g__Roseomonas
			f__Blastocatellaceae
			g__[Anaerorhabdus]_furcosa_group
			g__Hydrogenophaga
			g__Tepidiphilus
Summer	10		g__Rhodococcus
			g__Lachnospiraceae_NK3A20_group
			f__Aerococcaceae__uncultured
			g__Skermanella
			g__Actinomycespora
			f__Verrucomicrobiaceae__uncultured
			f__Erysipelotrichaceae__uncultured
			g__Candidatus_Alysiosphaera

		f__Bacillaceae
		g__Dyadobacter
		f__Sporichthyaceae__uncultured
SummerWithoutFrancisella	13	g__0319-7L14
		f__Beijerinckiaceae__uncultured
		f__Enterobacteriaceae
		g__Microvirga
		g__Marvinbryantia
		f__Gemmatimonadaceae
		g__Flavobacterium
		f__Micromonosporaceae
		g__Akkermansia
		g__TM7x
		g__Domibacillus
		g__Anaerofustis
		f__Microbacteriaceae
SummerWithoutRickettsia	22	f__Frankiales

f__WD2101_soil_group

f__Lachnospiraceae

g__Alphaproteobacteria__uncultured

f__Myxococcaceae

f__Microtrichales__uncultured

g__Candidatus_Nitrocosmicus

f__Bacillales

f__Saccharimonadales

f__Lachnospiraceae_XPB1014_group

f__Frankiales__uncultured

f__RF39

f__Clostridia_UCG-014

f__Family_XIII_UCG-001

g__Tychonema_CCAP_1459-11B

f__Lachnospiraceae_NK3A20_group

g__Conexibacter

g__Anoxybacillus

f__Family_XIII_AD3011_group

f__Clostridia

f__Vicinamibacteraceae

APPENDIX 9

Supplementary Table 8. Chemical composition of *Artemisia herba alba* essential oil.

Ret. Time	Peak Area	Compound Name	CAS
6,774	597405	α -Pinene	80-56-8
6,86	1101758,5	α -Pinene	80-56-8
8,27	19879054	Camphene	79-92-5
8,38	33372084	Camphene	79-92-5
8,745	5064251,5	2(5H)-Furanone, 5,5-dimethyl-	20019-64-1
8,83	11446444	2(5H)-Furanone, 5,5-dimethyl-	20019-64-1
9,691	6179713	β -Myrcene	123-35-3
10,031	3954857,25	o-Cymene	527-84-4
11,196	11269119	Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-	1461-27-4
11,591	226062528	Eucalyptol	470-82-6
12,112	8259069	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	1073-11-6
13,647	7854627	1,5-Heptadien-4-ol, 3,3,6-trimethyl-	27644-04-8
14,743	28344652	Bicyclo[3.1.0]hexan-3-one, 4-methyl-1-(1-methylethyl)-	1125-12-8
15,173	18688726	Thujone	546-80-5
15,643	634421632	Bicyclo[3.1.1]hept-2-en-6-one, 2,7,7-trimethyl-	473-06-3
16,083	14845964	Cyclohexane, 2-ethenyl-1,1-dimethyl-3-methylene-	95452-08-7
16,253	27753082	Isopinocarveol	6712-79-4
16,568	589438144	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	464-48-2
16,868	25041462	cis-p-mentha-1(7),8-dien-2-ol	
17,329	16494379	Pinocarvone	30460-92-5
17,449	64017696	endo-Borneol	507-70-0
17,899	36123544	Terpinen-4-ol	562-74-3
18,264	2255646	Tricyclo[4.3.0.0(3,8)]nonan-2-ol, 2-(aminomethyl)-, stereoisomer	
18,544	16288940	α -Terpineol	98-55-5
19,344	21757628	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, cis-	16721-38-3
19,84	30395826	Ethanol, 2-(3,3-dimethylbicyclo[2.2.1]hept-2-ylidene)-	2226-05-3
23,081	7372832,5	Thymol	89-83-8
25,612	59949252	3-Cyclohexene-1-methanol, $\alpha,\alpha,4$ -trimethyl-, acetate	80-26-2
27,728	39856092	3,5-Heptadienal, 2-ethylidene-6-methyl-	99172-18-6
28,138	31792098	3-Methyl-2-pent-2-enyl-cyclopent-2-enone	
35,621	32375940	(-)-Spathulenol	77171-55-2
36,086	1940056192	5-Hepten-3-one, 2-(5-ethenyltetrahydro-5-methyl-2-furanyl)-6-methyl-, [2S-[2 α (R*),5 α]]-	20482-11-5

Supplementary Table 9. Chemical composition of *Thymus vulgaris* essential oil.

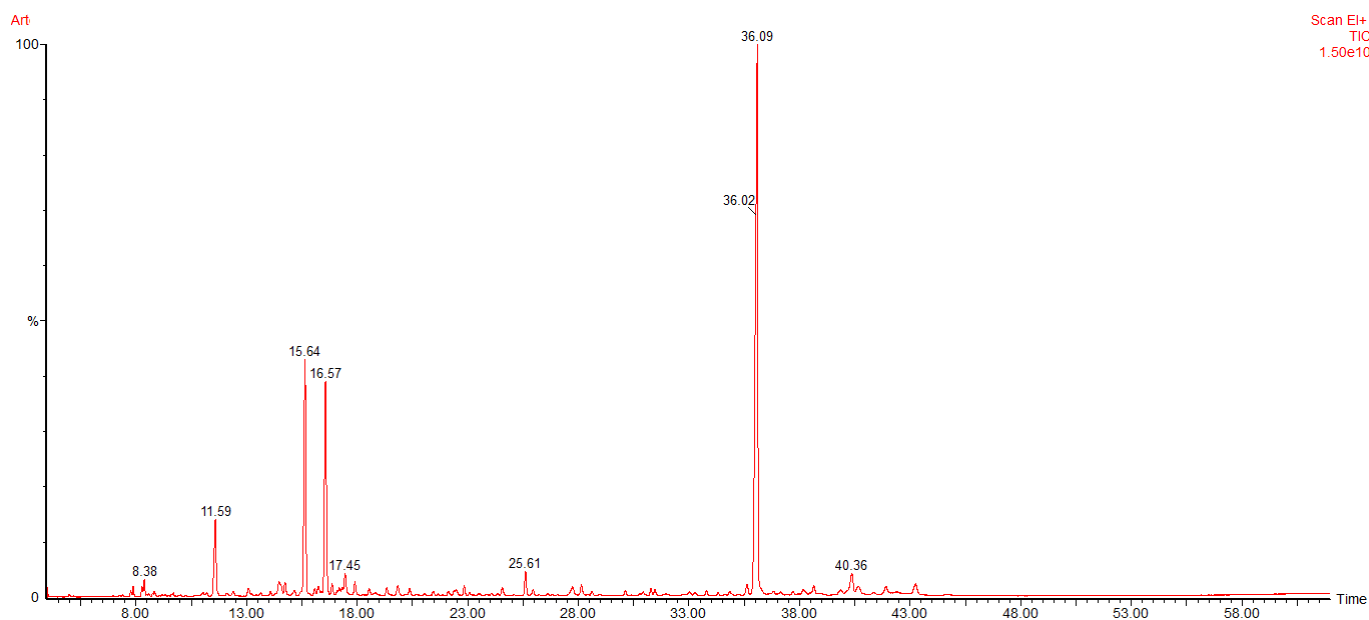
Ret. Time	Peak Area	Compound Name	CAS
7,65	11164848	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	28634-89-1
9,05	19424062	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	99-86-5
9,451	76065136	o-Cymene	527-84-4
9,706	19324154	β -Pinene	127-91-3
10,741	22251710	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	586-62-9
11,116	337069280	γ -Terpinene	99-85-4
11,631	11495556	Eucalyptol	470-82-6
12,417	252710592	γ -Terpinene	99-85-4
13,342	66370,25	1,6-Octadien-3-ol, 3,7-dimethyl-	78-70-6
13,437	35718600	1,6-Octadien-3-ol, 3,7-dimethyl-	78-70-6
15,688	95944,492	Camphor	76-22-2
16,583	15056538	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	464-48-2
16,853	9641252	1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol	10385-78-1
17,469	9444831	1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol	10385-78-1
23,361	1221142400	Phenol, 2-methyl-5-(1-methylethyl)-	499-75-2
23,536	1126824064	Phenol, 2-methyl-5-(1-methylethyl)-	499-75-2
35,316	7182558	(-)-Spathulenol	77171-55-2

Supplementary Table 10. Chemical composition of *Rosmarinus officinalis* essential oil.

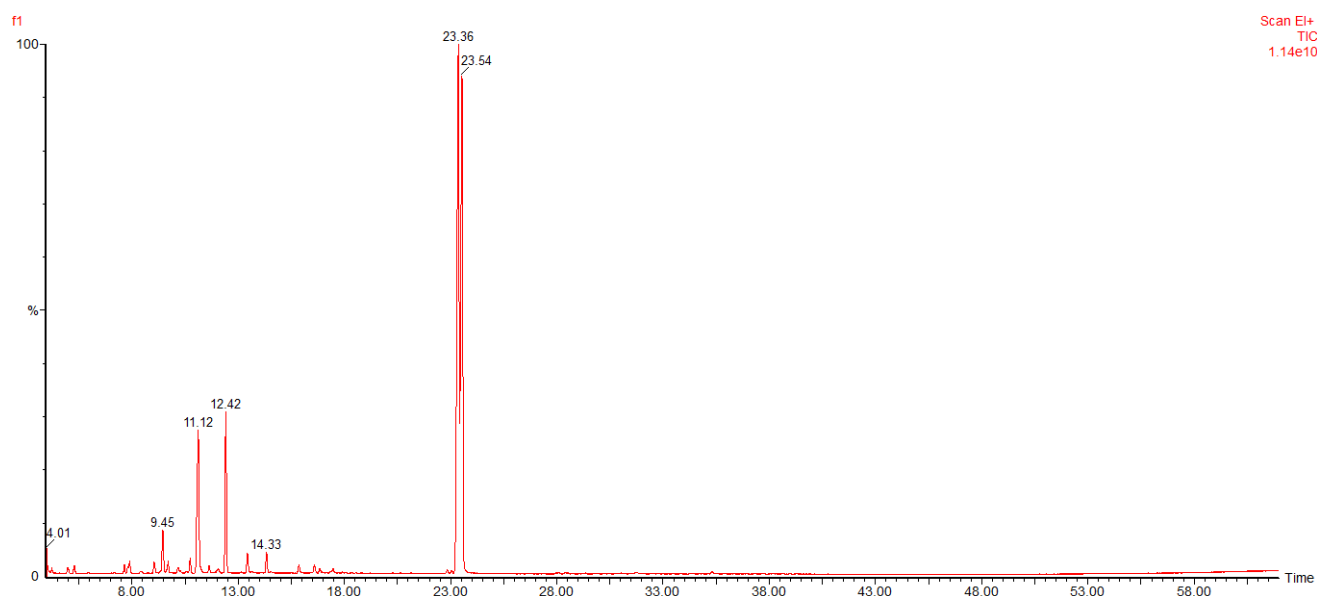
Ret. Time	Peak Area	Compound Name	CAS
7,27	1792320384	α -Pinene	80-56-8
7,835	2352991744	Camphene	79-92-5
8,35	306076224	Camphene	79-92-5
8,835	353389536	β -Pinene	127-91-3
9,29	108324584	β -Pinene	127-91-3
9,671	6638248	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	18172-67-3
9,856	32885934	α -Phellandrene	99-83-2
10,346	53435444	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	586-62-9

10,851	837816064	Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-	1461-27-4
11,261	2372425984	Eucalyptol	470-82-6
11,571	378010944	Eucalyptol	470-82-6
12,122	65768536	γ -Terpinene	99-85-4
12,387	9932264	γ -Terpinene	99-85-4
12,877	9750961	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1 α ,2 β ,5 α)-	15537-55-0
13,042	51326,941	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1 α ,2 β ,5 α)-	15537-55-0
13,427	37991840	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	586-62-9
13,662	6946979	4-Terpinenyl acetate	4821-04-9
14,157	15890431	1,6-Octadien-3-ol, 3,7-dimethyl-	78-70-6
14,328	11266261	exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	
14,868	21144510	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, (1R-endo)-	2217-02-9
15,073	1780572,25	Fenchol, exo-	22627-95-8
15,308	16199397	exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	
16,608	8764706816	Camphor	76-22-2
17,374	869458304	endo-Borneol	507-70-0
17,789	373520800	Terpinen-4-ol	562-74-3
18,154	18916832	Benzenemethanol, α,α ,4-trimethyl-	1197-01-9
18,444	372991488	α -Terpineol	98-55-5
18,764	9368504	(-)-Myrtenol	19894-97-4
19,229	3869339	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	29803-82-5
19,745	26541450	D-Verbenone	18309-32-5
21,56	5682243,5	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	89-81-6
22,746	122670960	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	92618-89-8
23,001	3490868	Cyclohexene, 2-ethenyl-1,3,3-trimethyl-	5293-90-3
23,181	5396025,5	Thymol	89-83-8
23,431	20808756	Phenol, 2-methyl-5-(1-methylethyl)-	499-75-2
23,906	9031220	6-Methyl-cyclodec-5-enol	
24,987	9673266	trans-p-Mentha-2,8-dienol	
25,867	7196165,5	Phenol, 2-methoxy-3-(2-propenyl)-	1941-12-4
26,142	6646721	α -ylangene	
26,532	28142894	alfa.-Copaene	
27,288	1676359,13	Geranyl isovalerate	109-20-6
27,918	11320493	Methyleugenol	93-15-2

