Characterization of the microbiome of human-relevant ticks and optimization of diagnostics of tick-associated pathogens

DISSERTATION

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Man muss an seine Berufung glauben und
alles daransetzen, sein Ziel zu erreichen.
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Abstract

Ixodes ricinus, the most common tick species in Germany, carries a diverse microbiome. The microbiome comprises a diverse microbial community, including bacteria, archaea, eukaryotes, and viruses. During the act of sucking on the human host, depending on the duration, potential or already proven tick-associated pathogens (TBPs) can be transmitted in addition to innocuous microorganisms. Thus, ticks pose a major health threat to humans. Thus, a better understanding of the composition of the tick microbiome and their interaction patterns and how biotic and abiotic environmental factors influence them is of enormous importance as it may affect the analytical outcome of TBPs. The newly gained knowledge should thus optimize the diagnosis of TBPs, allowing a better assessment of the risk of a potential disease. In this work, the bacterial microbiome of 1022 ticks was investigated.

Lyme borreliosis (LB) is caused by human pathogenic species of the *Borrelia burgdorferi* sensu lato (s. l.) complex (Bb). Therefore, the bacterial composition of the microbiome in Bb-positive (n = 82) and Bb-negative (n = 118) tick microbiomes was compared using 16S rRNA gene amplicon sequencing. Here, I investigated whether *Borrelia* spp. only occurs in a specific tick microbiome composition and whether the interaction patterns with other bacteria were affected. In addition, the influence of location on the composition of the tick microbiome was investigated for the two German locations Weiden in der Oberpfalz (Bavaria) and Esslingen (Baden-Württemberg). The presence of *Borrelia* altered the abundance of *Candidatus* Midichloria, *Rickettsia*, *Pseudomonas*, *Staphylococcus*, and *Neoehrlichia* and their topological roles in the tick microbiome. However, the location was less important for the composition of the tick microbiome but significantly shifted the abundance of *Pseudomonas* ($p = 2.07 \times 10^{-2}$) and *Wolbachia* ($p = 1.01 \times 10^{-3}$) and the topological role of bacterial members in microbial network analyses.

Since the relative abundances of potentially human pathogenic members of the order Rickettsiales differed between Bb-positive and Bb-negative members, the co-occurrence of Bb and members of the order Rickettsiales (*Rickettsia* spp., *Anaplasma phagocytophilum*, *Wolbachia pipientis*, and/or *Neoehrlichia mikurensis*) in the tick microbiome (n = 760) was further investigated. In addition, other factors, such as the location of the tick and the season in which the tick was collected, were added. While the occurrence of *Rickettsia* spp. (16.7%, n = 127) and

W. pipientis (15.9%, n = 121) was similar, *A. phagocytophilum* was found in only 2.8% (n = 21), and *N. mikurensis* in only 0.1% (n = 1) of all ticks. Bb was most common when co-occurring with *Rickettsia* spp. or *W. pipientis*, and the combination of all three species was also found. The results of *gltA* gene sequencing, which encodes citrate synthase and is suitable for species differentiation of *Rickettsia* spp. indicated that *Rickettsia helvetica* dominated the tick microbiomes. In addition, *Rickettsia monacensis* and *Rickettsia raoultii* correlated with autumn and southern location, respectively, and a negative Bb finding. Moreover, using Fisher's exact test, *Rickettsia aeschlimannii* correlated with a Bb-positive microbiome.

To determine the influence of the tick surface microbiome on the analytical result, decontamination of the tick surface (n = 62) was performed. Subsequent 16S rRNA gene amplicon sequencing demonstrated that ticks treated with 5% sodium hypochlorite had the lowest number of artificially added contaminants, followed by DNA Away, Reactive Skin Decontamination Lotion (RSDL), and 70% ethanol. In addition, the microbiomes of the ticks after decontamination with 5% sodium hypochlorite were comparable to those of the negative controls, which is why the efficiency of decontamination with 5% sodium hypochlorite was optimal.

The data basis of a combined diagnostic approach of qPCR and amplicon sequencing will allow valid decisions for adequate treatment of human pathogenic Bb species and other coincident pathogens, as they were site-specific. However, data such as species, sex, or stage of the tick should also be included in this process. Such data will allow the establishment of exclusion criteria for (potential) TBPs, which will subsequently facilitate the evaluation of critical test results with a very high probability. Furthermore, future testing should be performed with prior decontamination to prevent artificial bias of the microbial communities in a tick.

Zusammenfassung

Ixodes ricinus, die in Deutschland am häufigsten vorkommende Zeckenart, trägt ein vielfältiges Mikrobiom in sich. Das Mikrobiom umfasst eine vielfältige mikrobielle Gemeinschaft, darunter Bakterien, Archaeen, Eukaryoten und Viren zusammen. Während des Saugakts am menschlichen Wirt können je nach Dauer, neben ungefährlichen Mikroorganismen, auch potentielle oder bereits erwiesene zeckenassoziierte Krankheitserreger (TBPs) übertragen werden. Somit stellen Zecken eine große gesundheitliche Bedrohung für den Menschen dar. Ein besseres Verständnis der Zusammensetzung des Zeckenmikrobioms und deren Interaktionsmuster, sowie deren Beeinflussung durch biotischen und abiotische Umweltfaktoren ist somit von enormer Wichtigkeit, da es das Analyseergebnis von TBPs beeinflussen kann. Die neu gewonnenen Erkenntnisse sollten daher die Diagnose von TBPs optimieren und eine bessere Einschätzung des Risikos einer möglichen Erkrankung zulassen.

Im Rahmen dieser Arbeit wurde das bakterielle Mikrobiom von insgesamt 1022 Zecken untersucht.

Die Lyme-Borreliose wird durch humanpathogenen Spezies des *Borrelia burgdorferi* sensu lato (s. l.) Komplexes (Bb) verursacht. Deswegen wurde die bakterielle Zusammensetzung des Mikrobioms bei Bb-positiven (n = 82) und Bb-negativen (n = 118) Zeckenmikrobiomen mittels 16S rRNA Gen Amplikonsequenzierung verglichen. Hierbei wurde untersucht, ob Borrelien nur in einer bestimmten Zusammensetzung des Zeckenmikrobioms vorkommen und ob die Interaktionsmuster mit anderen Bakterien davon betroffen sind. Darüber hinaus wurde der Einfluss des Standorts auf die Zusammensetzung des Zeckenmikrobioms für die zwei deutsche Standorte Weiden in der Oberpfalz (Bayern) und Esslingen (Baden-Württemberg) untersucht. Die Anwesenheit von *Borrelia* veränderte die Häufigkeit von *Candidatus* Midichloria, *Rickettsia*, *Pseudomonas*, *Staphylococcus* und *Neoehrlichia* sowie deren topologische Rolle im Zeckenmikrobiom. Der Standort war jedoch weniger wichtig für die Zusammensetzung des Zeckenmikrobioms, verschob jedoch die Häufigkeit von *Pseudomonas* ($p = 2.07 \times 10^{-2}$) und *Wolbachia* ($p = 1.01 \times 10^{-3}$) sowie die topologische Rolle der bakteriellen Mitglieder in mikrobiellen Netzwerkanalysen erheblich.

Da sich die relativen Häufigkeiten von potenziell humanpathogenen Vertretern der Ordnung Rickettsiales zwischen Bb-positiven und Bb-negativen unterschied, wurde im weiteren Verlauf der Arbeit das gemeinsame Vorkommen von Bb und Mitgliedern der Ordnung Rickettsiales (*Rickettsia* spp., *Anaplasma phagocytophilum*, *Wolbachia pipientis* und/oder *Neoehrlichia mikurensis*) im Zeckenmikrobiom (n = 760) untersucht. Zusätzlich wurden weitere Faktoren, wie Standort der Zecke und Jahreszeit, in welcher die Zecke gesammelt wurde, berücksichtigt. Während das Vorkommen von *Rickettsia* spp. (16.7 %, n = 127) und *W. pipientis* (15.9 %, n = 121) ähnlich war, wurde *A. phagocytophilum* in nur 2.8 % (n = 21) und *N. mikurensis* in nur 0.1 % (n = 1) aller Zecken gefunden. Beim gemeinsamen Auftreten war Bb mit *Rickettsia* spp. oder *W. pipientis* am häufigsten, und auch die Kombination aller drei Arten wurde gefunden. Die Ergebnisse der *gltA*-Gen-Sequenzierung, welches für die Citrat-Synthase kodiert und sich zur Speziendifferenzierung von Rickettsia spp. eignet, zeigten, dass die Zecken von Rickettsia helvetica dominiert wurden. Außerdem korrelierte das Vorhandensein von *Rickettsia monacensis* und *Rickettsia raoultii* mit dem Herbst bzw. dem südlichen Gebiet und einem negativen Bb-Befund. Darüber hinaus korrelierte das Vorhandensein von R. aeschlimannii mit einem Bb-positiven Mikrobiom mittels exaktem Fisher-Test.

Um den Einfluss des Oberflächenmikrobioms der Zecke auf das Analyseergebnis zu bestimmen wurde eine Dekontamination der Zeckenoberfläche (n = 62) durchgeführt. Durch eine anschließende 16S rRNA Gen Amplikonsequenzierung wurde nachgewiesen, dass Zecken, die mit 5% Natriumhypochlorit behandelt wurden, die geringste Anzahl von künstlich hinzugefügten Kontaminanten aufwies, gefolgt von DNA Away, Reactive Skin Decontamination Lotion (RSDL) und 70% Ethanol. Außerdem waren die Mikrobiome der Zecken nach der Dekontamination mit 5% Natriumhypochlorit mit denen der Negativkontrollen vergleichbar, weswegen die Effizienz der Dekontamination mit 5% Natriumhypochlorit optimal war.

Die Datengrundlage eines kombinierten diagnostischen Ansatzes aus qPCR und Amplikonsequenzierung wird valide Entscheidungen für eine adäquate Behandlung humanpathogener Bb-Spezies und weiterer koinzidenter Erreger ermöglichen, da sie standortspezifisch waren. Allerdings sollte hierbei auch Daten wie Spezies, Geschlecht oder Stadium der Zecke hinzugezogen werden, da so Ausschlusskriterien für (potenzielle) TBPs

erstellt werden können, was anschließend die Auswertung kritischer Testergebnisse mit sehr hoher Wahrscheinlichkeit vereinfacht. Des Weiteren sollten zukünftige Untersuchung mit vorheriger Dekontamination durchgeführt werden, um einer künstlichen Verzerrung der mikrobiellen Gemeinschaften in der Zecke vorzubeugen.

List of abbreviation

ACE	abundance-based coverage estimator
ASA	amplicon sequencing approaches
Bb	*Borrelia burgdorferi* sensu lato complex
bp	base pair
Ca.	*Candidatus*
CSF	cerebrospinal fluid
Ct	cycle threshold
cDNA	complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
gDNA	genomic Deoxyribonucleic acid
HGA	human granulocytic anaplasmosis
IFAT	immunofluorescence antibody test
LB	Lyme borreliosis
LE	like-endosymbiont
MLST	multilocus sequence typing
MVZ	Medical Care Centre
NaOCl	sodium hypochlorite
NGS	Next Generation Sequencing
osp	outer surface protein
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
qPCR	quantitative real-time PCR
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
RSDL	reactive skin decontamination lotion
RT-PCR	reverse transcriptase PCR
S	Svedberg
s. l.	sensu lato

s. s.	sensu stricto
SSU	small subunit
TBPs	tick-borne pathogens

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1 Introduction

1.1 Vectors as carriers of disease

In order to transmit pathogens from one host to another, a vector is required, representing a living organism [1]. A distinction is made between biological and mechanical vectors. The host is only externally contaminated with the pathogen in mechanical vectors, and infection occurs through smear infection. The condition for successful transmission is that the pathogen is insensitive to air [2]. Biological vectors are mammals like humans, rats, dromedaries, horseshoe bats, or arthropods, such as fleas, lice, mosquitoes, or ticks. However, both types of vectors do not themselves cause disease and vary depending on location, climatic conditions, season, or pathogen load [2,3].

Diverse arthropods take one or more blood meals (hematophagy) from vertebrates of all sizes and species during their life cycle, while they are efficient vectors of disease [4]. As a result, they acquire many viral, bacterial, and eukaryotic pathogens in addition to other (beneficial for respective vector) microorganisms and transfer them to a new host during the next blood meal [4,5]. For example, fleas transmit plague (*Yersinia pestis*), mosquitoes transmit malaria (*Plasmodium* sp.), and ticks transmit early summer meningoencephalitis (arboviruses) [6,7].

Since various arthropods play an essential role as parasites of humans and animals and vectors, vectors (both types) represent a high health risk for humans and animals worldwide [6].

1.1.1 Taxonomy and geographical distribution of ticks

Ticks are taxonomically assigned to the class Arachnida and the order Ixodida. Within this order, the three families differ based on their morphology, habitat, and developmental cycle [8,9]. However, they all have in common that they feed on the blood of different vertebrates [10]. Except for the family *Nuttalliellidae* [11,12], which consists of only one species (*Nuttalliella namaqua*), the remaining approximately 900 classified tick species can be divided into the two large families *Argasidae* (soft ticks) consisting of 193 [13] and *Ixodidae* (hard ticks) with 707 species [8]. While the taxonomy of the *Ixodidae* has been sufficiently researched, the taxonomic

classification of the tick species in the family *Argasidae* is challenging as there are still no standardized morphological descriptions. Here, scientists still have elementary differences of opinion[8].

Tick species of both families occur worldwide. For example, ticks of the genera *Argas, Ornithodoros, Otobius, Amblyomma, Anomalohimalaya, Haemaphysalis, Hyalomma, Ixodes, Dermacentor*, or *Rhipicephalus* are found in part of North America [14], in Southeast Asia [15], in China [16], in Africa [17,18], in Turkey [19], in Italy [20], in France [21], and Germany [22]. In Germany, up to 21 species of ticks are present, see **Table 1**.

Table 1. Natural occurrence of 21 tick species in Germany [22]

Family	Genera	Species
Argasidae	*Argas*	*A. reflexus*
	Carios	*C. vespertilionis*
Ixodidae	*Ixodes*	*I. ricinus*
		I. hexagonus
		I. frontalis
		I. inopinatus
		I. canisuga
		I. trianguliceps
		I. arboricola
		I. rugicollis
		I. vespertilionis
		I. lividus
		I. acuminatus
		I. apronophorus
		I. ariadnae
		I. simplex
		I. uriae
	Dermacentor	*D. reticulatus*
		D. marginatus
	Haemaphysalis	*H.concinna*
		H. punctata

Furthermore, species of the genera *Hyalomma* (*H. marginatum* and *H. rufipes*) and *Rhipicephalus* (*R. sanguineus s.l.*) are repeatedly found. However, their occurrence can be

explained by migratory birds and dogs, respectively [22]. Nevertheless, *I. ricinus* remains the most widespread species in Germany [22–24]; and plays a central role in transmitting pathogens [25]; therefore, the following work focuses on *I. ricinus*.

The habitat of *I. ricinus* is located between the ground covered by foliage and on grasses and shrubs up to a maximum height of 1.5 meters. In addition, the tick always requires moderate temperatures and high humidity of about 80%. Thus, all deciduous and mixed forests, park and garden locations with hedges, and recreational locations provide optimal habitats for *I. ricinus* [26,27].

The average tick activity in Germany is from March to October. After the winter months, at temperatures above 4 °C, the winter torpor of *I. ricinus* ends, and the tick begins to search for hosts. Its activity ends when the temperature drops below 5°C. Consequently, there may be a change in seasonal activity due to climate. Furthermore, there is usually a brief dip in activity during the dry and hot summer months of July and August [28,29].

1.1.2 Development cycle and morphology of *I. ricinus*

Hard ticks of the genus *Ixodes* have three stages of development (**Figure 1**). This cycle begins with the larva, which hatches from the egg, develops into a nymph in the second stage, and finally into an adult, where the female needs a last blood meal to lay eggs again [8,30]. The ticks acquire a blood meal for each development step associated with a moulting process[31]. In this process, *I. ricinus* follows an exophilic behaviour, in contrast to ticks of the family *Argasidae* and individual species of the family *Ixodidae*. Thus, *I. ricinus* waits outside the burrow of its host for a suitable host, exposing the tick to all weather conditions [8]. The size and type of its hosts depend on the developmental cycle of *I. ricinus* (**Figure 1**). Thus, the host spectrum ranges from mice, birds, hedgehogs, and other small rodents to foxes, cats, dogs, deer, and horses. Thus, humans are not classic tick hosts but are also called dead-end hosts [32–34].

For host detection, *I. ricinus* uses its Haller's organ, located on the respective ends of the tick's anterior pairs of legs, carries specific chemoreceptors, and is present from the larval stage [35]. This organ can detect chemical compounds such as carbon dioxide, hydrogen sulfide, and

scents to locate potential hosts. Furthermore, they also use sensory stimuli, such as vibration and body heat, to locate hosts [36].

On the host, *I. ricinus* usually moves to warm and moist locations on the host's body. It then tears the host's skin with its chelicerae and pushes or stabs into the resulting wound with its hypostome (proboscis). During this process, *I. ricinus* releases saliva containing various proteins from the salivary gland into the wound [37]. On the one hand, this secretion helps prevent agglutination (clumping) of the blood platelets or blood clotting, thus, wound closure. On the other hand, it reduces or avoids the host's immune response[7,30]. During the sucking act, the absorbed blood is thickened via water extraction [31,38]. Therefore, the tick can absorb up to several milliliters of blood [31]. Thus, *I. ricinus* can now absorb blood from the host for several days. The water and other non-digestible components are returned to the host by regurgitation (reflux of fluids) [31,38]. Consequently, blood concentration and regurgitation transmit pathogens from *I. ricinus* to the host.

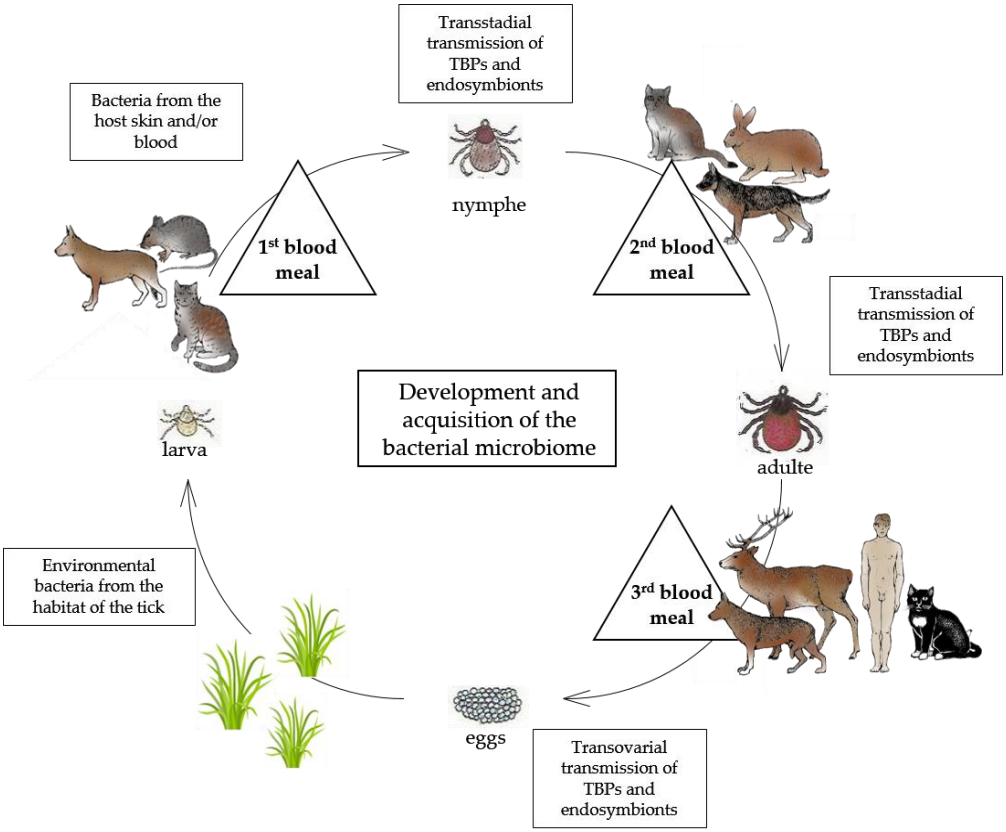

Figure 1. Life cycle of three-host tick *I. ricinus*. Each stage, i.e., larva, nymph, and adult, feeds on a (different) vertebrate host. Additionally, bacteria can be transmitted via transstadial or transovarial or acquired through the environment [39–41].

1.1.3 Bacterial microbiome of *I. ricinus*

Lederberg coined the term microbiome to describe the human body with an assemblage of microorganisms that colonize a wide variety of niches in the body [42,43]. The tick microbiome also consists of a diverse community of microorganisms, including bacteria, archaea, eukaryotes, and viruses [43]. These interact within the tick and with each other commensally, symbiotically, or mutualistically [44].

Depending on the study method, up to 133 different genera can be identified in the bacterial microbiome of *I. ricinus* [45]. The phylum *Proteobacteria* accounts for the majority, followed by the phyla *Actinobacteria*, *Spirochaetes*, *Firmicutes*, and *Bacteroidetes*. The phyla *Planctomycetes*, *Chloroflexi*, *Acidobacteria*, *Cyanobacteria*, *Deferribacteres*, and *Elusimicrobia*, were also observed, but in a much smaller proportion [43,44,46–48].

Pathogenic microorganisms, so-called tick-borne pathogens (TBPs), are present in the bacterial microbiome. Therefore, the bacterial microbiome is the focus of this work and will be denoted by the term "microbiome" in the following text. In addition, endosymbiotic bacteria, environmentally and skin-associated bacteria, and organ-associated bacteria are also found [44,49].

To infect the tick microbiome, TBPs, and endosymbiotic bacteria use various life strategies. Transmission can occur horizontally, where microorganisms are acquired through a tick bite or blood ingestion, or vertically, where microorganisms are acquired transovarially (maternally inherited) or transstadially, respectively [49]. Diverse microorganisms from the environment or the surface of hosts that colonize the tick internally and externally rarely become valid members of the tick microbiota [43].

A high phylogenetic similarity was found between transovarially transmitted endosymbionts or non-pathogenic microorganisms (*Coxiella*-like, *Rickettsia*-like, or *Francisella*-like) and TBPs as part of the tick microbiome [43,49]. Therefore, an origin development from a vertebrate pathogen acquired by a blood meal on an infected vertebrate can be suspected. Narasimhan and Fikrig (2015) describe two possible types of development for TBPs. While the pathogen adapted to the tick itself and its environment in one case, in the second case, it adapted to the tick and the vertebrate host and became virulent. On the other hand, there is

still the possibility that already existing commensal endosymbionts of the tick have evolved into pathogens for other hosts or ticks as hosts. Thus, endosymbionts harmless to the vertebrate host must be considered potentially pathogenic for other vertebrate hosts [43].

Compared to TBPs, endosymbionts are poorly studied. However, endosymbionts have become increasingly important in recent years, especially since they were transmitted to the host like TBPs and can act similarly to known TBPs [49].

Within *I. ricinus*, six different genera of maternally inherited endosymbionts co-exist. These include Candidatus (Ca.) *Midichloria*-like endosymbionts (Ca. *Midichloria*-LE), *Rickettsia*, *Spiroplasma*, *Coxiella*-like endosymbionts (*Coxiella*-LE), *Wolbachia*, and *Rickettsiella* [49]. However, many uncertainties are still present regarding the functions of certain endosymbionts, so only a few symbionts are known to be harmful or beneficial to the tick [4,49]. However, Ca. *Midichloria*-LE is known to play a role as a food symbiont in female *I. ricinus*, for example [50], as a supporter during tick moulting [4] or for egg-laying [51]. Concerning *Spiroplasma*, it is known from other insect species that *Spiroplasma* spp. produce sex ratio disruption by killing male *I. ricinus* [52]. In this context, recent findings by Lejal et al. (2019) and Aivelo et al. (2020) indicate the upregulation of *Spiroplasma* spp. represents a defense mechanism against TBPs in ticks [53,54]. In addition, symbionts of *Wolbachia* spp. and *Arsenophonus* spp. are found in *I. ricinus*, which are suspected of negatively affecting tick reproduction and fitness [55,56] or, like *Spiroplasma* spp. lead to disruption of sex ratios in ticks [43].

Furthermore, the diversity of the tick microbiome can be influenced by many other factors, such as tick species, season, the location where the tick was collected, availability of hosts and vegetation, stage of the tick, or nutritional status [43,49,57]. More detailed information on the influence of different abiotic and biotic factors on the tick microbiome can be found in chapter 1.3.4.

1.2 Tick-borne pathogenes and tick-borne diseases caused by *I. ricinus*

Various tick-associated pathogens are found worldwide. They differ according to tick species, region, reservoir, and host. The pathogenic bacterial species most frequently

transmitted by I. *ricinus* in Germany belong to the *Borrelia burgdorferi* sensu lato (s. l.) complex (Bb). For the sake of completeness, it should be mentioned here that viral as well as eukaryotic pathogens have also been found in the microbiome of *I. ricinus*. These include the tick-borne encephalitis virus (TBEV) or protozoa of the genus *Babesia* spp. [44], but are out of the focus of this thesis.

The diversity of bacterial pathogens in *I. ricinus* can be considered high since, on the one hand, pathogens can be found that cause only previously less studied infections, such as babesiosis (pathogen: *e.g.*, *Babesia divergens*) or tularemia (pathogen: *Francisella tularensis*) [24,58]. On the other hand, *I. ricinus* can also transmit relatively rare pathogens, such as *Coxiella burnetii* (disease: Q fever), *Ehrlichia canis* (disease: ehrlichiosis), or *Rickettsia conorii* (disease: Mediterranean spotted fever) [22,58].

The high diversity of different pathogens suggests that all the pathogens have evolved similar mechanisms to favour transmission to the vector or host and multiplication within the vector or host [4].

1.2.1 Tick-Host-Pathogen circulation of *I. ricinus*

Blood digestion begins from several weeks to months during the sucking action on the host. Pathogens pass through the midgut barrier during this process, possibly by using specific surface receptors [4]. They can enter the bloodstream through digestion and thus infect the tick's tissue cells. The tick then falls off its host, moults, and searches for a new host [8]. The frequency with which the tick feeds on a different host during its next blood-sucking act depends on two factors [25], which can be summarized as a tick-host relationship.

The first factor represents the host range and, thus, the suitability as a host for the tick. Compared to *I. hexagonus* or *I. canisuga*, *I. ricinus* has vast access to different hosts (see 1.1.2) [59]. The second factor represents the availability or abundance of suitable hosts within the habitat of *I. ricinus* [4,25]. As a result, the host range and the availability of *I. ricinus* impacts pathogen transmission [25]. Because of the wide host range, *I. ricinus* has broader accessibility to different pathogens [59]. However, this is countered by the fact that ticks of the family *Ixodes*

often have less host contact than ticks of the family *Argasidae* because they require fewer blood meals, which leads to a potentially lower frequency of pathogen transmission [4].

