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Chapter

Handling the Microbial Complexity Associated to Ticks

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Abstract

Ticks and the pathogens they transmit constitute a growing burden for human and animal health worldwide. In the last years, high-throughput detection and sequencing technologies (HTT) have revealed that individual ticks carry a high diversity of microorganisms, including pathogenic and non-pathogenic bacteria. Despite several studies have contributed to the availability of a catalog of microorganisms associated to different tick species, major limitations and challenges remain ahead HTT studies to acquire further insights on the microbial complexity associated to ticks. Currently, using next generation sequencing (NGS), bacteria genera (or higher taxonomic levels) can be recorded; however, species identification remains problematic which in turn affects pathogen detection using NGS. Microfluidic PCR, a high-throughput detection technology, can detect up to 96 different pathogen species, and its combination with NGS might render interesting insights into pathogen-microbiota co-occurrence patterns. Microfluidic PCR, however, is also limited because detection of pathogen strains has not been implemented, and therefore, putative associations among bacterial genotypes are currently unknown. Combining NGS and microfluidic PCR data may prove challenging. Here, we review the impact of some HTT applied to tick microbiology research and propose network analysis as an integrative data analysis benchmark to unravel the structure and significance of microbial communities associated to ticks in different ecosystems.

Keywords: high-throughput technologies, network analysis, ticks, tick-borne pathogens, microbiota

1. Introduction

Ticks are hematophagous ectoparasites of vertebrates that derive nutrition through blood feeding and are efficient vectors of major pathogens. Feeding habits and the process of blood digestion in ticks greatly differ from that in hematophagous insects (e.g. mosquitoes) and may influence pathogen acquisition and transmission. In ticks, digestion is a slow intracellular process [1, 2]. Argasidae, or "soft ticks," feed quickly and several times during their lifetime (approximately 40–60 minutes per feeding in most species). In adult soft ticks, full digestion only proceeds once mating occurs. In contrast to soft ticks, Ixodidae, or "hard ticks," feed for longer periods of time. Adult virgin females of Ixodidae Metastriate ticks attach to the host and take only a small quantity of blood before mating [3]. Mating

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induces females to fast feeding, increasing their weight approximately 100 times within few days [3]. Thus, feeding times in female hard ticks can last from few days to weeks depending on the stage and the availability of males. After hatching from the eggs, the three following developmental stages (i.e. larvae, nymphs and adults) of Prostriate *Ixodes* ticks feed on different hosts. Potentially, while feeding on a host, each of these stages can transmit and acquire new pathogens [4]. Once acquired, most, if not all, tick-borne pathogens (TBPs) are transmitted transstadially (i.e. the ability of a microorganism to pass from one to the next developmental stage of the vector), and thus, ticks are 'hubs' in pathogen's circulation cycles [5]. In consequence, a considerable proportion of ticks are found to be coinfected in field surveys [6–9]. The above characteristics, among others, enable ticks to transmit a great variety of pathogens, including bacteria, viruses, protozoa and helminths, which constitute a growing burden for human and animal health worldwide [4, 10]. Among arthropod vectors, ticks transmit the most diverse array of disease agents [11].

Despite tick biology favors the acquisition and transmission of a great diversity of pathogens, most studies on TBPs prevalence in ticks focused in single infections. This was probably influenced by technical limitations to detect multiple pathogens and, possibly, by the fact that initial discoveries on the role of ticks as vectors linked "one-pathogen" to "one-tick-species." After the first demonstration of pathogen transmission by ticks, when Smith and Kilbourne [12] demonstrated that *Rhipicephalus annulatus* transmit *Babesia bigemina*, several studies established the role of ticks as vectors of several pathogens including *Borrelia duttonii* transmitted by *Ornithodoros moubata* [13]; *Rickettsia rickettsii* transmitted by *Dermacentor andersoni* [14]; *Rickettsia conorii* transmitted by *R. sanguineus* [15]; and later, in the 1980s, *B. burgdorferi* s.l. responsible for Lyme borreliosis and transmitted by *Ixodes* spp. [16, 17]. These initial discoveries may have influenced the conception of a "single-pathogen" epidemiology. Thus, until recently, our experimental and theoretical models of pathogen transmission by ticks were limited because they frequently included single pathogen species [5]. Discoveries made using novel technologies [18], however, changed our current understanding of TBPs epidemiology: from the "single-pathogen" view, we are now at the bridge of unraveling the impact of "multiple-pathogen" in TBPs epidemiology. Coinfections, when multiple pathogen species coexist within an individual, are very common in ticks [9, 19, 20] and influence pathogen acquisition [21], transmission [19] as well as host infection risk [22]. When pathogens share a reservoir, they can interact directly via pathogen-pathogen interactions [23] and indirectly via host immune-mediation or they can also compete for host resources [24]. Within-host interactions are so strong that the dynamics of one pathogen, within a host and within a host population, cannot be understood without knowledge of other co-occurring pathogens [22, 25].

Pathogen coinfection in ticks can be studied by standard PCR using primers that detect known pathogens suspected to occur in a given tick species of a particular geographic region. This approach is the most frequently used; however, it is strongly biased and makes pathogen detection to be strongly influenced by particular research interests [5]. This may be the reason why one of the most studied coinfection is that between two of the most prominent TBPs, *Anaplasma phagocytophilum*, an intracellular bacterium that causes human granulocytic anaplasmosis (HGA), and *B. burgdorferi* s.l., an extracellular bacterium that produces Lyme borreliosis [6, 8, 21, 26, 27]. The approaches based on high-throughput technologies provided novel combinations of pathogen coinfections in ticks [9] with potential impact on vector competence. For example, Moutailler and colleagues [9] found 31 different pathogen confections in *Ixodes ricinus* ticks (see below and

Table A1). The most important realization of the recent research, however, is that most of the tick-associated microorganisms are not pathogens. Likely mirroring the revolution in microbiota research in model organisms [28–30], less than 10 years ago, tick researchers started applying next-generation sequencing (NGS) to explore the composition of tick microbiota [31]. The results showed a higher diversity of bacteria genera associated to ticks [32] compared to model organisms like *Drosophila melanogaster* [28]. This was surprising because while ticks have a restricted diet, *Drosophila* feed on a variety of decaying matter which could be the source of a complex microbiota. Possibly, allowing a high bacterial diversity is part of the evolutionary strategy of ticks to cope with their complex life cycle and metabolic deficiencies.