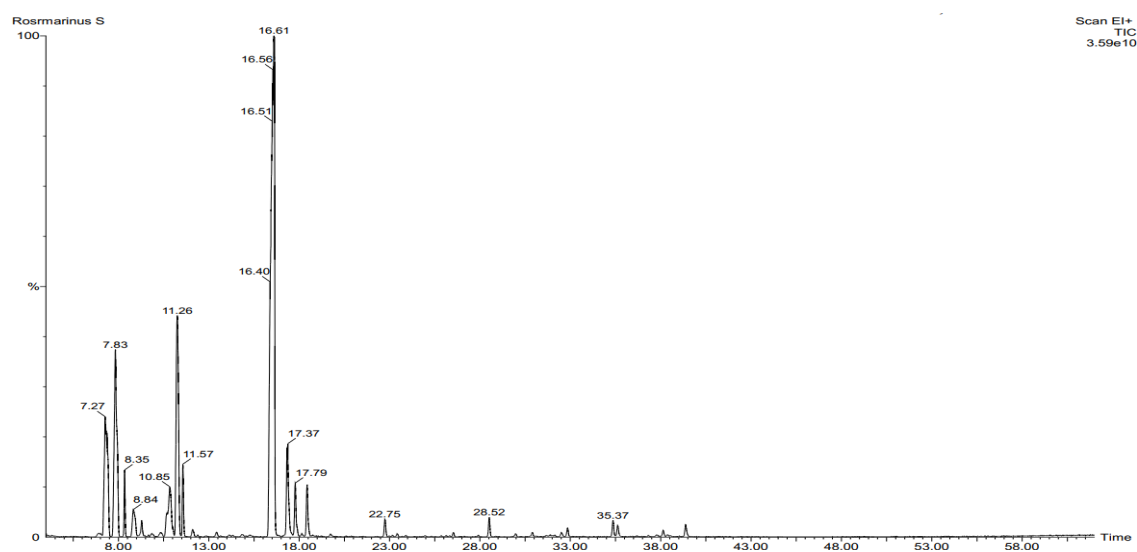
28,523	131788688	Caryophyllene	87-44-5
28,893	4678786,5	β -copaene	
29,984	25972328	Humulene	6753-98-6
30,609	1430640,75	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	719-22-2
30,904	35315244	γ -Muurolene	30021-74-0
31,904	11760922	α -Muurolene	31983-22-9
32,165	8734541	β -Bisabolene	495-61-4
32,51	35318380	γ -Muurolene	30021-74-0
32,855	63176020	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	483-76-1
33,735	4177015,5	9-Methoxycalamenene	
35,371	111612088	(-)-Spathulenol	77171-55-2
35,631	82860232	5-Hepten-3-one, 2-(5-ethenyltetrahydro-5-methyl-2-furanyl)-6-methyl-, [2S-[2 α (R*),5 α]]-	20482-11-5
37,332	8260035	cubedol	
38,142	45951828	Cyclopentaneacetic acid, 3-oxo-2-(2-pentenyl)-, methyl ester, [1 α ,2 α (Z)]-	42536-97-0
39,387	104688488	α -Bisabolol	515-69-5



Supplementary Figure 1. GC-MS chromatogram of *Artemisia herba alba* oil.



Supplementary Figure 2. GC-MS chromatogram of *Thymus vulgaris* oil.



Supplementary Figure 2. GC-MS chromatogram of *Rosmarinus officinalis* oil.

APPENDIX 10

Published Articles:

- Abdelali, S., Souttou, K., Kacimi-Elhassani, M., and Aissaoui, L. (2024). Assessment of the acaricidal efficacy of *Rosmarinus officinalis* essential oil against dog ticks, *Rhipicephalus sanguineus* (Acari: Ixodidae), and its chemical composition. *Revista de Ciências Agroveterinárias*, 23(3), 544-553. <https://doi.org/10.5965/223811712332024544>
- Abdelali, S. K., Aissaoui, L., Maitre, A., Piloto-Sardiñas, E., Julie, C., Foucault-Simonin, A., Moutailler, S., Galon, C., Mateos-Hernández, L., Obregon, D., Zając, Z., and Cabezas-Cruz, A. (2024). Guild Dynamics and Pathogen Interactions in *Hyalomma* Ticks From Algerian Cattle. *Transboundary and Emerging Diseases*, 2024(1), Article ID 5384559, 17 pages. <https://doi.org/10.1155/tbed/5384559>

Scientific Communication

International Seminars:

- El Oued (VBEH 2023)
Abdelali, S. K., Aissaoui, L., and Souttou, K. (May 10-11, 2023). *Chemical composition and acaricidal efficacy of Algerian Thymus vulgaris essential oil against dog ticks, Rhipicephalus sanguineus*. Poster presented at the 1st International Seminar on Valorization of Bioresources in Environment and Health (VBEH 2023), University of Echahid Hamma Lakhdar, El Oued, Algeria.
- Paris (Doctoral Day)
Abdelali, S. K. (September 16, 2024). *Unveiling pathogen interactions in Hyalomma ticks: Insights from central Algerian steppe regions*. Presentation at the Doctoral Day, École vétérinaire d'Alfort, Maisons-Alfort, Paris.

National Seminars:

- Souk Ahras (SNEE 2021)
Abdelali, S. K., Aissaoui, L., Belghouar, S., and Lamari, S. (November 10-11, 2021). *Veterinary-relevant ticks in the High Plateaus (Sétif)*. Presentation at the National Seminar on Ecophysiology and Environment (SNEE 2021), University of Med Chérif Messaadia, Souk Ahras, Algeria.

Assessment of the acaricidal efficacy of *Rosmarinus officinalis* essential oil against dogs' ticks, *Rhipicephalus sanguineus* (Acari: Ixodidae), and its chemical composition

Avaliação da eficácia acaricida do óleo essencial de *Rosmarinus officinalis* contra carrapatos de cães, *Rhipicephalus sanguineus* (Acari: Ixodidae), e sua composição química

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ABSTRACT

Ticks play the main role, in veterinary terms, in transmitting important pathogens. *Rhipicephalus sanguineus* is a widespread tick known for its ability to thrive in indoor domestic environments and could be the main reservoir host for many TBDs, which infest dogs living in urban areas. In this study, the acaricidal and larvicidal potential of *Rosmarinus officinalis* essential oil was evaluated against *R. sanguineus*. The aerial part of this plant was extracted by hydrodistillation and then analyzed by gas chromatography coupled with mass spectrometry (GC/MS). The yield obtained from this oil was 0.38%, its major chemical compounds were found to be Camphor (43.52%), Eucalyptol (13.66%), and Camphene (13.2%). The adult immersion test (AIT) using four concentrations (1µl/ml, 2µl/ml, 10µl/ml, 30µl/ml) revealed that this oil presented oviposition reduction percentages of 5.75%, 20.68%, 33.27%, and 46.84%, hatching reductions percentages of 5%, 15%, 35%, and 60%, and efficacy extract percentages of 10.46%, 32.58%, 56.63%, and 78.74%, respectively. Further, the larval immersion test (LIT) using five concentrations (0.5µl/ml, 1µl/ml, 2µl/ml, 3µl/ml, and 5µl/ml) revealed considerable larvicidal activities with LC50 and LC90 values of 2.286 µl/ml and 5.380 µl/ml, respectively. These results are encouraging and open interesting and promising horizons for its application as a bio-acaricide.

KEYWORDS: ticks; acaricide; essential oil; *Rosmarinus officinalis*; toxicological parameters.

RESUMO

Os carrapatos desempenham o papel principal, em termos veterinários, na transmissão de patógenos importantes. O *Rhipicephalus sanguineus* é um carrapato muito difundido, conhecido por sua capacidade de se desenvolver em ambientes domésticos e pode ser o principal hospedeiro reservatório de muitas DTAs que infestam cães que vivem em áreas urbanas. Neste estudo, o potencial acaricida e larvicida do óleo essencial de *Rosmarinus officinalis* foi avaliado contra o *R. sanguineus*. A parte aérea dessa planta foi extraída por hidrodestilação e depois analisada por cromatografia gasosa acoplada à espectrometria de massa (GC/MS). O rendimento obtido desse óleo foi de 0,38%, e seus principais compostos químicos foram a cânfora (43,52%), o eucaliptol (13,66%) e o canfeno (13,2%). O teste de imersão de adultos (AIT) usando quatro concentrações (1µl/ml, 2µl/ml, 10µl/ml, 30µl/ml) revelou que esse óleo apresentou porcentagens de redução de oviposição de 5,75%, 20,68%, 33,27% e 46,84%, porcentagens de redução de eclosão de 5%, 15%, 35% e 60% e porcentagens de eficácia do extrato de 10.46%, 32,58%, 56,63% e 78,74%, respectivamente. Além disso, o teste de imersão de larvas (LIT)

usando cinco concentrações (0,5 µl/ml, 1 µl/ml, 2 µl/ml, 3 µl/ml e 5 µl/ml) revelou atividades larvicidas consideráveis com valores de LC50 e LC90 de 2,286 µl/ml e 5,380 µl/ml, respectivamente. Esses resultados são encorajadores e abrem horizontes interessantes e promissores para sua aplicação como bioacaricida.

PALAVRAS-CHAVE: carrapatos; acaricida; óleo essencial; *Rosmarinus officinalis*; parâmetros toxicológicos.

INTRODUCTION

Ticks are obligate hematophagous ectoparasitic arthropods that depend entirely on one or more hosts to complete their life cycle; they are the most widespread arthropod, with more than 900 species worldwide (MANS & NEITZ 2004). Hard ticks (Ixodidae) are the dominant family of ticks, considering the number of species and their veterinary and medical importance (TSATSARIS et al. 2016).

The danger of these arachnids lies in their ability to transmit important pathogens (protozoa, bacteria and viruses) during bites (SONENSHINE et al. 2002). This transmission occurs through 3 routes: transstadial (from one life stage to another through molting), horizontal (through a host and during co-feeding) and transovarial transmission (from an infected female to her progeny). The latter (TOT) is the most important in maintaining the existence of a variety of pathogens (including *Rickettsia* spp. and *Babesia* spp. and many viruses). What makes ticks a reservoir of harmful vector-borne diseases (AZAD & BEARD 1998, BALASHOV 1999, BONNET et al. 2007, DANIELOVÁ et al. 2002).

The brown tick, *Rhipicephalus sanguineus* (LATREILLE 1806 – ROMA et al. 2013), is mainly an ectoparasite of dogs but is frequently associated with other animals, including humans, as hosts (SCHUSTER et al. 2009, KABIR et al. 2011, MENTZ et al. 2016). *R. sanguineus* is involved in the transmission of different etiological agents, such as *Babesia canis*, *Ehrlichia canis*, and *Rickettsia conorii*, which are the etiological agents of canine babesiosis, canine monocytic ehrlichiosis, and Mediterranean spotted fever, respectively (BRUMPT 1932, GROVES et al. 1975, REGENDANZ & MUNIZ 1936).

Unlike other species of exophilic ticks that live in open environments, pastures, or forests (PAROLA & RAOULT 2001), *Rhipicephalus sanguineus* is endophilic, known for its ability to thrive in indoor environments; the engorged female separated from the domestic dog can lay eggs in the residence (USPENSKY & IOFFE-USPENSKY 2002). Due to the high reproductive rate of ticks, their population can increase rapidly in a short period, resulting in severe residential infestation (KOCH 1982).

For a long time, the control of these arthropods was based on the use of synthetic acaricides, which offer relatively rapid and effective control of tick populations. The use of these chemical pesticides often results in many more problems than can be solved (SAVADOGO et al. 2016). The intensive and continuous application of ticks on the host and its surroundings creates toxicity problems for animals and humans, leading to environmental pollution and the development of tick resistance (DANDE 2015).

To reduce this dilemma, it becomes necessary to focus on natural plant compounds (ABDELALI et al. 2023) as essential oils that have been widely used in various fields (AISSAOUI et al. 2022). Furthermore, research on acaricidal plants in veterinary parasitology is a recent field of research worldwide; however, in Algeria, little work has been done in this context (ALIMI et al. 2022, DJEBIR et al. 2019).

Algeria is known for its richness in medicinal plants, considering its surface area and bioclimatic diversity (GHOMARI et al. 2014), among them *Rosmarinus officinalis* (Ikliil in Arabic), which is a species of flowering plant of the Lamiaceae family, exists in the Mediterranean region and grows wild in Algeria, France, Italy, Portugal, Morocco, and Spain, while it is cultivated in several countries such as the United States (VERMA et al. 2012). It is commonly used as a condiment and food preservative consisting of bioactive molecules and phytochemicals that are responsible for several pharmacological activities, such as anti-inflammatory activities (OLIVEIRA et al. 2019).

The aim of this study was to determine the chemical composition of the essential oil of the local plant *Rosmarinus officinalis* and evaluate the effects of different concentrations on larval mortality and reproductive aspects of females of *R. sanguineus*, initiating a biological control using an environmentally friendly and less harmful natural substance.

MATERIAL AND METHODS

Extraction of plant and essential oil

The aerial parts of *Rosmarinus officinalis* (Figure 1) were collected in May in the Djebel Hawas region (34° 41' N, 3° 09'02" E) in Djelfa, determined by comparison with a sample from the herbarium of the Missouri Botanical Garden, voucher number (3844178). The identification was confirmed by Mr. A. Brague, Chief Forest Inspector of the National Forestry Research Institute of Djelfa Province. The plant leaves were initially rinsed with distilled water and dried in the shade at room temperature. Next, 50 g of plant powder was hydrodistilled for 3 h using a Clevenger-type apparatus (CLEVENGER 1928) according to the recommendations of the Hellenic Pharmacopoeia (HELLENIC PHARMACOPOEIA 2002). The essential oil was dissolved in diethyl ether, dried over anhydrous magnesium sulfate MgSO_4 and stored in hermetically sealed sterile glass bottles, protected from light, at a temperature of 4 °C, until gas chromatographic analysis and toxicological study.

The essential oil yield was estimated using the formula given by FALLEH et al. (2008):

$$R (\%) = (\text{Mext} / \text{M'ech.}) \cdot 100.$$

Here, R is the yield in %. Mext is the mass of the extract (in g) after evaporation of the solvent. M'ech is the dry mass of the plant sample (in g).