Besides these two factors, the relationship between tick and pathogen or tick and host influences pathogens' acquisition, preservation, and transmission. For the tick-pathogen relationship, the innate immune response of the tick, which the pathogens have to bypass, the ability of the pathogens to colonize the midgut and thus evade its protective barrier to subsequently infect the salivary glands and/or ovaries of the tick, and the vector competence of the tick for a pathogen play a role. In contrast, for the host-pathogen relationship, the reservoir capacity of the host, the general susceptibility to a pathogen, and the duration and level of infection of the host with a pathogen matter [4,26]. Consequently, each host and tick can acquire, preserve, and transmit pathogens to varying degrees, continuing the tick-host pathogen cycle.

1.2.2 Occurrence and distribution of tick-borne pathogens

Since *I. ricinus* is distributed nationwide [22], the pathogens associated with it as a vector are also found nationwide. Nevertheless, the occurrence of the respective pathogens seems to depend on diverse factors, as the occurrence in the tick microbiome can vary strongly depending on the region [32,58]. For example, the occurrence of the Bb varies between 9 - 36.2%, of *A. phagocytophilum* between 2.9 - 9.0%, of *Neoehrlichia mikurensis* (*N. mikurensis*) between 8. 1 - 26.6%, and of *Rickettsia* spp. between 1.0 - 47.2% in questing *I. ricinus* depending on the city or region and the tick's corresponding habitat (forest, park, recreation location) [26,60].

There may be several reasons for the fluctuating occurrence. First, the spread, as well as density, of *I. ricinus* continues to increase. In addition, the distances between humans and wild animals are becoming smaller and smaller due to urbanization and increasing recreational activities in nature. Furthermore, suitable reservoir hosts, as well as a certain host density, have to be present for the successful transmission of TBPs to occur. In addition, TBPs are becoming better and more frequently detected due to advances in diagnostics [32,58,61].

1.2.3 Occurrence and risk of co-infection with tick-borne pathogens

I. ricinus exhibits high bacterial diversity in the microbiome due to its flexibility in host selection, which can harbour a variety of TBPs as a reservoir (see 1.1.3). This increases the risk of simultaneous transmission of different TBPs, as well as the risk of co-infection in humans from a tick bite [62,63].

In France, Poland, and Denmark, co-infections with a 3.2 - 45% prevalence rate have already been diagnosed by molecular biological approaches in questing *I. ricinus* [64–67]. However, multiple infections, i.e., infections with two or more pathogens from different genera [65], or mixed infections, i.e., infections with two or more pathogens from the same genus [65], have also been detected in questing *I. ricinus* in several studies in Germany. Tappe et al. (2014) detected double infections with *Borrelia* spp. and *Rickettsia* spp. in 7.3% [68], May et al. (2015) in 22.9% [69], and Schicht et al. (2012) in 10.7% [70] of the ticks examined. However, mixed infections with TBPs from the order Rickettsiales occurred less frequently (e.g., *A. phagocytophilum* and *Rickettsia* spp.: 0.6 - 2.8%). Furthermore, there is also a risk of triple or quadruple infections in Germany, confirming the broad vector competence of *I. ricinus* for tick-associated pathogens [70]. Infection rates with *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* between 0.1 - 1.3% have also been described in Germany [68–71].

TBPs can major impact bacterial fitness, transmission success, and disease progression through co-infection, which has already been reported in multiple infections with *A. phagocytophilum* and *B. burgdorferi*. Thus, infection with these two pathogens can lead to more severe arthritis than infection with *B. burgdorferi* alone [65]. Thus, based on the transmission of multiple TBPs, the severity of associated clinical symptoms of these mixed or multiple infections is also affected. In addition, such co-infection can also lead to problems, in the form of delays or even misdiagnosis, in diagnosis [62,65,72]. Therefore, it is essential to determine the occurrence or risk of co-infections to assess the population's health risk.

1.2.4 *Borrelia burgdorferi* sensu lato complex

Lyme borreliosis (LB) is a multisystemic infectious disease with the highest incidence in Europe and North America. In Germany, *I. ricinus* was associated with an average of 12,094

cases of LB from 2018 to 2022, with no apparent national distribution pattern [73–75]. Clinical symptoms infrequently occur, distinguishing between early localized, early disseminated, and late symptoms. These symptoms include skin manifestations (e.g., erythema migrans), neurological symptoms (e.g., early neuroborreliosis), and other manifestations (e.g., Lyme arthritis) [76]. Here, infection is caused by human pathogenic bacterial species of the genus *Borrelia* (order: Spirochaetales), grouped in the *Borrelia burgdorferi* s. l. complex. The first pathogen, *B. burgdorferi*, was discovered by Willy Burgdorfer in 1982 and officially classified as a new species of *Borrelia* in 1984. These are actively motile, helical, gram-negative bacterial species [77].

Of the at least 22 molecularly differentiable genospecies of the Bb, six are confirmed as human pathogenic species: *B. burgdorferi* sensu stricto (s. s.), *B. garinii*, *B. afzelii*, *B. spielmanii*, *B. mayonii*, and *B. bavariensis*. Furthermore, *B. valaisiana*, *B. lusitaniae*, and *B. bissetii* are also suspected pathogens of LB [78]. In addition to pathogenicity, differentiation can also be made based on the global distribution of species of the Bb. At the time, all known human pathogens are present in Europe, most commonly *B. afzelii* and *B. garinii*, only *B. burgdorferi* s. s. has been detected in the United States [79]. Furthermore, the density, as well as abundance, of diverse hosts at a location may also control the local occurrence of different genospecies in *I. ricinus* [61,80], as certain genospecies have defined host associations [81]. Thus, *B. afzelii* is found mainly in mice, hedgehogs, and other small rodents, and *B. garinii* in birds [81,82]. In contrast, the species *B. burgdorferi* s. s. is host non-specific and is considered a generalist. Since all three genospecies are omnipresent in Germany, ticks can thus become infected everywhere, including mixed infections with diverse pathogenic *Borrelia* species [83].

Nevertheless, ticks not infected with *Borrelia* are also found in different developmental stages. On the one hand, these are ticks, primarily in the larval stage, which have not yet become infected with *Borrelia*. On the other hand, these are ticks that have already been infected with certain genospecies in a previous stage but feed on a host that is zooprophylactic for the already acquired genospecies [80]. Zooprophylactic means infected ticks lose their *Borrelia* infection while feeding on a host, especially ruminants such as goats, deer, and cattle, and are subsequently *Borrelia*-free [84]. Presumably, the complement system of ruminants is

responsible for this. This system is considered an add-on strategy for the immune system. It consists of many diverse plasma proteins that react with each other to mark pathogens with antibodies and start a series of inflammatory reactions that help fight infections [85]. Different proteins can cover the surface of, e.g., pathogenic microorganisms and thus induce phagocytosis [34,86]. Once *I. ricinus* has become infected with a pathogenic genospecies of the Bb during a blood meal, the pathogen migrates to the midgut with the help of outer surface protein A (decoded by the *ospA* gene), which binds to the tick's *ospA*-specific receptor. The pathogen then accumulates in the freshly ingested blood during a future blood-sucking act. It migrates to the salivary glands with the help of other proteins, beginning the transmission of *Borrelia* [45,87]. The *ospC* produced by the tick during transmission prevents an immune response of the host by antibodies or the proteins controlled by the complement system by specific binding [4].

1.2.5 Human pathogenic species of the order Rickettsiales

The order Rickettsiales contains a variety of TBPs that can cause neoehrlichiosis (*N. mikurensis*), anaplasmosis (e.g., *Anaplasma phagocytophilum*), or rickettsiosis (e.g., *Rickettsia helvetica*) [26,88,89]. Therefore, knowing their occurrence and distribution in ticks is essential for risk assessment and disease prevention [90].

The genus *Rickettsia* of the family Rickettsiaciea includes a variety of obligate intracellular gram-negative species transmitted transstadially and/or transovarially in a vector population [26]. Consequently, ticks are not only vectors of *Rickettsia* but also reservoirs, contributing to maintaining bacterial species in the environment [89].

Of the at least 31 now recognized and fully named species [91], 25 are pathogenic [89]. Species are classified into four groups: Spotted Fever Group (SFG), Epidemic Typhus Group, the *Rickettsia bellii* Group, and the *Rickettsia canadensis* Group [88,92]. In addition to endosymbiotic *Rickettsia* species, the human pathogenic species *R. raoultii, R. massiliae, R. slovaca, R. aeschlimannii, R. monacensis*, and *R. helvetica* occur in Germany. Thereby, all species can be assigned to the spotted fever group. The most common *Rickettsia* species in *I. ricinus* is *R. helvetica*, but its pathogenicity for humans is increasingly discussed [88]. Nevertheless,

because rickettsioses are among the oldest tick-borne diseases, newly discovered rickettsial species should always be considered potentially pathogenic to humans [88,93]. However, pathogenic *Rickettsia* species have also been described in numerous vertebrates, such as birds (*R. aeschlimannii* and *R. helvetica*), reptiles (*R. helvetica* and *R. monacensis*), and mammals (*R. helvetica* and *R. monacensis*), among other reservoirs [72,88,89]. In this context, it should be noted that infection with *R. raoultii, R. massiliae, R. slovaca, R. aeschlimannii, R. monacensis*, or *R. helvetica* may be manifested by rickettsiosis-specific features in addition to typical symptoms, such as fever, rash, and headache [71,89].

Another TBP of the order Rickettsiales is the obligate intracellular species *A. phagocytophilum* from the family Anaplasmataceae, also gram-negative [71]. This pathogen also uses *I. ricinus* as a vector in Germany and many different vertebrates, such as wild boars, horses, and dogs, as reservoir hosts [94]. In this context, high host diversity is of enormous importance, as maintenance of the species occurs exclusively via transstadial transmission in a tick [94]. The rarely transmitted strains of *A. phagocytophilum* in Germany cause human granulocytic anaplasmosis (HGA), as circulating strains are classified as less pathogenic or non-pathogenic. Blazejak et al. showed that the infection rate of *I. ricinus* with *A. phagocytophilum* in Hannover remained constant over ten years (~3.8%) and can be considered an endemic and persistent health risk for humans. Because *A. phagocytophilum* affects the immune system response during disease, there is a potential risk for co-infection [71].

Also belonging to the family *Anaplasmataceae* are the potential human pathogenic species *Wolbachia pipientis* (*W. pipientis*) and the human pathogenic species *N. mikurensis*. *W. pipientis* is an endosymbiotic bacterium localized either as a commensal or symbiont in the Malpighian tubules and/or ovaries of ticks [56]. In addition, *W. pipientis* is a typical member of bacterial communities living in arthropods. However, no human infection has been reported, although it is consistently found in *I. ricinus.* However, it is known that *W. pipientis* significantly impacts the reproduction, sex determination, speciation, and behaviour of a wide range of invertebrates worldwide [95]. On the other hand, the impact of *N. mikurensis* on its vector *I. ricinus* is still unknown. As of 2019, "Candidatus" status is no longer applicable, as Wass et al. (2019) achieved the first successful isolation of the species in pure culture, which also

characterized that *N. mikurensis* uses vascular endothelial cells as target cells for infection in humans [96]. Nonetheless, the pathogen was previously detected in several patients with fever, rash, and asymptomatic infections [97]. In addition, it is now also known that mainly small mammals (yellow-necked mice, bank voles, common shrews) serve as reservoir hosts for Germany [97,98].

1.3 Current state of diagnostics of tick-borne pathogens in ticks

Different material is used to detect TBPs depending on the research issue. Either the nucleic acid (DNA/RNA) of the tick and/or organic material (cerebrospinal fluid (CSF), skin, blood) of the host is examined for TBPs.

In recent years, the incidence of TBPs has increased, partly due to more optimized, high-resolution, and, most importantly, diverse analytical methods [32,44]. A distinction is made between direct and indirect pathogen detection. For indirect pathogen detection, single or, in case of critical or uncertain results, successively combined (step-wise diagnostics) serological tests, such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT), or immunoblots, are used [99]. However, the disadvantage of serological tests is that detecting antibodies against the investigated pathogen from patient material alone through, e.g., ELISA and Western blot (example of an immunoblot) does not allow a reliable statement about a current infection. Only a definite diagnosis can be obtained if this evidence, other clinical results, and the medical history speak for an infection. In addition, *in vitro* culture systems are already available for a serological test to be developed, which presents difficulties, especially for newly discovered TBPs [96].

There are different methods for direct pathogen detection. Microscopy, growing by culture and xenodiagnosis, is used [99]. However, these are more traditional and usually very time-consuming and cost-intensive methods, which have been increasingly replaced in molecular diagnostics such as the nucleic acid-based polymerase chain reaction (PCR). Nevertheless, cultivating pathogens is indispensable when investigating their physiological properties, despite sometimes high safety levels and specific laboratory requirements. Nonetheless, infection cases remain hard to detect because they were not covered by cultivation-based

methods [96]. With the wide variety of emerging TBPs transmitted by *I. ricinus*, the need for comprehensive diagnostics of TBPs is apparent.

1.3.1 Polymerase chain reaction

The PCR revolutionized molecular biology in the 1980s through its inventor Kary Mullis [100]. Using PCR, it is now possible to detect the presence of sequence-specific deoxyribonucleic acid (DNA), regardless of the available test material [89].

Thus, monitoring the infection stage can also occur [99,101]. However, a PCR-positive result for a pathogen only confirms the presence of DNA obtained from the pathogen regardless of whether pathogens were viable [99]. In addition to PCR methods that test for a single pathogen (so-called singleplex PCRs), multiplex PCRs gradually become routine diagnostics. Reller and Dumler (2018) and Courtney et al. (2004) already developed assays for the parallel detection of *Ehrlichia* (*E.*) *chaffeensis* or *B. burgdorferi* and *A. phagocytophilum* [102,103]. However, multiplex PCR assays that examine up to nine TBPs simultaneously are permitted only for research purposes[99]. The primers most commonly used for PCR differ depending on the target sequence or gene. While the bacterial 16S rRNA gene is used as a template [104], other target genes as a template may differ depending on the pathogen. For example, primer sequences of the *glt*A, *omp*A, or *omp*B gene detect *Rickettsia* spp.. The *gltA* gene, which encodes citrate synthase, is an orthologous gene that is conserved across *Rickettsia* species and is therefore frequently used for species differentiation [99,158]. In contrast, the primer sequences of the *msp*2 gene are used for *A. phagocytophilum*, and primer sequences of the *groEL* gene are used for *N. mikurensis* detection [89,105–107].

1.3.2 Next-Generation Sequencing for bacterial microbiome analysis

Next-Generation Sequencing (NGS) is a quick and cost-effective technology that enhanced our knowledge of genes and genomes of single cells and compositions of complex microbial communities in the last decade [47]. Various sequencing technologies have already been used for microbiome investigation, such as Sanger sequencing, 454 pyrosequencing, Ion torrent or Illumina-based sequencing, and Whole Genome Shotgun [44]. In this work, only an Illumina-

based sequencing method was used. This system is based on sequencing by synthesis. As a result, the synthesis of a complementary DNA strand by fluorescent label-bearing dNTPs can be followed in real-time [108–111].

A major strength of NGS amplicon sequencing approaches (ASA) compared to PCR-based approaches is that ASA is not focused on detecting specific microorganisms but that the entire bacterial microbiome can be differentiated using the hypervariable regions of the 16S ribosomal ribonucleic acid (rRNA) gene.

The 16S rRNA gene has been frequently used as a target for amplicon sequencing to identify bacterial taxa [44,104]. This gene forms an essential component of the small subunit (SSU) of prokaryotic ribosomes, mitochondria, and chloroplasts [112,113]. Furthermore, the 16S rRNA gene fulfils several properties that predestine it as a marker gene for taxonomic study. First, the gene is ubiquitous, so it is possible to sequence all bacteria (and archaea). In addition, the average length of the 16S rRNA gene of about 1500 base pairs (bp) provides sufficient phylogenetic information.

Furthermore, the genetic variations due to the hypervariable regions of the gene are sufficient to determine the phylogenetic assignment of bacteria down to the genus level. Therefore, nine hypervariable regions (V1–V9) in the 16S rRNA gene have been assessed, of which regions V1–V4 are most commonly used to explore the bacterial sequence diversity in ticks [44]. Moreover, PCR can effortlessly amplify the gene because the conserved regions are used as diverse primer binding sites [104]. Last, the international accessibility of the gene sequences from different bacteria has resulted in an extensive sequence database that identifies almost all bacteria (and archaea) [112,114].

Thus, in recent years, thanks to NGS, new insights into the composition of the microbiome and the influence of biotic and abiotic factors on the tick microbiome have been repeatedly obtained. In addition, based on ASA, the determining bacterial diversity in the tick microbiome has allowed conclusions to be drawn about the host specificity of the tick. This host specificity raised many new questions related to the tick-host interaction and the interaction of the bacteria in the tick organism [44,45,49,57,115–117].

1.3.3 Multivariate statistics and network analysis

Different methodologies of multivariate statistics are applied to interpret the results of the ASA. These include various types of correspondence analyses (CA), hierarchical cluster analyses (e.g., Euclidean distances), and calculations of diversity indices (e.g., Shannon-/ Simpson-Index, Pielou's evenness index) [118–121]. These methodologies of multivariate statistics allow differences or similarities between samples to be summarized and presented concisely without significantly reducing the included variables or observations (Operational taxonomic unit (OTU)). To investigate the dependencies and effects of variables, such as location or season, Fisher Exact Tests with Bonferroni correction is used [118,122]. However, suppose direct knowledge about the interaction patterns of bacteria in the tick microbiome is of interest. In that case, new software such as MENA (Molecular Ecological Network Analysis) or CoNet (Co-occurrence Network inference) is increasingly applied for network analysis [123,124].

The resulting microbial networks are composed of nodes and edges. While nodes always represent a taxonomic category (here: OTU), edges act as connections between nodes. Edges are distinguished in copresences (positive interaction) and mutual exclusions (negative interaction). However, it should be noted that the length of the edges does not allow any conclusions about the intensity of the interaction because the assumption is based on statistical calculations and, therefore, cannot serve as real evidence.

Nevertheless, a mathematical procedure can be used to calculate the topological roles of the nodes. The intramodule connectivity (Z) and the intermodule connectivity (P) are calculated based on the connection pattern of a node. These values can assign a node to one of four defined roles. Peripherals: Less interaction with other nodes, Connectors: High importance due to linking of different modules with each other, $z_i < 2.5$ and $P_i > 0.625$; Module hubs: High importance for the own module, $z_i > 2.5$ and $P_i < 0.625$ and network hubs: High importance for module and the overall network, $z_i > 2.5$ and $P_i > 0.625$ (**Figure 2**). So a node can be assigned a defined range within the network and thus a weighting[125]. This classification can be used to understand how bacteria interact within the tick microbiome. In other words, whether the

simultaneous presence of certain bacteria brings advantages, leads to a rearrangement of interaction partners, or results in the addition of a bacterial species.

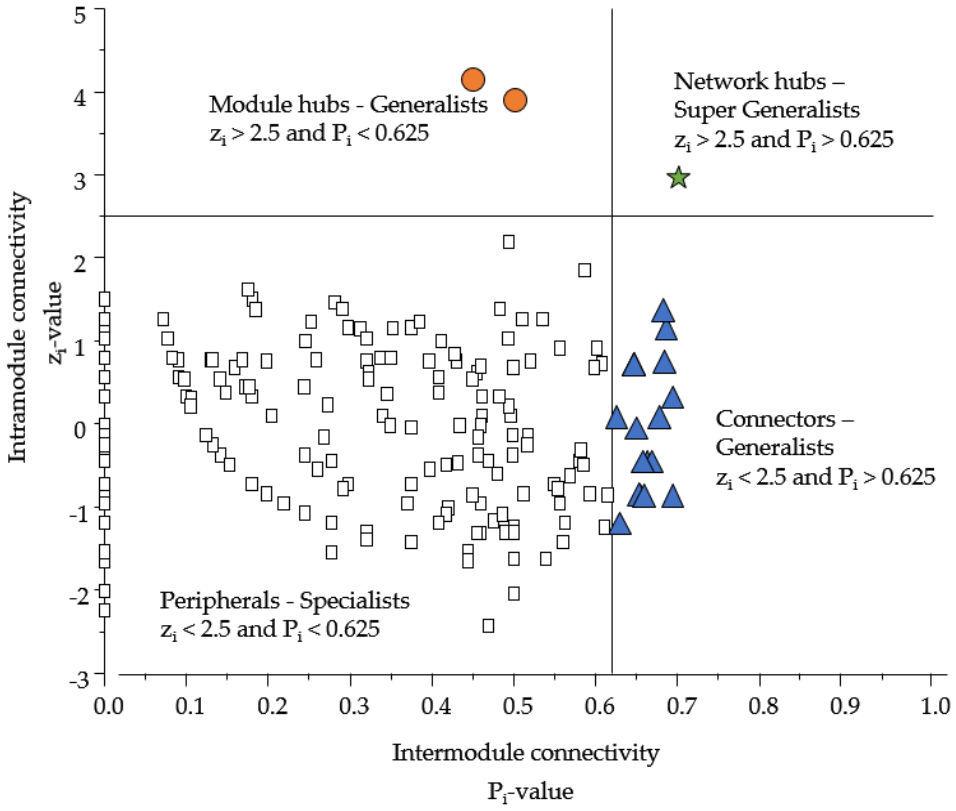

Figure 2. Based on the topological z_i and p_i values, defined areas result, leading to a classification of nodes into four areas: Peripherals, Connectors, Module hubs, and Network hubs as introduced by Oleson et al. (2007).

1.3.4 Influence of abiotic and biotic factors on tick microbiome

Narasimhan and Fikrig (2015) compared the microbiomes of wild-caught and laboratory-reared ticks (*I. scapularis*). They found differences in the microbiome composition explained by different biotic and abiotic environmental factors [43]. While abiotic factors are understood as non-living parameters, such as temperature, humidity, or light, biotic factors summarize living parameters that interact with each other, such as tick-host interaction, suitable hosts, or reservoir competence for TBPs. Thus, these environmental factors influence the distribution and density of ticks and their (reservoir) hosts, which has implications for the distribution and occurrence of TBPs in the environment [32,64,126].

If suitable hosts are separated from ticks by landscape fragmentation, such as in cities, tick density and the continuous circulation of TBPs will be affected [32,127,128]. Thus, habitat structure seems to affect host diversity and abundance, which in turn impacts the composition of the tick microbiome. However, whether the consequences are more negative or positive is still debated. Because while Pfäffle et al. (2013) state that habitat fragmentation tends to work against the establishment of a well-established vector population, Perez et al. (2016) suggest more favorable conditions for generalists and only disadvantages for specialists due to fragmentation but also emphasize that the effects of fragmentation depend on the tick-host interaction patterns [126,129].

In a review, Pollet et al. (2020) summarized the temporal and spatial factors that may influence the composition of the tick microbiome, noting that ticks, and most likely their microbiome, rely on vertebrate movement to survive as a meta-population [57]. This spatial scale implies that location drives variability in the tick microbiome and the abundance of *I. ricinus*. In addition, regional conditions of tick reservoirs and habitats have been tested to be critical drivers of bacterial colonization and shifts in the tick microbiome, including tick-associated human pathogens [32,57,130].

To determine location (in)dependent correlations between TBPs and non-pathogenic bacteria, additional information, such as the location of tick collection, should be added to the dataset. Such information will allow an even better understanding of the bacterial composition of the microbiome.

Another example of how bacterial diversity in the tick microbiome is affected can be demonstrated using temperature. For instance, a mild winter can result in extended or continuous tick activity and increase or prolong the probability of transmission of TBPs [4,126].

Furthermore, there is also evidence that the sex and life stage of the tick impacts the microbial composition. For example, Aivelo et al. (2019) demonstrated that endosymbionts, such as *Spiroplasma*, *Rickettsiella*, *Lariskella*, and *Rickettsia* spp., were lower in adult female ticks [53]. In contrast, Carpi et al. (2011) demonstrated the influence of the life stage on the microbiome. In this context, he attributed the higher bacterial diversity of nymphs than adults

to the difference in host selection. It is known that nymphs have a broader host range available [46].

1.3.5 Influence of contaminants on tick surface on the analysis result

TBPs and endosymbiotic bacteria are part of a diverse tick microbiome that consists of different bacterial diversity unevenly distributed throughout tick organs, like the external surface of ticks (skin) [43,44,131]. The diversity and abundance of bacteria in the internal tick microbiome are essential for the tick's life cycle, including fitness, survival, and immunity [43]. In addition, ubiquitous bacteria are always found, which are more associated with the soil, plants, or the host surface. Therefore the external tick microbiome is transmitted and influenced, on the one hand, by the tick-host interaction during blood meals and/or by environmental microorganisms from the tick's habitat [44,132]. Narasimhan and Fikrig (2015) suggest that the external host microbiome is also involved in the bacterial diversity of the tick gut [43].

Nevertheless, the external tick microbiome and its influence on the tick and diagnostic analysis are still too unexplored. Thus, microbiome studies need more attention [132]. However, the external microbiome of other arthropods and insects has already been studied, which is why it is known that such cuticular microbiomes were essential for protecting the vector from environmental stressors and/or valuable for host recognition [133–135].

In contrast, the microbial diversity of the external human microbiome is well known and includes approximately $1x\ 10^6$ bacteria per cm^2 [136]. Lotions and agents, including povidone-iodine, chlorhexidine, ethanol, bleach, and reactive skin decontamination lotion (RSDL), are commonly used to decontaminate the skin surface [137,138].

The host skin microbiome (e.g., that of mammals) is potentially part of the external tick microbiome, influencing analyses of the entire tick microbiome. Such contamination can lead to misleading detection of pathogens with therapeutic implications. Nonetheless, a review of 30 studies that addressed the internal tick microbiome found that only 11 studies preceded a decontamination assay. Greay et al. (2018) reported that decontamination of the tick surface microbiome was performed heterogeneously rather than homogeneously. When

decontaminated the tick surface in advance, 70% to 100% ethanol was used in most cases [44,139,140]. In contrast, only a few studies used decontamination with bleach solutions such as sodium hypochlorite [140–143]. In principle, ethanol and various bleaching agents are equally effective in killing bacteria. However, a decontamination procedure based on ethanol still allows the detection of this bacterial DNA by PCR or ASA, whereas bleach simultaneously denatures the DNA [144]. It also becomes problematic when the remaining external DNA is counted as part of the diversity of the internal tick microbiome due to missing or incorrect decontamination [132].

1.4 Motivation and hypotheses

The microbiome of *I. ricinus* carries a complex bacterial community dominated by non-pathogenic bacteria and TBPs. As a result, TBPs frequently interact with non-pathogenic bacteria, affecting the fitness, abundance, spread, and transmission of TBPs within the tick and to a host. Thus, it is evident that much work remains to be done to understand the complex and particular interactions between TBPs and non-pathogenic bacterial species within the microbiome of *I. ricinus*. In this case, the optimal starting point for diagnostics is the tick. The tick itself is usually available since tick bites rarely go unnoticed and can therefore be examined. The microbiome of the tick can be assessed, and thus the incidence of known single or multiple TBPs can be estimated.