A major challenge of high-throughput data is data analysis, and therefore, integrative analytical tools are needed to improve our current understanding of tick-pathogen-microbiota interactions. Network analysis, a branch of graph theory, is a mathematical tool for the analysis of complex systems composed of many components which may interact with each other. Network analysis has been used to unravel complex microbial communities such as those present in soil [33], water [34] and human [35, 36] and tick microbiota [37]. This chapter focuses on the impact of high-throughput technologies in the current understanding of the microbial complexity associated to ticks. In addition, we propose to combine high-throughput data with network analysis to gain new insights into the structure of microbial communities associated to ticks and their impact on pathogen circulation. Throughout this review, we will use the term "microbiota" as "the microbial taxa associated with a given host" and "microbiome" as "the catalog of these microbes and their genes." A distinction can be established between these terms, while the microbiome includes information about the microbiota composition, the latest term does not necessarily includes information about gene composition.

2. New technologies and the microbial universe of ticks

2.1 Microfluidic PCR

2.1.1 General background on the technology

Frequently, studies on TBPs prevalence in ticks focused mainly on bacteria and parasites and only few species or genera are targeted in each study. Detection assays (e.g. PCR, nested PCR or real-time PCR) are designed to detect a restricted number of pathogens that are known or suspected to be transmitted by particular tick species collected at a particular location. In addition to the "*research interest*" bias, using standard PCR methods, only few microliters of total DNA are available per sample, which limits the number of pathogens that can be tested in each sample and confirmation by sequencing becomes difficult. Ideally, to better understand the epidemiology of TBPs, researchers should be able to detect in each sample (i.e. individual ticks or tick pools) most of the pathogens that ticks could potentially transmit, regardless of the tick species or the location. For this purpose, Michelet and collaborators [18] have developed a new high-throughput tool to detect a high number of TBPs in a high number of samples by real-time PCR in a single experiment [18]. Briefly, they developed a chip (BioMark™ dynamic arrays, Fluidigm Corporation) targeting TBPs (bacteria and parasites) of worldwide distribution. The designed epidemiologic arrays may detect simultaneously 48 pathogens in 48 samples (or potentially 96 pathogens in 96 samples) corresponding to 2304 real-time microfluidic PCRs (or potentially 9216 real-time microfluidic PCRs). Specific

primers and TaqMan probes were designed for each pathogen, and their specificity was tested *in silico* using Blast.

A brief workflow of the microfluidic PCR is provided **Figure 1**. Firstly, ticks are homogenized in cell culture medium (i.e. D-MEM) completed with 10% of fetal calf serum to preserve viral particles and separated into three aliquots: one dedicated to total DNA extraction, one to total RNA extraction and one conserved at −80°C for back-up. Secondly, RNAs are reverse transcribed into cDNA using random primers (only 1 µL of RNA is used per reaction), and then cDNA and DNA are preamplified with a pool of primers/probe targeting TBPs to increase the signal of TBPs relative to the signal of tick RNA/DNAs. Remarkably, only 1.25 µL per sample are needed to test all the pathogens simultaneously. Two different chips were run in the BioMark™ dynamic array system: one to detect RNA viruses using the preamplified cDNAs and the other to detect DNAs from bacteria/parasites using the preamplified DNAs. In the chip, samples and primers/probes are added into the right and left wells, respectively. Pressure and oil allow the distribution of each sample and primers/probe sets into the microfluidic PCR chambers in the middle of the chip. Each sample will be mixed with all the primers/probes sets and each primers/probe set will be mixed with all samples, allowing 2304