Figure 1. *Rosmarinus officinalis*.

Chemical analysis

The chemical composition of the essential oil was analyzed by gas chromatography coupled to mass spectrometry (GC/MS), which allowed the qualitative and quantitative determination of most of the compounds in the sample (2-5 µl). The essential oil was transferred to a gas chromatograph vial, diluted in

hexane (1-2 ml), and sealed with a high-performance septum (DELAZAR et al. 2004). Constituents were identified by comparing their mass spectra with those stored in the NIST/EPA/NIH mass spectral database (version 2.0 as of May 19, 2011).

Rhipicephalus sanguineus

Engorged females of *R. sanguineus* were collected from naturally infested domestic dogs shortly after they began to abandon the host to ensure uniformity. These hosts did not receive any acaricide treatment for at least 45 days to avoid any negative interference in many farms in the municipality of Ain Maabed (34° 48' 17" N, 3° 07' 46" E), Djelfa, Algeria.

Ticks were stored in cooled plastic boxes ($\approx 15^{\circ}\text{C}$) to reduce their activity and immediately transported to the laboratory, where they were carefully washed with distilled water and dried on paper towels. Species were identified using binocular magnifying glass according to the keys and descriptions provided by WALKER et al. (2003).

Preparation of the toxicological test

This test was performed in two stages: engorged females and larvae, using an immersion test (AIT/LIT). *Rosmarinus officinalis* essential oil was dissolved and serially diluted in 1 ml of ethanol. Preliminary tests with different doses were performed to select a range of concentrations before starting the toxicity test. Four concentrations (1 $\mu\text{l/ml}$, 2 $\mu\text{l/ml}$, 10 $\mu\text{l/ml}$, 30 $\mu\text{l/ml}$) were chosen for AIT, and five concentrations (0.5 $\mu\text{l/ml}$, 1 $\mu\text{l/ml}$, 2 $\mu\text{l/ml}$, 3 $\mu\text{l/ml}$, 5 $\mu\text{l/ml}$) for LIT. For each concentration, three replicates were maintained, as in the control.

Adult immersion test

AIT was performed as described in the literature (DRUMMONDS et al. 1973, FAO 2004) with minor modifications. In groups of fifteen engorged female ticks, each was weighed individually to obtain groups with similar weights (0.5 ± 0.1 g). The different groups of ticks were immersed in 10 ml of each concentration for 5 min. All tests were replicated three times. After exposure, engorged females were removed, dried, and placed in Petri dishes, which were incubated for 15 days at $27 \pm 2^{\circ}\text{C}$ and 80% relative humidity. Tick death was confirmed based on signs of hemorrhagic skin lesions, cuticular darkening, and absence of Eustachian tube movement. After 2 weeks, the eggs were weighed, transferred to tubes, and placed in an incubator under the same hatching conditions as the larvae.

The egg production index (EPI), hatching reduction (RE), oviposition reduction (RO), reproductive efficiency index (REI), and extract efficiency (EP) were calculated using the following formulas:

$\text{EPI (\%)} = (\text{weight of eggs/weight of engorged female}) \times 100$ (BENNETT 1974)

$\text{RO (\%)} = [(\text{EPI control group} - \text{EPI experimental group}) / \text{EPI control group}] \times 100$ (ROULSTON et al. 1968)

$\text{HR (\%)} = [(\text{hatching rate in control group} - \text{hatching rate in experimental group}) / \text{hatching rate in control group}] \times 100$ (GONZALES 2003)

$\text{REI} = (\text{egg mass weight} \times \% \text{ egg hatch/weight of engorged females}) \times 20,000$ (DRUMMONDS et al. 1973)

$\text{EP (\%)} = [(\text{REI control} - \text{REI treated}) / \text{REI control}] \times 100$ (DRUMMONDS et al. 1973)

Larval immersion test (LIT)

LIT is not recommended or standardized by FAO. Therefore, the following protocol was modified from a previous test described by (RIBEIRO et al. 2011). The larvae used in this test (LIT) were from eggs provided by untreated engorged females; larval treatments were performed on the 15th day after total larval hatching.

A number of 100 larvae were immersed for 5 min in tubes containing 10 mL of different concentrations of *Rosmarinus officinalis* essential oil. The tubes were closed, shaken vigorously for a few seconds, and then gently shaken for 5 min.

The larvae were then transferred with a brush to dry on a paper towel. They were then placed on filter paper (8.5×7.5 cm) (Whatman No. 1), which was folded and closed with clips to form a packet. The packages were incubated at 27°C – 28°C and $\geq 80\%$ relative humidity.

Live and dead larvae were counted after 24 h, 48 h, and 72 h of exposure (three packages per treatment) for subsequent calculation of LC50, LC90, LT50, and LT90 for each group.

Statistical analysis

The mortality values obtained at the different concentrations were considered averages. These results were subjected to probit analysis to calculate lethal concentrations and lethal times (LC50% LC90%, LT50% and LT90%). This analysis was performed using IBM SPSS Statistics23 software on Windows.

RESULTS

Yield and chemical composition of essential oils from *Rosmarinus officinalis*

The oil yield of *Rosmarinus officinalis* was 1.49%. The chemical composition by GC-MS (Table 1) revealed 50 compounds with a total percentage of 100%. Five main components were identified:

Camphor (43.52%), Eucalyptol (13.66%), Camphene (13.2%), α -Pinene (8.9%), endo-Borneol (4.32%), Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)- (4.16%), β -Pinene (2.29%), and the other proportions ranging from 1.85% to 0.01% (Table 1, Figure 2).

Acaricidal effects of *Rosmarinus officinalis* essential oil:

All tested concentrations of *Rosmarinus officinalis* essential oil showed considerable efficiency from 10.76% to 78.74%, which resulted in a significant reduction in the egg mass of engorged females from 33.82% to 16.41% with a significant reduction in the reproductive efficiency index compared to the control group.

As a result, the egg production of *R. sanguineus* was reduced by a ratio of 5.75% to 46.84% from the minimum to maximum concentration.

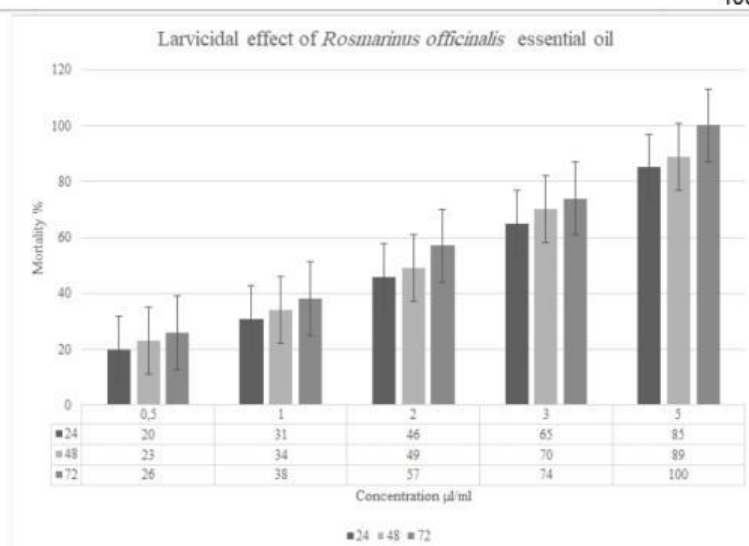
In addition, a high proportion of egg hatching inhibition was obtained using this essential oil at a maximum concentration of 60% egg hatching; however, the newly hatched larvae did not survive and died within a few hours after hatching (Table 2).

Table 1. Abundance (%) of *Rosmarinus officinalis* essential oil components determined using gas chromatography-electron impact mass spectrometry (GC-MS).

No.	RT	Compound name	Abundance %
1	7.27	α -Pineno	8.90
2	8.35	Camphene	13.20
3	9.29	β -Pinene	2.29
4	9.671	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene, (1S)-	0.03
5	9.856	α -Phellandrene	0.16
6	10.85	Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-	4.16
7	11.57	Eucalyptol	13.66
8	12.38	γ -Terpinene	0.38
9	13.04	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1 α ,2 β ,5 α)-	0.05
10	13.42	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.46
11	13.66	4-Terpinenyl acetate	0.03
12	14.15	1,6-Octadien-3-ol, 3,7-dimethyl-	0.08
13	14.32	exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	0.06
14	14.86	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, (1R-endo)-	0.10
15	15.07	Fenchol, exo-	0.01

16	15.30 8	exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	0.08
17	16.60 8	Camphor	43.52
18	17.37 4	endo-Borneol	4.32
19	17.78 9	Terpinen-4-ol	1.85
20	18.15 4	Benzenometanol, $\alpha,\alpha,4$ -trimetil-	0.09
21	18.44 4	α -Terpineol	1.85
22	18.76 4	(-)-Myrtenol	0.05
23	19.22 9	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-	0.02
24	19.74 5	D-Verbenone	0.13
25	21.56	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	0.03
26	22.74 6	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	0.61
27	23.00 1	Cyclohexene, 2-ethenyl-1,3,3-trimethyl-	0.02
28	23.18 1	Thymol	0.03
29	23.43 1	Phenol, 2-methyl-5-(1-methylethyl)-	0.10
30	23.90 6	6-Methyl-cyclodec-5-enol	0.04
31	24.98 7	trans-p-Mentha-2,8-dienol	0.05
32	25.86 7	Phenol, 2-methoxy-3-(2-propenyl)-	0.04
33	26.14 2	α -ylangene	0.03
34	26.53 2	α -Copaene	0.14
35	27.28 8	Geranyl isovalerate	0.01
36	27.91 8	Methyleugenol	0.06
37	28.52 3	Caryophyllene	0.65
38	28.89 3	β -copaene	0.02
39	29.98 4	Humulene	0.13
40	30.60 9	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	0.01
41	31.90 4	α -Muurokene	0.06
42	32.16 5	β -Bisabolene	0.04
43	32.51	γ -Muurokene	0.36

44	32.85	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	0.31
45	33.73	9-Methoxycalamenene	0.02
46	35.37	(-)-Spathulenol	0.55
47	35.63	5-Hepten-3-one, 2-(5-ethenyltetrahydro-5-methyl-2-furanyl)-6-methyl, [2S-[2 α (R*),5 α]]-	0.41
48	37.33	Cubedol	0.04
49	38.14	Cyclopentaneacetic acid, 3-oxo-2-(2-pentenyl)-, methyl ester, [1 α ,2 α (Z)]-	0.23
50	39.38	α -Bisabolol	0.52
Total			100

Figure 2. Chromatographic profile of *Rosmarinus officinalis* essential oil analyzed by GC-SM.Table 2. Effects of *Rosmarinus officinalis* essential oil on the reproductive characteristics of *R. sanguineus* females.

Concentration $\mu\text{l/ml}$	EPI (%)	RO (%)	KING	EP (%)	Hatching (%)	HR (%)
1	33.82	5.75	642579.37	10.46	95	5
2	25.15	20.68	427529.76	32.58	85	15
10	20.44	33.27	265659.4	56.63	65	35
30	16.41	46.84	131256.16	78.74	40	60
Control	32.27 $\pm$ 1.23	0	645394.34 $\pm$ 24527.54	0	100 $\pm$ 0	0

On the other hand, the essential oil of *Rosmarinus officinalis* showed a larvicidal effect against *R. sanguineus* larvae, with a mortality rate that varied between 20% after 24 h for the lowest concentration (0.5 $\mu\text{l/ml}$) and up to 100% after 72 h when the larvae were exposed to the highest concentration (5 $\mu\text{l/ml}$) (Figure 3). This efficiency increased as the exposure time and oil concentration increased; furthermore, the

correlation coefficients R recorded in Table 3 confirm this strong positive correlation between the recorded mortality rates and the exposure time and/or the essential oil concentration.

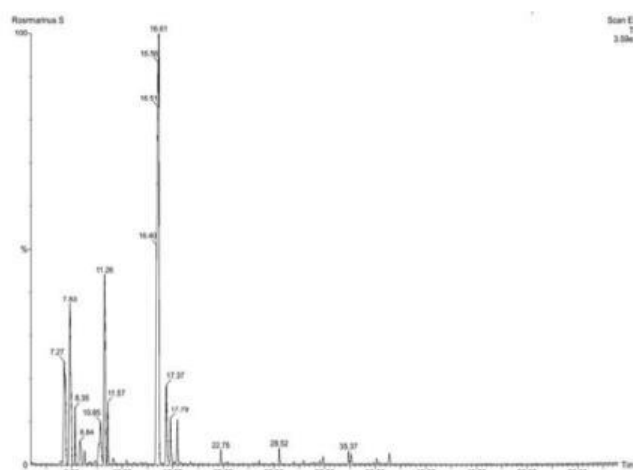


Figure 3. Evolution of the mortality rate of *R. sanguineus* larvae treated with different concentrations of *Rosmarinus officinalis* essential oil

Table 3. Toxicological parameters of *Rosmarinus officinalis* essential oil in *R. sanguineus* larvae.

A					
Time (hours)	24	48	72		
Regression line	E = -0.95+0.41x	E = -0.88+0.43x	E = -0.85+0.51x		
CL = 50% (µl/ml)	2.286	2.021	1.635		
LC 90% (µl/ml)	5.380	4.935	3.783		
95% Confidence Interval	[0.334 0.495]	[0.356 0.524]	[0.487 0.706]		
Chi-square value	1.416	1.109	4.45		
P-value	0.702	0.775	0.217		
R	0.988	0.991	0.995		
B					
Concentration (µl/ml)	0.5	1	2	3	5
Regression line	Y=-0.94+413E-3x	Y=-0.59+3.97E-3x	Y=-0.26+5.77E-3x	Y=0.26+5.38E-3x	Y=0.85+7.92E-3x
LT50% (hours)	227.572	149.898	45.076	*	*
LT90% (hours)	538.028	472.656	267.411	189.917	38.561
95% Confidence Interval	[-0.004 0.012]	[-0.003 0.011]	[-0.001 0.015]	[-0.001 0.013]	[-0.002 0.013]
Chi-square value	0	0.006	0.005	0.168	0.004
P-value	0.983	0.941	0.946	0.682	0.95
R	1	0.995	0.935	0.998	1

*Calculation was not performed.