Furthermore, it allows exploratory detection of TBPs in ticks that have not previously been associated with ticks. Indeed, consequently, there is an opportunity to disrupt the identified interactions, thereby reducing tick burdens on the one hand and the incidence or occurrence of TBPs on the other. Therefore, no matter how small, any further knowledge represents a potential key to reducing the suitability of ticks as vectors of TBPs. This knowledge can be used as a new diagnostic approach to avoid further false positive or negative results, including in the host.

The current state of research on the microbiome of *I. ricinus* can therefore be summarized as follows:

1) Lyme borreliosis is the most common disease in humans following a tick bite, with no obvious national distribution pattern [73-75]. However, the differences in the composition of the microbiome of Bb-positive and Bb-negative ticks are unknown.
2) TBPs of the order Rickettsiales are well researched [26,88,89]. However, the influence of Bb on the (co-)occurrence of TBPs of the order Rickettsiales is unknown.
3) The influence of the tick's external microbiome, acquired through its habitat, as well as through various host-tick interactions, on the results of the tick's microbiome analysis is unknown [44,132]. Nevertheless, the analysis of tick microbiomes usually takes place without decontamination of the external microbiome.

Since Lyme borreliosis occurs throughout Germany, the location of exposure of an infected person cannot be used to clarify and confirm the LB diagnosis. However, in order to characterize the tick microbiome and optimize diagnostics, it is important to know to what extent the presence or absence of Bb affects the diversity of the microbiome or the interaction of microorganisms, and whether this influence remains independent of location.

In contrast, TBPs of the order Rickettsiales have been well researched. Nevertheless, uncertainties remain regarding the risk of co-infections in humans through tick bites. According to current studies, co-infections of TBPs of the order Rickettsiales are rare. This suggests that members of the Rickettsiales order can be considered weak competitors and are displaced by species of the Bb in tick microbiomes.

Greay et al. (2018) showed that there is no homogeneous approach to decontaminating the external microbiome of ticks when the internal microbiome is to be analyzed. Since numerous studies have already found ubiquitous bacteria that are more likely to be associated with the soil, plants, or the surface of the host, it can be assumed that these bacteria are also included in a microbiome analysis. However, the microorganisms can distort the actual microbiome analysis in terms of diversity and abundance.

Based on the present knowledge, the following hypotheses were postulated and answered with biological questions in the following chapters:

Hypothesis 1: Is there a difference in the composition of the microbiome of *Borrelia*-positive and *Borrelia*-negative ticks?

The occurrence of *Borrelia* is location independent and coupled with the presence of other tickborne microorganisms. The bacterial richness of the tick microbiome is reduced if *Borrelia* is present. (3.1)

a) Does the presence or absence of *Borrelia* affect the bacterial community composition, the bacterial richness and the abundance of tick microbiomes? (3.1.1)
b) Does the presence of *Borrelia* affect the bacterial abundance of tick microbiomes regardless of the location? (3.1.1)
c) Does the location affect the bacterial community composition of tick microbiomes regardless of the presence of *Borrelia*? (3.1.2)
d) Do genera have the same topological roles in microbial networks when *Borrelia* is present or absent on the tick microbiomes? (3.1.3)
e) Are there different interaction patterns of bacteria with an important topological role between Bb-positive and Bb-negative tick microbiomes? (3.1.3)
f) Does the Bb or the genus *Borrelia* play an important topological role on Bb–positive tick microbiomes? (3.1.3)

Hypothesis 2: Does *Borrelia* influence the (co-)occurrence of TBPs of the order *Rickettsiales*?

Members of the order *Rickettsiales* have been exemplified as weak competitors and outcompeted by *Borrelia* in tick microbiomes. Therefore, the frequency of members of the order Rickettsiales is expected to be reduced once Bb-positive are compared with Bb-negative tick microbiomes. Furthermore, additional variables such as season and location influence the bacterial diversity of the tick microbiome, affecting the diversity of hosts. Thus, other microorganisms, in varying abundance, are available. (3.2.)

a) Does the (co-)occurrence and sequence read abundances of tick-associated pathogens of the order *Rickettsiales* significantly correlate with the presence or absence of Bb in the bacterial tick microbiome? (3.2.1)

b) Is there a seasonal or location-specific relationship to the (co-)occurrence of tick-associated pathogens of the order Rickettsiales? (3.2.1)

c) Is there a specific rickettsial species that dominates the tick microbiome? (3.2.2)

d) Is the (co-)occurrence of species of the genus *Rickettsia* spp. independent of the variables` location or season? (3.2.3)

Hypothesis 3 (H3): Does decontamination of the external microbiome prior to microbiome analysis influence the outcomes?

Insufficient or no decontamination of the tick surface microbiome will bias the results of the microbiome diversity analysis, as contaminants from the environment or the host are included in the analysis. These contaminants may lead to an incorrect conclusion regarding the microbial composition of the tick microbiome and bias the result. (3.3)

a) Does the decontamination efficiency of gram-positive and gram-negative contaminants differ between the decontamination treatments? (3.3.1)
b) Does the bacterial richness of tick microbiomes differ between DNA and complementary DNA (cDNA) samples with or without respective decontamination treatment? (3.3.2)
c) Does the bacterial community composition of tick microbiomes differ with and without various decontamination treatments of the tick surface? (3.3.3)
d) To what extent does the bacterial richness and the sequence read abundance of the tick microbiome differ after respective decontamination treatment of the tick surface? (3.3.3)
e) To what extent does the bacterial richness, as well as the sequence read abundance of the tick microbiome differ after respective decontamination treatment of the tick surface concerning the DNA and cDNA samples? (3.3.3)

2 Material and Methods

In the following, all the methods used in this work are explained in detail according to the research topic, and the equipment, materials, chemicals, and reagents required for them are listed in **Table 2** and **Table 3**.

2.1 Equipment and materials

All equipment and materials are shown below (**Table 2**).

Table 2. Overview of the equipment and materials used.

Equipment	Manufacturer
µDrop-Platte	Thermo Fisher Scientific Inc., MA, USA
chemagic™ MSM I instrument	PerkinElmer chemagen Technologie GmbH
CFX 96 Touch™ Real-Time PCR Detection System	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
GenoPlex Geldokumentationssystem	VWR International GmbH, Darmstadt, Germany
Heraeus Multifuge X1R	Thermo Fisher Scientific Inc.
MaxQ™ 6000 Shaker	Thermo Fisher Scientific Inc.
Multiskan GO	Thermo Fisher Scientific Inc.
mySPIN™ 12 Mini Centrifuge	Thermo Fisher Scientific Inc.
Neubauer-improved counting camber	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
PowerPac Universal Power Supply	Bio-Rad Laboratories GmbH
SpeedVac Concentrator plus	Eppendorf, Hamburg, Deutschland
Steel bead (5mm)	Qiagen GmbH, Hilden, Germany
Sub-Cell®GT Agarose Gel Electrophoresis Systems	Bio-Rad Laboratories GmbH
T100™ Thermal Cycler	Bio-Rad Laboratories GmbH
TissueLyser II	Qiagen GmbH
Thermomixer comfort	Eppendorf
Zirconium beads, Triple-Pure starter kit	Merck KGaA, Darmstadt, Germany

2.2 Chemicals

The following is an overview of the chemicals used in this work (**Table 3**).

Table 3. Overview of the chemicals used.

Chemicals	Manufacturer
Agar-Agar	Merck KGaA
Agarose Standard	Carl Roth GmbH & Co. KG., Karlsruhe, Germany
Agarose Standard	Carl Roth GmbH & Co. KG.
chemagic™ Viral DNA/RNA kit	PerkinElmer chemagen Technologie GmbH
DNA Away	VWR International GmbH
DNA ladder 100 bp - 1 kb	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
DNA ladder 250 bp - 10 kb	Biozym Scientific GmbH
DNA ladder 50 bp - 1.5 kb	Biozym Scientific GmbH
dNTP Mix (10 mM each)	Thermo Fisher Scientific Inc.
Ethanol (70%, 100%)	Carl Roth GmbH & Co. KG.
Ethidium bromide	Carl Roth GmbH & Co. KG.
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific
HF buffer (5x)	Thermo Fisher Scientific
Hydrochloric acid 0.1 M	Carl Roth GmbH & Co. KG.
Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific Inc.
iTaq™ Universal SYBR Green Supermix (2x)	Bio-Rad Laboratories GmbH
Loading Dye (6x)	Biozym Scientific GmbH
Molecular BioProducts ™ DNA AWAY	Thermo Fisher Scientific Inc.
PCR Enhancer (5x)	Biozym Scientific GmbH
Phusion™ High-Fidelity DNA Polymerase (2 U/µL)	Thermo Fisher Scientific Inc.
Reactive Skin Decontamination Lotion (RSDL)	Amazon Europe Core S.à r.l., Luxemburg, Luxemburg
RINGER tablets for the preparation of RINGER solution	Thermo Fisher Scientific Inc.
RQ1 RNase-Free Dnase	Promega GmbH, Walldorf, Germany
Sodium hypochlorite solution (5%)	Carl Roth GmbH & Co. KG.
Standard culture medium I for microbiology	Carl Roth GmbH & Co. KG.
TAE-Puffer (50x)	Carl Roth GmbH & Co. KG.

2.3 Sampling and metadata management

2.3.1 Nucleic acid extracts provision and tick collection

To analyze TBPs (e.g., human pathogenic species of the Bb., *Rickettsia* spp., and-tick-borne encephalitis), 2,029 *I. ricinus* ticks were sent by physicians or directly by clients (human hosts) to the accredited tick laboratory of Synlab Medical Care Centre (MVZ) Weiden from January to December of 2018. Subsequently, nucleic acids of each tick were extracted by Synlab MVZ to analyze the TBPs requested by the physicians or clients. After nucleic acid extraction, the presence of human pathogenic Bb species was tested by a real-time TaqMan PCR with the primer pair (5′-AATATTTATTGGGAATAGGTCTAA-3' and 5′-CACCAGGCAAATCTACTGA-3') and probe (tm-FA TTAATAGCATGYAAGCAAAATGTTAGCA) as reported earlier [145]. Most of the prior screened nucleic acid extracts of ticks by Synlab MVZ were provided to our institute and were stored at −20 °C until the further investigation (**H1** and **H2**).

Since whole ticks and no nucleic acid extracts of ticks were still needed for the decontamination experiment (**H3**) at the beginning, these ticks were collected independently.

2.3.2 Metadata management and sample selection

For each tick sent to the laboratory, patient or personal data such as name, address with postal code, and submission date were deposited. To compare a high amount of tick extracts, the regional postal codes of nucleic acid extracts from ticks available for the study from 2018, were mapped on a map of Germany using Rstudio and the packages sf, tmap, tmaptools, dplyr, and ggplot [146–150].

Based on the resulting map (**Figure S1**), Neustadt an der Waldnaab with the independent city of Weiden in der Oberpfalz (Upper Palatinate, Bavaria) and Esslingen (Stuttgart, Baden-Wuerttemberg) were selected, as both locations offered a high number of Bb-positive and Bb-negative tick extracts for detailed microbiome analyses (**H1**). In addition, the nucleic acid extracts with the human pathogenic Bb species were confirmed by a multilocus sequence typing (MLST) with eight housekeeping genes and a *p41* gene [151–153]. Therefore, the nucleic acid extracts from the ticks were grouped into Esslingen Bb-positive tick extracts (EP, n = 38),

Esslingen Bb-negative tick extracts (EN, n = 62), Weiden Bb-positive tick extracts (WP, n = 44), and Weiden Bb-negative tick extracts (WN, *n* = 56).

To analyze TBPs of the order Rickettsiales (**H2**), 760 tick extracts, consisting of 50 µL each, were selected arbitrarily based on the variables of location, season, month, and Bb findings and were used for a more detailed analysis (**Table 4 and Figure SE1**).

Subsequently, 2 µL of nucleic acid extract from each of the 10 ticks was pooled, and each was sorted according to Bb findings (negative or positive), site of discovery, and month of submission date. However, since I already received extracted DNA from ticks, this study could not consider morphological information. Thus, 76 individual tick nucleic acid extract pools (760 ticks) were created, of which 38 were Bb-positive, and 38 were Bb-negative (**Table SE1**).

To test the suitability of four different decontamination agents for ticks, a total of 62 host-seeking individual specimens of adult *I. ricinus* were collected in September 2018 at the Hofgarten Coburg (50°15′39″, 10°58′24″). All ticks were collected by flagging a 1m^2 white cloth [154]. Each tick was transferred with a tweezer, which was freshly disinfected after each tick, to a 1.5 mL sterile reaction tube. The tubes were immediately transported to the lab and stored for 16 h at 4 °C until further processing.

Table 4. Characteristics of the variables of each tick nucleic acid extract for subsequent Fisher exact test with Bonferroni correction. Seasons were categorized according to the meteorological calendar (spring: March 1st, summer: June 1st, autumn: September 1st); n indicates how many tick nucleic acid extracts were available per characteristic.

Variable	Characteristics
Bb finding	Positive (n = 380), negative (n = 380)
Location	South (n = 120), southeast (n = 560), west (n = 80)
Month	May (n = 180), June (n = 380), July (n = 140), September (n = 60)
Season	Spring (n = 180), summer (n = 520), autumn (n = 60)

2.4 Contamination and decontamination of ticks

The bacterial strains *Escherichia coli* (*E. coli*; DSM 423), *Pseudomonas fluorescens* (*P. fluorescens;* DSM 4358), and *Micrococcus luteus* (*M. luteus;* DSM 20030) were chosen based on different cell wall constitutions for artificial spiking to the external tick microbiome to evaluate the efficiency of decontamination methods. In addition, dog saliva and human sweat were artificially spiked to the external tick microbiome to add complex microbial communities of potential hosts. The microbiome of dog saliva and human sweat has been frequently reported. The most abundant bacterial genera of its compositions were not found in tick microbiomes [155,156], which is also true for this study.

Ticks (n = 62) were randomly subdivided into nine treatments (**Table 5**). Except for ticks of the negative control (DKA 1 NC, DKA 2 NC, DKA 3 NC, DKA 4 NC; *n* = 12), each tick (*n* = 50) was separately contaminated by placing [140] in a 1.5 mL tube for 5 min at room temperature containing 45 µL of a defined contamination solution of *E. coli*, *P. fluorescens*, *M. luteus,* dog saliva and human sweat (**Table 6**).

Contamination solution was discarded thereafter, and contaminated ticks, except for ticks of the positive control (PC; *n* = 10), were decontaminated by placing them for 5 min at room temperature in a 1.5 mL tube containing 50 µL of (i.) 70% ethanol, (ii.) DNA Away, (iii.) 5% sodium hypochlorite, or (iv.) RSDL (**Table 5**). Afterward, the decontamination solution was discarded, and SpeedVac completely evaporated the remaining solution.

2.5 Nucleic acid extraction of collected ticks

Nucleic acid extraction from each treated whole tick (**Table 5**) was carried out as explained in hypothesis 1 at our cooperation partner Synlab MVZ Weiden. Briefly, chemagic™ Viral DNA/RNA kit (PerkinElmer chemagen Technologie GmbH, Baesweiler, Germany) on the chemagic™ MSM I instrument (PerkinElmer chemagen Technologie GmbH) as specified by the manufacturer. Briefly, each tick was homogenized by adding 50 µL of isotonic saline solution (0.9% 154 mM NaCl; pH 5.7) and one 5 mm steel bead (Qiagen GmbH, Hilden, Germany) by a TissueLyser II (Qiagen GmbH) for 4 min. After that, each homogenized tick

was lysed by a lysis buffer of the chemagic™ Viral DNA/RNA kit (including protease and Poly (A) RNA), and nucleic acids were automatically extracted using magnetic beads as explained

Table 5. Decontamination strategies to assess the efficiency of four decontamination solutions.

Decontamination solution (5min)	Abbreviation	Contamination	Number of independent ticks
70% ethanol	DKA 1	yes	10
DNA Away	DKA 2	yes	10
5% sodium hypochlorite	DKA 3	yes	10
RSDL	DKA 4	yes	10
70% ethanol	DKA 1 NC	no	3
DNA Away	DKA 2 NC	no	3
5% Sodium hypochlorite	DKA 3 NC	no	3
RSDL	DKA 4 NC	no	3
no	PC	yes	10

Table 6. Composition of defined contamination solution.

Contaminants	Cell Number [mL^{-1}]	Volume of Solution (in Total)	Percentage Volume in Solution per Tick
Escherichia coli	6.5×10^9	650 µL	28.9%
Pseudomonas fluorescens	1.4×10^9	650 µL	28.9%
Micrococcus luteus	1.1×10^9	650 µL	28.9%
Human sweat	not determined	200 µL	8.9%
Dog saliva	not determined	100 µL	4.4%

by the manufacturer. Nucleic acid extractions were carried out at Synlab MVZ (Weiden in der Oberpfalz, Germany). Nucleic acid extract from each tick was equally divided into two parts; one part was used for genomic DNA (gDNA) analyses, while the other was used for RNA

analyses. The latter was treated with DNase for 30 min at 37 °C (RQ1 RNase-Free DNase, Promega GmbH, Walldorf, Germany), and the success of DNA degradation was checked by PCR, as explained earlier [157]. Afterward, RNA was transcribed into cDNA by a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc., Applied Biosystems™, Waltham, MA, USA), as specified by the manufacturer.

2.6 PCR amplification of environmental, tickborne nucleic acid extracts

2.6.1 Qualitative PCR amplification

For detailed microbiome analyses of Bb-negative and Bb-positive tick (**H1**), the V3-V4 region of the bacterial 16S rRNA gene (primers: 341f: 5′-CCTACGGGNGGCWGCAG-3′ and 785r: 5′-GACTACHVGGGTATCTAATCC-3′ [104]) of nucleic acid extracts from each tick were amplified by PCR. 13.5 µL UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific Inc.), 10 µL 5× HF buffer (Thermo Fisher Scientific Inc.), 2 µL forward (5 µM), and 10 µL reverse primer (5 µM), 1 µL dNTP Mix [10 mM] (Thermo Fisher Scientific Inc.), 0.5 µL Phusion high-fidelity polymerase (Thermo Fisher Scientific Inc.), 10 µL 5× PCR-Enhancer (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and 3 µL (5 to 75 ng gDNA) of tick nucleic acid extract were applied for each PCR reaction. For negative control, UltraPure™ water (Thermo Fisher Scientific Inc.) and a nucleic acid extract of *E. coli* (DSM 423) was used for positive control. PCR thermal profile was an initial denaturation at 98 °C for 2 min followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 57.4 °C for 30 s, extension at 72 °C for 30 s, and the final elongation at 72 °C for 10 min in a T100™ Thermal Cycler (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). PCR products were checked with a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light (Genoplex, VWR International GmbH, Darmstadt, Germany).

Conventional PCR screened the pools of the nucleic acid extracts of ticks (H2) for *A. phagocytophilum*, *N. mikurensis*, *Rickettsia* spp., and *W. pipientis*. For a 50 µL reaction, 10 µL 5x HF buffer (Thermo Fisher Scientific Inc.), 1 µL dNTP Mix [10 mM] (Thermo Fisher Scientific Inc.), 0.5 µL Phusion high-fidelity polymerase (Thermo Fisher Scientific Inc.), 10 µL 5x PCR-

Enhancer (Biozym Scientific GmbH), 5 µL respective primer pairs (details see **Table 7**), 16.5 µL UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific Inc.) and 2 µL (5 to 75 ng gDNA) for each tick nucleic acid extract were applied per PCR reaction. UltraPure™ water (Thermo Fisher Scientific Inc., Inc.) was used as a negative control. Nucleic acid extracts of *A. phagocytophilum* (extracted from *I. ricinus*), *N. mikurensis* (extracted from *Apodemus agrarius*), *R. helvetica* (extracted from *I. ricinus*), or *W. pipientis* (extracted from *Culex pipiens* laboratory colony) were used as positive controls. The PCR thermal profiles of *W. pipientis*, *Rickettsia* spp., or *A. phagocytophilum* specific PCR reactions were an initial denaturation at 98 °C for 2 min followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 56.2 °C (wsp-81f/wsp-691r [95]), 54.3 °C (RH314f/RH654r [158]) or 64.3 °C (ApMSP2f/ApMSP2r [102]) for 30 s, extension at 72 °C for 30 s, and the final elongation at 72 °C for 10 min in a T100™ Thermal Cycler (Bio-Rad Laboratories GmbH). For *N. mikurensis*, the thermal profile of PCR had an initial denaturation at 98 °C for 2 min followed by 55 cycles of denaturation at 98 °C for 15 s, annealing at 59 °C (NM-128f/NM-1152r [105]) for 30 s, extension at 72 °C for 45 s, and the final elongation at 72 °C for 10 min in a T100™ Thermal Cycler (Bio-Rad Laboratories GmbH). PCR amplicons were checked on a 3% agarose gel, stained with ethidium bromide, and visualized by UV light (Genoplex, VWR International GmbH).

Whenever a nucleic acid extract pool was tested positive for one of the respective species, the pool was split separately under the same PCR thermal profile for the respective pathogen(s). In addition, tick extracts that tested positive for *Rickettsia* spp. were sent to LGC Genomics GmbH (Berlin, Germany) for Illumina MiSeq paired-end sequencing using the same PCR primer pairs (RH314f/RH654r, **Table 7**) as sequencing primers.

2.6.2 Quantitative PCR amplification

The qPCR for DNA and cDNA samples (**H3**) of each nucleic acid extract was performed to quantify the contaminants *E. coli* (primers: 395f: 5′-CATGCCGCGTGTATGAAGAA-3′ and 470r: 5′-CGGGTAACGTCAATGAGCAAA-3′ [159]), *P. fluorescens* (primers: 433f: 5′-CTGACACCAAGGCTATCG-3′ and 576r: 5′-GCCTTCTACAACCGACAG-3′ [160]), and *M. luteus* (primers: 172f: 5′-AACCGTTAGACTCCGAGCAC-3′ and 393r: 5′-

CAGGAGCGTATTGCCGATGA-3′, this thesis). Primer pairs were evaluated as outlined previously [161]. Twofold concentrated iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories GmbH), 1 µL of the template (1:1, 1:10, and 1:100 dilution in three replicates) or nuclease-free master mix were run as a negative control for qPCR in a final volume of 20 µL. CFX96™ Real-Time System C1000™ Thermal Cycler (Bio-Rad Laboratories GmbH) was used

Table 7. Primer pairs were used in this study to detect species of the order Rickettsiales in tick nucleic acid extracts.

Bacterial species/ genus	Target gene	Amplicon length [bp]	Final Primer Concentration (nM)	Primer set*	References
A. phagocytophilum	*msp2*	77	400	**ApMSP2f:** 5′-ATGGAAGGTAGTGTTGGTTATGGTATT-3′ **ApMSP2r:** 5′-TTGGTCTTGAAGCGCTCGTA-3'	[102]
N. mikurensis	*groEL*	1024	500	**NM-128f:** 5′-AACAGGTGAAACACTAGATAAGTCCAT-3′ **NM-1152r:** 5′-TTCTACTTTGAACATTTGAAGAATTACTAT-3′	[105]
***Rickettsia* spp.**	*gltA*	340	400	**RH314f:** 5′-AAACAGGTTGCTCATCATTC-3' **RH654r:** 5′-AGAGCATTTTTTATTATTGG-3′	[158]
W. pipientis	*wsp*	591	500	**wsp-81f:** 5′-TGGTCCAATAAGTGATGAAGAAAC-3′ **wsp-691r:** 5′-AAAAATTAAACGCTACTCCA-3′	[95]

* f, forward and r, reverse. Primers were named as introduced in the respective reference.

for qPCR with the following thermal conditions: initial denaturation at 95 °C for 3 min for DNA samples or 30 s for cDNA samples followed by 35 cycles of denaturation at 95 °C (5 s),

annealing at 48 °C for *E. coli*, 46 °C for *P. fluorescens* and 50 °C for *M. luteus* (30 s), extension 60 °C (30 s) and the final elongation at 72 °C (10 min).

To calculate the gene copy numbers, the initial cell number of *E. coli*, *M. luteus,* and *P. fluorescens* were microscopically estimated in a Neubauer counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), followed by a nucleic acid extraction as explained above. Thereafter, a quantity of gDNA of each strain was used as a standard to correlate the PCR-cycle threshold values of nucleic acid extracts of each sample to respective gene copy numbers. The gDNA concentration per PCR reaction of *E. coli*, *M. luteus,* and *P. fluorescens* standard ranged from 6×10^9 to 6×10^1, 8×10^8 to 8×10^4, and 2×10^8 to 2×10^2 gene copies, respectively. Multiple dilutions were run simultaneously to check for inhibitors in qPCR assays. Based on these results, non-diluted DNA and cDNA extracts were best suited for qPCR analyses (data not shown). Cycle threshold and efficiency were calculated by the Bio-Rad software CFX manager version 3.1.

2.7 Amplicon sequencing

2.7.1 Bacterial 16S rRNA gene amplicon sequencing

To create amplicon sequencing libraries (**H1**, **H3**), the V3–V4 region of the bacterial 16S rRNA gene was amplified with the primer set (341f: 5′-CCTACGGGNGGCWGCAG-3′ and 785r: 5′-GACTACHVGGGTATCTAATCC-3′ [104]) of each of the 62 gDNA and 62 cDNA extracts, as well as the 200 Bb-negative and Bb-positive gDNA extracts of Weiden and Esslingen. Amplicon sequence libraries were made by adding inline barcodes and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) and MiSeq Reagent Kit v3 600 cycles (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. PCR products for library preparation were purified by AMPure XP beads (Beckman Coulter, Brea, CA, USA), and 5 µL of DNA or cDNA was equimolar pooled for each library (up to 96 libraries) with unique indices for each tick and treatment (**Table 5**). The sequencing of libraries was performed by 300 bp paired-end sequencing on an Illumina MiSeq platform (Illumina MiSeq V3; Illumina) based on a standard protocol from the

manufacturer. Amplicon sequencing and a basic sequence quality check were carried out by LGC Genomics GmbH (Berlin, Germany).

2.7.2 G*ltA* gene sequencing

Tick extracts that tested positive for *Rickettsia* spp. (**H2**) were also sent to LGC Genomics GmbH (Berlin, Germany) for Illumina MiSeq paired-end sequencing. The *gltA* gene-based amplicon sequencing library preparation was identical to the 16S rRNA gene amplicon sequencing approach, except for the sequencing primers (RH314f/RH654r, **Table 7**) used here.