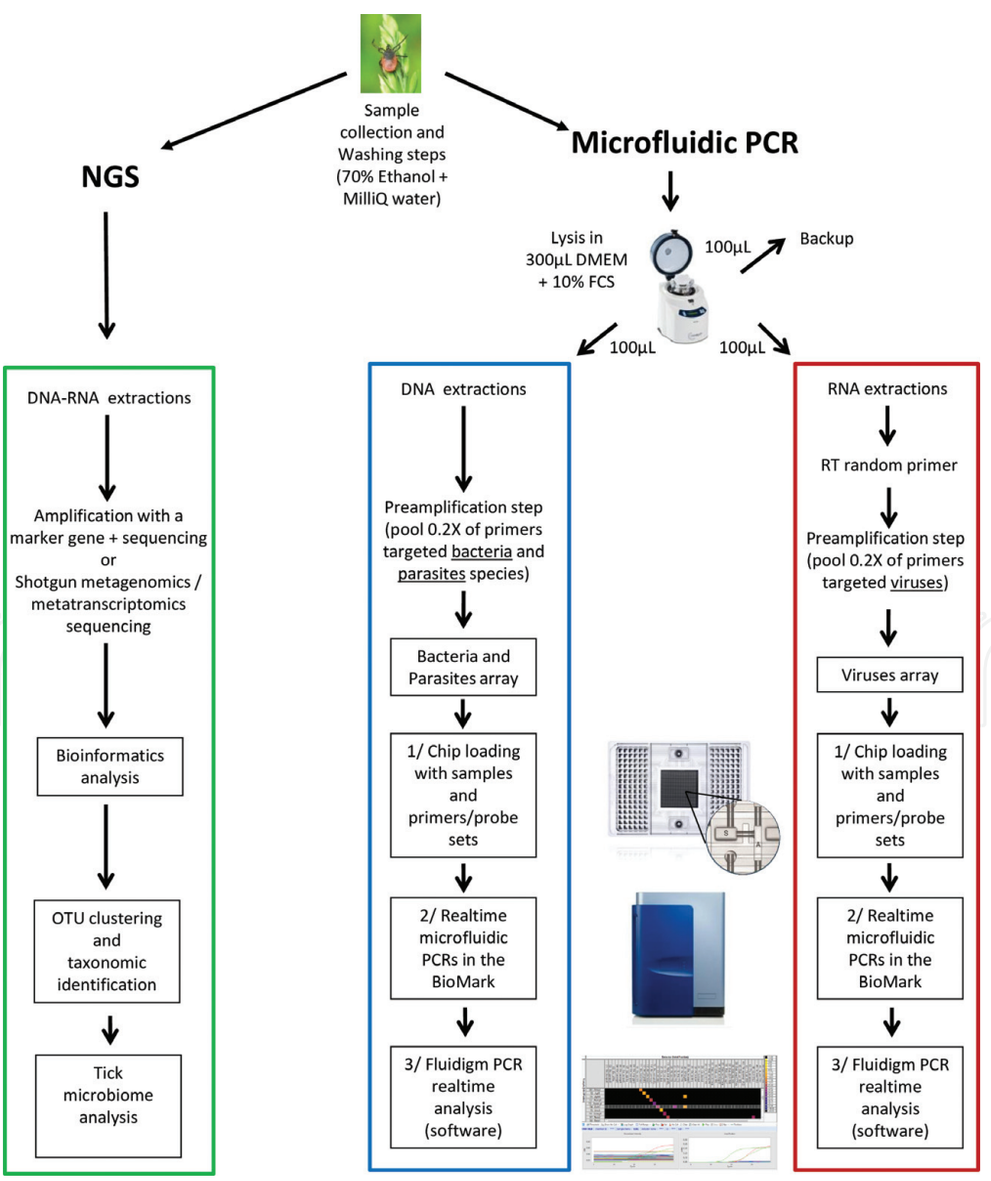

Figure 1.
General workflows of high-throughput screening of ticks using the real-time microfluidic PCR system and NGS.

individual real-time PCRs at a final volume of six nanoliters per reaction. For further details, we refer the reader to [18].

2.1.2 Tick-borne pathogen coinfections revealed by microfluidic PCR

The first application of microfluidic PCR targeted 37 pathogens including *Francisella tularensis*, *Coxiella burnetii*, *Candidatus* Neoehrlichia mikurensis, five species of *Anaplasma*, three species of *Ehrlichia*, eight species of *Borrelia* (seven from the Lyme borreliosis group and one, *B. miyamotoi*, from the relapsing fever group), two species of *Bartonella*, four species of *Rickettsia*, ten species of *Babesia* and two species of *Theileria* [18]. To confirm the morphological characterization of the tick species analyzed and to control the quality of DNA extraction, primers specific to five species of ticks, including three species of *Ixodes* and two species of *Dermacentor*, were tested. Sensitivity of primers and probes was tested on a dilution range of reference DNAs of the targeted pathogens on a Lightcycler 480 real-time PCR system. Then, the specificity was tested on the BioMark™ dynamic array system. The resulting chip was further evaluated on field samples corresponding to 47 pools of 25 *I. ricinus* nymphs each collected in two sites per country in France, The Netherlands and Denmark, 7050 samples in total. Several pathogens were successfully detected, and the prevalence of *A. phagocytophilum*, *Ca*. N. mikurensis, *Rickettsia helvetica*, *Bartonella henselae*, five different genospecies of *B. burgdorferi* s. l., *B. miyamotoi*, *B. divergens* and *B. venatorum* was determined [18]. Positive samples were validated by PCR amplification and sequencing of selected gene fragments [18]. Notably, this study revealed for the first time the presence of five pathogens previously unreported in Denmark. This work highlighted the potential of unbiased pathogen detection. A similar tool targeting 22 tick-borne viruses (TBVs) has also been developed and evaluated on European ticks (unpublished data). These fast and low-cost tools allow comprehensive testing of TBPs and can be customized to fit regional demands or to accommodate new or emerging pathogens. Indeed, specific sets of primers/probe are continuously designed by our team. These tools represent a major improvement for surveillance and future epidemiological studies.

This new high-throughput technology has been used mainly during epidemiological studies of TBPs in specific countries with different tick species screened as *I. ricinus* in Ireland [38] and Denmark [39], *Ornithodoros* spp. in France [40], *Rhipicephalus microplus* in Galápagos Islands [41] and TBVs in *Hyalomma* spp. ticks collected on migratory birds in Sweden [42]. Remarkably, this allowed the detection of expected pathogens (i.e. *Borrelia* species in *I. ricinus*), rare (i.e. *Bartonella* species in *I. ricinus* and *Borrelia* from the relapsing fever group in *Ornithodoros* spp.), or unexpected pathogens (i.e. Alkurma virus in *Hyalomma* spp.) in different regions.

Moreover, these high-throughput screenings of TBPs in individual ticks have highlighted the co-occurrence of several pathogens in one tick, known as tick coinfections. Before the use of this novel technique, tick coinfections were evaluated by classical PCR, nested PCR or real-time PCR, and related publications focused in few pathogens, less than 10 different genera screened per publication [43–59]. After the year 2016, two publications have demonstrated the presence of up to five and four different pathogen species in *I. ricinus* female ticks collected in France and Romania, respectively, using this high-throughput system [9, 20]. The advantages of microfluidic PCR over classical PCR detection methods (i.e. qualitative PCR, nested PCR, or real-time PCR) can be summarized: (i) small amount of sample is needed for detection of tens of microorganisms, (ii) convenient and easy to implement when thousands of samples are to be tested and (iii) price per sample run is lower. Tick coinfections among bacteria, parasites and/or viruses described in the literature in the last 4 years are listed in **Table A1**. Not surprisingly, the most

commonly found coinfections are those between *Borrelia* spp. and *A. phagocytophilum* or *Rickettsia* spp. as well as between different species of *Borrelia* included in the Lyme borreliosis group. Nevertheless, this result could reflect the reality or could be a bias resulting from the high quantity of research projects focusing on the above bacteria.

2.1.3 Challenges and perspectives

Unfortunately, only few publications are available regarding coinfection by bacteria and parasites or bacteria and viruses or parasites and viruses in ticks [49, 50, 52, 54, 60]. To solve this gap of information regarding inter-taxa coinfections, a system to detect simultaneously bacteria, parasites and viruses will be, without any doubt, an improvement of available tools. Nevertheless, even if this high-throughput system allows a rapid detection of numerous pathogens present in a high number of samples, confirmation of doubtful results or presence of unexpected pathogens should be confirmed by classical or nested PCR. Knowing the fact that for each pathogen different genotypes/strains could exist, this confirmation step could allow us to sequence different genes per pathogen leading to a better characterization of the epidemiological history of TBPs present in the targeted region/ecosystem.