After 24 h, a concentration of 2,286 $\mu\text{l/ml}$ guarantees 50% mortality of the larval stage; furthermore, to guarantee 90% mortality, the concentration of *R. officinalis* must be equal to 5,380 $\mu\text{l/ml}$. After 48 and 72 h of treatment, the LC50% is 2.021 $\mu\text{l/ml}$ and 1.635 $\mu\text{l/ml}$, respectively, whereas the LC90% is 4.935 $\mu\text{l/ml}$ and 3.783 $\mu\text{l/ml}$ (Table 3).

The concentrations of 0.5 $\mu\text{l/ml}$, 1 $\mu\text{l/ml}$ and 2 $\mu\text{l/ml}$ *Rosmarinus officinalis* eliminated 50% of the *R. sanguineus* population at 9.48, 6.25, and 1.88 days. Furthermore, when the five concentrations of *R. officinalis* were applied, the LT90% was 22.42 days, 19.69 days, 11.14 days, 7.91 days, and 1.6 (Table 3).

DISCUSSION

Yield and chemical characterization of *Rosmarinus officinalis* essential oil

The yield of essential oil from *R. officinalis* was 1.49%, higher than many other works carried out, noting that the yield of oil collected in Kenya was 0.59% (MWITHIGA et al. 2022), in Portugal it was 0.3–0.7% (SERRANO et al. 2002), and in Türkiye it was 0.71–0.94% (GURBUZ et al. 2016). However, it is lower compared to those collected in Algeria, where the essential oil yield in Tbessa was 1.85–2.29% (BOUTABIA et al. 2016). The chemical composition of this oil differs from those obtained by BAKKALI et al. (2018) in Morocco, where 17 compounds represented about 75.6% of the total. The main constituents are α pinene (32.64%), β humulene (8.71%), and camphene (5.95%).

The essential oil of Indian rosmarinus was also enriched with alpha-pinene (31.91%) and 1,8-cineole (14.66%). However, in France, KALOUSTIAN et al. (2002) recorded a camphor chemotype with high-level (30–45%). Furthermore, in Algeria, BOUTABIA et al. (2016) showed that 1,8-cineole is the predominant chemotype of essential oil from *Rosmarinus officinalis*. However, Lograda et al. (2013) observed that the chemical composition of rosemary essential oils collected in five regions of eastern Algeria is dominated by camphor (42.7%).

Acaricidal effect of *Rosmarinus officinalis* essential oil:

For a long time, resource-poor farmers in Africa and Asia have practiced traditional medicine based on the use of plant materials to treat livestock endo- and ectoparasites, including ticks (MONDAL et al. 2013). The first intensive tests on acaricidal activity were launched by KHAIDAROV (1971), which evaluated 84 plants; currently, at a global level, 200 plant species have been registered for their repellent or acaricidal properties (ADENUBI et al. 2016). The orientation toward biocides is due to the abundance of secondary plant metabolites with toxicological activity, their low cost, and relatively lower toxicity to the environment and hosts (BORGES et al. 2011), in addition to the slow development of resistance due to the variability of active agents with different mechanisms of action (BALANDRIN et al. 1985, CHAGAS et al. 2002, OLIVO et al. 2009), making plant extracts a better alternative for controlling tick populations (OLIVEIRA et al. 2016).

The toxicological tests of the present study revealed a considerable and variable sensitivity of *R. sanguineus* to the essential oil of *Rosmarinus officinalis*, as indicated by a significant reduction in the egg mass of engorged females from 33.82% to 16.41% with a significant reduction in the reproductive efficiency index compared with the control group. Furthermore, the essential oil was also toxic to larvae, expressed by low to very high mortality rates, which correlates with increasing time from one concentration to another, with LC50 of 2.286 $\mu\text{l/ml}$ for 24 h, 2.021 $\mu\text{l/ml}$ for 48 h, and 1.635 $\mu\text{l/ml}$ for 72 h.

In comparison with the tick species chosen for this work, DAEMON et al. (2009) and MONTEIRO et al. (2009) showed the effectiveness of thymol on the larvae and pupae of *R. sanguineus*, with a mortality rate that reached 100% at concentrations of 2% and 0.5%, respectively. However, in the case of non-engorged *R. sanguineus* larvae, only 37.7% mortality was recorded at a concentration of 2% thymol (DAEMON et al. 2009), besides GODARA et al. (2013) showed the in vitro efficacy of the chloroform extract obtained from *Artemisia absinthium* on adults, eggs, and larvae using the adult immersion test (AIT) causing a mortality rate of up to 93.3% with LC50 and LC95 values of 8.793% and 34.59%, the egg hatch test (EHT)

reducing egg production to 85.1% with complete inhibition of hatching, and the larval packaging test (LPT) causing 100% mortality of larvae with LC50 and LC95 values of 1.11% and 2.37%.

In Algeria, few studies have investigated the control of ticks using plant extracts. There are two studies on the same species *Hyalomma scupense* revealing considerable toxic activity, the first by DJEBIR et al. (2019) evaluating the acaricidal activity of six aromatic plants belonging to the Lamiaceae and Myrtaceae families through an adult immersion test (AIT) and a larval immersion test (LIT), and the second by ALIMİ et al. (2022) evaluating the acaricidal activity of *Ocimum basilicum* essential oil and its main constituents through the adult immersion test (AIT) and the larval package test (LPT).

Notably, the toxicity of different extracts of some plants is not only limited to mortality, but can also affect the fecundity and hatching rate of female eggs (ELLSE & WALL 2014), while altering the morphophysiology of some important organs (CAMARGO-MATHIAS 2018), such as the ovaries (KONIG et al. 2020), salivary glands (REMEDIO et al. 2016), and the nonganglion (ROMA et al. 2013).

However, variations between the methods used and the conditions for testing the repellent and acaricidal effects of certain plant extracts, such as the choice of test type, test duration, presence or absence of the index host, species, and stage of ticks, as well as the plant, extraction type, and solvent, made it difficult to compare studies and select the best plant species.

CONCLUSION

In conclusion, this toxicological study showed that the distilled essential oil of *R. officinalis* has high in vitro acaricidal activity against larvae, in addition to strongly affecting the reduction of the hatching and egg-laying capacity of engorged females of *R. sanguineus*.

These results provide interesting horizons for its application as a potential alternative to synthetic acaricides for the control of animal ticks. However, in vivo clinical studies under practical external conditions are also necessary to validate this control strategy to standardize experimental control design, establish the correct doses to be administered to animals, and determine side effects related to phytotoxicity.

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Research Article

Guild Dynamics and Pathogen Interactions in *Hyalomma* Ticks From Algerian Cattle

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Ticks are pivotal in transmitting a variety of pathogens that affect both humans and animals. These pathogens often occur in guilds, groups of species that exploit similar resources in similar ways. Although the composition of tick-borne pathogen (TBP) guilds is well-documented, the interactions among pathogens within these guilds remain poorly understood. We hypothesized that abiotic and biotic factors significantly influence the patterns of occurrence and interactions among pathogens within these guilds. To investigate this, we analyzed microfluidic-based high-throughput data on microorganisms from 166 *Hyalomma excavatum* ticks (94 male and 72 female) collected across different seasons from cattle in the central Algerian steppe using network analysis to uncover complex pathogen–pathogen interaction patterns. We found that female ticks had a higher infection rate (63.9%) with common pathogens such as *Rickettsia slovaca* (26.4%), unclassified Apicomplexa (22.2%), and *Borrelia afzelii* (19.4%). Male ticks showed a 56.4% infection rate, with *Rickettsia* (31.1%) and *R. slovaca* (16%) being the most prevalent. Notable pathogen–pathogen interactions within guilds were identified, with positive associations such as between *R. slovaca* and *Rickettsia conorii* in males, and *B. afzelii* and *Borrelia spielmanii* in females, indicating cooperative interactions. Conversely, negative associations, such as between *Anaplasma phagocytophilum* and *Francisella tularensis*, suggested competitive exclusion. The observed variation in interaction patterns under different conditions indicates that ecological determinants, both biotic and abiotic, influence pathogen association dynamics within guilds. These findings have significant implications for understanding disease transmission and developing control strategies.

Keywords: cattle; *Hyalomma*; network analysis; tick-borne pathogen interactions; ticks

1. Introduction

The rise in emerging zoonotic diseases, particularly tick-borne diseases, not only threatens public health but also has significant economic implications for the agricultural

sector [1, 2]. As nearly 80% of the world's livestock is affected by ticks and the pathogens they carry, these diseases contribute to increased costs and production losses, especially in regions heavily reliant on cattle farming [3–7].

In Algeria, where cattle farming is a key industry, the prevalence of ticks, particularly those from the *Hyalomma* genus, including species like *Hyalomma anatolicum*, *Hyalomma excavatum*, and *Hyalomma marginatum*, poses a considerable challenge [8–11]. The documented presence of 24 tick species [8, 12, 13] underscores the ongoing risk to both animal and human health, reinforcing the need for integrated disease management effort. Previous studies have primarily focused on identifying these species, but fewer have explored the interactions among pathogens they transmit, which is essential for predicting outbreaks and managing disease risks.

Pathogen–pathogen interactions during coinfections can have ecological and epidemiological consequences [14], including increased virulence [15], gene transfer [16], and altered immune responses in hosts that exacerbate disease progression [17, 18]. Building on previous work [19, 20], this study uses advanced methods to explore these interactions in more detail, expanding on traditional pathogen identification by utilizing high-throughput microfluidic PCR and network analysis.

High-throughput PCR allows for simultaneous detection of multiple pathogens, offering deeper insights into pathogen diversity within individual ticks or populations than older methods like serology or standard PCR [21, 22]. Meanwhile, network analysis enables the mapping of pathogen co-occurrence and interaction patterns, which are crucial for understanding the ecological networks that shape disease transmission [23]. These tools have been successfully employed in recent studies to map tick-borne pathogen (TBP) communities across various ecosystems, revealing complex interspecies interactions [24, 25].

The ecological relevance of the ticks and pathogens is significant [21, 26]. *Hyalomma* ticks are known vectors for multiple zoonotic pathogens, including *Rickettsia* spp. and *Theileria* spp., which impact both human and animal health [5, 7]. Their feeding behavior, extended in female ticks, increases the efficiency of pathogen transmission [22]. Additionally, the microbiota of ticks influences pathogen survival and interaction, adding another layer of complexity to TBP dynamics [27]. Abiotic factors such as temperature and humidity also play a crucial role; higher temperatures have been shown to accelerate pathogen replication [28], while seasonal variations affect both tick activity and pathogen prevalence [2, 29].

This study aims to deepen the understanding of pathogen dynamics in ticks by exploring TBP guilds (TBPGs). In ecological terms, a guild refers to species that utilize the same types of resources in similar ways [30], and in TBPs, these guilds often involve pathogens sharing a host [31], such as *Hyalomma* ticks. These interactions, whether competitive or cooperative, shape community structure and influence disease transmission dynamics [32, 33]. For example, pathogens may compete for limited resources, such as access to the tick's immune system, resulting in competitive exclusion [34], or they may cooperate and enhancing each other's survival and transmission through coinfection [35].

We hypothesize that TBP interactions are influenced by tick sex, with male and female *Hyalomma* ticks showing different pathogen assemblages and interaction patterns. Additionally, seasonal changes are expected to affect TBP

composition and interactions. Conducted in the Djelfa region of Algeria, this research utilizes advanced molecular techniques, including high-throughput PCR and network analysis, to provide the first comprehensive examination of TBP communities in *Hyalomma* ticks in the country. By investigating the combined effects of biotic (tick sex) and abiotic factors (seasonal changes), this study not only identifies the presence of multiple pathogens, but also reveals how their interactions within shared ecological niches (guilds) influence disease dynamics. These findings offer critical insights into the ecological and epidemiological drivers of disease transmission and the evolutionary strategies pathogens employ within the tick–host system, with significant implications for controlling tick-borne diseases in Algeria and similar regions.

2. Materials and Methods

2.1. Tick Collection. A total of 166 ticks (94 males and 72 females) were collected throughout the year 2021–2022 from 60 local breed cattle across different seasons in the province of Djelfa, Algeria (winter: 14 females and 24 males; spring: 15 females and 23 males; summer: 25 females and 23 males; autumn: 18 females and 24 males). Djelfa, positioned at 34°40'00"N and 3°15'00"E and known as the steppe capital of Algeria, provided a unique setting for this study due to its distinct environmental conditions. The semiarid climate, marked by hot summers, cold winters, sparse vegetation, and varying altitude, influences the behavior and survival of tick populations, making it an ideal location for observing how these factors affect tick–host interactions. Each cattle underwent meticulous manual inspections to ensure all ticks adhering to the skin were thoroughly removed and preserved in 70% ethanol for further analysis.

The ticks were accurately identified using a binocular magnifier (Optika, Ponteranica, Italy), with identification aided by the detailed keys from Walker et al. [36], and confirmed through sequencing of the 16S rRNA gene by molecular PCR tools. This comprehensive approach allowed for detailed study of the ticks in an environment where their natural behaviors are notably influenced by the climatic and ecological conditions of the region and confirmed through sequencing of the 16S rRNA gene by molecular PCR tools.

2.2. Nucleic Acid Extraction. Before extracting nucleic acids, each tick was meticulously washed with sterile milli-Q water to ensure cleanliness. DNA extraction followed, using the Nucleo Spin tissue kit for Genomic DNA from tissue (Macherey-Nagel, Düren, Germany). The manufacturer's "Standard protocol for human or animal tissue and cultured cells" was employed with minor modifications tailored to our specific requirements. After disinfecting the ticks, they were carefully sectioned into quarters on a sterile petri dish using a sterile scalpel blade and then, transferred to the extraction tube that contained the provided lysis buffer. The lysis process was completed in these prefilled tubes, followed by centrifugation to separate the DNA-containing supernatant. This supernatant was then used for DNA quantification, performed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) at an absorbance ratio

of A260/A280 to ensure purity. Finally, the extracted DNA was stored at -20°C for subsequent analyses, maintaining its integrity for future genetic examinations.