2.7.3 Raw data pre-processing of bacterial 16S rRNA gene amplicon sequencing

Raw data pre-processing (**H1** and **H3**) with demultiplexing, sorting, adapter trimming, and merging reads were assembled using the Illumina bcl2fastq conversion software v2.20 and BBMerge [162]. Afterward, the sequence quality of the reads was checked with the FastQC software, version 0.11.8 [163]. Sequence pre-processing was carried out separately for DNA and cDNA samples, as Buettner and Noll (2018) described, with minor modifications [164]. Sequence pre-processing and OTUs picking from amplicons were performed using Mothur 1.35.1 [165]. Sequences with an average Phred quality score over 33 were aligned against the 16S Mothur-Silva SEED r119 reference alignment [166]. Short alignments were filtered, and sequencing errors were reduced by pre-clustering, where a maximum of one nucleotide mismatch per 100 nucleotides in a cluster was allowed. Singletons and chimeras were eliminated with the UCHIME algorithm [167]. For picking OTUs, sequences were classified taxonomically against the Silvia references classification and were removed from other life domains. By using the cluster.split method, OTUs were picked and assigned to a taxonomic level by clustering at the 97% identity level [168], leading to OTU tables.

2.7.4 *GltA* gene-based sequence analyses

For the *gltA* gene-based sequencing data analysis (**H2**), a reference database was created by downloading 13 *gltA* gene-based sequences described within the genus *Rickettsia* and transmitted by ticks, fleas, or mites from the National Center for Biotechnology Information

(NCBI) web-based database (https://www.ncbi.nlm.nih.gov/; last data update for this study was carried out August 13th 2021) [169,170]. The obtained *gltA* gene sequences were trimmed at the *gltA* primer pair sequences (**Table 7**) and were used for alignment by using the MEGA X software version 10.2.3 [171] and the MUSCLE algorithm [172]. While *R. helvetica*, *R. massiliae*, *R. monacensis*, *R. raoultii*, *R. slovaca,* and *R. aeschlimannii* were already described to be transmitted by ticks, *R. felis* and *R. akari* were included as outgroups of the dataset as both species were transmitted by fleas or mites instead of ticks [88,169]. In addition, *R. sibirica, R. africae, R. japonica,* and *Rickettsia* endosymbiont of *I. scapularis* were used as outgroups, as these *Rickettsia* species were so far found only outside of Europe (**Table S1**) [89,173].

The tick-borne *gltA* gene-based sequences were assembled and aligned to the *gltA* sequence database on the Galaxy training platform [174,175]. As Galaxy is used so far as the 16S rRNA gene pipeline, a few steps were modified to analyze *gltA* sequences. Data cleanup was conducted with a maximum sequence length of 325 bp. When sequences were poorly aligned between the *gltA* gene-based sequence's start and endpoint, including homopolymers with a length greater than or equal to six bases, they were removed from the dataset. Thereafter sequence alignment was pre-clustered to assemble sequences that were nearly identical to each other. Sequences with a difference of one in 100 bases potentially represent sequencing errors, not biological variability (here, three out of 300 bp). According to the Galaxy training platform, after removing chimeras, OTU clustering was performed on the obtained sequences [174].

OTUs were sorted in decreasing order according to the number of *gltA* gene-based amplicon sequence reads. Subsequently, all OTUs with a read number of fewer than three reads (singletons and doubletons) were removed from the dataset. Taxonomic classification was performed using NCBI BLAST [176]. An OTU count table with 24 OTUs was obtained (**Table SE2**). Based on the final alignment, the neighbor-joining method calculated a DNA-based phylogenetic tree using the Tamura–Nei model in MEGA X [171]. In total, 1397 positions in the final alignment were used for tree construction.

2.8 Statistics

Correspondence analysis (CA) was performed with the converted OTU tables (binary and relative sequence read abundances) summarized on location and assignment to human pathogenic *Borrelia* species presence (EN, EP, WN, WP; **H1**)) using RStudio and the package FactoMineR [177] to get the first insight into differences in the bacterial community compositions. Cluster analyses were carried out with a Euclidian distance according to the ward.D2 method between the composition of tick microbiomes (first two dimensions of CA) by using the functions dist and hclust in the package stats [147] and visualized by the package dendextend [178]. The ten most abundant genera were illustrated using Origin 2017 (OriginLab Corporation, Northampton, MA, USA) to compare the bacterial community composition between groups. Significant effects ($p < 0.05$) between the relative frequencies of the ten most common genera between groups (EN, EP, WN, WP) were calculated using Kruskal–Wallis ANOVA by using Origin 2017 (OriginLab Corporation) to exclude the apparent significance of bacterial genera.

The PCR results of each tick nucleic acid extract (**H2**) were analysed using the Fisher exact test with Bonferroni correction for each comparison of occurrence with other variables (Bb finding, location, month, season, (**Table 4**)) to determine which variable affected the TBP occurrence significantly. A Fisher exact test with Bonferroni correction was also performed to examine the occurrence of *Rickettsia*-associated OTUs in nucleic acid extracts as revealed by the *gltA* gene-based amplicon sequencing data. All tests were performed with the R software version 3.5.2. The significance level for the Fisher exact test was set as $\alpha = 0.05$. All statistical analyses were performed by RStudio, and Fisher exact test data were illustrated using Origin 2017 (OriginLab Corporation).

Rarefaction analysis, the estimation of alpha diversity (OTU richness, Shannon index, Pielou's Evenness), and OTU richness estimators (Chao1 and an abundance-based coverage estimator (ACE)) were performed for DNA and cDNA samples (**H3**) in RStudio, and the packages vegan 2.5-4. [147,179]. CA was performed with transformed bacterial OTU matrices (taxonomically summarized on genus level and additionally summarized on decontamination strategies). The Bray Curtis similarity heatmap and cluster analysis of OTU matrices were

calculated with the packages vegan 2.5-4. and gplots 3.0.3. [179,180]. As explained earlier, relative OTU abundances were calculated for each tick extract (Noll et al., 2005). OTUs were taxonomically summarized on a genus level, and the ten most abundant genera were visualized with Origin 2017 (OriginLab Corporation). Significant effects ($p < 0.05$) between bacterial OTUs, gene copy numbers, DNA and cDNA samples, and respective decontamination treatment were calculated by one-way ANOVA with a post hoc adjusted Tukey test in Origin 2017 (OriginLab Corporation).

The network analyses (**H1**) were performed using CoNet App, an integrated app in Cytoscape version 3.7.2 [124,181]. The networks were calculated separately for each group (EN, EP, WN, and WP), with an individual OTU table with relative abundances. Then, Pearson and Spearman correlation, Bray Curtis and Kullback–Leibler dissimilarity, and Hellinger distance were combined to create a robust network. 3000 edges (top and bottom) were chosen as the threshold for network calculation [182,183]. Finally, only edges were presented supported by at least three of these five statistical methods. Finally, the statistical evaluation of the obtained networks was achieved by calculating 100 random networks using the permutation and bootstrap method [184]. The topological roles of each node in all four networks were defined as previously described [125] (**Figure 2**).

Furthermore, the average closeness centrality and the average clustering coefficient of all four networks were determined. The closeness centrality of a node is a measure of centrality in a network and can be interpreted as measuring the distance between one node to another [185]. The clustering coefficient, in turn, measures the degree of interconnectivity in a node's neighborhood [186].

2.9 Availability of the generated data files

The bacterial 16S rRNA gene sequences for tick samples (**H1**) were deposited in the NCBI nucleotide sequence databases under accession PRJNA698232.

The *gltA* gene sequences for tick samples (**H2**) were deposited in the NCBI nucleotide sequence databases under accession PRJNA839573.

The bacterial 16S rRNA gene sequences for DNA and cDNA samples (**H3**) were deposited in the NCBI nucleotide sequence databases under accession PRJNA631133.

2.10 Statement of collaboration

My tasks during the project period were the formulation of the hypotheses to be investigated, as well as the planning, execution, or coordination in case of supervision thesis and (partial) evaluation of the experiments. These tasks included the preparation of a research plan at the beginning of the project, as well as the organization of laboratory materials and reagents. The 16S rRNA gene (**H1** and **H3**) and the *gltA* gene (**H2**) amplicon sequencing were carried out by the service provider LGC Genomics GmbH. Raw data preprocessing (see 2.7.3) of the 16S rRNA gene amplicon sequencings was also performed by LGC Genomics GmbH, while a separate raw data preprocessing pipeline (see 2.7.4) was developed for the analysis of the *gltA* gene sequencing data with the support of student Julia Braun to obtain her bachelor's degree.

Statistical analyses, as well as the corresponding visualization of the results, were then carried out either by myself or as part of the students' final theses. The student Tobias Rodiek contributed to calculating the co-occurrence networks (H1) and the first interpretations of possible interactions between Borrelia spp. and other bacterial genera. Regarding **H2**, I received support from the students' Marina Maier and Julia Braun. Through their research by Ms. Maier on specific genes and primer pairs for detecting TBPs of the order Rickettsiales, specific genes were validated, and primer pairs were established for each species under investigation at Coburg University. Subsequently, with the help of Ms. Braun, sample pools prepared from tick extracts were screened for human pathogens of the order Rickettiales using specific genes by PCR (**H2**). Pathogen-positive pools were separated after consultation with me to determine the tick extracts that had human pathogens. Thanks to the student Nadine Regnet, doubts about the choice, as well as the reactions of PCR for the investigation of **H3**, were revised, but their resolution is not part of the work.

Subsequently, the results were presented by myself through publications and presentations or posters (see 9) at symposia, congresses, or within the working group. In addition, interim or final reports were prepared by me periodically.

During my thesis, there was a continuous professional exchange with the Bavarian State Office for Health and Food Safety (LGL) and the integrated national reference center for Borrelia. With Dr. med. Volker Fingerle and his decades of experience with tick-associated pathogens, especially the genus *Borrelia*, expert knowledge was always available. The project was also supported by Dr. med. Fingerle and his team performed a multilocus sequence typing (MLST) to confirm the *Borrelia* findings (**H1**, 2.3.2). Furthermore, he contributed to all three publications through constructive criticism, as well as review and editing as a co-author.

In addition, all tick nucleic acid extracts used here (except the tick from **H3**), including the associated metadata, were made available to me by Dr. Thomas Müller from the company SYNLAB. Furthermore, I was supported by the possibility of sharing the extraction instrument, as well as the associated reagents for the extraction of the nucleic acids of the ticks from **H3**.

3 Results

After investigating the impact of human pathogenic *Borrelia* species on the bacterial community composition in the tick microbiome via 16S rRNA gene amplicon sequencing (see 3.1), different abundances were observed for the order Rickettsiales (see 3.2). Furthermore, different decontaminants were tested to assess the influence of artificially added contaminants on the results (see 3.3).

3.1 Influence on tick microbiome by presence of *Borrelia* species

Using 16S rRNA gene amplicon sequencing of Bb-negative and Bb-positive ticks, it was examined whether *Borrelia's* presence or absence affects the general composition of the bacterial community in the tick microbiome (3.1.1). Subsequently, the influence of *Borrelia* on the abundance of the different bacterial genera between Bb-negative and Bb-positive ticks was determined (3.1.1), and whether this influence occurs independently of the location (3.1.1). On the other hand, the role of location in the composition of the tick bacterial community was also evaluated independently of Bb findings (3.1.2). Network analysis was then used to make topological assignments of bacteria at the genus level (3.1.3) and to identify interaction patterns of bacterial genera with important topological roles in *Borrelia*'s presence or absence (3.1.3).

3.1.1 Bacterial community composition differed by the presence of *Borrelia* species

A total of 17,696,484 sequences were obtained after filtering, corresponding to 1944 bacterial OTUs (**Table S2** and **Table SE3**). All singletons were filtered from the OTU table, and OTUs were summarised on the genus level (**Table SE4**).

The presence of *Borrelia* species shifted the bacterial community composition in both locations (**Figure 3A**); EP and WP were clustered more closely than EN and WN (**Figure 3B**).

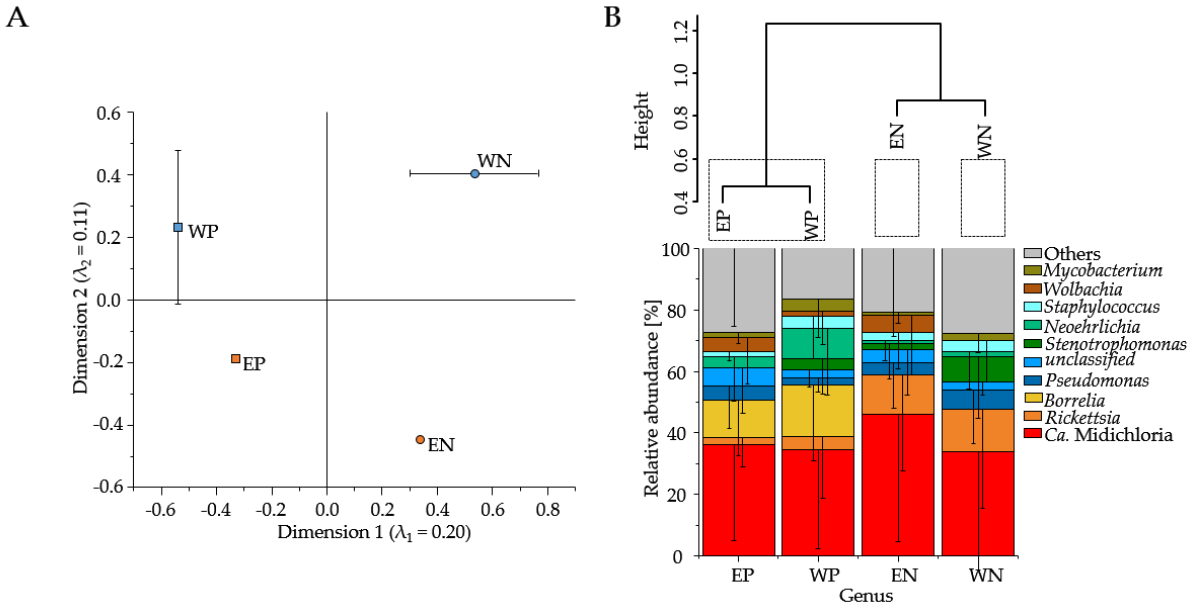

Figure 3. Correspondence analysis of bacterial community compositions (A), Euclidean distance matrix based on ward.D2 method (B above), and stack columns of the bacterial community composition (B below) of Bb-negative ticks retrieved from Esslingen (EN; n = 62; orange circle) or Weiden (WN; n = 56, blue circle) and Bb-positive ticks retrieved from Esslingen (EP; n = 38; orange square) or Weiden (WP; n = 44; blue square). Bacterial community composition is based on genus level with relative abundance data. The eigenvalues of both axes and standard error (SE) are shown (A and B). For clusters, heights of 0.6 were chosen and denoted in dashed boxes (B above). Bacterial community composition of the ten most abundant genera is shown, and other genera are summarized as "others". For colors and patterns, see figure legend.

Moreover, the Euclidean distances showed that *Borrelia* species (EP and WP) coincided in one cluster. In contrast, the absence of *Borrelia* species (EN and WN) leads to distinct clusters independently if binary or relative abundance data sets were used (**Figure 3B**, **Figure S2**, and **Figure S3**).

The shift in the bacterial community composition by the presence of *Borrelia* species was caused by higher sequence read abundances of *Ca.* Midichloria, *Rickettsia*, *Pseudomonas*, *Staphylococcus,* and an unclassified bacterial OTU in Bb-negative tick extracts and *Ca.* Midichloria, *Borrelia*, *Neoehrlichia,* and another bacterial unclassified OTU in Bb-positive tick extracts (**Figure 4**). While *Ca.* Midichloria was still present in each tick-borne bacterial community composition (EP, WP, EN, and WN) with approximately similar high sequence

read abundances; there was a noticeable reduction of the genus *Rickettsia* in the Bb-positive tick extracts (EP: 2.2%, WP: 4.3%, EN: 12.8% and WN: 13.9%) (**Figure3B and Figure 4**).

Moreover, *Neoehrlichia* showed higher sequence read abundances in Bb-positive (EP, 3.7%; WP, 9.8%) than Bb-negative tick extracts (EN, 0.9%; WN, 1.9%). Abundant bacterial genera differed in their relative abundances in each tick-borne bacterial community composition (EP, WP, EN, and WN). These included the genera *Stenotrophomonas* for WN and WP, *Coxiella* for WN, *Arsenophonus* and *Wolbachia* for EN, *Rickettsia*, *Staphylococcus*, and *Mycobacterium* for WP and *Sphingomonas*, *Spiroplasma*, *Wolbachia* as well as *Pseudomonas* for EP (**Figure 4**). The bacterial genera *Mycobacterium* (p = 1.35 × 10^{-3}), as well as Borrelia (p = 2.02 × 10^{-41}) and Neoehrlichia (p = 9.19 × 10^{-3}), were significantly more abundant in Bb-positive compared to Bb-negative tick extracts.

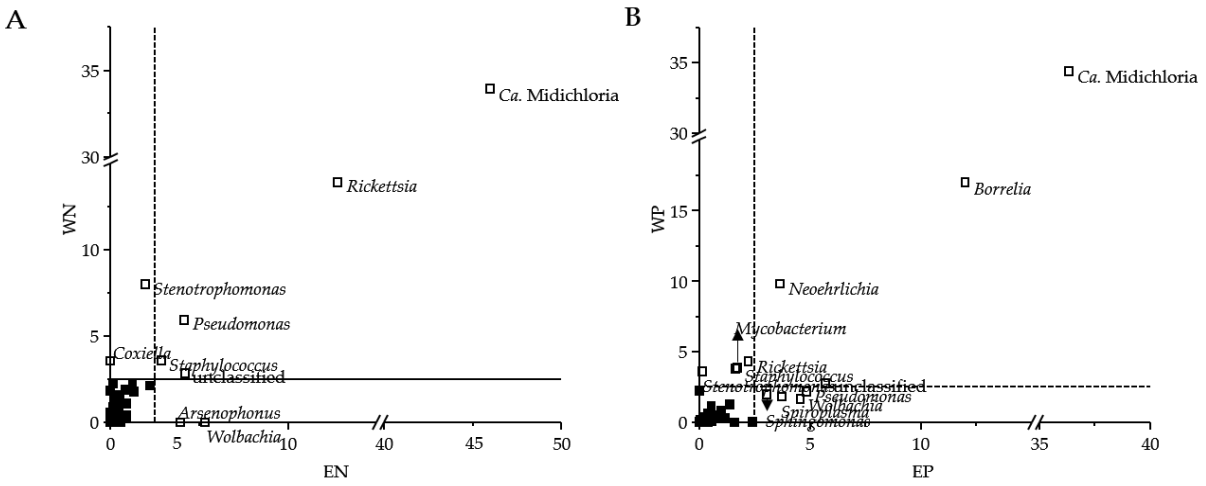

Figure 4. Differences in the sequence read abundances of bacterial genera obtained from tick-borne Bb-negative (A) (EN, n = 62; WN, n = 56) and Bb-positive bacterial community compositions (B) (EP; n = 38; WP; n = 44). Only bacterial genera were named with a mean relative abundance of at least 2.5% in Esslingen (E) or Weiden (W).

The richness (p = 1.62 × 10^{-3}) and Shannon index (p = 2.58 × 10^{-5}) were significantly lower in Bb-negative than in Bb-positive tick extracts, as well as Pielou's evenness (p = 4.74 × 10^{-42}) were significantly higher in Bb-positive compared to Bb-negative tick extracts. However, these indices were non-significantly different between both locations (**Table S2**).

3.1.2 Bacterial community composition differed between two locations

The bacterial community compositions of ticks retrieved from Esslingen (EN and EP) differed from those from Weiden (WN and WP) (**Figure 3**). The differences in the bacterial community composition between Esslingen and Weiden were based on the sequence read abundances of *Ca*. Midichloria, an unclassified bacterial OTU, *Pseudomonas*, and *Wolbachia* in ticks retrieved from Esslingen (EN vs. EP) and *Ca*. Midichloria, *Rickettsia*, *Staphylococcus*, *Stenotrophomonas*, and the same unclassified OTU in ticks retrieved from Weiden (WN vs. WP) (**Figure 3B** and **Figure 5**). Furthermore, EP and WP were governed by the bacterial genera *Borrelia* and *Neoehrlichia*, while *Spiroplasma* and *Sphingomonas* specifically dominated EP and *Mycobacterium* WP. On the other hand, *Staphylococcus*, *Arsenophonus*, and *Rickettsia* were more frequently abundant for EN, Coxiella, and *Pseudomonas* for WN. The sequence read abundances of the bacterial genera *Wolbachia* ($p = 1.01 \times 10^{-3}$) and *Pseudomonas* ($p = 2.07 \times 10^{-2}$) were significantly higher from ticks retrieved from Esslingen compared to those from Weiden. However, the bacterial genera *Stenotrophomonas* ($p = 0.085$), *Staphylococcus* ($p = 0.369$), as well as *Rickettsia* ($p = 0.730$) were non-significantly higher in ticks obtained from Weiden compared to those from Esslingen.

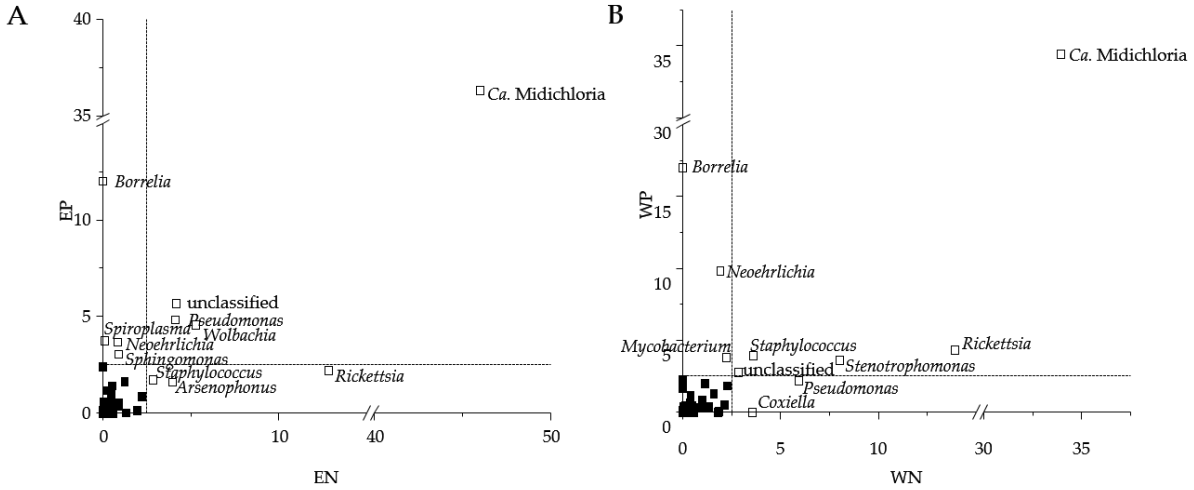

Figure 5. Differences in the sequence read abundances of bacterial genera obtained from tick-borne bacterial community compositions from Esslingen (EN, Bb-negative, n = 62; EP, Bb-positive, n = 38) and Weiden (WN, Bb-negative, n = 56; WP, Bb-positive; n = 44). Only bacterial genera were named with a mean relative abundance of at least 2.5% in Esslingen or Weiden (A and B).

3.1.3 Bacterial differences in co-occurrence networks between two locations and the presence of *Borrelia* species

The co-occurrence networks differed in their number of nodes and edges between EN, EP, WN, and WP, and the networks derived from EN and WN were more complex than those from EP and WP based on the number of clusters (**Table S3**). Networks derived from EN and

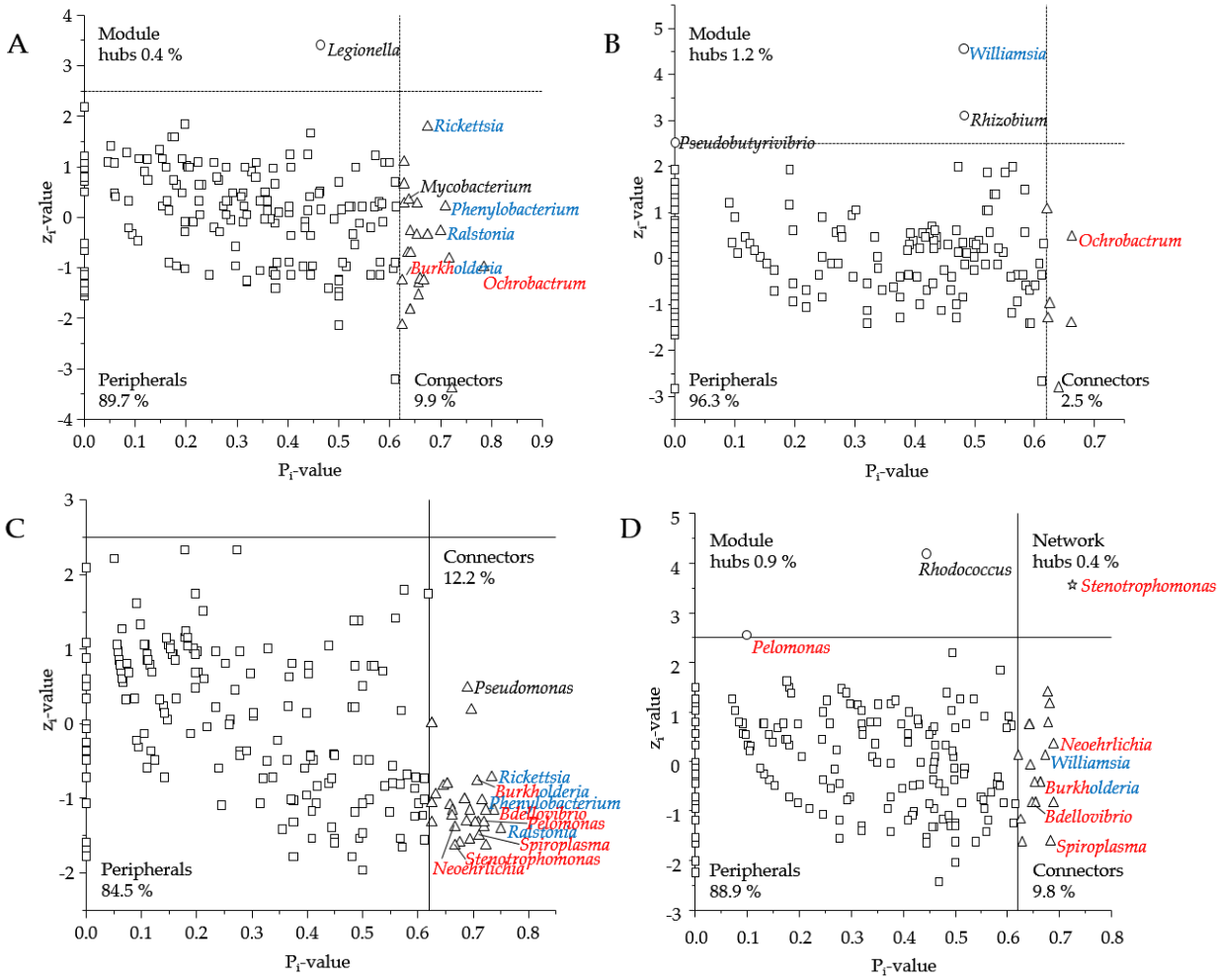

Figure 6. Topological roles of each node (bacterial genus) of a microbial network analyses for EN (A; n = 62), EP (B; n = 38), WN (C; n = 56) and WP (D; n = 44) according to their z_i and P_i values. All nodes of the networks were categorized into one of four groups according to their z_i and P_i values, as suggested [125]. Module hubs are marked as circles, network hubs as stars, peripherals as squares, and connectors as triangles. The blue-labeled genera occurred in two networks of the same locus (EN and EP or WN and WP) with the same or changed topological role (blue), while the red-labeled genera occurred in two networks of the different locus (EN and WN or EP and WP) with the same or changed topological role. An overview of the co-occurrence patterns of all nodes can be found in the appendix (Table SE5, Table SE6, Table SE7, and Table SE8).