High-throughput identification of pathogen strains would be also a significant improvement to current microfluidic PCR protocols. Genetic diversity of bacteria species resulting in novel strains can be associated to changes in pathogenicity, virulence and host specificity. A classic example of this is that different strains of the bacterium *Escherichia coli* can provide health benefits or produce deadly diseases. In particular, *E. coli* strain Nissle 1917 is used as a probiotic [61] and *E. coli* strain O157:H7 has been responsible for a number of deadly food-borne pathogen outbreaks [62]. It has been reported that multiple strains of *A. phagocytophilum* circulate in Europe, with minimal overlap in their reservoir associations [63]. One of these strains is a generalist infecting a wide range of mammalian species, including livestock and other domestic animals [64–66]. A second strain appears to specialize almost exclusively on roe deer [63]. Both of these strains are transmitted by *I. ricinus* and both could affect humans. A third strain has a host range restricted to rodents and is circulated by *I. trianguliceps* [64]. Targeting different *A. phagocytophilum* strains in a high-throughput system may allow studying not only tick vector specificity of this bacterium but also coinfections among and between strains of *A. phagocytophilum* and other pathogens. Thus, systematic detection of pathogen strains using high-throughput approaches would provide a more comprehensive view of TBPs diversity and may inform on host specificity and the emergence of novel TBPs. By including primers/probe sets targeting pathogen strain-specific markers, current microfluidic PCR protocols can be updated for strains detection and identification.

An additional challenge to high-throughput detection is how to detect novel strains or species. The emergence of novel pathogens is a dynamic process. For example, a novel species of *Ehrlichia*, *E. minasensis* [67], evolved from variable strains of the pathogen *E. canis* [68], and it was associated to new invertebrate and vertebrate hosts. While the common tick vector for *E. canis* is *R. sanguineus* s.l. [69], *E. minasensis* was isolated from *R. microplus* hemolymph [70], and while *E. canis* is mainly pathogenic for dogs [71], *E. minasensis* was found to be pathogenic for cattle [67, 72]. An alternative for the detection of novel pathogen strains or novel pathogens closely related to recognized pathogen species is the amplification and sequencing of genetic markers followed by phylogenetic analysis to assess strain diversity in samples positive to given pathogens. Emergence of novel strains is frequently associated with

genetic variability in surface proteins which can be used as genetic markers to assess strain diversity [68, 71].

Finally, high-throughput quantification of TBPs in tick organs could be a useful approach to assess some components of tick vector competence, for example, vector colonization by pathogens. It is known that the simple detection of pathogen DNA in a tick does not demonstrate the vector competence of this tick species for this pathogen. Vector competence depends effectively on genetic factors determining the ability of a vector to transmit a pathogen and has to be demonstrated under controlled conditions [10]. A typical TBP colonizes tick midgut and migrates to salivary glands to be transmitted with tick saliva to the host. The detection and quantification of the pathogen in different organs including midgut and salivary glands could be a step forward from pathogen detection to tick vector competence assessment. As an example, Berggoetz et al. [73] detected different pathogens (i.e. *Babesia*,*Theileria*, *Anaplasma* and *Ehrlichia*) with variable prevalence in the salivary glands of four tick species (*Rhipicephalus evertsi evertsi*, *Rhipicephalus decoloratus*, *Amblyomma hebraeum* and *Hyalomma rufipes*) collected in ruminants. In addition to describe new vector-pathogen combinations, this approach using tick organs allowed to detect *Theileria bicornis*,*Theileria* sp. (giraffe), *Theileria* sp. (Kudu) and *Babesia* sp. (sable) for the first time in ticks and more precisely in salivary glands suggesting vector competence of the studied tick species. As another example, Budachetri et al. [74] detected *Rickettsia parkeri*, known to cause human rickettsiosis, in the midgut, salivary glands and the saliva of questing ticks *Amblyomma maculatum*. Detection and quantification of TBPs in tick organs can provide new insights into the distribution of pathogens within ticks in different ecological settings. High pathogen levels relative to negative controls and in salivary glands relative to midgut may inform on pathogen replication in tick tissues and thus vector colonization by pathogens. The BioMark™ dynamic array system offers the possibility to achieve this by using a specific chip dedicated to digital PCR. This technology has been used to quantify viruses in food and/or in different organs of mice, and it can be adapted to TBPs detection and quantification in different tick organs [9, 75].

2.2 Next-generation sequencing

2.2.1 General background on the technology

During the past decade, NGS technologies have provided new insights into microbial community dynamics and ecology. These tools allow high-throughput analysis of complex and diverse microbial communities in multiple ecosystems such as soils and aquatic systems or in the microbiota of host organisms such as plant, animals and humans. With the development of these new sequencing approaches, it has definitively become faster and more economical to comprehensively evaluate the complexity of microbial species and strains in various ecosystems. Three main sequencing strategies are commonly used to study microbial communities: (i) marker gene approaches (i.e. SSU rRNA genes) with amplicon sequencing to identify microbiota composition (the 16S rRNA gene being the most used), (ii) shotgun metagenomics to characterize the functional potential of the microbiome and (iii) shotgun metatranscriptomics to determine actively expressed genes [76]. For further details on these different sequencing approaches, the reader is referred to [77, 78].

2.2.2 Tick microbial communities revealed by NGS

While ticks are known to be one of the main vectors of various pathogenic agents [4, 9, 10, 20, 73, 79, 80], it is now recognized that TBPs in ticks coexist with

microorganisms considered non-pathogenic for humans. Studies using NGS have shown that specific TBPs are frequently found together with other pathogens, symbionts and commensals [81]. This tick microbial complex, recently named "pathobiome" [82, 83], is influenced by the environment, and the interactions between its different components might influence pathogen acquisition by ticks and transmission to the host. In this context, the identification and characterization of tick microbiota has become essential to understand tick-pathogen interactions [84, 85]. While at the beginning of the twenty-first century, some studies started to characterize microbial communities associated to ticks using fingerprinting approaches (e.g. [86, 87]), the development of NGS technologies allowed higher resolution in the identification of tick microbiota bacteria and revealed an