2.3. DNA Preamplification for Microfluidic Real-Time PCR. To enhance the detection of the pathogen's genetic material relative to the host's, the DNA was preamplified using the Standard BioTools preamplification kit (Standard BioTools, CA, USA). Following the manufacturer's guidelines, the process began by preparing a 0.2x pool, and then conducting PCR preamplification. Primers were combined in equal volumes to create a pooled primer mix with a final concentration of 200 nM. The preamplification reaction was performed in a 5 μl volume, comprising 1 μl of PreAmp Master Mix, 1.25 μl of the pooled primer mix, 1.5 μl of distilled water, and 1.25 μl of DNA. The thermocycling program initiated with an initial cycle at 95°C for 2 min, followed by 14 cycles of 95°C for 15 s and 60°C for 4 min. After completion, the amplification products were diluted to a 1/10th concentration and stored at -20°C to minimize contamination risks, ensuring the integrity of the samples for subsequent analysis.

2.4. Microfluidic Real-Time PCR Assay. Michelet et al. [37] extensively detailed the techniques utilized in their study, which focused on detecting tick-borne microorganisms. The primary method employed 48.48 Dynamic Array IFC chips (Standard BioTools, CA, USA) used within the BioMark real-time PCR system. These chips allow for the separation of 48 PCR assays and 48 samples into individual wells where real-time PCR reactions occur in separate chambers thanks to an on-chip microfluidics assembly. Each chip also includes a negative water control (Milli-Q water) to ascertain the absence of contaminants, and DNA from the *Escherichia coli* strain EDL933 (Milli-Q water and DNA diluted to 1/10) serves as an internal inhibition control in the assay plate to validate the absence of PCR inhibitors, using specific primers and a probe targeting the *E. coli* gene.

Once loaded, the BioMark real-time PCR system was programmed with parameters as reported in earlier studies [38]. Throughout this process, stringent sterility measures are maintained to ensure accurate results. Post-run analysis was conducted using the "Fluidigm Real-Time PCR Analysis" software, and results were annotated in Excel. The genes targeted and the primer sequences employed for amplification are detailed in Supporting Information 1: Table S1. This investigation cataloged a comprehensive range of tick-borne microorganisms, including 27 bacterial species such as *Borrelia burgdorferi*, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitanae*, *B. spielmanii*, *B. bissettii*, *B. miyamotoi*, *Anaplasma marginale*, *A. platys*, *A. phagocytophilum*, *A. bovis*, *A. centrale*, *A. ovis*, *Ehrlichia canis*, *N. mikurensis*, *R. conorii*, *R. slovaca*, *R. massiliae*, *R. helvetica*, *R. aeschlimannii*, *R. felis*, *Bartonella henselae*, *Francisella tularensis*, *Francisella*-like endosymbionts (FLEs), *Coxiella*-like endosymbionts (CLEs), and *Coxiella burnetii*. Additionally, seven parasite species were identified, including *Babesia microti*, *B. canis*, *B. ovis*, *B. divergens*, *B. bovis*, *B. caballi*, and *Babesia* sp. EU1. The bacterial genera included were *Bartonella*, *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, and *Mycoplasma*, and parasite taxa encompassed

Apicomplexa, *Theileria*, and *Hepatozoon*, providing a thorough overview of the pathogens present in tick populations.

2.5. Confirmation of Pathogen Presence Using Conventional PCR. TBPs were detected through conventional and nested PCR assays, with the cycling conditions and primers detailed in Supporting Information 2: Table S2. Additional PCR assays, utilizing species-specific primers, further confirmed the presence of certain target TBPs identified in the initial analysis. This crucial confirmation step strengthens the accuracy and reliability of the findings by providing an additional layer of validation [23].

2.6. DNA Sequencing Analysis. The PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany), and the sequences were assembled using BioEdit software from Ibis Biosciences in Carlsbad, CA, USA. Our findings were then compared against publicly available sequences in GenBank using the online BLAST tool provided by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), available at <http://www.ncbi.nlm.nih.gov/blast>.

2.7. Phylogenetic Analysis. A phylogenetic analysis of TBPs associated with *Hyalomma* species was performed, grouping them into 10 guilds based on collection seasons and sexes (M: males, MW: males collected in winter, MSP: males collected in spring, MSU: males collected in summer, MA: males collected in autumn, F: females, FW: females collected in winter, FSP: females collected in spring, FSU: females collected in summer, and FA: females collected in autumn). The details about pathogens identified in these TBPGs are provided in Supporting Information 3: Table S3. For this purpose, reference sequences of the 16S rRNA (bacterial pathogens) and 18S rRNA (eukaryotes) genes fragments were searched in the National Library of Medicine database; NCBI (accessed 13 June 2024). Then all sequences of particular species showing similarity to the reference ones were downloaded from the Blast database. Finally, sequences of up to 1800 nucleotides in length, excluding redundant ones underwent initial alignment using the online MAFFT tool [39]. Next, obtained set of sequences was analyzed using the MUSCLE algorithm in MEGA 11 [40]. Phylogenetic trees were then constructed using the Tamura-Nei model with Gamma distribution (TN93+G) and the Tamura 3-parameter model (T92) for the 16S rRNA and 18S rRNA gene, respectively.

Moreover, our aim was to investigate whether there is a consistent pattern of genetic distances between TBPs within each guild and whether this pattern holds across guilds. To this end, the pairwise distance between sequences within each guild was calculated (as *p*-distance) in MEGA 11. Furthermore, the statistical significance of differences in *p*-distance between the studied groups (guilds) was calculated using the Mann-Whitney *U* test, while the significance of differences in *p*-distance within particular guilds was calculated using the Wilcoxon test. Statistical calculations were performed using GraphPad 8.0 (Prism, Massachusetts, USA).

2.8. Statistical Analysis. The gathered data were assembled using Microsoft Excel 2016. Prevalence rates and 95% binomial confidence intervals (CIs) for each TBP infection and

coinfection were calculated based on microfluidic real-time PCR amplification results. Chi-square tests (χ^2) were conducted to compare TBP prevalence between males and females, a p value <0.05 was considered significant, the calculations were performed using SPSS software version 22.

2.9. Coinfections and Network Interactions Between Microorganisms. Investigations into pathogen associations within ticks have utilized a modeling approach based on binary presence/absence data. In the dataset, ticks are represented in columns and the microorganisms tested are represented in rows, where 0 indicating the absence and 1 indicating the presence of pathogen. This analysis employed Yule's Q statistic, defined for 2×2 contingency tables as:

$$\text{Yule's } Q = (ad + bc) / (ad - bc).$$

'a' and 'd' denote the number of concordant pairs (where both microorganisms are either present or absent), while 'b' and 'c' represent the number of discordant pairs (where one pathogen is present while the other is absent). Statistical analysis was conducted using the *igraph* package [41] implemented in R version 4.3.3 [42] and performed using RStudio [43].

Interaction networks were constructed using results from high-throughput microfluidic analyses, allowing simultaneous detection of multiple pathogens in ticks. The presence of some of these pathogens was confirmed by nested PCR. Only edges with weights of 1 and -1 were included. The resulting association networks, visualized as R plots, were constructed and refined using Gephi [44]. In each network, node color and size were indicative of modularity class and eigenvector centrality. The network's spatial layout was optimized using Yifan Hu and Fruchterman Reingold parameters within Gephi. Positive and negative interactions were determined from the correlation coefficients of abundance data. Network complexity was evaluated by examining the number of nodes, edges, and overall interaction patterns. Nodes within the network represent microorganisms, while blue and red edges denote positive and negative associations, respectively. An R script detailing the calculation of Yule's Q and the construction of the co-occurrence network is provided as additional material (Supporting Information 4: File S1).

3. Results

3.1. Tick Morphological and Genetic Classification. The ticks were morphologically identified as *H. excavatum*. To confirm this identification with higher precision, advanced PCR techniques were applied. Subsequent sequencing of the 16S rRNA gene definitively confirmed the presence of *H. excavatum*. The phylogenetic relationships of the sequences obtained further supported this identification (Figure 1). The sequences were submitted to GenBank and assigned the following accession numbers: PP800859, PP800860, PP800863, PP800864, PP800865, and PP800866. This multitiered approach of

morphological examination followed by genetic verification ensured a robust classification of the tick specimens.

3.2. Diversity of TBPs in Ticks. The diversity of TBPs was analyzed in 166 *Hyalomma* ticks, consisting of 72 females and 94 males. Overall, 63.9% of female ticks (46/72; Table 1) and 56.4% of male ticks (53/94; Table 2) tested positive for at least one pathogen. Single infections were more common in males (45.8%, 43/94) than females (19.4%, 14/72), while coinfections were more frequent in females (44.4%, 32/72; Table 1) compared to males (10.6%, 10/94; Table 2).

Across both sexes, *Rickettsia* spp. dominated the pathogen landscape, with *R. slovaca* most prevalent in females (26.4%) and *Rickettsia* spp. highest in males (31.1%). Other notable pathogens in females included Apicomplexa (22.2%) and *Borrelia afzelii* (19.4%; Table 1), while males showed lower prevalence for Apicomplexa (5.3%) and *R. slovaca* (15.1%; Table 2).

A χ^2 test ($\chi^2 = 62.94$, $p < 0.001$) confirmed significant differences in TBP diversity between sexes, suggesting distinct transmission dynamics and ecological exposures for males and females. Pathogens such as *A. phagocytophilum*, *B. afzelii*, and *B. spielmanii* were detected only in females, while *Ehrlichia* was found exclusively in males, further highlighting sex-specific pathogen associations.

3.3. Coinfections Between Tick-Borne Microorganisms. Coinfections were more frequent in females (44.4%, 32/72; Table 1) compared to males (10.6%, 10/94; Table 2). In females, coinfections involving two pathogens occurred in 15.3%, while coinfections of three to eight pathogens were also observed, with *R. slovaca*, *R. conorii*, and Apicomplexa being the most frequent combination (4.2%, 3/72; Table 1). In males, coinfections typically involved two pathogens (9.6%), with the most common pairing being Apicomplexa and *Rickettsia* (3.2%, 3/94; Table 2).

3.4. Influence of Biotic and Abiotic Ecological Determinants on Microbe–Microbe Interactions

3.4.1. Tick Sex as a Biotic Ecological Determinant of Microbe–Microbe Interactions. Network analysis of *Hyalomma* ticks revealed sex-specific pathogen interactions (Figure 2a,b). In females, negative associations between *A. phagocytophilum*, *B. afzelii*, and *F. tularensis* (Figure 2a) indicated competitive exclusion, where one pathogen's presence inhibits others. In males, strong negative interactions were found between *Anaplasma*, *Ehrlichia*, *N. mikurensis*, and *Rickettsia* species (Figure 2b), suggesting competition for resources or immune evasion strategies.

FLEs and CLEs played a central role in both sexes, showing positive associations with multiple pathogens, possibly facilitating their coexistence. Moderate positive associations, such as between *R. conorii* and *R. slovaca* in males and between *B. afzelii* and *B. spielmanii* in females, further suggest reduced competition in some coinfections.

3.4.2. Seasonal Changes as an Abiotic Ecological Determinants of the Pathogen–Pathogen Interaction. The co-occurrence networks reveal clear seasonal differences in pathogen interactions

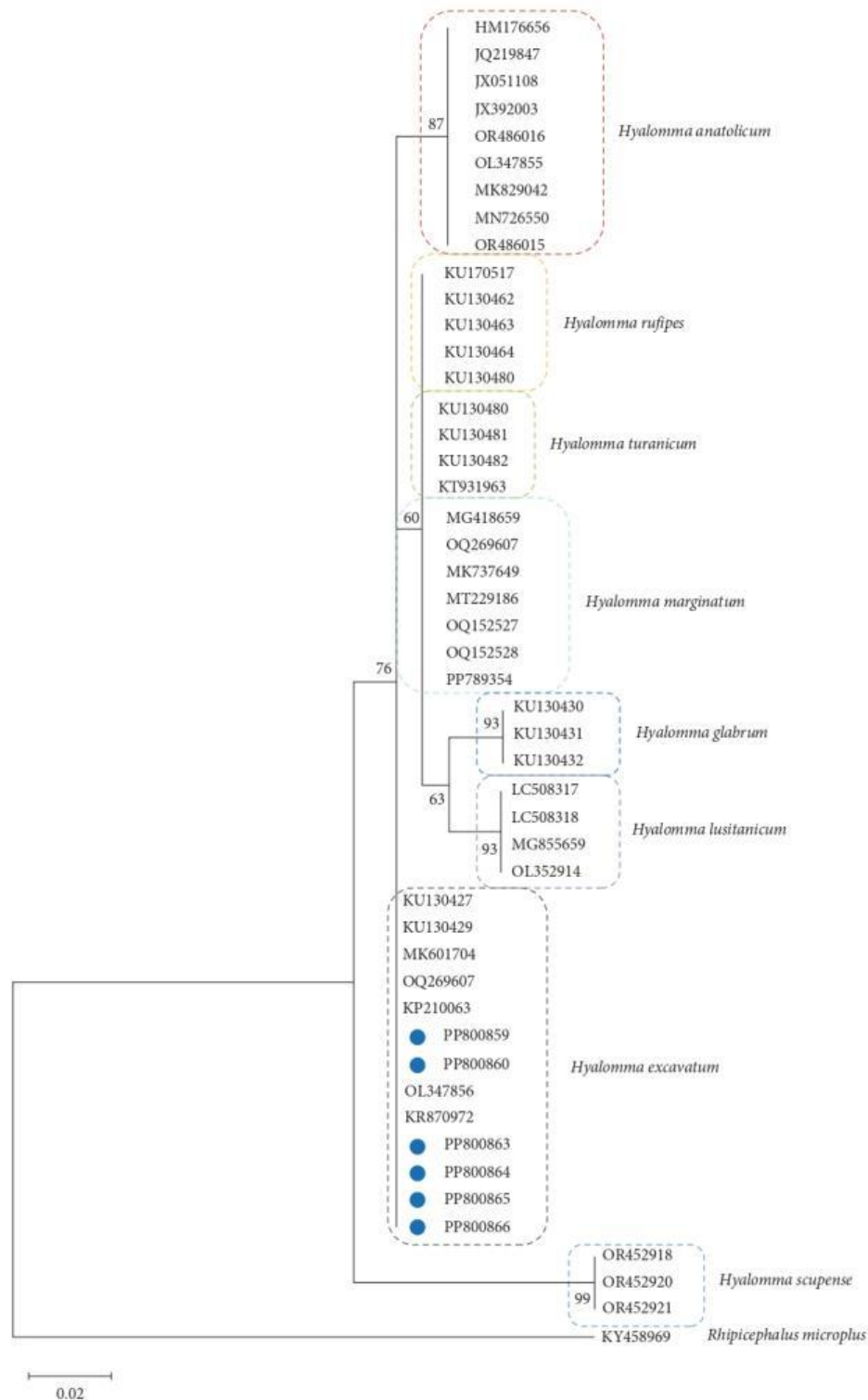


FIGURE 1: Phylogeny of the genus *Hyalomma* based on 16S rRNA gene. The evolutionary history was inferred by using the maximum likelihood method and the Tamura 3-parameter model (T92). The analysis contains sequences identified in the current study (marked with

blue dot) and retrieved from GenBank database. Accession numbers of sequences are given. Bootstrap values are represented as percentage of internal branches (1000 replicates), and values lower than 50 are hidden. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Rhipicephalus microplus* sequence KY458969 was used to root the tree.