WN showed a higher clustering coefficient than EP and WP. Regardless, a similar closeness centrality was found for all four networks. Networks derived from EN and WN had a higher proportion of connectors (9.9% and 12.2%) compared to EP (2.5%), while the number of module hubs was two and three times higher for EP and WP than for EN and WN, respectively (**Figure 6**). Overall, one network hub (*Stenotrophomonas* in WP) was identified (**Figure 6D**).
The network analyses showed that most bacterial genera co-occurred in the same patterns independently of *Borrelia* species or location, which was reflected in their respective topological role (**Figure 6**). However, the bacterial genera *Ralstonia*, *Clostridium*-sensu-stricto-19, *Rickettsia*, and *Phenylobacterium* were identified as connectors in the Bb-negative networks of EN and WN (**Figure 6A, C**). In contrast, no such observation was obtained in EP and WP networks. However, the topological role of *Williamsia* from a module hub in EP was found to be a connector in the WP network **Figure 6B, D**). Some bacterial genera obtained the same topological role as connectors in multiple networks, such as *Ochobactrum* for Esslingen networks (EN, EP) and *Spiroplasma*, *Bdellovibrio*, *Burkholderia* as well as *Neoehrlichia* for Weiden networks (WN, WP). Furthermore, the topological roles of *Pelomonas* and *Stenotrophomonas* were classified as connectors in WN but were module hub and network hub in WP, respectively (**Figure 6C, D**).

3.2 Co-infection of the order Rickettsiales on Bb-negative and Bb-positive tick microbiomes

PCRs were used to investigate the (co-)occurrence and sequence read abundances of (potential) TBPs of the order *Rickettsiales* and if they significantly correlate with the presence or absence of Bb in the bacterial tick microbiome (3.2.1). Furthermore, it was determined whether a seasonal or location-specific influence leads to the (co-)occurrence of (potential) TBPs of the order Rickettsiales via Fisher exact test (3.2.1). A 340 bp-sized *gltA* gene-based sequencing approach was performed to identify *Rickettsia* species that dominate the tick microbiome (3.2.2). Subsequently, the *Rickettsia* species identified here were tested for their independent occurrence with respect to the variables of location and season via Fisher exact test with Bonferroni correction (3.2.3).

3.2.1 *Rickettsia* spp. and *W. pipientis* showed the highest frequency in tick extracts

Among the 760 tick nucleic acid extracts tested, 16.7% were *Rickettsia* spp. positive, and 15.9% were *W. pipientis* positive. In addition, 2.8% of the 760 ticks were positive for *A. phagocytophilum* and 0.1% for *N. mikurensis*. The occurrence of *Rickettsia spp.* and *W. pipientis* was similar when comparing Bb-positive (*n* = 380) and Bb-negative (n = 380) tick nucleic acid extracts (**Table 8**).

Furthermore, 62 (8.1%) tick nucleic acid extracts were characterized with more than one member of the order Rickettsiales or with additional co-occurrence of Bb, of which two (n = 60/7.9%) or three (n = 2/0.3%) TBPs were found simultaneously (**Table 9**).

In particular, a high ratio of co-infection of Bb and *Rickettsia* spp. or *W. pipientis* was identified, and a significant effect between the co-infection of Bb and *Rickettsia* spp. ($p = 4.15*10^{-2}$) as well as *W. pipientis* ($p = 4.71*10^{-2}$) was observed (**Figure 7** and **Table 9**). Bb with *Rickettsia* spp. (64.9%) and Bb with *W. pipientis* (36.4%) co-occurred particularly frequently in the location of Weiden i. d. Oberpfalz (n = 242) and Neustadt a.d. Waldnaab (n = 229), respectively, as part of the southeast location (**Table SE1**).

Table 8. Presence of *A. phagocytophilum*, *N. mikurensis*, *Rickettsia* spp., and *W. pipientis* in individual tick nucleic acid extracts with Bb-negative (n=380) and Bb-positive (n=380) findings. Frequency is indicated by numbers and their relative frequency in percentage (n=760).

Bacterial species or genus	Bb finding	Frequency	Total frequency
A. phagocytophilum	negative	13 (1.7%)	21 (2.8%)
	positive	8 (1.0%)	
N. mikurensis	negative	1 (0.1%)	1 (0.1%)
	positive	0 (0.0%)	
***Rickettsia* spp.**	negative	53 (7.0%)	127 (16.7%)
	positive	74 (9.7%)	
W. pipientis	negative	50 (6.6%)	121 (15.9%)
	positive	71 (9.3%)	

Table 9. Co-occurrence of Bb, *A. phagocytophilum*, *N. mikurensis*, and/or *Rickettsia* spp. of individual nucleic acid extracts from ticks. Frequency is indicated by numbers and their relative frequency in percentage (n=760).

Frequency of co-infection	Bacteria species or genus	Frequency	Total frequency
Double	Bb + *A. phagocytophilum*	3/0.4%	60 (7.9%)
	Bb + *Rickettsia spp.*	57/7.5%	
Triple	Bb + *A. phagocytophilum* + *Rickettsia spp.*	2/0.3%	2/0.3%
			62/8.1%

The variables location, month, and season affected the occurrence of respective potential TBPs of the order Rickettsiales in the tick nucleic acid extracts. There was a significantly higher occurrence of *W. pipientis* in the location southeast (n = 69) compared to the locations south (n = 33) and west (n = 19). Further, *W. pipientis* was affected by season (highest in spring) or month (highest in May), while *A. phagocytophilum* and *Rickettsia* spp. showed no significance against any of the variables investigated (**Figure 7**).

However, when examining the influence of variables on the 76 pools, neither the Bb finding nor any other variable significantly affected the (co-)occurrence of the analysed species of Rickettsiales (**Figure S4**, **Table S4**, **and Table SE1**).

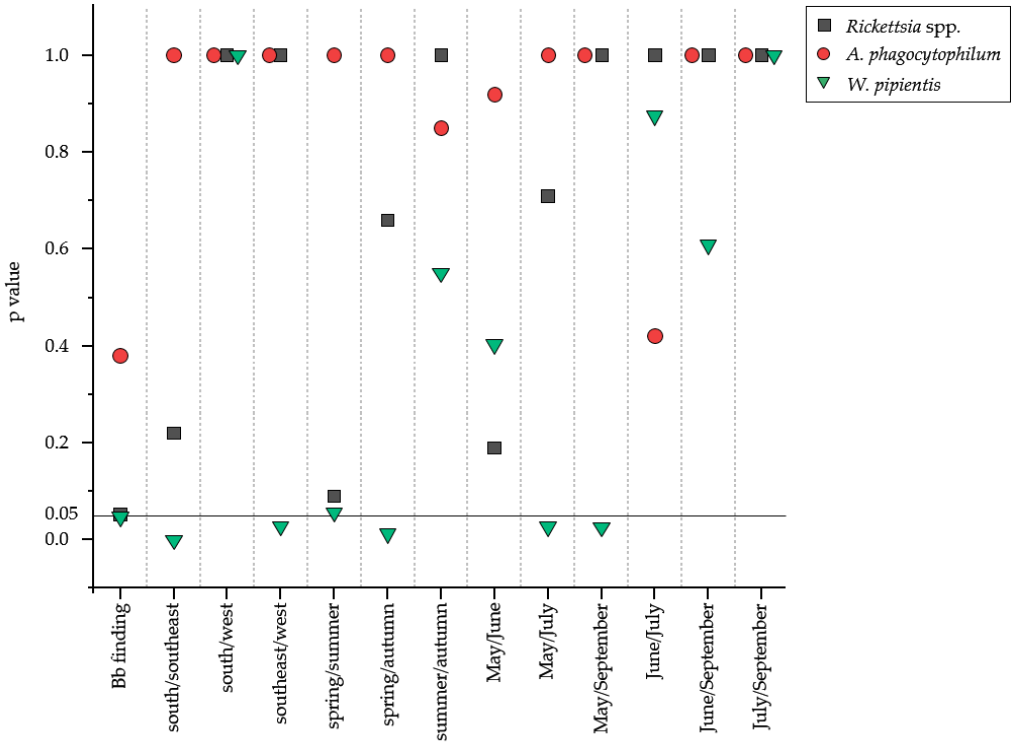

Figure 7. Effect of environmental variables on the occurrence of *Rickettsia* spp., *A. phagocytophilum*, *N. mikurensis*, and *W. pipientis* as revealed by Fisher exact test with Bonferroni correction. Symbol legend for each species is included in the figure. Details of the characteristics of the variables can be found in Table 4. A significance level of 0.05 is indicated.

3.2.2 *Rickettsia*-positive ticks were dominated by *R. helvetica*

The *gltA* gene was sequenced from 127 *Rickettsia*-positive nucleic acid extracts by Illumina MiSeq paired-end sequencing, and 24 different OTUs remained after filtering procedures. As a result of blasting the sequences of the OTUs, five different *Rickettsia* species were identified (**Figure 8** and **Table SE2**), and *R. helvetica* dominated 20 out of the 24 OTUs (94.4% of 2805 total sequence reads). Subsequently prevalent were *R. aeschlimannii* and *R. yenbekshikazakhensis*, respectively, each with two OTUs (OTU 2 and OTU 8, both 2.7%), *R. raoultii* with one OTU (OTU 3, 1.8%), and R. monacensis with one OTU (OTU 4, 1.0%) (**Figure 8** and **Table SE2**).

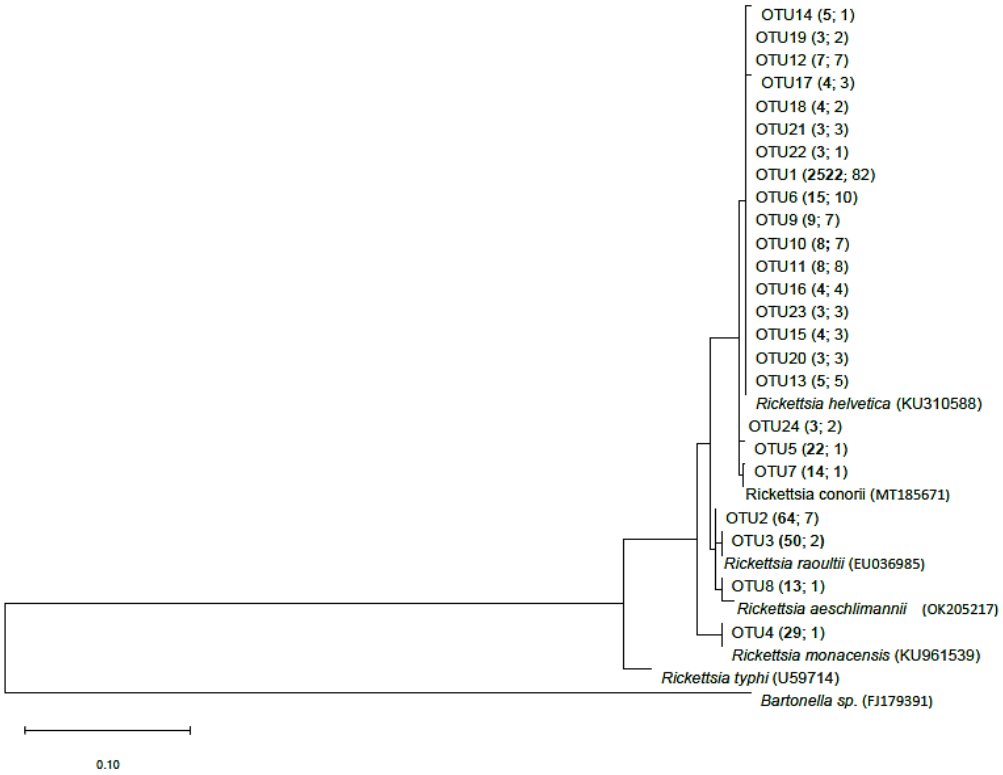

Figure 8. DNA-based neighbor-joining tree of partial *gltA* gene sequences. Environmental sequences retrieved from this study are indicated as—OUT with their total sequence read numbers in bold and frequency of OTUs both in brackets. The *gltA* gene sequences from reference organisms were retrieved from GenBank and their accession numbers are indicated in brackets. The scale bar represents 0.1 nucleotide substitution per site. For a sample-specific overview, see Table SE2.

3.2.3 Occurrence of *Rickettsia* species was affected by Bb finding

The identified *Rickettsia* species were then tested for significance to the presence of Bb, the variables location, month, and season (**Figure 9**). However, no association with any environmental variable was detected for *R. aeschlimannii*, but a significant effect was identified with Bb ($p = 0.011$). For *R. helvetica*, on the other hand, no significant effect was found for any of the environmental variables tested.

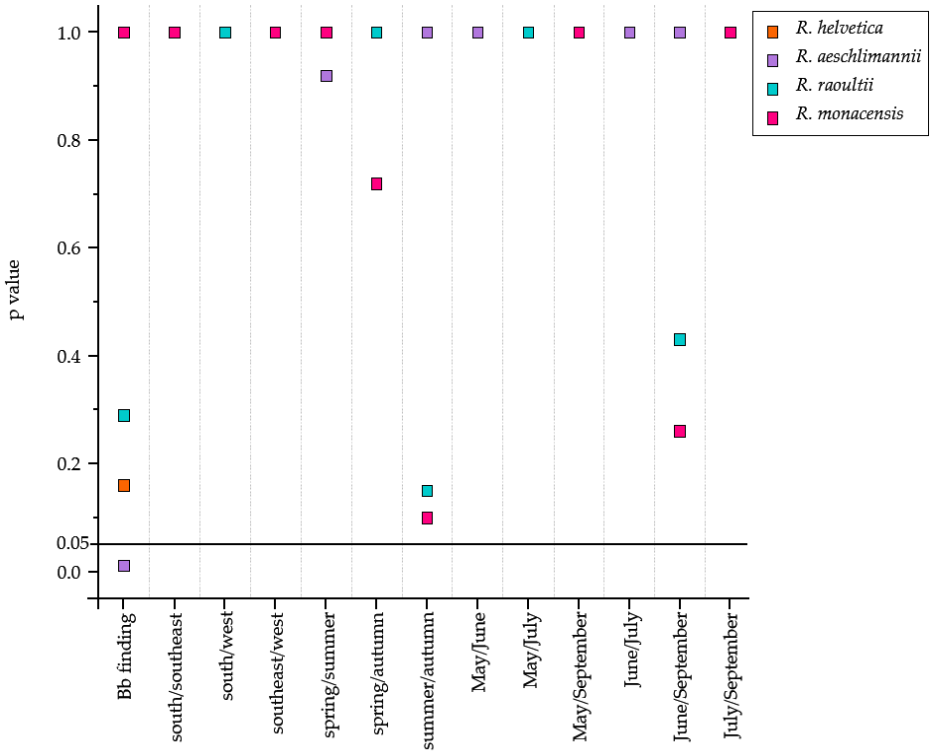

Figure 9. Effect of environmental variables on the occurrence of *R. helvetica*, *R. aeschlimannii*, *R. raoultii*, and *R. monacensis* as revealed by Fisher exact test with Bonferroni correction. Symbol legend for each species is included in the figure. Details of the characteristics of the variables can be found in Table 4. A significance level of 0.05 is indicated.

3.3 Survey for the decontamination of tick surfaces

To determine the influence of bacterial contaminants on the tick surface in microbiome studies, four different decontamination treatments ((i.) 70% ethanol, (ii.) DNA Away, (iii.) 5% sodium hypochlorite, and (iv.) RSDL) were tested. Differences in decontamination efficiency between gram-positive and gram-negative bacteria (3.3.1) or between DNA and cDNA samples (3.3.2) after decontamination treatment were observed. Additionally, the different decontamination treatments (3.3.3) affected the bacterial community composition, demonstrated by varying bacterial diversity and abundance according to decontamination treatments in DNA and cDNA (3.3.3).

3.3.1 Different reductions of artificial bacterial contaminants

A decontamination treatment with 5% sodium hypochlorite (DKA 3) was most efficient, followed by DNA Away (DKA 2), RSDL (DKA 4), and 70% ethanol (DKA 1) for DNA samples (**Figure 10A**). Regardless of the respective decontamination treatment, *M. luteus* was significantly less efficiently removed compared to *P. fluorescens* ($p = 2.34 \times 10^{-10}$) and *E. coli* ($p = 7.79 \times 10^{-12}$) for DNA samples (**Figure 10A**). Efficiency in removing *P. fluorescens* and *E. coli* was similar irrespective of decontamination treatment ($p = 0.650$) (**Figure 10A**). Decontamination efficiency was different between DNA and cDNA samples for particular contaminant strains. *M. luteus* was significantly less efficiently removed for cDNA samples compared to *E. coli* ($p = 0.024$) but not compared to *P.* fluorescens ($p = 0.999$) (**Figure 10B**). The decontamination efficiency of *P. fluorescens* was similar ($p = 0.057$) for cDNA samples compared to DNA samples, whereas *E. coli* ($p = 0.031$) and *M. luteus* ($p = 0.011$) differed for both sample types.

3.3.2 Decontamination treatment shifted bacterial contamination diversity

A total of 3,005,661 and 1,389,711 sequences were obtained for DNA or cDNA samples, corresponding to 2699 or 2256 bacterial OTUs, respectively. 1756 bacterial OTUs were found in both sample types, while 943 and 500 OTUs were solely present for DNA or cDNA samples, respectively. The ACE, Shannon index, as well as evenness were significantly different for DNA ($p = 0.001$, $p = 1.0 \times 10^{-6}$, $p = 3.0 \times 10^{-9}$) as well as for cDNA samples ($p = 0.002$, $p = 1.2 \times 10^{-4}$, $p = 9.1 \times 10^{-10}$) between the decontamination methods (DKAs) (**Table 10**). The OTU richness of respective DKA was significantly different for DNA samples ($p = 6.8 \times 10^{-5}$) but not for cDNA samples ($p = 0.124$). In turn, S.chao1 was not significantly different for DNA samples ($p = 0.102$) but for cDNA samples ($p = 0.001$).

3.3.3 Effect of decontamination treatment on tick-borne microbiome

The composition of the tick-borne microbiome was highly impacted by respective decontamination treatments for DNA and cDNA samples (**Figure 11**). While the microbiome composition after DKA 1, DKA 2, and DKA 4 was more similar to the positive control, the DKA 3 (5% sodium hypochlorite) clustered closer to the negative controls. These results were

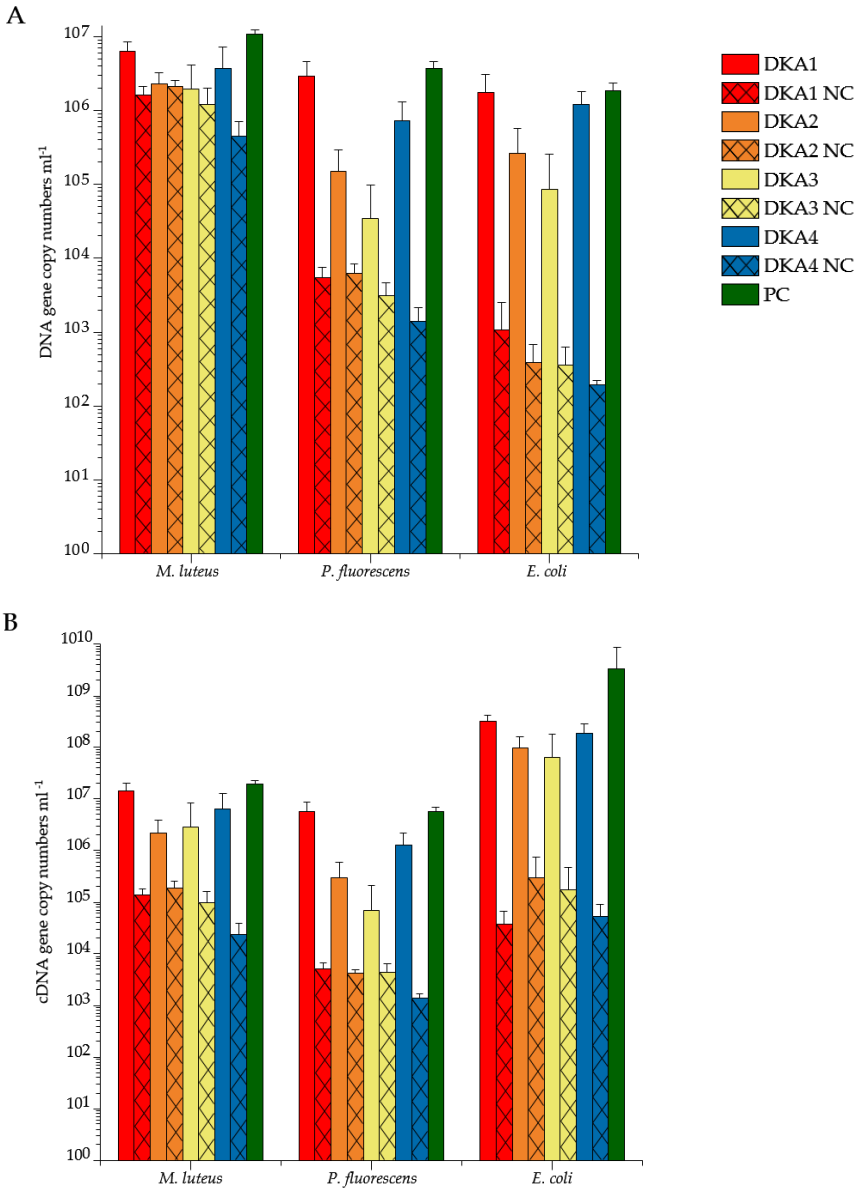

Figure 10. Reduction of artificial external tick-microbiome contaminants after decontamination with 70% ethanol (DKA 1), DNA Away (DKA 2), 5% sodium hypochlorite (DKA 3), and RSDL (DKA 4); positive control (PC), negative controls (NC) for DNA (A) and cDNA samples (B). For colors and patterns, see figure legend. Error bars indicate SE: n = 10 (each treatment, PC) or n = 3 (NC). The details of treatments and replicates are summarized in Table 5.

Table 10. OTU diversity indices from the tick microbiome after decontamination treatment with 70% ethanol (DKA 1), DNA Away (DKA 2), 5% sodium hypochlorite (DKA 3), and RSDL (DKA 4) for DNA (A) and cDNA samples (B). OTUs ≥ 1% of relative abundances were included. Mean values are indicated; n = 10 or n = 3. The details of treatments and replicates are summarized in Table 5.

A	DNA	OTU richness	Shannon	Pielou´s Evenness	S.chao1*	S.ACE**
	DKA 1	86 ± 17	0.9 ± 0.1	0.2 ± 0.0	359 ± 95	371 ± 118
	DKA 2	101 ± 50	1.0 ± 0.2	0.2 ± 0.1	1229 ± 1750	697 ± 599
	DKA 3	43 ± 22	1.6 ± 0.5	0.5 ± 0.2	136 ± 147	124 ± 122
	DKA 4	99 ± 65	0.8 ± 0.2	0.2 ± 0.1	536 ± 563	384 ± 378
	DKA 1 NC	35 ± 8	1.8 ± 0.2	0.5 ± 0.1	44 ± 16	41 ± 12
	DKA 2 NC	22 ± 4	1.4 ± 0.5	0.5 ± 0.2	26 ± 6	27 ± 6
	DKA 3 NC	17 ± 1	1.8 ± 0.2	0.6 ± 0.1	21 ± 3	21 ± 3
	DKA 4 NC	29 ± 3	1.9 ± 0.9	0.6 ± 0.3	42 ± 8	41 ± 6
	PC	112 ± 31	1.1 ± 0.1	0.2 ± 0.0	530 ± 270	512 ± 205

B	cDNA	OTU richness	Shannon	Pielou´s Evenness	S.chao1*	S.ACE**
	DKA 1	101 ± 60	1.0 ± 0.3	0.2 ± 0.1	370 ± 298	389 ± 348
	DKA 2	31 ± 24	1.3 ± 0.2	0.5 ± 0.2	44 ± 34	46 ± 32
	DKA 3	44 ± 24	2.0 ± 0.5	0.6 ± 0.1	88 ± 65	78 ± 52
	DKA 4	104 ± 144	1.6 ± 1.0	0.4 ± 0.1	168 ± 239	145 ± 194
	DKA 1 NC	12 ± 6	1.7 ± 0.6	0.8 ± 0.2	20 ± 10	28 ± 7
	DKA 2 NC	60 ± 29	2.7 ± 0.7	0.7 ± 0.1	75 ± 31	77 ± 32
	DKA 3 NC	20 ± 12	1.8 ± 0.6	0.6 ± 0.1	27 ± 10	33 ± 6
	DKA 4 NC	20 ± 8	1.9 ± 0.8	0.6 ± 0.2	34 ± 3	39 ± 2
	PC	92 ± 49	0.8 ± 0.3	0.2 ± 0.1	300 ± 162	313 ± 162

*Bias-Corrected Chao1, ** Abundance-Based Coverage estimator.

found for DNA as well as for cDNA samples (compare **Figure 11A** with **Figure 11B**).