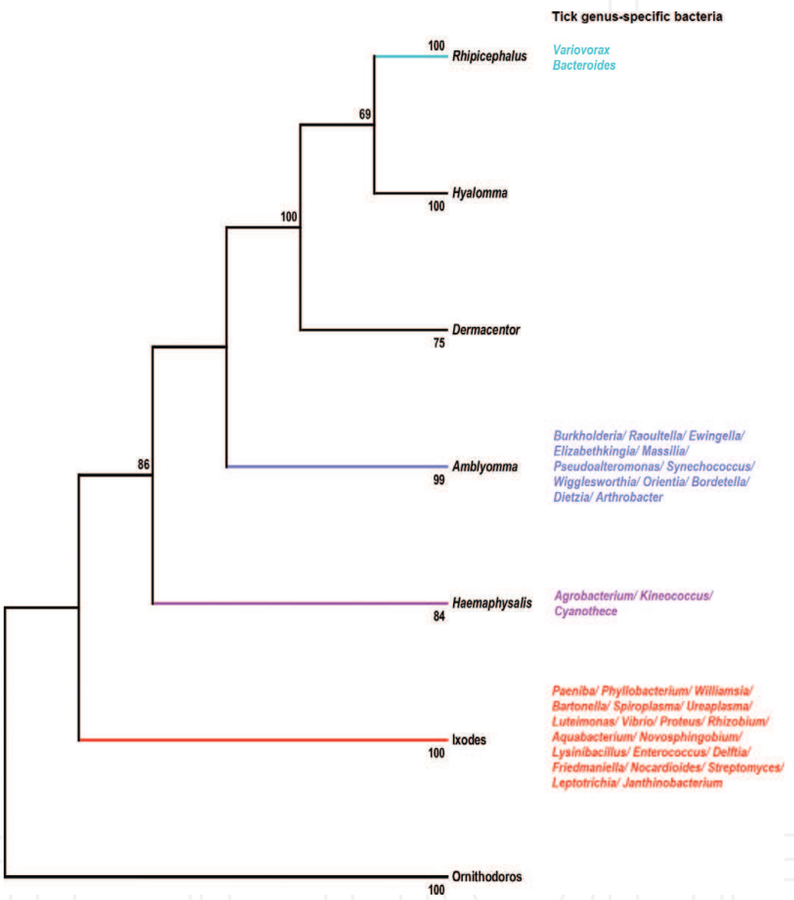

Figure 2.
Bacteria genera found across tick genera. The figure is a cladogram displaying the phylogenetic relation among major tick genera. Information on bacteria genera specific to each tick genus was collected from published data available in Table A2. The cladogram is based on a maximum parsimony phylogenetic tree of subolesin nucleotide sequences that were aligned using MAFFT followed by codon alignment. The final alignment contained 576 total sites of which 329 were gap-free. Bootstrap values (500 replicates) are shown next to the branches. Branches were collapsed at the genus level. Sequences were collected from GenBank and transcriptome projects, and accession numbers are as follow: Ixodes scapularis (AY652654), I. persulcatus (KM888876), I. ricinus (JX193817), I. ariadnae (KM455971), I. hexagonus (JX193818), Rhipicephalus evertsi (JX193846), R. appendiculatus (DQ159967), R. microplus (EU301808), R. sanguineus (JX193845), R. haemaphysaloides (KP677498), R. annulatus (JX193844), R. decoloratus (JX193843), R. zambeziensis (GFPF01005851), R. bursa (GFZJ01017781), R. pulchellus (GACK01006228), Dermacentor silvarum (JX856138), D. sinicus (KM115649), D. marginatus (KU973622), D. variabilis (AY652657), D. reticulatus (JX193847), Amblyomma variegatum (JX193824), A. hebraeum (EU262598), A. cajennense (JX193823), A. americanum (JX193819), A. maculatum (JX193825), A. aureolatum (GFAC01005925), A. triste (GBBM01002796), A. sculptum (GFAA01000261), Hyalomma anatolicum (KT981976), H. rufipes (JX193849, H. marginatum (DQ159971), H. excavatum (GEFH01000904), Haemaphysalis longicornis (EU289292), Hae. elliptica (JX193850), Hae. qinghaiensis (EU326281), Hae. flava (KJ829652), Hae. punctata (DQ159972), Ornithodoros moubata (JX193852), O. savignyi (JX193851), O. turicata (GDIE01114362), O. erraticus (HM622148), and O. rostratus (GCJJ01005500).

unexpected microbial diversity in these arthropods [88–90]. The general workflow commonly used to study tick microbiota using NGS is presented in **Figure 1**.

Since the first study using NGS to describe the bacterial diversity in the cattle tick *R. microplus* [91], different NGS technologies have been applied to identify the microbiota of various tick species. In consequence, the microbiota of several tick species of the genera *Ixodes*, *Dermacentor*, *Haemaphysalis*, *Rhipicephalus* and *Amblyomma* has been studied, and its composition in different locations was reported. A review of studies using these tools and describing tick microbial community composition at the genera level is presented in **Table A2**. Focusing on metagenomics approaches, both Illumina MiSeq and 454 pyrosequencing represented the most used sequencing techniques, even though the Illumina chemistry is now the most used due to the higher number of sequences generated by this approach. Most of our knowledge about tick microbial diversity and composition comes from sequencing the 16S rRNA gene based on DNA extracts (**Table A2**). Interestingly, the diversity of genus-specific microorganisms detected in ticks varies among the main tick genera (**Figure 2**). While a large number of bacterial genera are exclusively associated with *Ixodes*, not a single bacteria genus was found yet to be exclusively associated to *Dermacentor* (**Figure 2**). Whether this is related with the fact that more studies are available on *Ixodes* spp. (i.e. [17]) than on *Dermacentor* (i.e. [8]), microbiota is unknown; however, this finding warrants further research. Not only *Ixodes* has the highest number of genus-specific microorganisms (**Figure 2**), but it can also accommodate most of the bacteria found in other tick genera (**Figure 3**). Despite clear differences in the microbial communities of different tick genera (**Figures 2** and **3**), several bacteria genera were shared by all tick genera including *Rickettsia*, *Pseudomonas*, *Acinetobacter*, *Coxiella and Flavobacterium*. These findings should be approached under the hypothesis that these bacteria have a deep influence on the physiological processes of the tick or they would be not tightly associated to such diverging tick genera [81].