TABLE 1: TBP detected in female ticks collected from cattle using microfluidic PCR.

Vector-borne pathogen(s)	Total	Prevalence rate (%)	95% CI
Total infected ticks (≥ 1 pathogen)	46	63.89	51.65–74.63
<i>R. slovaca</i>	19	26.39	17.01–38.31
Apicomplexa	16	22.22	13.61–33.85
<i>B. afzelii</i>	14	19.44	11.41–30.80
<i>Rickettsia</i> sp.	13	18.06	10.33–29.26
<i>R. conorii</i>	12	16.67	9.27–27.70
<i>N. mikurensis</i>	11	15.28	8.23–26.12
<i>B. spielmanii</i>	10	13.89	7.22–24.52
<i>Anaplasma</i> sp.	8	11.11	5.26–21.26
<i>Hepatozoon</i> sp.	6	8.33	3.43–17.88
<i>R. aeschlimannii</i>	5	6.94	2.58–16.14
<i>Mycoplasma</i> sp.	2	2.78	0.48–10.58
<i>Theileria</i> sp.	2	2.78	0.48–10.58
<i>A. phagocytophilum</i>	1	1.39	0.07–8.54
<i>Bartonella</i> sp.	1	1.39	0.07–8.54
<i>F. tularensis</i>	1	1.39	0.07–8.54
Single infections	14	19.44	11.41–30.80
<i>Rickettsia</i> sp.	5	6.94	2.58–16.14
Apicomplexa	2	2.78	0.48–10.58
<i>N. mikurensis</i>	2	2.78	0.48–10.58
<i>R. slovaca</i>	2	2.78	0.48–10.58
<i>B. spielmanii</i>	1	1.39	0.07–8.54
<i>Mycoplasma</i> sp.	1	1.39	0.07–8.54
<i>Anaplasma</i> sp.	1	1.39	0.07–8.54
Mixed infections	32	44.44	32.90–56.59
Mixed infection with two pathogens	11	15.28	6.22–22.90
<i>R. slovaca</i> + <i>R. aeschlimannii</i>	2	2.78	0.48–10.58
<i>R. slovaca</i> + <i>R. conorii</i>	1	1.39	0.07–8.54
<i>R. slovaca</i> + Apicomplexa	1	1.39	0.07–8.55
<i>Rickettsia</i> sp. + <i>B. spielmanii</i>	1	1.39	0.07–8.54
<i>B. afzelii</i> + <i>B. spielmanii</i>	1	1.39	0.07–8.55
<i>A. phagocytophilum</i> + <i>Rickettsia</i> sp.	1	1.39	0.07–8.56
<i>B. afzelii</i> + <i>Rickettsia</i> sp.	1	1.39	0.07–8.57
<i>N. mikurensis</i> + Apicomplexa	1	1.39	0.07–8.58
<i>R. slovaca</i> + <i>F. tularensis</i>	1	1.39	0.07–8.59
<i>B. afzelii</i> + <i>Anaplasma</i> sp.	1	1.39	0.07–8.60
Mixed infection with three pathogens	9	12.50	6.22–22.90
<i>R. slovaca</i> + <i>R. conorii</i> + Apicomplexa	3	4.17	1.08–12.50
<i>R. slovaca</i> + <i>R. conorii</i> + <i>N. mikurensis</i>	1	1.39	0.07–8.54
<i>N. mikurensis</i> + Apicomplexa + <i>R. conorii</i>	1	1.39	0.07–8.55
<i>R. slovaca</i> + <i>R. conorii</i> + <i>Anaplasma</i> sp.	1	1.39	0.07–8.56
<i>B. afzelii</i> + <i>B. spielmanii</i> + <i>R. slovaca</i>	1	1.39	0.07–8.57
<i>N. mikurensis</i> + Apicomplexa + <i>Rickettsia</i> sp.	1	1.39	0.07–8.58
<i>R. slovaca</i> + <i>R. conorii</i> + <i>B. afzelii</i>	1	1.39	0.07–8.54
Mixed infection with four pathogens	6	8.33	3.43–17.88
<i>Rickettsia</i> sp. + <i>Bartonella</i> sp. + <i>B. afzelii</i> + Apicomplexa	1	1.39	0.07–8.54
<i>R. slovaca</i> + <i>R. conorii</i> + <i>R. aeschlimannii</i> + <i>Hepatozoon</i> sp.	1	1.39	0.07–8.55

TABLE 1: Continued.

Vector-borne pathogen(s)	Total	Prevalence rate (%)	95% CI
<i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Rickettsia</i> sp. + <i>Anaplasma</i> sp.	1	1.39	0.07–8.56
<i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Anaplasma</i> sp. + Apicomplexa	1	1.39	0.07–8.57
Apicomplexa + <i>Mycoplasma</i> sp. + <i>Theileria</i> sp. + Hepatozoon sp.	1	1.39	0.07–8.58
<i>B. afzelii</i> + <i>N. mikurensis</i> + <i>Rickettsia</i> sp. + Hepatozoon sp.	1	1.39	0.07–8.59
Mixed infection with five pathogens	4	5.56	1.79–14.35
<i>B. afzelii</i> + <i>Anaplasma</i> sp. + <i>N. mikurensis</i> + <i>Rickettsia</i> sp. + Apicomplexa	1	1.39	0.07–8.54
<i>R. slovaca</i> + <i>B. spielmanii</i> + <i>R. conorii</i> + Apicomplexa + Hepatozoon sp.	1	1.39	0.07–8.55
<i>B. afzelii</i> + <i>Anaplasma</i> sp. + <i>N. mikurensis</i> + <i>R. aeschlimannii</i> + Hepatozoon sp.	1	1.39	0.07–8.56
<i>R. slovaca</i> + <i>R. conorii</i> + <i>B. afzelii</i> + <i>B. spielmanii</i> + <i>N. mikurensis</i>	1	1.39	0.07–8.57
Mixed infection with six pathogens	1	1.39	0.07–8.58
<i>R. slovaca</i> + <i>R. conorii</i> + <i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Theileria</i> sp. + Apicomplexa	1	1.39	0.07–8.59
Mixed infection with eight pathogens	1	1.39	0.07–8.60
Hepatozoon sp. + Apicomplexa + <i>R. slovaca</i> + <i>R. aeschlimannii</i> + <i>B. afzelii</i> + <i>B. spielmanii</i> + <i>Anaplasma</i> sp.	1	1.39	0.07–8.61
Not detected	26	36.11	25.37–48.35

Abbreviations: CI, confidence interval; TBPs, tick-borne pathogens.

TABLE 2: TBPs detected in male ticks collected from cattle using microfluidic PCR.

Vector-borne pathogen(s)	Total	Prevalence rate (%)	95% CI
Total infected ticks (≥1 pathogen)	53	56.38	45.78–66.46
<i>Rickettsia</i> sp.	30	31.91	22.89–42.44
<i>R. slovaca</i>	15	15.96	9.5–25.27
<i>R. conorii</i>	6	6.38	2.62–13.91
Apicomplexa	5	5.32	1.97–12.55
<i>Anaplasma</i> sp.	1	1.06	0.06–6.62
<i>Bartonella</i> sp.	1	1.06	0.06–6.62
<i>Ehrlichia</i> sp.	1	1.06	0.06–6.62
<i>F. tularensis</i>	1	1.06	0.06–6.62
<i>N. mikurensis</i>	1	1.06	0.06–6.62
<i>R. Aeschlimannii</i>	1	1.06	0.06–6.62
<i>Theileria</i> sp.	1	1.06	0.06–6.62
Single infections	43	45.74	35.54–56.3
<i>Rickettsia</i> sp.	27	28.72	20.09–39.12
<i>R. slovaca</i>	11	11.70	6.27–20.38
<i>R. conorii</i>	2	2.13	0.37–8.21
<i>Ehrlichia</i> sp.	1	1.06	0.06–6.62
<i>N. mikurensis</i>	1	1.06	0.06–6.62
<i>R. aeschlimannii</i>	1	1.06	0.06–6.62
Mixed infections	10	10.64	5.5–19.12
Mixed infection with two pathogens	9	9.57	4.74–17.85
Apicomplexa + <i>Rickettsia</i> sp.	3	3.19	0.83–9.71
<i>R. slovaca</i> + <i>R. conorii</i>	3	3.19	0.83–9.71
Apicomplexa + <i>Theileria</i>	1	1.06	0.06–6.62
<i>Anaplasma</i> sp. + <i>F. tularensis</i>	1	1.06	0.06–6.62
<i>Bartonella</i> sp. + <i>Rickettsia</i> sp.	1	1.06	0.06–6.62
Mixed infection with three pathogens	1	1.06	0.06–6.62
Apicomplexa + <i>R. slovaca</i> + <i>R. conorii</i>	1	1.06	0.06–6.62
Not detected	41	43.62	33.54–54.22

Abbreviations: CI, confidence interval; TBPs, tick-borne pathogens.

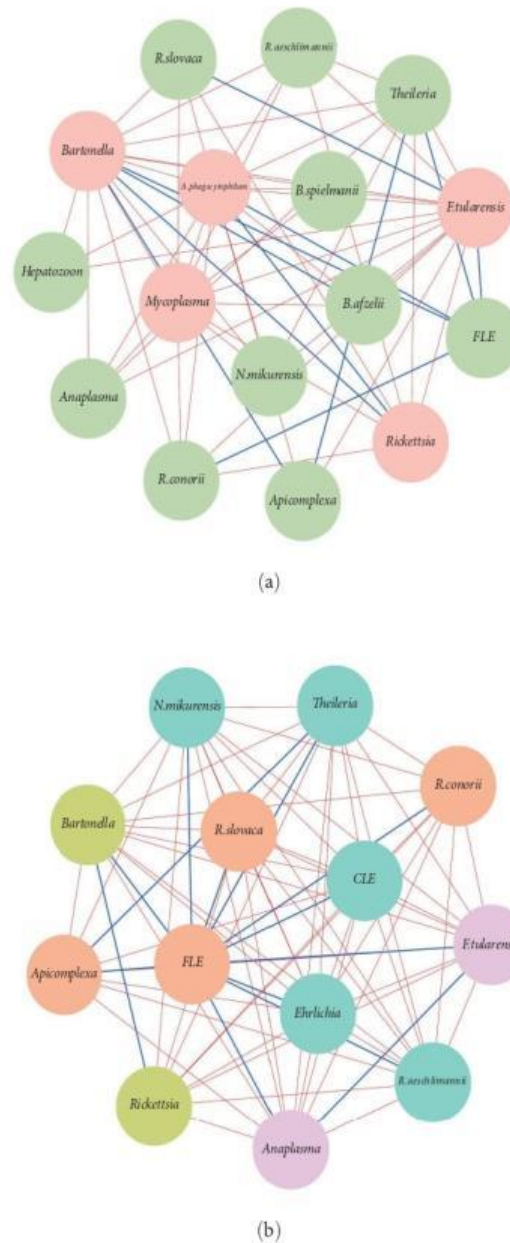


FIGURE 2: Microbial co-occurrence networks. Meaningful connections between pairs of microbial species using Yule's Q statistic: (a) female network and (b) male network. Nodes represent distinct microbial taxa, including pathogens and symbionts, while edges signify statistically significant associations with weights between 1 and -1 . The colors of nodes are based on modularity class metric, and the size is proportional to the eigenvector centrality value of each taxon. Blue edges denote positive connections, while red edges represent negative ones. CLE, *Coxiella*-like endosymbionts; FLE, *Francisella*-like endosymbiont.

between female and male *Hyalomma* ticks (Figure 3a–h). In winter, female networks show balanced interactions between *Rickettsia* species and FLE (Figure 3a), while male networks exhibit more competitive dynamics, such as negative interactions between *R. slovaca* and Apicomplexa (Figure 3b).

In spring, female networks are more complex, dominated by positive interactions suggesting cooperation (Figure 3c), while male networks are simpler and more competitive, with taxa like *Bartonella* absent from females but present in males (Figure 3d).