The Euclidean distances revealed five bacterial clusters for DNA and cDNA samples, similarly organized in clusters 1 to 3 (**Figure 12**). However, negative controls (DKA 3 NC, DKA 1 NC, and DKA 4 NC) clustered differently between DNA and cDNA samples. Bacterial community compositions retrieved from ticks after 5% sodium hypochlorite treatment (DKA

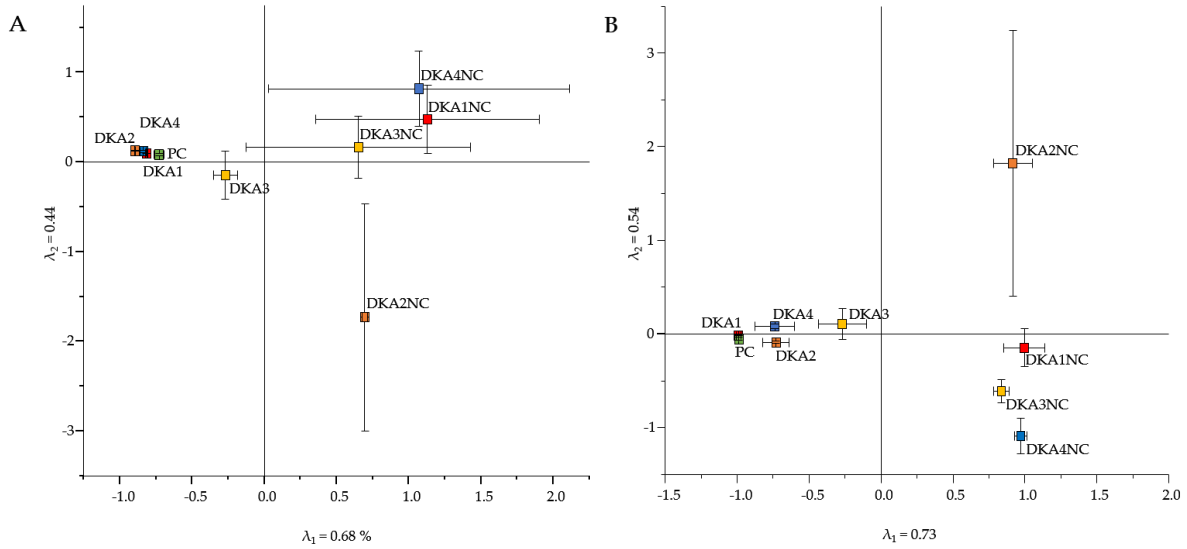

Figure 11. Correspondence analysis of bacterial community compositions for DNA (A) and cDNA samples (B) of 70% ethanol (DKA1), DNA Away (DKA 2), 5% sodium hypochlorite (DKA 3) and RSDL (DKA 4) decontaminated ticks. PC (positive control) without decontamination and NC (negative control) ticks without contamination. The error bars represent the SE: n = 10 (each treatment, PC) or n = 3 (NC). The details of treatments and replicates are summarized in Table 5.

3) clustered individually, whereas ticks after DKA 1, DKA 2, and DKA 4 treatment clustered with PC (cluster 2) (**Figure 12A**).

Bacterial community compositions of ticks after 5% sodium hypochlorite treatment (DKA 3) had high abundances of bacterial genera that were not part of the contamination solution (36.1% for DNA samples and 52.3% for cDNA samples) (**Figure 12** and **Table 11**). Bacterial community compositions of non-treated but contaminated ticks (PC) were characterized by low abundances of non-contaminants (3.5% for DNA samples and 3.7% for cDNA samples). In comparison, bacterial community compositions of non-contaminated but decontaminated ticks (NC) consisted of high abundances of non-contaminants (83.9% ± 7.0% for DNA samples and 97.25% ± 2.5% for cDNA samples) (**Figure 12** and **Table 11**). For detailed analyses, which treatment and replicate clustered together, see **Figure S5**.

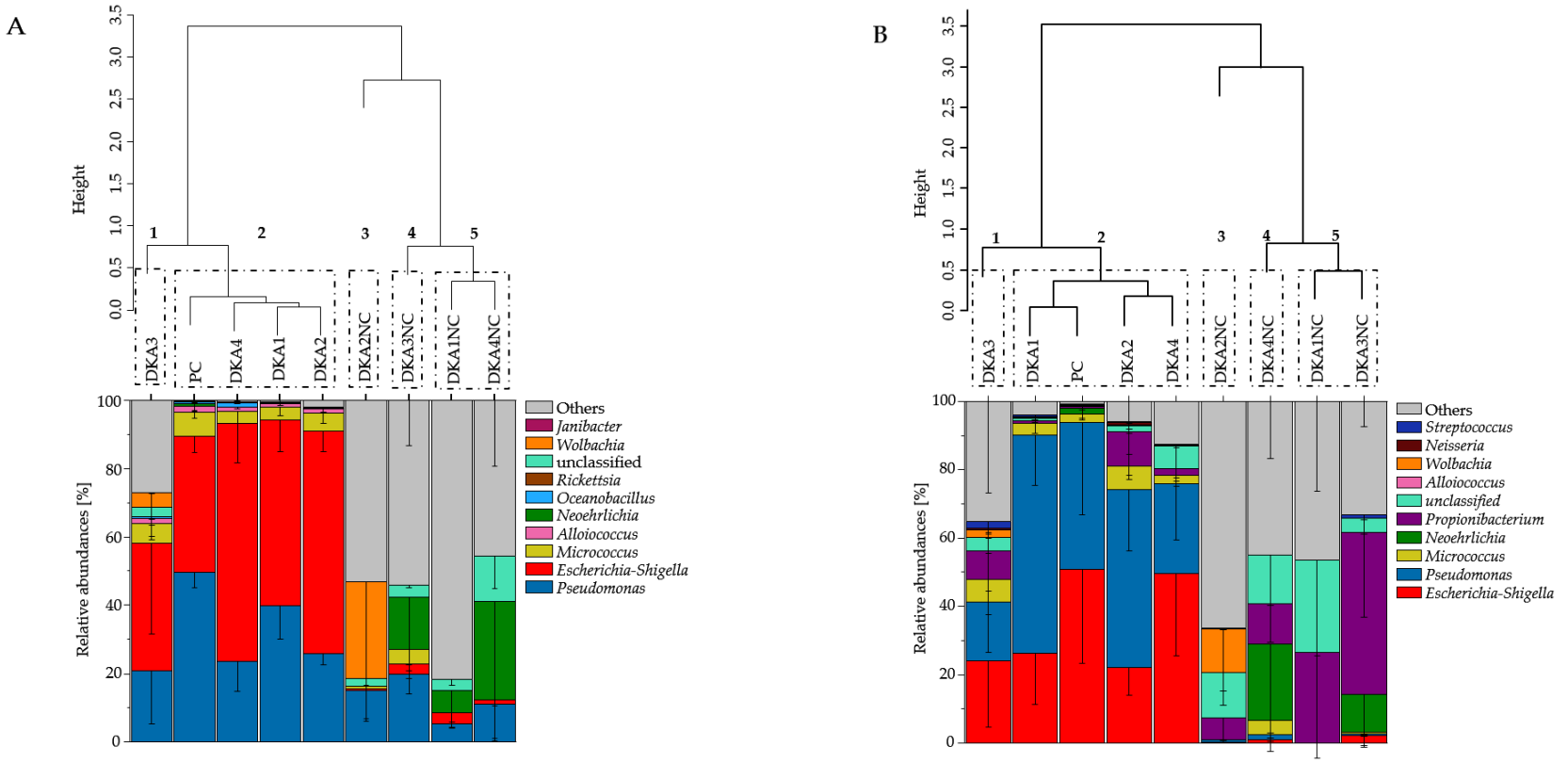

Figure 12. Euclidean distance matrix based on the ward.D2 method (above) and bacterial community composition on genus level (below) for the DNA (A) and cDNA samples (B) of 70% ethanol (DKA 1), DNA Away (DKA 2), 5% sodium hypochlorite (DKA 3) and RSDL (DKA 4) decontaminated ticks. PC (positive control) without decontamination and NC (negative control) ticks without contamination. For colors and patterns, see figure legend. The bacterial community composition of the ten most abundant genera is denoted, and other genera are summarized as "others". The composition of each sample can be seen in Figure S3. A height of 0.5 was chosen, numbered, and denoted in dashed boxes for clusters. The details of treatments and replicates are summarized in Table 5.

Table 11. Relative OTU abundances from the tick microbiome assigned to *M. luteus, P. fluorescens,* and *E. coli* after decontamination treatment with 70% ethanol (DKA 1), DNA Away (DKA 2), 5% sodium hypochlorite (DKA 3) and RSDL (DKA 4) for DNA (A) and cDNA samples (B). Mean values are indicated; n = 10 or n = 3. Details of treatments and replicates are summarized in Table 5. Relative OTU abundances were calculated for each tick and summarized decontamination strategies by the OTU count table for DNA or cDNA samples (Table SE9 and Table SE10).

A	DNA	Relative Abundance of Sequences of Bacterial Contaminants [%]		
		E. coli	*P. fluorescens*	*M. luteus*
	DKA 1	54.6	39.7	3.6
	DKA 2	65.2	25.8	5.1
	DKA 3	37.4	20.8	5.7
	DKA 4	69.7	23.5	3.5
	DKA 1 NC	3.1	5.3	0.1
	DKA 2 NC	0.6	15.1	0.7
	DKA 3 NC	3.0	19.7	4.4
	DKA 4 NC	1.2	11.0	0.0
	PC	39.7	49.8	7.0

B	cDNA	Relative Abundance of Sequences of Bacterial Contaminants [%]		
		E. coli	*P. fluorescens*	*M. luteus*
	DKA 1	26.2	64.1	3.4
	DKA 2	22.0	52.2	6.8
	DKA 3	24.0	17.1	6.6
	DKA 4	49.6	26.4	2.3
	DKA 1 NC	0.0	0.0	0.0
	DKA 2 NC	0.2	0.8	0.1
	DKA 3 NC	2.1	0.6	0.5
	DKA 4 NC	1.1	1.3	4.3
	PC	50.8	43.1	2.4

4 Discussion

The 16S rRNA gene amplicon sequencing results indicated that the presence of *Borrelia* species shifted sequence read abundances, especially those of the order Rickettsiales, and important topological roles in the tick microbiome (3.1.1 and 3.1.3). In addition, it was documented that location was less important for the bacterial composition of the tick microbiome but influenced the abundance of sequence reads (species of the order Rickettsiales). Suggesting that location characteristics altered bacterial interaction patterns, including location-dependent topological roles (3.1.2).

Species-specific PCRs and *gltA* gene sequencing data for differentiation of *Rickettsia* spp. were used to investigate the (potential) TBPs of the order Rickettsiales in more detail. It was shown that every fifth tick was infected with more than one TBP of the order Rickettsiales (3.2.1) and that the species *R. helvetica* dominates the genus *Rickettsia* spp. (3.2.2). Regarding the variables to be investigated here, it was noticeable that the Bb finding influenced the occurrence of *Rickettsia* species (3.2.3).

To determine the impact of environmental or host surface contaminants on tick diagnostics, four different decontaminants (**Table 5**) were applied to tick surfaces. Their nucleic acids were subsequently analyzed via 16S rRNA gene amplicon sequencing. Although 70% ethanol is well established for the decontamination of surfaces, 5% sodium hypochlorite was superior to all other decontamination assays in this study (3.3.1 and 3.3.2). While sodium hypochlorite may negatively impact sequencing results, the results indicate no loss of common sequences or altered community composition compared to the negative controls (3.3.3).

4.1 Presence of human pathogens of the *Borrelia burgdorferi* s. l. complex on tick microbiomes in two locations

4.1.1 Shifted sequence read abundances of bacterial genera

The microbiome of Bb-negative and Bb-positive ticks differed significantly as they revealed distinct clusters regardless of location (**Figure 3A**). The presence of *Borrelia* species in the tick microbiome was only weakly associated with shifts in the bacterial community composition. However, it was highly associated with shifts in sequence read abundances of the bacterial

genera in Bb-negative and Bb-positive tick microbiomes, as revealed when all microbiomes were analyzed, excluding *Borrelia* sequences (**Figure S6**). My findings of lower versus higher sequence read abundances of bacterial taxa in the presence of *Borrelia* species in the microbiome of *I. ricinus* were in line with results of other tick species (*I. scapularis* (laboratory-reared and field-collected) and *Amblyomma americanum*) [187–189]. These findings indicate that the presence of *Borrelia* species shifted the tick-borne community composition, interaction with *Borrelia* species, and interaction patterns within the tick-specific microbial community.

Results showed that the sequence read abundances of *Neoehrlichia* and *Mycobacterium* were significantly higher in the Bb-positive microbiomes (**Figure 3B** and **Figure 4A**, **B**), suggesting a clinical threat of co-infection with *Borrelia*, as *N. mikurensis* and *B. afzelii* have already been described in Romania [190]. The occurrence of *N. mikurensis* (8.4% [191]) and *B. afzelii* (30.5% [192]) can be estimated as very high in Germany, indicating that these species are common in tick microbiomes. This positive association between the two species is because *N. mikurensis* is a TBP that uses rodents as a reservoir host [191]. Similarly, the Bb contains numerous rodent-adapted genospecies, such as *B. afzelii* and *B. bavariensis* [60]. Ticks that take their larval and/or nymphal blood meals from rodent hosts are likely to be infected with these two pathogens.

In contrast, ticks that feed on other vertebrate hosts, such as deer, incompetent hosts for *B. afzelii* and *B. bavariensis*, will not be infected with these pathogens. Therefore, researchers should consider whether a strong positive association is present when analyzing the ticks, excluding morphological data (tick species, life stage, sex, engorged/flat) or whether it reflects the fact that the ticks have fed on different vertebrate hosts that differ in their competence to maintain TBPs. In contrast to *Neoehrlichia*, *Mycobacterium* is widely distributed in various environments such as soil, water, human and animal hosts [193]. Most tick-borne microbiome analyses based on 16S rRNA gene surveys have frequently reported worldwide members of the genus *Mycobacterium* [189,194,195]. Therefore, these members were also common in tick microbiomes. For example, *Mycobacterium smegmatis* possesses specific regulators capable of generating novel survival-related tick morphotypes during *B. burgdorferi* migration in nymphs [194,195].

4.1.2 Tick microbiome was dominated by *Rickettsia*, *Wolbachia*, *Coxiella*, *Spiroplasma,* and *Arsenophonus* regardless of the Bb finding

The tick microbiome was strongly dominated only by a few bacterial genera (**Figure 3B**). Most genera (*Rickettsia*, *Wolbachia*, *Coxiella*, *Spiroplasma,* and *Arsenophonus*) were classified as endosymbionts in one of the following hard ticks *I. pacificus*, *I. angustus*, *D. variabilis*, *D. occidentalis*, *D. albipictus,* and *Haemaphysalis leporispalustris* [196]. Such endosymbionts play an important role in the physiology of the tick and are believed to be essential for its survival, for instance, in vitamin synthesis [115]. Moreover, these endosymbiotic genera interact with co-present human pathogenic bacterial taxa (*Anaplasma*, *Borrelia*) of the tick microbiome [196]. In addition, the colonization of *A. phagocytophilum* in the microbiome of *I. scapularis* caused a decreasing occurrence of members of the genera *Rickettsia* and *Enterococcus* but an increase of *Pseudomonas* [197,198]. My study showed that the sequence read abundances of the genus *Rickettsia* were particularly higher in Bb-negative than Bb-positive microbiomes (**Figure 3B** and **Figure 4A**, **B**), indicating a correlation between *Borrelia* and *Rickettsia* in the tick microbiome. Kowalec et al. (2019) found a significant positive correlation between the genus *Rickettsia* and Spirochaetes in *I. ricinus* nymphs [127]. However, the pathogens of these genera occur in different inner organs of the tick. Therefore, it remains to be clarified how these pathogens can interact and what advantages they may gain. Next to *Rickettsia*, *Pseudomonas* and *Staphylococcus* revealed high sequence read abundances in the Bb-negative but not in the Bb-positive microbiomes (**Figure 4A**), which is in line with previous results from *I. scapularis* [189]. Moreover, both genera were described to inhibit *Borrelia* transmission next to other microbiome microbes [189]. Another study supports my findings of abundant *Pseudomonas* in Bb-negative microbiomes as they carry a Type VI secretion system, supporting antagonistic interactions with *Borrelia* [199].

Furthermore, the genus *Staphylococcus* was also characterized to interact antagonistically with *Borrelia* [189]. In addition, the infection of *I. scapularis* with *A. phagocytophilum* altered sequence read abundances in the tick-borne microbiome and the ability of *Staphylococcus* to form biofilms in the tick gut, which was assigned to be beneficial to inhibit the transmission of

A. phagocytophilum [197]. However, the role of other members of the tick microbiome on the *Borrelia* colonization or their presence has not yet been sufficiently investigated [115].

Nevertheless, it should be noted that a 450 bp nucleic acid sequence generated by 16S rRNA gene amplicon sequencing does not provide robust reliability for the identification of pathogens, and therefore the results of the sequencing can only be presented at the genus level [109,113].

4.1.3 Importance of location for tick microbiome composition

As the LB is almost equally distributed in Germany [75], I hypothesized that the bacterial tick microbiome, including the Bb findings, is location-independent. However, this hypothesis was rebutted as I found significant differences in the sequence read abundances of *Wolbachia* between both locations (**Figure 5A**, **B**). Members of the genus *Wolbachia* were described as mutualistic and capable of infecting mainly arthropods, including insects and nematodes. Members of the genus *Wolbachia* can be transmitted by endoparasitoid wasps *Ixodiphagus hookeri* (*I. hookeri*) to the ticks by oviposition [95], while ticks without contact with *I. hookeri* were free from *Wolbachia* [95,200]. However, such transfer by *I. hookeri* is expected to be at least 20% in the natural populations of *I. ricinus* in France [95]. Similar findings can be expected in this study for *I. ricinus* as *I. hookeri* is domestic in Germany.

As mentioned, Pollet et al. (2020) summarised in a review the temporal and spatial scales that affect tick microbiome compositions and found that ticks and, most likely, their microbiome rely on vertebrate movement to prevail as a meta-population [57]. This finding implies that location drives variability in the tick microbiome. So the regional composition or structure of vegetation (more natural, less fragmented sylvatic environment, or a more fragmented sylvatic environment) is essential for ticks and their hosts [57]. These studies concluded that landscape topography, climatic conditions, and vegetation are strongly associated with tick and host development [53,57,201–203], indicating why the tick microbiome composition between Esslingen and Weiden differed. Thus, an explanation of the significant differences in sequence read abundances of *Pseudomonas* and *Wolbachia* in Esslingen (**Figure 5A**) is the different regional conditions as well as the geographical separation from

each other with different flora and fauna. Therefore, both locations differed in their connectivity patterns of the landscape, which in turn influenced the presence or absence of hosts (variable host composition). Secondly, the spatial diversity within a location (microclimate) thus affects the tick density, leading to additional variability of the tick microbiome [57,130,187]. Finally, regional conditions contribute to microbial interaction patterns, shifting the acquisition/refusal of additional members into the tick microbiome (see 4.1.2), thereby playing a crucial role in the tick microbiome's composition and sequence read abundances[53].

However, besides regional structuration, many other tick-associated factors influence and cause variation in the tick microbiome [202,203]. First and foremost, the female tick plays a crucial role in the microbiome's composition by transferring beneficial microbes, important endosymbionts, and pathogens to the eggs or larvae by vertical transmission [115,204]. Furthermore, tick species, life stage and sex, nutritional status (fed/unfed), and host as the source of blood meal play key roles in the diversity of the tick microbiome [53,202–206]. These factors contribute to a regionally defined tick microbiome, and more research is needed to unravel the importance of each factor in the composition and abundance of the tick microbiome. Unfortunately, there was a lack of such information in my study, as I received already extracted tick nucleic acids. Therefore, it would be advantageous to repeat and support my hypotheses with complete morphological information, such as the life stage, sex, or status of engorgement of each tick.

4.1.4 Influence of *Borrelia* species on bacterial network

High bacterial variability within the microbiomes of diverse tick species was found [116,189,196]. Based on the co-occurrence networks of these microbiomes, I was interested in elucidating the location and Bb finding specific topological roles (**Figure 2**) of the respective bacterial members. Strikingly, none of the four networks was characterized by particularly high numbers of mutual exclusion (**Figure SE2**, **Figure SE3**, **Figure SE4**, and **Figure SE5**), suggesting that the tick microbiomes were not strongly characterized by microbial competition, which is in line with previous findings [204]. Furthermore, large nodes were

absent, indicating a robust and resilient co-occurrence network, as removing one node would only slightly affect the connectivity of the others. Therefore, these network topologies indicate the lack of trophic dependences or competition between co-occurring bacterial genera, which is in line with previous findings [116]. The average cluster coefficient of all four networks was similar (EN: 0.69 ± 0.27, EP: 0.57 ± 0.29, WN: 0.70 ± 0.27, WP: 0.58 ± 0.28), which contradicts the drawbacks of the "small world" theory [207] and supports the findings mentioned earlier [208].

Most mutual exclusions were found by members of the genus *Rickettsia* (**Figure SE6**, **Figure SE7**, **Figure SE8**, and **Figure SE9**), which is in line with previous findings of the tick microbiome of *I. pacificus* [204]. However, it is still unclear whether *Rickettsia* directly displaces or promotes other microorganisms or is independently present while other bacteria decreased over time in the tick microbiome [196,204]. Members of the genus *Rickettsia* played particularly in the Bb-negative microbiomes an important topological role as a connector (**Figure 2** and **Figure 6A**, **C**). Connectors are generalists with little to no specialization in their network behavior and can use many different resources [125]. Furthermore, the set of connectors in a network can be used to estimate modularity as they relate to each other [125]. Thus, modularity increases with the specificity of the connections, as shown by the high number of modules (clusters, **Figure SE10**, **Figure SE11**, **Figure SE12**, and **Figure SE13**), as well as the high proportion of connectors in the Bb-negative microbiome of Esslingen and Weiden (**Figure 6A**, **C**). These findings imply that the presence of Bb species by feeding into the tick microbiome caused a shift and rearrangement of interactions within the tick microbiome. Thus, the natural tick microbiome is disturbed, reflected in more unspecific interactions between the bacteria and a lower number of clusters (**Table S6**).

Members of the genus *Rickettsia* were connectors in Bb-negative microbiomes (**Figure 6A**, **C**) but were peripherals in the Bb-positive microbiomes. Therefore, any acquirements of *Borrelia* species lead to less prominent role members of the genus *Rickettsia* within the tick microbiome. This indicates that the sequence read abundances were reduced, and the bacterial interactions and functions were displaced. In addition, members of the genus *Ochrobactrum* were characterized as connectors in the microbiomes from Esslingen (**Figure 6A**, **B**), which co-occurred with less mutual exclusions if members of the genus *Rickettsia* were present in high

sequence read abundances in EN (**Figure SE14**). However, high mutual exclusions, if members of the genus *Rickettsia* were present in the extremely low sequence, read abundances in EP (**Table S6**, **Table SE6**, and **Figure SE15**). Thus, members of the genera *Rickettsia* and *Ochrobactrum* faced intense competition once *Borrelia* was present. If *Borrelia* was absent, both genera had more freedom over each other, and required resources could be more likely shared. Reconstruction of genomic information from members of the genus *Rickettsia* showed that all relevant genes for folic acid biosynthesis are present. Due to its unbalanced diet, the supply of folic acids is essential for the tick[49]. As the resource requirements for folic acid production in a substrate-limited tick environment [115] are demanding, the higher frequency of mutual exclusions of *Rickettsia* can be explained by bacterial competition for resources. These findings from Esslingen were in line with those from Weiden; however, networks revealed fewer mutual exclusions (**Figure SE16** and **Figure SE17**). Moreover, the WN network revealed an increase in mutual exclusions in the genus *Stenotrophomonas* compared to the EN network (**Figure SE18**, **Figure SE19**, **Figure SE20**, and **Figure SE21**), which is most properly based on the higher sequence read abundances of members of the genus *Stenotrophomonas* in Weiden (**Figure 3B** and **Figure 5B**), as well as the negative interactions (mutual exclusions) of members of the genus *Ochrobactrum*.

Thus, it can be hypothesized that members of the genus *Rickettsia* provide important defensive endosymbionts that protect the tick from colonization from members of the genus *Borrelia* [49]. If members of the genus *Rickettsia* were absent in the tick microbiome, such a protective function was likely to be carried out by members of the genus *Stenotrophomonas*, which is supported by the change in the topological role of *Stenotrophomonas* from a connector in WN to a network hub in WP.

4.2 Co-infection of potential tick-borne pathogens and *Borrelia burgdorferi* s. l. complex and their link to season and location in Germany

4.2.1 Co-infection of potential TBPs are season dependent

This study used PCR to investigate the diversity and occurrence of *Rickettsia* spp., *A. phagocytophilum*, *W. pipientis*, and *N. mikurensis* in 760 nucleic acid extracts of *I. ricinus* ticks

detached from humans, which were previously tested for the presence of human pathogenic species of Bb. Results were correlated to the variables season, location, as well as Bb finding of each tick extract via the Fisher exact test with Bonferroni correction. The occurrence of *Rickettsia* spp. in ticks varies widely within Europe (0.5 - 66%) and also within Germany (1 - 30.4%), depending on the infected host species [26,127]. Thus, the prevalence rate of *Rickettsia* spp. determined here is 16.7%, within the expected range [68,70,139,209].

In contrast, as described herein in the study, a similarly high occurrence of *W. pipientis* has not yet been described in the literature. Evidence suggests these endosymbionts significantly impact their vectors' fitness, reproduction, immunity, and other characteristics [62,139,210]. Thus, the viability of the tick and TBPs appears to depend on members of the genus *Wolbachia* [62,95]. In addition, using the Fisher exact test, a significant difference was found in the occurrence of *W. pipientis* in the variables of the season (spring (14.0%) and autumn (12.4%)), and month (May (14.0%) and July (24.8%) or rather September (12.4%)) (**Figure 8**). The tick infection can argue the reason with *W. pipientis* as the ticks are usually parasitized by the wasp *I. hookeri*, which carries *Wolbachia* [95,139,211]. Since the wasps lay their eggs in *I. ricinus* in summer and the development of *I. hookeri* continues only with the blood meal of the nymph in summer, an increased occurrence of *W. pipientis* in summer and autumn (June/July and September) and a flattening of the *Wolbachia* infection over the winter months is plausible [139,211].

On the other hand, A. phagocytophilum has been adequately described and is reported to have a prevalence rate of 1 - 17.4% in questing *I. ricinus* ticks in Germany [26]. The 2.8% prevalence rate detected here is thus low. According to Svitalkova *et al.*, the developmental stage of the tick itself influences the occurrence of *A. phagocytophilum* [212]. They observed a high frequency of *A. phagocytophilum,* especially in adult *I. ricinus,* whereas the prevalence rate in nymphs was low [139,212]. However, since the physicians or the diagnostic laboratory prepared no morphological data on the ticks sent in, it can only be assumed that they were nymphs rather than adults.

The simultaneous occurrence of two or three potential TBPs in 7.9% (n = 760) of the examined ticks indicates a potential contact of the ticks with a broad spectrum of pathogens

and/or hosts (**Table 9**) [70]. Klitgaard *et al.* (2019) summarized that double infection occurs in 1 – 22% of the examined *I. ricinus* ticks in Europe, and *Borrelia* spp. is involved in most co-infections [65]. A high frequency of co-infection of Bb and *Rickettsia* spp. or *W. pipientis* was also shown here (**Table 9**), supported by a significant effect (**Figure 8**). Concerning *Borrelia* spp. and *Rickettsia* spp., a similarly high frequency of co-infection [68], as well as a positive association [127], have already been described in the literature. However, the benefit of both genera remains unclear to date. The effects of co-infection of potentially pathogenic bacteria in a tick may imply beneficial and harmful properties for the pathogen itself and the infected tick. This means that, on the one hand, the virulence of the pathogens and, on the other hand, the severity and duration of the disease after successful transmission to a vertebrate host can be influenced [65]. However, co-infection suggests that using a common vertebrate host offers advantages in bacterial maintenance in a tick and spreads by the tick to circumvent the dilution effect. Indeed, it is already known that when host density is lower or decreases, ticks spread to fewer hosts, increasing the co-infection of (potential) TBPs [60]. The impact of the co-infection and potential bacterial interactions in the tick microbiome is unclear, including the significant effect of Bb finding on the endosymbiont *W. pipientis* (**Figure 8**). Nevertheless, as described above, there is evidence that the endosymbionts significantly influence their vector with their characteristics, which may explain an increased occurrence of *W. pipientis* with species of Bb.