2.2.3 Challenges and perspectives

NGS methods have improved increasing in sequencing depth (i.e. a higher number of sequences obtained per sample) and thus a better estimation of the microbial diversity. However, the read length of the most widely used sequencing

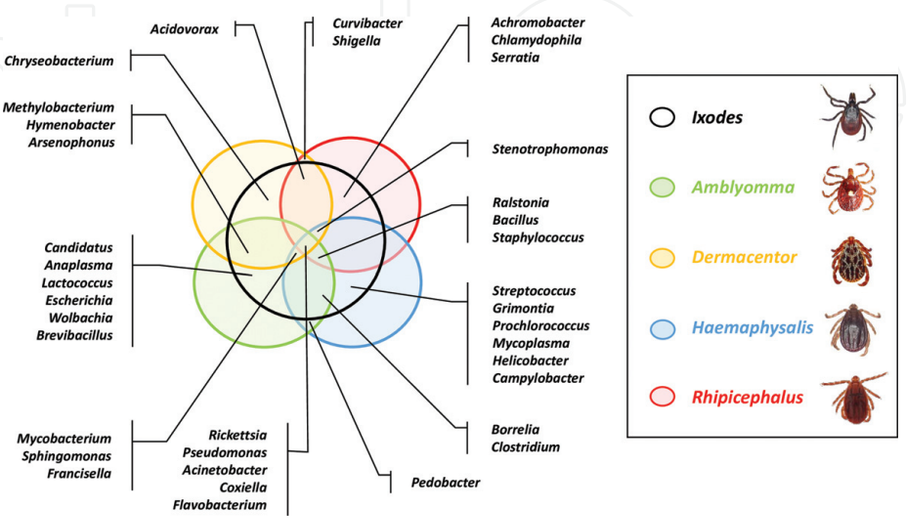

Figure 3.
Bacteria genera shared by major tick genera. Information on bacteria genera shared by more than one tick genera was collected from published data available in Table A2. For figure display reasons, the bacteria genera shared by Ixodes, Rhipicephalus and Amblyomma are not shown. These three tick genera share bacteria of the genera Corynebacterium and Propionibacterium.

platforms today is very short (few hundreds base pairs) and requires the researchers to choose a region of the 16S rRNA gene to sequence. For NGS purposes, the 16S rRNA gene is divided into nine regions (i.e. V1–V9). Most of the previous studies that used the 454 pyrosequencing approach amplified the V1–V3 region (**Table A2**). Studies that used the MiSeq approach mainly amplify the second part of the 16S rRNA gene with the V3–V4/V3–V5 or V5–V6 regions (**Table A2**). In this context, many bacteria genera may share the same amplified region, and the taxonomic resolution of profiling is inherently limited with incomplete information on tick microbial composition at the species level. There is a need for a simple 16S rRNA gene-based profiling approach that avoid the short read length to provide a much larger coverage of the gene to obtain higher taxonomic resolution in tick microbiota identification. The limitation of 16S rRNA gene sequencing (DNA-based) for microbial community analyses is the inability to differentiate between active and non-active cells. In comparison, 16S rRNA sequencing (RNA-based) can target metabolically active cells which produce rRNA. It is thus essential to include RNA and metatranscriptomic approaches to characterize the tick microbiota [92–94]. In addition, limitations linked to the 16S rRNA gene sequencing include polymerase chain reaction (PCR) bias, resulting, as previously mentioned, in low taxonomic resolution (typically genus-level) and limited functional insight into the microorganisms. These limitations hamper our ability to investigate how the non-pathogenic members of the tick microbiota interact with the pathogens and influence their presence and transmission. One way to avoid these biases is to use whole genome sequencing (WGS) to sequence thousands of genes from hundreds of microorganisms in a given sample. By gaining access and annotating the whole genome, it would become possible to reconstruct the putative metabolism of individual microbial species and gain insight into their potential role in tick-borne pathogens and diseases.

Using NGS techniques, many studies described tick microbial community composition and diversity and reported lists of microorganisms associated to several tick species. However, as underlined by Shade [95], diversity and composition without context provide limited insights into the mechanisms underpinning community patterns. Measurement of microbial diversity should be the starting point for further inquiry of ecological mechanisms rather than the "answer" to community outcomes [95]. Studying microbial communities associated to ticks needs thus contextual data, and it appears crucial to know the dynamics in space and time of these communities and the influence of environmental factors on their dynamics. In addition to factors associated with tick biology, the composition of tick microbial communities can be highly variable due to environmental factors such as biogeography, temperature, light-dark cycles, hygrometry, and vegetation [87–89, 96, 97]. Future studies on tick microbiota will have to consider these different variables and define more deeply their role in the dynamics of microbial communities associated to ticks. Biotic interactions are also important drivers of diversity, and the nature and strength of interactions can result in complex multimember interactions. Considering the pathobiome concept, one additional challenge for the understanding and control of tick-borne diseases is to increase the measurements of microbial diversity and calls for identifying potential associations/interactions between pathogens and other tick microbes. Finally, after identifying the tick microbiota including symbionts, it becomes crucial to determine the relationships between ticks and these bacteria. Ticks are strict hematophagous arthropods, and this specific diet is limited in B vitamins. Duron et al. [98] have recently demonstrated that the exploit of this unbalanced diet is possible because an intracellular bacterial symbiont of the genus *Francisella* supplies missing nutrients and that this nutritional symbiont is essential for tick development and survival to adulthood.

Similar studies have to be carried out in the future to better understand the complex roles of these symbionts in tick ecology.

3. Network analysis

3.1 General background on network analysis methodology

Networks are formed by components, known as nodes, and the relationships between these components are named links (**Figure 4**). The network may be undirected (there is not directionality in the link) or directed (there is directionality in the link). In microbial networks, each node represents a species and each link, representing co-occurring bacteria, resulting in undirected networks. Directed networks would be those resulting from, for example, parasites "on" vectors or microbes "in" a reservoir. The complete set of records can be then weighted according to the number of times one node is linked to another node (**Figure 4**). Several indices can be used to measure network properties from which the relationships among the co-occurring bacteria are derived. The degree centrality (DC, i.e. number of links connecting a given node to other nodes) is the most basic measure of a network and is calculated after weighting the total number of records containing this interaction. The DC provides an estimation of the strength of the association but does not evaluate the importance of each node in the context of the network. The node betweenness centrality (NBC) indicates how often a node is found on the shortest path between two nodes in the network [99, 100]. The implicit meaning of the NBC in microbial networks is the importance of a node in the flow of other components of the network and is considered a basic index defining the relative importance of a node in an ecological network. The PageRank