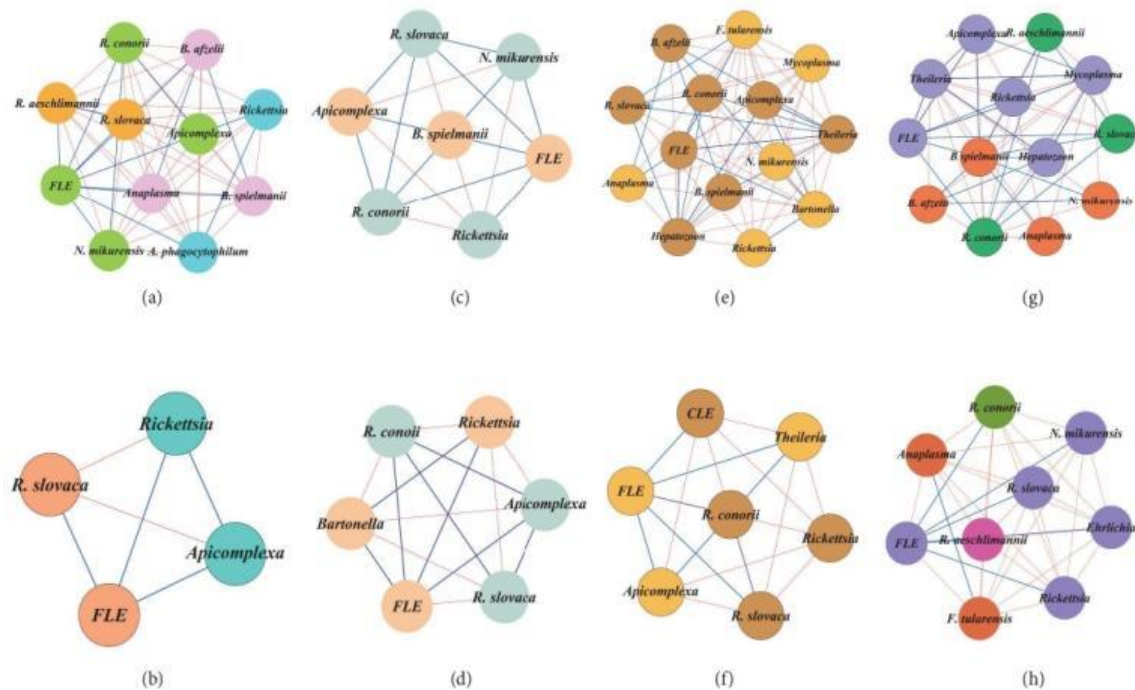


FIGURE 3: Microbial co-occurrence networks across seasons and sexes. Using co-occurrence networks, we analyzed the complex dynamics of microbe-microbe interactions in male and female *Hyalomma* ticks across different seasons. The figure includes separate networks for each season, presented as follows: winter networks in (a) for females and (b) for males; spring networks in (c) for females and (d) for males; summer networks in (e) for females and (f) for males; and autumn networks in (g) for females and (h) for males. The visualization showcases significant connections between pairs of microbes using Yule's Q statistic. Each node symbolizes a unique microbe, with edges indicating statistically significant associations with weights between 1 and -1 . Blue edges denote positive connections, while red edges represent negative ones. The color and size of nodes reflect modularity class and eigenvector centrality, respectively. CLE, *Coxiella*-like endosymbiont; FLE, *Francisella*-like endosymbiont.

In summer, females show a more diverse and complex network, with largely positive interactions and the presence of *F. tularensis* (Figure 3e), while males display stronger negative interactions, particularly between *Rickettsia* and *Theileria* (Figure 3f).

Autumn networks reflect similar patterns, with females showing more balanced interactions (Figure 3g), while males demonstrate stronger competitive pressures, particularly between species like *Anaplasma* and *R. conorii* (Figure 3h).

In pathogen-pathogen co-occurrence network of the same guild, the nodes all maintained the same value of degree centrality, suggesting the same numbers of connections for each node within the network regardless of the differences in the nature and preference of interaction (Supporting Information 5: Table S4). On the other hand, the degree centrality values of the shared nodes varied between the TBPGs networks for the same node, demonstrating that tick sex and seasonal changes influence not only the nature of interaction but also the number of associations that a taxon can establish within one condition (Supporting Information 6: Table S5).

Overall, while both female and male networks display seasonal variations in species composition and interaction patterns, males tend to exhibit more pronounced competitive interactions,

particularly in summer and autumn. Females show a similarly dynamic but slightly less competitive network structure, indicating subtle differences in ecological strategies and adaptations between the sexes throughout the year.

3.5. Genetic Diversity and Variation in Pathogen Guilds. Significant genetic diversity was observed among sequences within guilds composed of bacterial pathogens (16S rRNA), surpassing that found within guilds grouping eukaryotic microorganisms (18S rRNA; Figures 4 and 5). *Rickettsia slovaca* was the only pathogen identified in all TBPGs, while *Ehrlichia* sp. was only identified in M and MA guilds (Figure 4a). The rest of the bacterial pathogens were identified in both F and M guilds and in at least one corresponding to a seasonal change guild (Figure 4a). Protozoan pathogens presented lower genetic diversity, Apicomplexa (other) was identified in a greater number and variety of guilds followed by *Hepatozoon* sp., while *Theileria* sp. was only identified in F, FSU, and FA guilds (Figure 4b).

Analysis revealed that the majority of studied guilds, with the exception of MSP 16S rRNA, displayed statistically significant variations ($p < 0.05$) in genetic distances among their constituent sequences (Figure 5, Tables 3 and 4). This trend

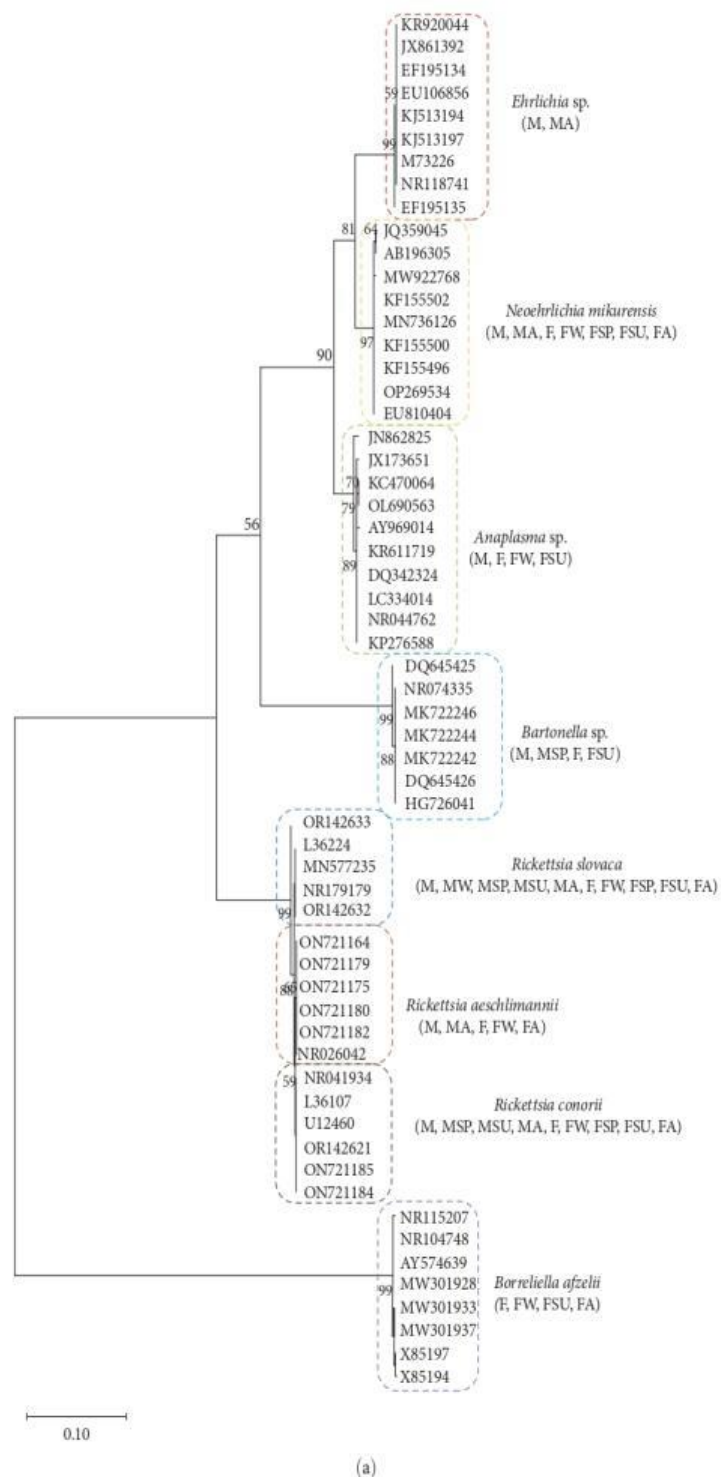


FIGURE 4: Continued.

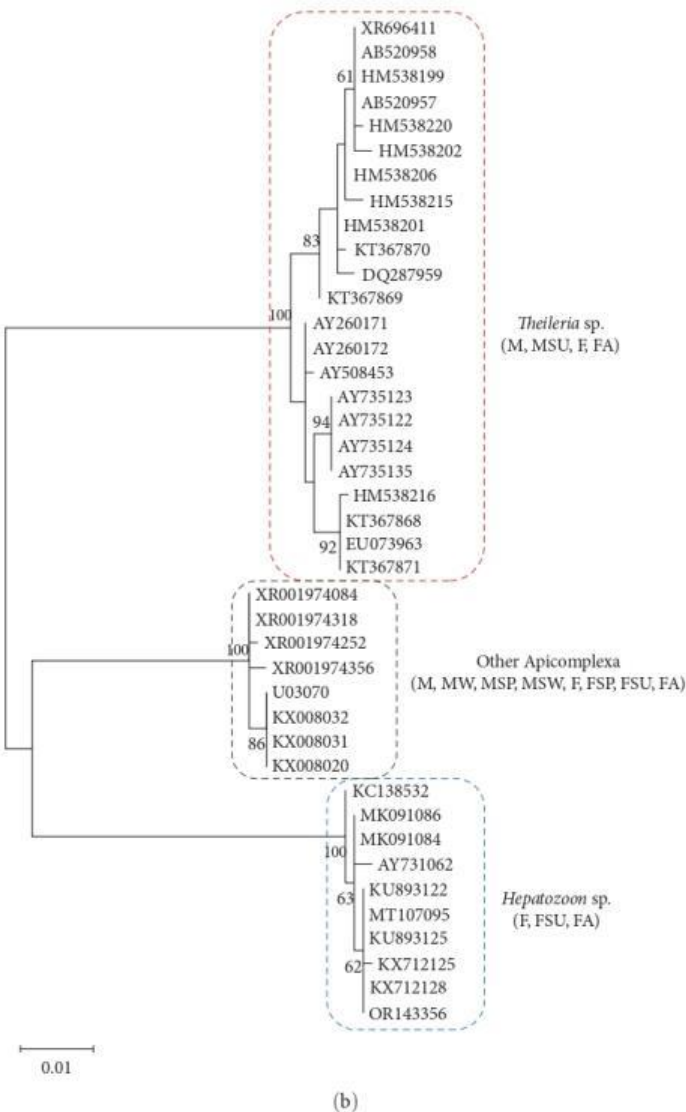


FIGURE 4: Distribution of guilds across the phylogenetic trees of tick-borne pathogens (TBPs) linked with *Hyalomma excavatum*. (a) Tick-borne bacteria associated with *H. excavatum*. The phylogram was constructed from the 16S rRNA gene, and the evolutionary history was inferred using the maximum likelihood method with the Tamura–Nei model and Gamma distribution (TN93+G). (b) Tick-borne protozoa associated with *H. excavatum*. The phylogram was constructed from the 18S rRNA gene, and the evolutionary history was inferred using the maximum likelihood method with the Tamura 3-parameter model (T92). For both trees, accession numbers of sequences are given. Bootstrap values are represented as percentages of internal branches (1000 replicates), with values lower than 50 hidden. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Letters represent different guilds: F, females; FA, females collected in autumn; FSP, females collected in spring; FSU, females collected in summer; FW, females collected in winter; M, males; MA, males collected in autumn; MSP, males collected in spring; MSU, males collected in summer; MW, males collected in winter.

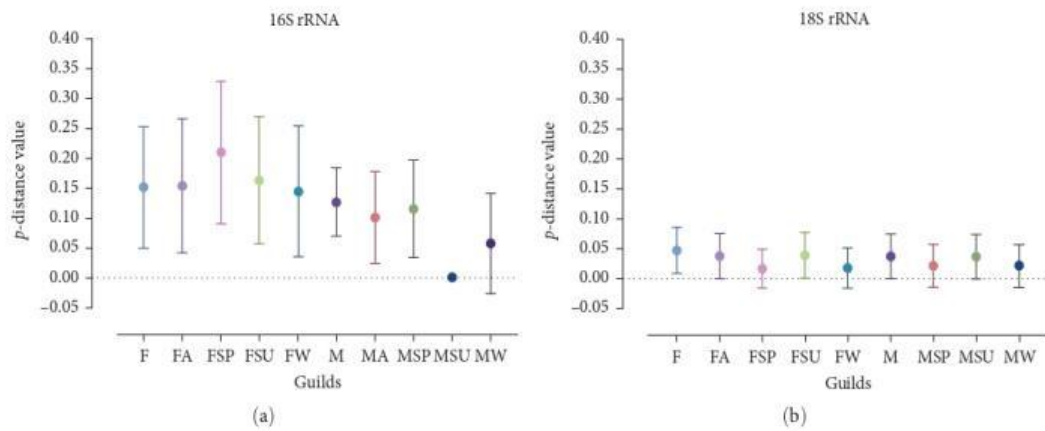


FIGURE 5: Genetic distances between sequences grouped into different guilds. (a) Genetic distances between 16S rRNA sequences grouped into different guilds. (b) Genetic distances between 18S rRNA sequences grouped into different guilds. The genetic distances were calculated as pairwise distances. The diagram shows the mean p-distance values and standard deviation ranges within each studied guild. The guilds are represented by the following abbreviations: F (females); FA (females collected in autumn); FSP (females collected in spring); FSU (females collected in summer); FW (females collected in winter); M (males); MA (males collected in autumn); MSP (males collected in spring); MSU (males collected in summer); MW (males collected in winter).