The prevalence rate of 0.4% for Bb and *A. phagocytophilum* co-infection is in line with other studies from Germany [68]. However, a higher rate could have been expected, as it is known that *A. phagocytophilum* increases the colonization ability of *Borrelia* spp. and leads to more severe disease courses with diverse disease manifestations through immunosuppressive effects [65,72]. However, the detected co-occurrence seems logical since a relatively low A. phagocytophilum occurrence was obtained here.

It should be mentioned that the observed co-occurrences here should also be considered purely incidental. First, nucleic acid extracts from ticks infected with species of Bb were directly detected. Second, the occurrence of each pathogen was significantly higher as a co-infection; therefore, I consider an interaction between the pathogens likely.

4.2.2 Occurrence of *R. helvetica* to environmental variables

I. ricinus is dominated by *R. helvetica* in Europe and Germany [88,127] compared to other continents like North America, where *R. rickettsii* is the predominant species of the genus *Rickettsia* [89]. In addition, 94.4% of the *gltA* gene sequencing reads of *Rickettsia*-positive ticks were identical to sequences of *R. helvetica* (**Figure 8**, **Table S5** and **Table SE2**). Since *I. ricinus* simultaneously serves as a vector of *R. helvetica*, and additional highly efficient transovarial transmission is likely here, *R. helvetica* can be permanently maintained in the tick life cycle [26,71]. Additionally, *Rickettsia* spp. can be passed on to the eggs during fertilization by male ticks [71]. The high reservoir competence of diverse vertebrate hosts, like deer, mice, or hedgehogs, ensures geographic distribution and availability in diverse habitats [88]. The assignment of OTU 2 and OTU 8 is ambiguous as both OTUs matched 100% to both *R. aeschlimannii* and *Ca.* R. yenbekshikazakhensis (**Table S5** and **Table SE2**). Turebekov *et al.* described how *R. aeschlimannii* and *Ca.* R. yenbekshikazakhensis carry identical *gltA* gene sequences. Therefore, further investigations on these species in the future should be aimed at completely excluding the occurrence of *Ca.* R. yenbekshikazakhensis in Germany. Nevertheless, the occurrence of Ca. R. yenbekshikazakhensis in Germany is improbable since it has been found only in Kazakhstan [139,213]. Moreover, the associated tick species *Haemaphysalis punctata* was identified as a probable vector for *Ca.* R. yenbekshikazakhensis and this tick species is not part of the natural German tick diversity today [139,213,214]. However, *R. aeschlimannii* has occasionally been detected before in the microbiome of *I. ricinus* but was found to be transmitted by ticks of the genus *Hyalomma* in the African region [139,215,216]. An occurrence of these *Rickettsia* species in Germany is nevertheless possible, as *R. aeschlimannii* infected *Hyalomma* ticks have already been collected from several migratory birds, which occur in Germany and elsewhere [88]. *R. monacensis* in 1.0% of the reads seems realistic as the species is distributed on one side throughout Europe and associated with *I. ricinus* [26,70,139,216]. However, the host's risk of *R. monacensis* manifesting infection can be considered low, as only four clinical cases have been described recently.

Thus, while *I. ricinus* mainly harbors the *Rickettsia* species *R. helvetica*, *R. aeschlimannii*, and *R. monacensis* [26], the presence of *R. raoultii* in the dataset (**Figure 8** and **Table SE2**) is novel. *R. raoultii* has already been described in Germany [15] but is only connected with *D. reticulatus* and *D. marginatum* [71,88]. *R.raoultii* was present in 2.2% of the examined ticks. As *R. helvetica* and *R. monacensis* are mainly associated with *I. ricinus*, *R. raoultii* is strongly connected to *Dermacentor* ticks. As species identification was not carried out, the identified *R. raoultii* must likely have been harboured by an individual belonging to the genus *Dermacentor*. Therefore, molecular biological confirmation should be performed in the future to confirm the morphological identification of the tick. The results of the Fisher exact test for the *Rickettsia* species support my assumption that *Rickettsia* species are adapted to the environmental variables as they revealed no dependencies with respect to the variables tested in this study (**Figure 9**). Moreover, the problem of PCR inhibition is crucial for both PCR and sequencing approaches, which have already been observed in the tick microbiome studies [98]. Therefore, upcoming studies should be aware of such PCR drawbacks.

4.3 Analysis of Tick Surface Decontamination Methods

4.3.1 Difference in decontamination strategy

Most previous studies assessed the internal tick microbiome without any decontamination strategy for the external tick microbiome. Studies with a decontamination strategy mainly applied ethanol-based decontamination [46,48,217], which is methodologically similar to my approach (DKA 1). However, my decontamination efficiency tests revealed that 5% sodium hypochlorite was the most efficient agent for tick surface decontamination, followed by DNA Away, RSDL, and 70% ethanol (**Figure 11** and **Figure 12**). Sodium hypochlorite was mainly used to eliminate DNA contaminants and dental infections and for laboratory surface decontamination [218,219]. My literature research revealed that only one study used bleach solutions to decontaminate the external tick microbiome [140], while two other studies combined bleach and ethanol [141,142]. However, these studies did not address the decontamination efficiency of bleach solutions or compare different decontamination approaches, but they did reveal meaningful conclusions. Sodium hypochlorite is known to

randomly disrupt cellular metabolic processes resulting in the degeneration of phospholipids. This causes oxidative reactions with irreversible enzymatic inactivation, forming cytoplasmic chloramines that finally inhibit cellular metabolism, leading to a degradation of lipid and fatty acids [144,220]. Suppose 5% sodium hypochlorite was used as the primary reactive substance in the decontamination solution. In that case, degradation of nucleic acids is expected, which can cause a lower DNA and RNA content in the external tick microbiome.

4.3.2 Differences between gram-positive and gram-negative contaminants

The highest quantitative removal of *E. coli*, *P. fluorescens*, and *M. luteus* as contaminants were achieved by a 5% sodium hypochlorite treatment (**Figure 10**), which is in line with previous results from biofilm removal [219]. The removal efficiency was similar to DNA and cDNA samples for all tested contaminants (**Figure 10**). However, the decontamination of Gram-positive *M. luteus* was significantly less efficient than other contaminants, which is in line with previous sodium hypochlorite treatments [221]. Moreover, Gram-positive bacteria and their nucleic acids were more challenging to eliminate. These bacteria withstood sodium hypochlorite treatments of different concentrations, including 5% sodium hypochlorite and an incubation period from 1 to 10 min [221–223]. Besides, other decontamination treatments (DKA 1, DKA 2, and DKA 4) were also less efficient in removing the contaminant strain *M. luteus* than Gram-negative contaminants, such as *E. coli* and *P. fluorescens*. However, a direct comparison of gene copy numbers and sequence reads is inappropriate for estimating the removal efficiency. Different primer sets and PCR conditions were employed, and these methodological implications were addressed previously [224].

4.3.3 Ticks treated with 5% sodium hypochlorite displayed the lower richness

Ticks treated with 5% sodium hypochlorite revealed lower richness than other DKAs, as the number of singletons and doubletons was much lower (**Table SE9** and **Table SE10**). Therefore, 5% sodium hypochlorite treatment reduced the number of rare sequences and the overall read coverage compared to the other DKAs (**Table 10**). In turn, 5% sodium hypochlorite-treated tick microbiomes had a more even distribution of sequences as the

number of rare sequences of other DKAs caused uneven distributions. The effect of sodium hypochlorite on amplicon sequencing results can be based on (i) a higher degradation of free and less abundant nucleic acid sequences, (ii) the Illumina-based synthesis reaction during sequencing, or (iii) or Illumina-based dye reactions. Interestingly, sodium hypochlorite concentrations between 0.9 to 6% (vol./vol.) caused no negative effect on the PCR amplification process [225,226]. The MiSeq system guide of Illumina routinely recommends a solution containing 1.25% (vol./vol.) sodium hypochlorite in a washing step after post-run to eliminate contaminations of previous sequencing runs [227].

Furthermore, the manufacturer recommends being careful in this washing step since high sodium hypochlorite concentrations lead to failures in cluster generation in subsequent runs [227]. Therefore, a carryover of even trace amounts of sodium hypochlorite into the sequence reactions can significantly affect sequencing results. However, in my experiments, I included in each DKA a strict regime to remove DKA components by (i) discarding the supernatant after DKA treatment, (ii) evaporating the remaining solution in SpeedVac, and (iii) washing nucleic acid extracts three times with RNAse and DNAse free water. After amplification, amplicons were desalted before sequence reactions, which removed potential traces of sodium hypochlorite or other DKA compounds.

Microbiomes retrieved from ticks decontaminated with 5% sodium hypochlorite clustered with NCs (**Figure 11**). However, some NCs contained very low sequence read amounts of OTUs affiliated with *P. fluorescens* and *E. coli* (**Table 11**), similar to my contamination solution sequences (**Table 6**). Likewise, identical sequences were also found as part of the tick microbiome in previous studies without contamination [197,228], indicating that these OTUs are part of the indigenous tick microbiome. Variation in the bacterial community composition of the tick microbiome has been frequently found [229]; therefore, standardised microbiomes from the NCs were not expected and revealed a high variance (**Figure 11**). The microbiomes of ticks decontaminated with DNA Away, RSDL, or 70% ethanol were highly biased by sequences of the contamination solution and clustered with the PCs (**Figure 12**).

4.4 Conclusion

4.4.1 *Borrelia* influences the distribution of sequence reads in the tick microbiome depending on location

The presence of *Borrelia* species significantly shifted the sequence read abundances and important topological roles of the tick microbiome. Moreover, the location was less important in the tick microbiome composition, and sequence read abundances were important in the location-dependent topological network role, indicating that location characteristics changed bacterial interaction patterns. Since infection with human pathogenic Bb species and other tick-associated pathogens varies regionally, I suggest that a bacterial 16S rRNA gene-based microbiome analysis should be included for both tick and host in the case of Bb-positive findings. The data foundation of a combined diagnostic approach of qPCR and amplicon sequencing will enable valid decisions for adequate treatment of *Borrelia* species and additional co-occurring pathogens, as they were location-specific.

4.4.2 Every fifth tick carried at least two of the (potential) pathogens

The risk of co-infection of several potential TBPs in tick microbiomes was high in the studied locations of Germany. Almost every fifth tick collected from humans carried at least two of the (potential) pathogens or genera (i.e., *Rickettsia* spp.) containing human pathogenic species investigated here. In addition, a few rarer *Rickettsia* species (*R. monacensis*, *R. raoultii*) were identified as not established in vector *I. ricinus*, suggesting that they use other vectors and live in smaller ecological niches. Thus, in contrast to the other human pathogenic species of the genus, the predominant species in Germany, *R. helvetica*, seems to have found better mechanisms to establish itself in *I. ricinus* and its hosts, thus ensuring its survival.

Nevertheless, the lack of morphological determination of the tick in the diagnostic laboratory must be considered here. In the future, this should be integrated into the analysis process. Only based on the data, such as species, sex, or stage, can exclusion criteria of (potential) TBPs be created, simplifying the evaluation of critical test results with a very high probability.

4.4.3 5% sodium hypochlorite treatment as superior decontamination solution

The decontamination of ticks is commonly carried out with ethanol, which was the most inefficient agent in my study. Strategic rethinking in the decontamination of ticks is needed as sodium hypochlorite treatment was superior to other DKAs. Although sodium hypochlorite may negatively affect sequencing results, my findings suggest no loss in frequent sequences or shifted community composition compared to the NCs. In this study, I focused on adult *I. ricinus* individuals as decontamination targets, and upcoming approaches should address the transferability of my methodology to decontaminate other plant or animal targets (including larvae or nymphs of *I. ricinus* or other *Ixodes* species).

5 Outlook

Through this study, it became clear that the presence of species of Bb impacted the abundance of specific bacterial genera in ticks. Furthermore, the presence of *Borrelia* even caused shifts in the topological roles in the network. Here, the results must be first independently verified and cross-checked in the microbiomes of other tick vectors to underline the conclusions of this work.

Also, location dependence was shown to impact the sequence read abundance and the presence of bacterial genera rather than the bacterial presence in the tick microbiome. This location dependence consequently affected the topological roles and associated interaction patterns, as revealed earlier by *Borrelia*'s presence. This result of the work supports the idea of using bacterial genera or species with important topological roles as biomarkers, as further differentiation based on the location of the tick finding seems unnecessary. However, it should be noted here that the findings on location (in)dependence and their results refer exclusively to the tick species *I. ricinus* established in Germany. Thus, a database expansion for different tick vectors in other or larger locations would be necessary so that the bacterial community compositions found can also be used as reliable biomarkers beyond the German borders or for other tick vectors far less established in Germany (*D. reticulatus or Haemaphysalis punctata*).

Another interesting question would be to find out which *Borrelia* load in a tick after a tick bite leads to a confirmed *Borrelia* infection (including symptoms). While the *Borrelia* load can be measured with a quantitative PCR method, the results can be matched with patient data, such as gender, age, and previous illnesses. In addition, the patient's antibody titer could be measured and monitored by ELISA and included in the evaluation of *Borrelia* infection.

In the further course of the work, it was found that every fifth tick (n = 760) carried at least two of the (potential) TBPs investigated here. However, since only TBPs of the order Rickettsiales were examined in more detail, an extension of the analysis of other TBPs occurring in Germany (e.g., *Coxiella brunetii* or *Francisella tularensis*) would be necessary to determine a risk assessment for these as well. Subsequently, the results could be used to establish a multiplex PCR. This type of PCR would have the advantage that not only are individual pathogens tested, but at the same time, the risk of co-infection can be excluded,

which in turn can influence the course of the disease, as well as the treatment options. In addition, the costs, benefits, and time required after successful establishment would be significantly lower. For this purpose, automation could even be used for PCR preparation and sample processing, which have been increasingly found in diagnostic laboratories since the onset of the 2020 pandemic and should subsequently be used for other samples, tasks, or analyses.

Species differentiation of the genus *Rickettsia* spp. in addition to the dominant species *R. helvetica*, other species are also present, although in lower abundance, such as *R. monacensis* and *R. raoultii*. Surprisingly, the *Ca.* R. yenbekshikazakhensis was found for the first time in Germany. This finding should be followed up to verify whether *Ca*. R. yenbekshikazakhensis is a chance finding or a new species that could become established in Germany.

Regarding the suitability of decontaminants for cleaning the tick surface, 5% sodium hypochlorite was superior to the other decontaminant agents tested (70% ethanol, DNA Away, and RSDL). Nevertheless, different concentrations should be tested in advance (e.g., 0.5 - 10%). Furthermore, the duration of exposure of the decontaminant to the tick surface could play another important role in removing environmental contaminants. If the results are identical or similar to this study, sodium hypochlorite should be used in tick diagnostics. In conclusion, analysis results would be even more reliable, and possible false negative or false positive results of TBPs would be reduced, influencing the further (dispensable) procedure in treating the "patient."

Machine learning, in particular deep-learning algorithms, could provide another approach to studying the diverse microbiome of *I. ricinus*. These have made significant advances in automated clinical diagnostics (e.g., lung cancer, heart disease, or stroke), making diagnostics cheaper, more efficient, faster, and less error-prone. Furthermore, increasingly voluminous and validated datasets are available for machine learning, which makes the algorithms better and better at pattern recognition. Subsequently, a database of bacteria with a specific topological role in the presence or absence of *Borrelia* could be created and act as a kind of biomarker in tick diagnostics. More precisely, this means that the bacterial species identified as biomarkers can serve as a detectable parameter in the case of an uncertain *Borrelia* finding.

Thus, these bacterial species have a diagnostic significance and can be used in case of a questionable *Borrelia* finding. This database should then be freely accessible to all diagnostic laboratories to advance the optimization of Bb diagnosis and ensure a uniform analysis strategy ideally organized in DIN standards.

The added value or relevance of the topic for society, as well as for industry, is considerable. The introduction of high-throughput sequencing methods in routine diagnostics and the resulting data sets can not only make treatment success more feasible in complex microbial backgrounds but also avoid misdiagnosis, as well as resulting incorrect therapeutic approaches or treatments in the future. Since antibiotic resistance in patients has risen sharply in recent decades, the "aimless" use of this group of drugs must be regarded as absolutely avoidable. The microbiome and its beneficial interaction with humans is disturbed by this systemic antibiotic use, and subsequent implications are caused. By establishing this methodology in diagnostics, including the machine-learning approach of sequencing data sets regarding the microbiome of different habitats, personalized therapy approaches can be made possible, thus reducing side effects.

6 References

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7 Acknowledgment

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8 Supplementary data

8.1 Figures

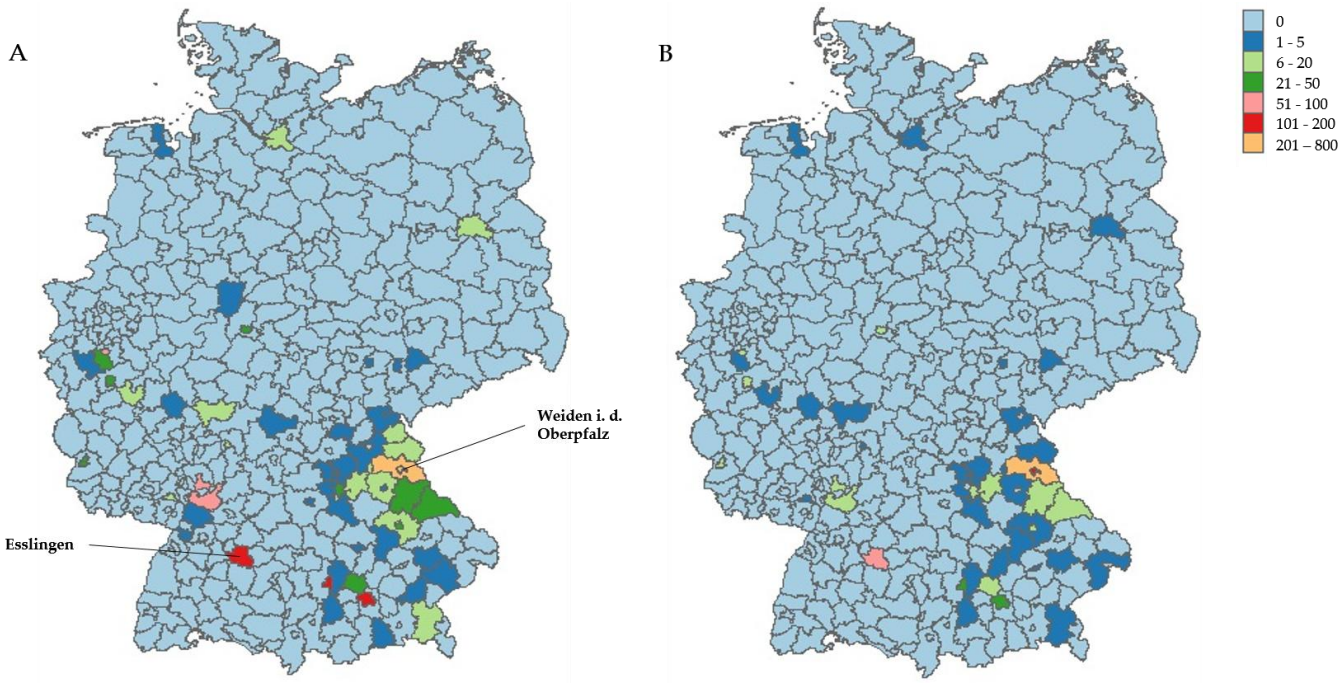

Figure S1. Mapped Bb-negative findings (A) and Bb-positive findings (B) on a map of Germany with identification of the districts and district-free cities for 2018. Colors of the legend mark the available tick nucleic acid extracts to the corresponding Bb finding in the figure.

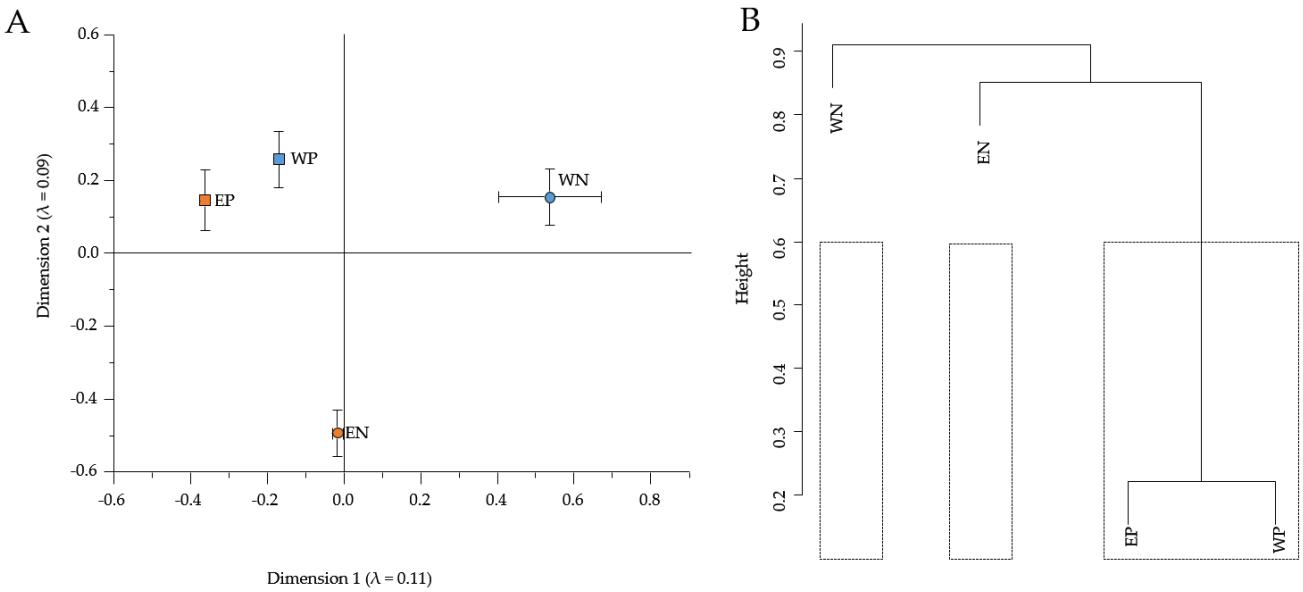

Figure S2. Correspondence analysis of bacterial community compositions (A) and euclidean distance matrix based on ward.D2 method (B) of Bb-negative ticks retrieved from Esslingen (EN; n=62; orange circle) or Weiden (WN; n=56, blue circle) and Bb-positive ticks retrieved from Esslingen (EP; n=38; orange square) or Weiden (WP; n=44; blue square). Bacterial community composition is based on genus level with binary data. The eigenvalues of both axes and SE are shown (A). For clusters, heights of 0.6 were chosen and denoted in dashed boxes (B).

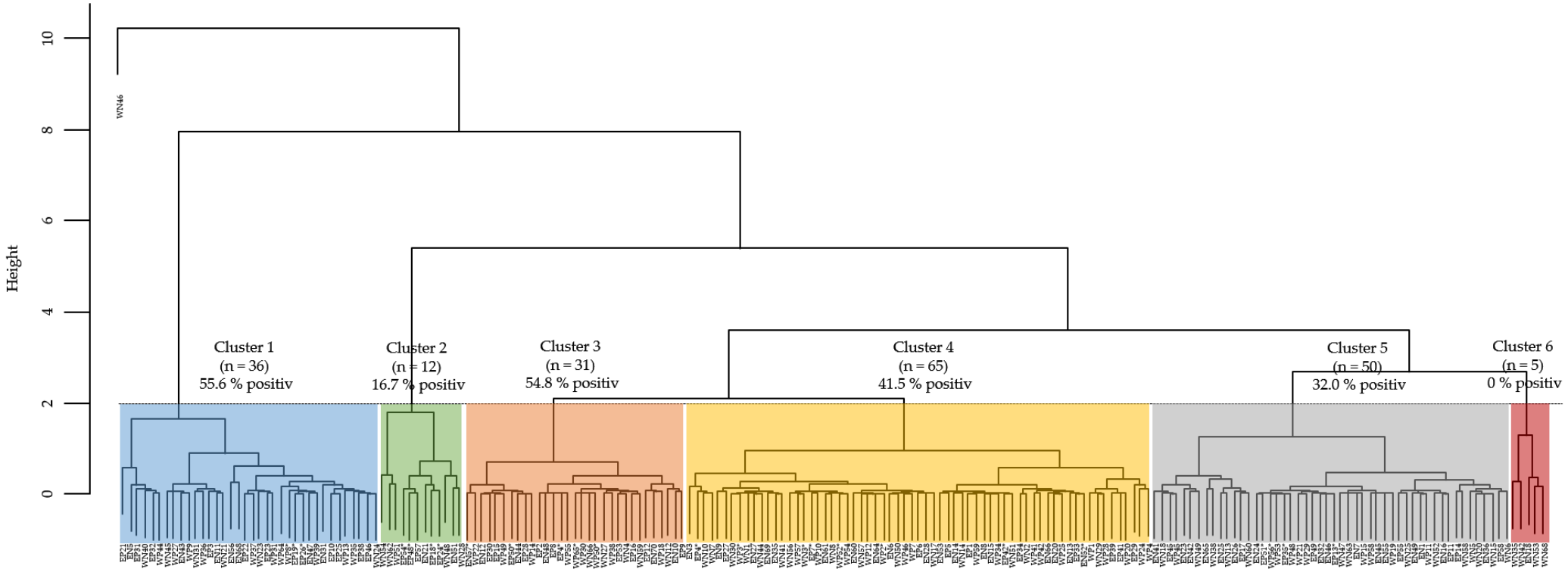

Figure S3. Euclidean distance matrix based on ward.D2 method on genus level with binary data of Bb-negative ticks retrieved from Esslingen (EN; n=62) or Weiden (WN; n=56) and Bb positive ticks retrieved from Esslingen (EP; n=38) or Weiden (WP; n=44). For clusters, heights of 2 were chosen and denoted in colored boxes.