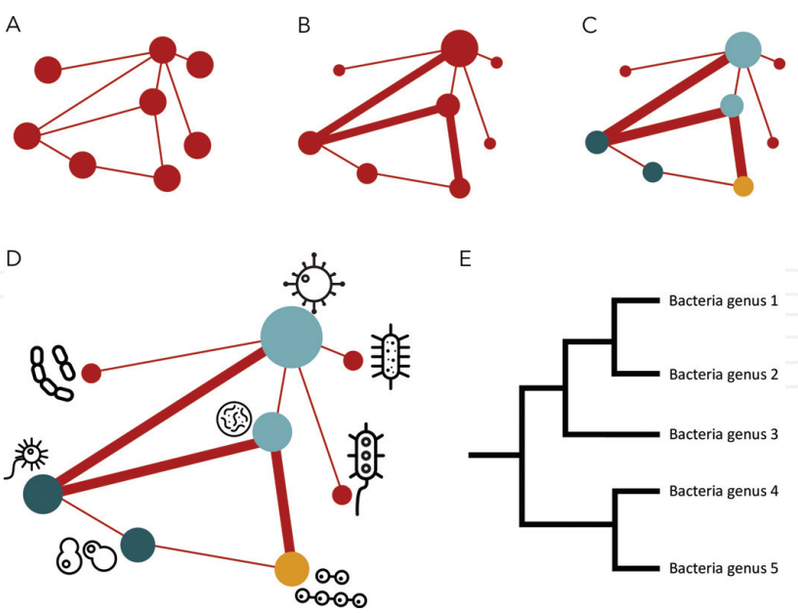

Figure 4.
A schematic explanation of the construction of networks for co-occurring bacteria in the microbiome. (A) A network is composed of nodes (circles) and links (lines). Each pair of bacteria that co-occur is connected by a link. The absence of a link means that a given pair of bacteria was not found to co-occur in any carrier. (B) The relative importance of each bacterial taxa and the importance of the links between co-occurring bacteria can be measured with indexes of centrality. In the schematic representation, larger circles mean higher centrality and wider links mean frequently detected co-occurring bacteria. Then, clustering algorithms (C) can detect communities of co-occurring bacteria (randomly colored in the figure). Once the complete network is built (D), results can be translated to a phylogenetic tree of the detected taxa to obtain important indexes of phylogenetic diversity and tracking the phylogenetic signal of the quantitative traits of the network (E).

(PR) is an index of centrality that assigns a universal rank to nodes based on the importance of the other nodes to which it is linked. Therefore, the NBC and PR are complementary measures for capturing the importance of each node in the linkage of other nodes throughout the network. These three indexes capture the ecological relationships between the interacting partners.

Real-world networks have been shown to separate into logical clusters in which nodes are tightly connected to each other but only loosely connected to nodes outside of their module [101]. They thus represent sets of organisms that interact more among them than with the others. This modularity separates the complete network into compartments that can be observed as naturally segregated niches in which a subset of taxa has a statistically higher affinity among them than with other species in the network.

3.2 Network analysis to disentangle the microbial complexity associated with ticks

The important value of the tick microbiota is the ecological interpretation of the associations or co-occurrence rates of the microorganisms detected in a collection of ticks. Whether these ticks were collected in different ecosystems, or associated to different hosts, or surveyed at different time intervals, the most important purpose is capturing the ecological meaning of these associations among the detected bacteria. Therefore, it is necessary to determine the relationships among the microorganisms, identify 'dominant' taxa in the microbiota and to study how they interact.

It is logical to assume that microorganisms that co-occur in the network are those that "overlap in the habitat" provided by the carrier of a given microbiota. This high co-occurrence likely ensures cohesiveness and persistence of the network improving the circulation of the microorganisms. Most important, a phylogenetic tree of the detected bacteria can be built, and the indexes of centrality can be tracked over the branches of the resulting tree (**Figure 4**). This is commonly known as "tracking the phylogenetic signal of quantitative traits" [102]. A common empirical observation for organisms is that continuous traits (i.e. morphological features, or the occupancy of ranges of the variables shaping its environmental niche) of closely related species in a phylogeny are often similar, meaning that these traits are under selection pressure. The link between phylogeny and continuous trait values is commonly referred in the literature as the phylogenetic signal. Therefore, it is possible to test the phylogenetic signal of the network indexes, which are actually quantitative traits, over the branches of the tree. Several indexes and dedicated computer packages are available to measure the phylogenetic signal [102]. Tracking these indexes on the phylogenetic tree explains the relative importance of the taxa of the microbiota and how it is organized in a population of ticks. The phylogenetic distance of the microorganisms detected in ticks can be calculated. This could be used to evaluate the phylogenetic diversity carried by ticks according to the habitat, the season of the year or the environmental conditions driving the tick phenology and survival. It is necessary to stress that an index of phylogenetic distance, together with the centrality indexes of the realized network, provides ecological or possibly physiological information of the microbiota composition. This cannot be achieved by listing bacterial taxa.

Most of the guidelines expressed above have been addressed in a recent study on the microbiota of *Ixodes ricinus* ticks and one of its main hosts, the vole *Myodes glareolus* [37]. In this study, NGS was combined with network analysis to measure the impact of the ecosystem in the composition of tick and vole microbiota. One of the main conclusions of the study is that the similarity of the microbiota between ticks and hosts is low, with a clear impact on the type of ecosystem in which ticks

were collected on the resulting microbiota. These findings could be a consequence of the different range of hosts available for the tick in two different ecosystems. Regardless of the causes of these findings, the study demonstrated that the tick microbiota seems to be optimized for the co-occurrence of bacteria with low phylogenetic similarities. This could be interpreted in two ways: (i) the high phylogenetic diversity of bacteria in ticks evolved to decrease the competition for the 'tick niche' of closely related taxa, since it is expected that largely divergent taxa would have very different requirements in the tick and (ii) the microbiome is organized to provide the tick with a large number of bacterial metabolic routes that benefit the physiological processes of the tick; therefore, a high diversity of taxa in a tick would ensure a high diversity of these 'physiological complementarity' supporting the physiology of the tick in many different ways. The lack of empirical data in this field warrants further research, either from field studies or from laboratory controlled studies.