TABLE 3: Statistical significance of genetic distances calculated as pairwise distance between particular 16 rRNA sequences grouped into guilds.

Guilds	Guilds and <i>p</i> values								
	F	FA	FSP	FSU	FW	M	MA	MSP	MSU
FA	0.862	—	—	—	—	—	—	—	—
FSP	<0.001*	<0.001*	—	—	—	—	—	—	—
FSU	0.154	0.185	<0.001*	—	—	—	—	—	—
FW	0.131	0.124	<0.001*	0.004*	—	—	—	—	—
M	0.009*	0.007*	<0.001*	<0.001*	0.525	—	—	—	—
MA	0.003*	<0.001*	<0.001*	<0.001*	0.036*	0.211	—	—	—
MSP	0.049*	0.041*	<0.001*	0.814	0.099	<0.001*	0.3811	—	—
MSU	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	—
MW	0.008*	0.005*	<0.001*	0.002*	0.009*	0.012*	0.001*	0.002*	0.189

Note: *p*-level of statistical significance.

Abbreviations: F, females; FA, females collected in autumn; FSP, females collected in spring; FSU, females collected in summer; FW, females collected in winter; M, males; MA, males collected in autumn; MSP, males collected in spring; MSU, males collected in summer; MW, males collected in winter.

*Statistically significant.

TABLE 4: Statistical significance of genetic distances calculated as pairwise distance between particular 18S rRNA sequences grouped into guilds.

Guilds	Guilds and <i>p</i> -Values							
	F	FA	FSP	FSU	FW	M	MSP	MSU
FA	0.006*	—	—	—	—	—	—	—
FSP	<0.001*	<0.001*	—	—	—	—	—	—
FSU	0.010*	0.753	<0.001*	—	—	—	—	—
FW	<0.001*	<0.001*	0.887	<0.001*	—	—	—	—
M	0.005*	0.904	<0.001*	0.665	<0.001*	—	—	—
MSP	<0.001*	<0.001*	0.534	<0.001*	0.627	<0.001*	—	—
MSU	0.003*	0.816	<0.001*	0.591	<0.001*	0.913	<0.001*	—
MW	<0.001*	<0.001*	0.5344	<0.001*	0.6275	<0.001*	0.999	<0.001*

Note: *p*-level of statistical significance.

Abbreviations: F, females; FA, females collected in autumn; FSP, females collected in spring; FSU, females collected in summer; FW, females collected in winter; M, males; MA, males collected in autumn; MSP, males collected in spring; MSU, males collected in summer; MW, males collected in winter.

*Statistically significant.

was consistently observed across comparisons between different guilds (Figures 4 and 5 and Tables 3 and 4).

3.6. Confirmation of Pathogen Presence Using Conventional PCR. Utilizing conventional PCR techniques, specific genetic targets were amplified to confirm the presence of selected pathogenic species. Amplification of the 18S rRNA gene generated fragments of 1258 and 1373 bp, indicating the presence of *Babesia occultans* (accession numbers: P809771 and PP809772) in two out of nine samples tested. For *Rickettsia* species, PCR assays targeting the *gltA* and *ompB* genes produced amplicons of 282, 380, 173, and 169 bp, respectively. These results confirmed two distinct *Rickettsia* sequences in 2 out of 15 samples tested (PP828624 (282 bp) and PP828625 (380 bp)). Further analysis specifically identified *Rickettsia sibirica* (PP828626 (173 bp)) and *Rickettsia africae* (PP828627 (169 bp)) in 2 out of 18 samples tested. Additionally, two samples tested positive for *E. tularensis* subsp. *holarctica* via PCR in two out of two samples tested. However, sequencing of these PCR products was not attempted. The utilization of species-specific primers in these PCR assays ensured accurate identification of the target pathogens, thereby, enhancing the reliability and robustness of the study's findings.

4. Discussion

Traditional TBP detection methods in North Africa, like PCR and real-time PCR, are limited to identifying single pathogens [43]. Recent studies emphasize the importance of coinfections in pathogen transmission and disease severity [45–47]. This study utilizes microfluidic PCR and network analysis to examine interactions among 43 microorganisms in *Hyalomma* ticks infesting cattle in Algeria's steppe region. This innovative approach reveals the prevalence and diversity of pathogens while highlighting the complex dynamics of coinfections, providing crucial insights into pathogen community structures and their influence on disease transmission in North Africa.

One of the key findings of this study is the significant difference in pathogen prevalence and coinfection patterns between male and female ticks. These variations are likely influenced by several factors. Female ticks, which typically have longer feeding periods and consume larger blood meals compared to males, face increased exposure to pathogens [48]. Krawczyk et al. [22] suggest that this extended feeding duration, coupled with physiological differences like hormonal variations, enhances females' susceptibility to infections, such as *B. burgdorferi* and increases their likelihood of harboring multiple pathogens. Hormones like ecdysteroids and juvenile hormones, which vary between sexes, are believed to modulate immune responses and pathogen susceptibility in arthropods [48, 49]. Additionally, these physiological differences may alter the tick microbiome, potentially impacting pathogen colonization and persistence [50].

The presence of unique pathogens in female (e.g., *B. afzelii*, *B. spielmanii*, *Hepatozoon*, and *Mycoplasma*) and male (e.g., *Ehrlichia*) ticks suggests sex-specific ecological niches and behaviors that influence pathogen acquisition and transmission. These observations are consistent with findings from

studies by Treuren et al. [51] and Benyedem et al. [52], which highlighted sex-specific differences in bacterial communities within ticks.

Ecological factors are crucial in the epidemiology of zoonotic diseases [53]. Climate change, marked by increased heat waves, heavy rains, and droughts, alters environmental conditions [54], affecting animal distribution and, in turn, the biology and redistribution of ticks [29]. As ticks expand, the pathogens they carry follow [2]. Tick life cycles, primarily driven by heat, rely on favorable conditions like humidity and host availability to support egg development and larval metamorphosis [55]. High temperatures can also accelerate pathogen replication, as seen with *Theileria parva*, which causes East Coast fever in cattle, while reducing transmission time in infected ticks [28]. In North Africa, *Hyalomma excavatum* is active year-round, with developmental rates peaking during warmer months [56]. This tick follows either a two- or three-host life cycle depending on host availability, adding complexity to its seasonal development [13]. Larvae and nymphs may feed on different hosts or the same one before molting into adults, creating a fluctuating landscape for pathogen transmission [36]. Seasonal peaks in tick activity often coincide with higher pathogen presence in large mammals, particularly in summer when adult ticks are most active [57].

Moreover, this study underscores the crucial role of FLEs in supporting pathogen coexistence in *Hyalomma* ticks, particularly with *Rickettsia*. FLE enhance the stability of tick microbial communities, promoting coinfections and pathogen persistence. This aligns with findings from Kumar et al. [58], who highlighted the competitive advantage of FLE over ancient endosymbionts in *Amblyomma americanum*, suggesting their ecological dominance. Azagi et al. [59] also found that imported *Hyalomma* ticks may exhibit different endosymbiont–pathogen relationships, indicating that geographical factors influence disease transmission dynamics. The evolutionary link between FLE and pathogens is further supported by Gerhart, Moses, and Raghavan [60], who showed that a FLE evolved from a mammalian pathogen, emphasizing its role in pathogen interactions. Additionally, Sesmero-García, Cabanero-Navalon, and Garcia-Bustos [61] discuss how climate change could enhance FLE's role in disease transmission, as they may help *Hyalomma* ticks adapt to changing environments.

Hussain et al. [62] propose that targeting FLE could serve as an effective tick management strategy by disrupting their symbiotic relationships, thereby, reducing tick fitness and pathogen transmission. Developing anti-microbiota vaccines to target FLE presents a promising strategy to influence tick microbiota and reduce pathogen transmission. For example, vaccination of mice against a commensal *Escherichia* in *Ixodes ricinus* altered the tick microbiota [63], leading to decreased levels of *B. afzelii* [64]. Similarly, vaccination of alpha-gal knockout mice with the same commensal decreased tick survival [65]. Additionally, microbiota-driven vaccination in soft ticks, such as *Ornithodoros moubata*, has demonstrated implications for survival, fitness, and reproductive capabilities [66]. In another study, vaccination of

birds against a commensal in *Culex quinquefasciatus* effectively reduced *Plasmodium* colonization in the mosquito [67]. These results support the concept that vector microbiota manipulation by host antibodies can be utilized as a strategy to develop transmission-blocking vaccines [68].

The observed coinfections reveal important insights into disease dynamics, particularly the positive associations between pathogens like *Rickettsia conorii* and *R. slovaca* in male ticks and *B. afzelii* and *Borrelia spielmanii* in female ticks, indicating a lack of competition. Moutailler et al. [47] found a strong association between *Borrelia garinii* and *B. afzelii*, suggesting that biological interactions may promote their coinfection. Similarly, *R. conorii* and *R. slovaca* have been found to coexist without competition, as noted by Torina et al. [69]. These interactions may contribute to more complex infection patterns, influencing the epidemiology of tick-borne diseases.

Pathogens can cooperate by producing shared resources, or “common goods,” essential for their collective growth and survival. In bacterial communities, for instance, siderophores are produced to capture iron from the environment, a critical element for bacterial growth. These siderophores benefit multiple strains within the population, enhancing the overall fitness and survival of the community [70, 71]. Additionally, such cooperative behaviors are often regulated by quorum sensing, where bacteria use chemical signals to coordinate the production of these shared resources, further demonstrating the intricate cooperation among pathogens [72].

In contrast, strong negative associations between pathogens like *Anaplasma phagocytophilum* and *F. tularensis* suggest mutual exclusion. Competition among parasites within a host can lead to varied evolutionary outcomes, driven by different mechanisms [34]. Exploitation competition occurs when parasites compete for the host's limited resources by occupying overlapping ecological niches, intensifying during coinfections [73, 74]. Apparent competition, on the other hand, arises from cross-reactive immune responses, where the host's nonspecific defenses affect the abundance and success of different parasites [75, 76]. Last, interference competition involves direct suppression, where parasites actively inhibit their rivals through chemical or mechanical means [16, 77]. These competitive interactions may limit the co-occurrence of certain pathogens, impacting disease prevalence and influencing control strategies.

Interactions between pathogens in multi-infections significantly influence the evolution of virulence. Pathogens may compete for resources or cooperate to enhance survival and share resources. The observed sex-specific and seasonal variations in these interactions provide important insights into tick-borne disease dynamics. These findings highlight the need to consider both biotic and abiotic factors when developing control strategies. By combining molecular techniques with ecological and epidemiological approaches, this study enhances the understanding of TBPs and improves predictions and management strategies for their spread, leading to more effective public health interventions. While the study provides valuable insights, its findings may be constrained by the limited sample size, focus on specific tick species, and potential geographical biases. These limitations

should be considered when interpreting the results and applying them to broader ecological or epidemiological contexts.

5. Conclusion

This study provides valuable insights into the complex interactions between TBPs within *Hyalomma excavatum* tick populations. The significant differences in pathogen prevalence and interactions between male and female ticks, along with seasonal variations, underscore the multifaceted nature of tick-borne disease ecology. These findings emphasize the need for sex-specific and seasonally tailored approaches in disease surveillance and control.

Future research should prioritize the development of targeted disease control strategies that consider seasonal and sex-based differences in tick behavior and pathogen interactions, allowing for more tailored and effective management practices. Integrating molecular diagnostics with ecological and network analyses will further advance our understanding of pathogen dynamics and support the design of innovative control strategies. One promising approach is the development of anti-microbiota vaccines, which aim to disrupt key microbial communities within ticks. By destabilizing tick microbiomes, these vaccines could reduce pathogen transmission and serve as a valuable complement to existing control measures. Emphasizing these research directions will strengthen our capacity to address the complexities of tick-borne diseases across varied ecological settings.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

Ethics Statement

All procedures performed in this study are in accordance with the ethical standards of the institutional and/or national research committee. We further declare that no animal was harmed during this study.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Alejandro Cabezas-Cruz, Salma Kaoutar Abdelali, and Lynda Aissaoui: conceptualization. Salma Kaoutar Abdelali, Lourdes Mateos-Hernández, Angélique Foucault-Simonin, and Clemence Galon: investigation. Salma Kaoutar Abdelali, Apolline Maitre, Elianne Piloto-Sardiñas, and Zbigniew Zając: formal analysis. Lourdes Mateos-Hernández and Angélique Foucault-Simonin: data Curation. Apolline Maitre, Constance Julie, Sara Moutailler, and Dasiel Obregon: methodology.

Salma Kaoutar Abdelali and Zbigniew Zając: visualization. Alejandro Cabezas-Cruz: supervision. Sara Moutailler and Alejandro Cabezas-Cruz: resources. Salma Kaoutar Abdelali, Elianne Piloto-Sardiñas, and Alejandro Cabezas-Cruz: writing—original draft. All authors contributed to writing—review and editing.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. Table S1: List of primer/probe sets used in the BioMark real-time PCR system.

Supporting Information 2. Table S2: Primer pairs and PCR conditions for selected pathogens and tick species confirmation.

Supporting Information 3. Table S3: Tick-borne microorganisms identified in TBPGs associated with *Hyalomma excavatum*.

Supporting Information 4. File S1: R script detailing the calculation of Yule's Q and the construction of the co-occurrence network.

Supporting Information 5. Table S4: Centrality measures of pathogens in TBPGs networks.

Supporting Information 6. Table S5: Centrality measures of shared pathogens across TBPGs networks.

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