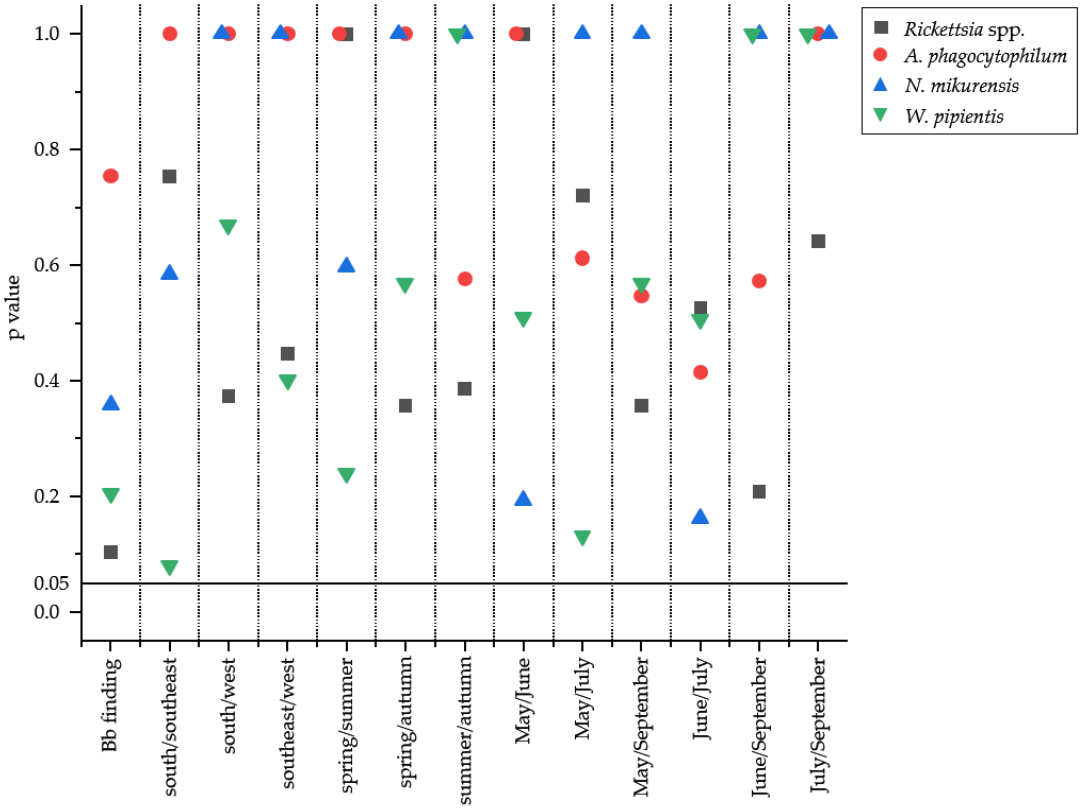

Figure S4. Effect of the environmental variables on the occurrence of *Rickettsia* spp., *A. phagocytophilum*, *N. mikurensis*, and *W. pipientis* as revealed by Fisher exact test on the 76 tick pools (Table S4 and Table SE1). Symbol legend for each species is included in the figure. Details of the characteristics of the variables can be found in Table 4. A significance level of 0.05 is indicated.

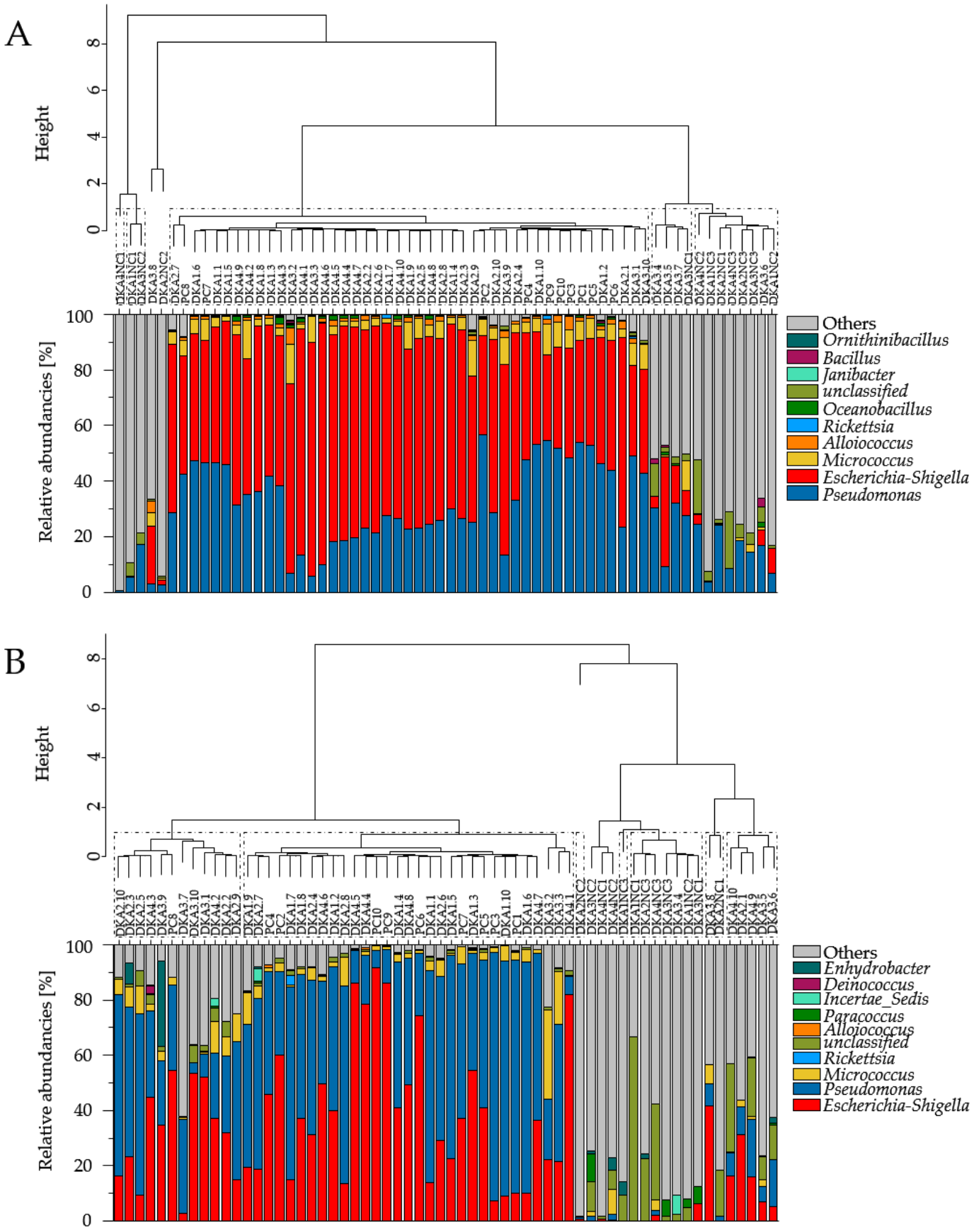
A
Height
0
2
4
6
8
Relative abundancies [%]
0
20
40
60
80
100
Others
Ornithinibacillus
Bacillus
Janibacter
unclassified
Oceanobacillus
Rickettsia
Alloiococcus
Micrococcus
Escherichia-Shigella
Pseudomonas
B
Height
0
2
4
6
8
Relative abundancies [%]
0
20
40
60
80
100
Others
Enhydrobacter
Deinococcus
Incertae_Sedis
Paracoccus
Alloiococcus
unclassified
Rickettsia
Micrococcus
Pseudomonas
Escherichia-Shigella

Figure S5. Euclidean distance matrix based on ward.D2 method and bacterial community composition on genus level for DNA (A) or cDNA (B) samples of 70% ethanol (DKA 1), DNA Away (DKA 2), 5% sodium hypochlorite (DKA 3) and RSDL (DKA 4) decontaminated ticks. PC (positive control) without decontamination and NC (negative control) ticks without contamination. For colors and patterns, see figure legend. Bacterial community composition of contaminants on the genus level is denoted, and other genera are summarized as "others" for non-contaminants. For clusters, heights of 1 were chosen and denoted in black boxes. Replicates of each treatment are numbered after the respective acronym. Details of treatments and replicates are summarized in Table 5.

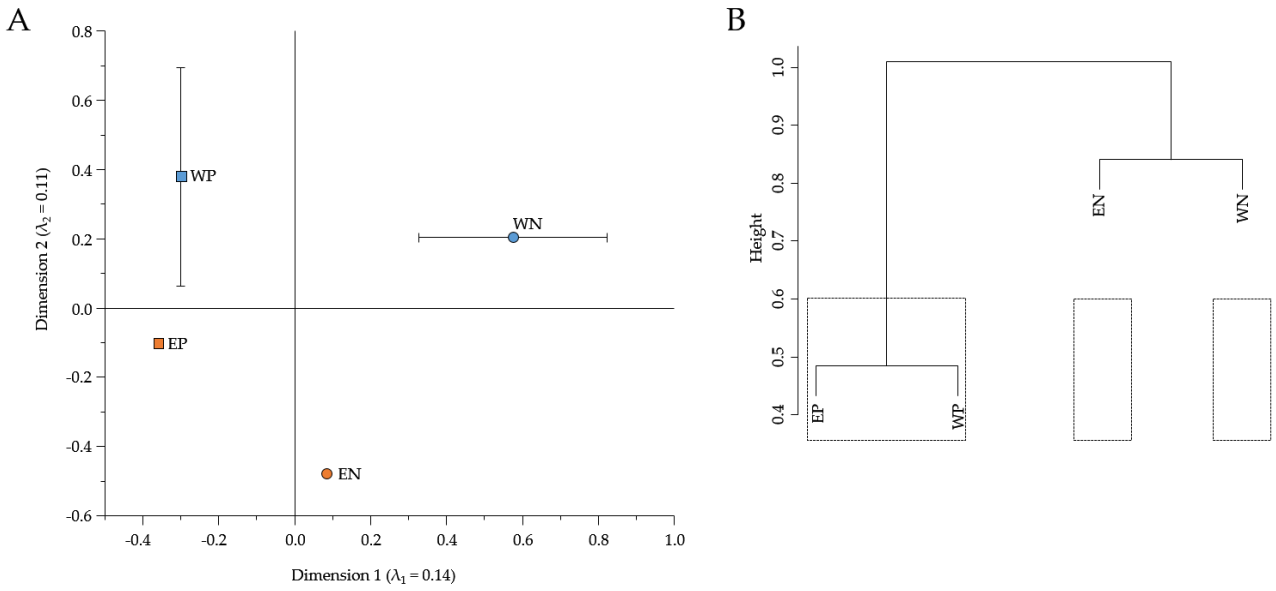

Figure S6. Correspondence analysis of bacterial community compositions (A) and euclidean distance matrix based on ward.D2 method (B) of Bb-negative ticks retrieved from Esslingen (EN; n = 62; orange circle) or Weiden (WN; n=56, blue circle) and Bb-positive ticks retrieved from Esslingen (EP; n = 38; orange square) or Weiden (WP; n = 44; blue square) after removal of the *Borrelia* sequences. Bacterial community composition is based on genus level with relative abundance data. The eigenvalues of both axes and SE are shown (A). For clusters, heights of 0.6 were chosen and denoted in dashed boxes (B).

8.2 Tables

Table S1. Overview of the *Rickettsia* species included in the *gltA* gene database and their distribution (Dobler & Wölfel, 2009; Kurtti et al., 2015; Parola et al., 2013).

Species	Distribution	Host	Accession numbers
Rickettsia felis	Germany; Europe; worldwide	fleas	NC_007109.1
Rickettsia helvetica	Germany; Europe; Africa; Asia	ticks	NZ_CM001467.1
Rickettsia massiliae	Germany; Europe; Africa; Asia; America	ticks	NC_009900.1
Rickettsia monacensis	Germany; Europe; Africa	ticks	LN794217.1
Rickettsia raoultii	Germany; Europe; Africa; Asia	ticks	DQ365803.1
Rickettsia slovaca	Germany; Europe; Asia	ticks	MT667405.1
Rickettsia aeschlimannii	Europe; Africa	ticks	HQ335153.1
Rickettsia conorii	Europe; Africa; Asia	ticks	EU716648.1
Rickettsia sibirica	Europe; Africa; Asia	ticks	KY780024.1
Rickettsia africae	Africa; North and Central America, Caribbean	ticks	NC_012633.1
Rickettsia japonica	Asia	ticks	NZ_AP017574.1
Rickettsia endosymbiont of *I. scapularis*	North America	ticks	CM000770.1
Rickettsia akari	worldwide	mites	U59717.1

Table S2. OTU diversity indices from the tick-borne Bb-negative (EN, n = 62; WN, n = 56) and Bb-positive microbiome (EP, n = 38; WP, n = 44) derived from Esslingen (E) or Weiden (W).

	OTU Richness	Shannon	Pielou's Evenness
EN	23 ± 15	0.8 ± 0.8	0.3 ± 0.2
EP	34 ± 20	1.4 ± 0.7	0.4 ± 0.2
WN	24 ± 14	1.0 ± 0.8	0.3 ± 0.2
WP	28 ± 16	1.1 ± 0.7	0.3 ± 0.2

Table S3. Parameters and topological roles from the tick-borne Bb-negative (EN; WN) and Bb-positive co-occurrence networks (EP; WP) derived from Esslingen (E) or Weiden (W). The EN, WN, EP, and WP network consisted of 62, 56, 38, and 44 bacterial community compositions, respectively.

		EN	EP	WN	WP
Network parameters	**Nodes**	253	243	271	225
	Edges	2871	2967	2855	2692
	GlayCluster †	7	3	7	4
	Average clustering coefficient	0.69 ± 0.27	0.57 ± 0.29	0.70 ± 0.27	0.58 ± 0.28
	Average closeness centrality	0.54 ± 0.15	0.51 ± 0.17	0.55 ± 0.17	0.54 ± 0.15
z_i/P_i -Plot	**Peripherals**	227	234	229	200
	Connectors	25	6	33	22
	Module hub	1	3	0	2
	Network hub	0	0	0	1

† connected components

Table S4. Presence of *A. phagocytophilum, N. mikurensis, Rickettsia* spp., and *W. pipientis* in pooled tick nucleic acid extract pools with Bb-negative (n=38) and Bb-positive (n=38) findings. Frequency is indicated by numbers and their relative frequency in percentage (n = 76).

Bacteria species or genus	Bb finding	Frequency	Total frequency
A. phagocytophilum	negative	7 (9.2%)	12 (15.8%)
	positive	5 (6.6%)	
N. mikurensis	negative	1 (1.3%)	1 (1.3%)
	positive	0 (0%)	
***Rickettsia* spp.**	negative	18 (23.7%)	44 (57.9%)
	positive	26 (34.2%)	
W. pipientis	negative	8 (10.5%)	22 (28.9%)
	positive	14 (18.4%)	

Table S5. Summarized OTU count table of *gltA* gene sequencing data. For a sample-specific overview, see Table SE2.

#OTU ID	total reads	Number of OTUs	Bacterial species
OTU00001	2522	82	*Rickettsia helvetica*
OTU00002	64	7	*Rickettsia aeschlimannii/ R. yenbekshikazakhensis*
OTU00003	50	2	*Rickettsia raoultii*
OTU00004	29	1	*Rickettsia monacensis*
OTU00005 - 00007	51	12	*Rickettsia helvetica*
OTU00008	13	1	*Rickettsia aeschlimannii/ R. yenbekshikazakhensis*
OTU00009 - 00024	76	61	*Rickettsia helvetica*

Table S6. Overview of interaction patterns of bacterial genera with important topological roles from the tick-borne Bb-negative (EN; WN) and Bb-positive (EP; WP) co-occurrence networks obtained from Esslingen (E) and Weiden (W), respectively. The EN, WN, EP, and WP networks were composed of 62, 56, 38, and 44 bacterial community assemblages, respectively.

	EN		EP		WN		WP	
Interacting Bacterial Genera	Mutual Exclusions	Co-Occurrence	Mutual Exclusions	Co-Occurrence	Mutual Exclusions	Co-Occurrence	Mutual Exclusions	Co-Occurrence
Borrelia	/	/	/	1	/	/	1	1
Rickettsia	106	0	2	0	25	3	8	0
Mycobacterium	13	36	14	5	8	29	1	11
Phenylobacterium	14	10	11	8	5	4	1	8
Ralstonia	5	5	15	5	12	11	2	18
Burkholderia	1	3	2	6	23	8	7	15
Ochrobactrum	6	12	56	6	1	23	7	3
Legionella	3	14	0	20	0	35	3	10
Williamsia	2	36	41	24	10	35	5	37
Rhizobium	0	37	31	19	2	13	20	8
Pseudobutyrivibrio	/	/	7	22	2	37	2	18
Pseudomonas	8	2	5	0	16	5	5	6
Bdellovibrio	1	6	/	/	7	11	10	16
Pelomonas	1	8	5	8	3	9	17	22
Spiroplasma	2	0	7	3	4	3	13	3
Stenotrophomonas	2	4	3	10	36	9	60	3
Neoehrlichia	2	1	5	4	9	2	25	6
Rhodococcus	0	32	4	27	4	22	30	41

8.3 Electronic data

Due to their large size, the following figures and tables can be found on the CD provided with this work:

8.3.1 Electronic figures

Figure SE1. Mapped nucleic acid extracts of ticks on a map of Germany to create pool compositions with similar characteristics of the variables (**Table 4**). Colors of the legend mark the number of ticks per postcode area. For further information, see **Table SE1**.

Figure SE2. Bb-negative network from Esslingen (EN; *n* = 62). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE3. Bb-positive network from Esslingen (EP; *n* = 44). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE4. Bb-negative network from Weiden (WN; *n* = 56). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE5. Bb-positive network from Weiden (WP; *n* = 44). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE6. Interaction of *Rickettsia* in the Bb-negative tick microbiome from Essling (EN; *n* = 62). Red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE7. Interaction of *Rickettsia* in the Bb-positive tick microbiome from Essling (EP; *n* = 62). Red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE8. Interaction of *Rickettsia* in the Bb-negative tick microbiome from Weiden (WN; *n* = 56). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE9. Interaction of *Rickettsia* in the Bb-positive tick microbiome from Weiden (WP; *n* = 44). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE10. Representation of the Bb-negative network by community cluster (GLay) from Esslingen (EN; *n* = 62). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE11. Representation of the Bb-positive network by community cluster (GLay) from Esslingen (EP; *n* = 38). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE12. Representation of the Bb-negative network by community cluster (GLay) from Weiden (WN; *n* = 56). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE13. Representation of the Bb-positive network by community cluster (GLay) from Weiden (WP; *n* = 44). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE14. Interaction of *Ochrobactrum* in the Bb-negative tick microbiome from Essling (EN; *n* = 62). Red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE15. Interaction of *Ochrobactrum* in the Bb-positive tick microbiome from Essling (EP; *n* = 62). Red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE16. Interaction of *Ochrobactrum* in the Bb-negative tick microbiome from Weiden (WN; *n* = 56). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE17. Interaction of *Ochrobactrum* in the Bb-positive tick microbiome from Weiden (WP; *n* = 44). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE18. Interaction of *Stenotrophomonas* in the Bb-negative tick microbiome from Essling (EN; *n* = 62). Red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE19. Interaction of *Stenotrophomonas* in the Bb-positive tick microbiome from Essling (EP; *n* = 62). Red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE20. Interaction of *Stenotrophomonas* in the Bb-negative tick microbiome from Weiden (WN; *n* = 56). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

Figure SE21. Interaction of *Stenotrophomonas* in the Bb-positive tick microbiome from Weiden (WP; *n* = 44). Green lines indicate co-occurrence links, while red lines indicate mutual exclusion links. The different colors of the squares with the bacterial genera indicate different cluster membership.

8.3.2 Electronic tables

Table SE1. Composition of the pools with associated PCR results for the corresponding human pathogen from Rickettsiales, including co-occurrence information.

Table SE2. OTU count table of the *Rickettsia* spp. *gltA* amplicon sequencing.

Table SE3. OTU count table from the tick-borne Bb-negative (EN, n=62; WN, n=56) and Bb-positive microbiome (EP, n=38; WP, n=44) derived from Esslingen (E) or Weiden (W).

Table SE4. OTU count table summarized on genus level from the tick-borne Bb-negative (EN, n=62; WN, n=56) and Bb-positive microbiome (EP, n=38; WP, n=44) derived from Esslingen (E) or Weiden (W).

Table SE5. OTU count table summarized on genus level with the relative sequence read abundance and topological role in the network of Bb-negative ticks from Esslingen.

Table SE6. OTU count table summarized on genus level with the relative sequence read abundance and topological role in the network of Bb-positive ticks from Esslingen.

Table SE7.OTU count table summarized on genus level with the relative sequence read abundance and topological role in the network of Bb-negative ticks from Weiden.

Table SE8. OTU count table summarized on genus level with the relative sequence read abundance and topological role in the network of Bb-positive ticks from Weiden.

Table SE9. OTU count table for DNA samples of 70% ethanol (DKA1), DNA Away (DKA2), 5% sodium hypochlorite (DKA3), and RSDL (DKA4) decontaminated ticks. PC (positive control) without decontamination and NC (negative control) ticks without contamination. Replicates of each treatment are numbered after the respective acronym. Details of treatments and replicates are summarized in **Table 5**.

Table SE10. OTU count table for cDNA samples of 70% ethanol (DKA1), DNA Away (DKA2), 5% sodium hypochlorite (DKA3), and RSDL (DKA4) decontaminated ticks. PC (positive control) without decontamination and NC (negative control) ticks without contamination. Replicates of each treatment are numbered after the respective acronym. Details of treatments and replicates are summarized in **Table 5**.

9 Publications with a declaration of contribution, Presentations, and Posters

Parts of this work have already been published or are under review as follows:

Publication 1:

Hoffmann, A.; Fingerle, V.; Noll, M. Analysis of Tick Surface Decontamination Methods. *Microorganisms* **2020**, *8*, 987.

Authors: Hoffmann, A. (A.H.); Fingerle, V. (V.F.); Noll, M. (M.N.)

Title: Analysis of Tick Surface Decontamination Methods.

Journal and status: Published in the journal *MDPI Microorganisms* on June 30, 2020.

Own Contribution: Methodology 90%, formal analysis 90%, validation 90%, investigation 80%, writing—original draft preparation 70%, writing—review and editing 50%, visualization 100%

Author Contribution: Conceptualization, M.N., and V.F.; methodology, A.H.; validation, A.H.; formal analysis, A.H.; investigation, A.H. and M.N.; resources, M.N.; writing—original draft preparation, A.H. and M.N.; writing—review and editing, A.H., V.F., and M.N.; visualization, A.H.; supervision, M.N.; project administration, M.N.; funding acquisition, M.N.

Publication 2:

Hoffmann, A.; Müller, T.; Fingerle, V.; Noll, M. Presence of Human Pathogens of the *Borrelia burgdorferi* sensu lato Complex Shifts the Sequence Read Abundances of Tick Microbiomes in Two German Locations. Microorganisms **2021**, 9, 1814.

Authors: Hoffmann, A. (A.H.); Müller, T. (T.M.); Fingerle, V. (V.F.); Noll, M. (M.N.)

Title: Presence of Human Pathogens of the *Borrelia burgdorferi* sensu lato Complex Shifts the Sequence Read Abundances of Tick Microbiomes in Two German Locations.

Journal and status: Published in the journal *MDPI Microorganisms* on August 26, 2021.

Own Contribution: Methodology 80%, formal analysis 90%, validation 90%, investigation 80%, writing—original draft preparation 80%, writing—review and editing 50%, visualization 100%

Author Contribution: Conceptualization, M.N., and V.F.; methodology, A.H. and T.M.; validation, A.H.; formal analysis, A.H.; investigation, A.H. and M.N.; resources, M.N.; writing—original draft preparation, A.H. and M.N.; writing—review and editing, A.H., V.F., T.M. and M.N.; visualization, A.H.; supervision, M.N.; project administration, M.N.; funding acquisition, M.N.

Publication 3:

Hoffmann, A.; Müller, T.; Fingerle, V.; Silaghi, C.; Noll, M. Co-Infection of Potential Tick-Borne Pathogens of the Order Rickettsiales and *Borrelia burgdorferi* s. l. and Their Link to Season and Area in Germany.*Microorganisms* **2023**, 11, 157.

Authors: Hoffmann, A. (A.H.); Müller, T. (T.M.); Fingerle, V. (V.F.); Silaghi, C. (C.S.); Noll, M. (M.N.)

Title: Co-Infection of Potential Tick-Borne Pathogens of the Order Rickettsiales and *Borrelia burgdorferi* s. l. and Their Link to Season and Area in Germany

Journal and status: Published in the journal *MDPI Microorganisms* on January 7, 2023.

Own Contribution: Methodology 80%, formal analysis 70%, validation 70%, investigation 70%, writing—original draft preparation 80%, writing—review and editing 50%, visualization 100%

Author Contribution: Conceptualization, M.N., and V.F.; methodology, A.H., T.M., J.B; validation, A.H.; formal analysis, A.H.; investigation, A.H. and M.N.; resources, M.N.; writing—original draft preparation, A.H. and M.N.; writing—review and editing, A.H., V.F., C.S., T.M., and M.N.; visualization, A.H.; supervision, M.N.; project administration, M.N.; funding acquisition, M.N. All authors have read and agreed to the published version of the manuscript.

Participation in conferences or symposia with posters or presentations:

Hoffmann, A.; Müller, T.; Fingerle, V.; Noll, M. (2019) Analysis of skin surface decontamination methods to assess unbiased tick-borne microbiomes. 13th International Symposium on Ticks and Tick-borne Diseases, Weimar, Germany, 28th – 30th March 2019, Poster T&O – 14

Hoffmann, A.; Müller, T.; Fingerle, V.; Noll, M. (2020) Decontamination of ticks to assess unbiased internal tick-borne microbiome. 6th Joint Conference of the DGHM & VAAM, Leipzig, Germany, 8th – 11th March 2020, Poster 320-MSHP

Hoffmann, A.; Müller, T.; Fingerle, V.; Noll, M. (2021) Presence of *Borrelia* species are linked to the composition of tick microbiomes in two German counties. 14th International Symposium on Ticks and Tick-borne Diseases, online, 24th – 26th March 2021, Talk LBT – 5

10 (Eidesstattliche) Versicherung und Erklärung

(§ 9 Satz 2 Nr. 3 PromO BayNAT)

Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 97 Abs. 1 Satz 8 BayHIG).

(§ 9 Satz 2 Nr. 3 PromO BayNAT)

Hiermit erkläre ich, dass ich die Dissertation nicht bereits zur Erlangung eines akademischen Grades eingereicht habe und dass ich nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden habe.

(§ 9 Satz 2 Nr. 4 PromO BayNAT)

Hiermit erkläre ich, dass ich Hilfe von gewerblichen Promotionsberatern bzw. -vermittlern oder ähnlichen Dienstleistern weder bisher in Anspruch genommen habe noch künftig in Anspruch nehmen werde.

(§ 9 Satz 2 Nr. 7 PromO BayNAT)

Hiermit erkläre ich mein Einverständnis, dass die elektronische Fassung meiner Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung unterzogen werden kann.

(§ 9 Satz 2 Nr. 8 PromO BayNAT)

Hiermit erkläre ich mein Einverständnis, dass bei Verdacht wissenschaftlichen Fehlverhaltens Ermittlungen durch universitätsinterne Organe der wissenschaftlichen Selbstkontrolle stattfinden können.

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Ort, Datum, Unterschrift