The current impossibility to obtain germ-free ticks is a gap in this field of study. Colonization of ticks with single species of bacteria could help to understand the contribution of individual bacteria to tick physiology. However, accumulating evidence demonstrated that most of these bacteria are fundamental for tick physiological processes and survival in the environment. Therefore, the information about the ecological and physiological relationships between the tick and the microbiome must be obtained from field surveys and subjected to big data analysis as proposed before. We firmly believe that the next step forward in the field of tick microbiome must be a change of paradigm from 'taxonomical listing' to the functional characterization of tick microbiome in the environment. Classic statistics can be of little help in such task.

4. Conclusions

High-throughput technologies have improved our current understanding of the microbial complexity associated to ticks. These technologies allowed us to move from the "one-tick-one-pathogen" paradigm to the "one-tick-many-microorganisms" paradigm. This new concept can be summarized: ticks are associated with complex microbial communities, including pathogenic and non-pathogenic microorganisms, which interact between them and with the vector and are together under the influence of the environment. Future developments may be related with the characterization of tick microbiome at the species level and with inclusion of strain diversity analysis in high-throughput pathogen detection. Finally, high-throughput data analysis could benefit from tools assessing the relevance and contribution of individual nodes of the microbial network. Network analysis can be used to calculate co-occurrence patterns and centrality indexes that may assist in the identification of highly important members of tick microbiota.

Acknowledgements

The authors thank the members of their laboratories for fruitful discussions.

Conflict of interest

The authors declare that they have no competing interests.

A. Appendix

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
Ixodes ricinus	Nymphs/ adults	*Borrelia burgdorferi s.l.* + *Rickettsia spp. (R. helvetica* mainly)	NT	NT	7.3	Realtime PCR (5S and 23S rRNA genes of Intergenic Spacer region)	NE	Germany	[44]
		B. burgdorferi s.l. + *Anaplasma phagocytophilum*	NT	NT	0.3				
		B. burgdorferi s.l. + *Rickettsia spp. (R. helvetica mainly)* + *A. phagocytophilum*	NT	NT	0.1				
	Adults	—	*Babesia microti* + *Toxoplasma gondii*	NT	42	Nested PCR (conservative regions of the flagelline gene)	E	Poland	[45]
		—	*B. microti* + *T. gondii*	NT	32		NE		
	Adults	*B. burgdorferi s.l.* + *Rickettsia spp.*	NT	NT	12.7	Realtime PCR (5S and 23S rRNA genes of Intergenic Spacer region)	NE	Germany	[58]
	Nymphs	*B. burgdorferi s.l.* + *Rickettsia spp.*	NT	NT	12.7				
	Nymphs/ adults	*Different genospecies of B. burgdorferi s.l. (detail not provided)*	NT	NT	3.6				
	Nymphs	*B. afzelii* + *Ca. N. mikurensis*	NT	NT	3.3	Realtime PCR (16S rRNA and *hbb* gene)	NE	Norway	[59]
	Nymphs/ adults	*Different genospecies of B. burgdorferi s.l. (detail not provided)*	NT	NT	2.1	Realtime PCR (*fla* gene fragment)	NE	Poland	[46]
	Nymphs/ adults	*B. burgdorferi s.l.* + SFG *Rickettsia*	NT	NT	3.7	Realtime PCR (*opsA* and flagelin genes)	NE	The Netherlands	[57]
	Adults	*R. helvetica* + *A.phagocytophilum*	NT	NT	0.4	Realtime Microfluidic PCR (16S rRNA encoding *rrs* genes)	NE	France	[9]
		R. helvetica + *B. afzelii*	NT	NT	0.4				
		R. helvetica + *B. garinii*	NT	NT	0.4				
		R. helvetica + *B. valaisiana*	NT	NT	0.7				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
		R. helvetica + *Bartonella henselae*	NT	NT	3.0				
		B. burgdorferi + *B. valaisiana*	NT	NT	0.4				
		B. garinii + *B. afzelii*	NT	NT	0.7				
		B. garinii + *B. burgdorferi*	NT	NT	0.4				
		B. garinii + *B. henselae*	NT	NT	0.4				
		B. miyamotoi + *B. henselae*	NT	NT	0.4				
		Candidatus Neoehrlichia mikurensis + *B. miyamotoi*	NT	NT	0.7				
		An. phagocytophilum+ *B. henselae*	NT	NT	0.4				
		B. spielmanii + *B. henselae*	NT	NT	0.4				
		R. helvetica + *B. afzelii* + *B. garinii*	NT	NT	0.7				
		R. helvetica + *B. valaisiana* + *B. burgdorferii*	NT	NT	0.4				
		R. helvetica + *An. phagocytophilum* + *B. afzelii*	NT	NT	0.4				
		B. garinii + *B. afzelii* + *B. spielmani*	NT	NT	0.4				
		B. garinii + *B. afzelii* + *B. valaisiana*	NT	NT	0.7				
		B. garinii + *B. burgdorferi* + *B. valaisiana*	NT	NT	0.7				
		B. garinii + *B. burgdorferi* + *B. henselae*	NT	NT	0.4				
		B. garinii + *B. afzelii* + *B. henselae*	NT	NT	1.1				
		B. garinii + *B. miyamotoi* + *B. henselae*	NT	NT	0.4				
		B. garinii + *R. helvetica* + *B. henselae*	NT	NT	0.4				
		B. garinii + *B. afzelii*	*B. divergens*	NT	0.4				
		B. garinii + *B. afzelii* + *Ca. N. mikurensis*	NT	NT	0.7				
		R. helvetica + *B. afzelii* + *B. garinii* + *A. phagocytophilum*	NT	NT	0.4				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
		B. afzelii + *B. garinii* + *B. burgdorferi* + *B. henselae*	NT	NT	0.4				
		B. afzelii + *B. garinii* + *B. burgdorferi* + *R. helvetica*	NT	NT	0.4				
		B. afzelii + *B. garinii* + *B. burgdorferi* + *B. spielmanii*	NT	NT	0.7				
		R. helvetica + *B. afzelii* + *B. garinii* + *B. valaisiana* + *B. burgdorferi*	NT	NT	0.4				
		B. henselae + *B. afzelii* + *B. garinii* + *B. spielmanii* + *B. burgdorferi*	NT	NT	0.4				
	Nymphs/ adults	*Ca.* N. mikurensis + *A. phagocytophilum*	NT	NT	0.1	Realtime PCR	NE	Slovakia	[48]
	Nymphs/ adults	*B. miyamotoi* + *B. burgdorferi s.l.*	NT	NT	0.29	Realtime PCR (*glpQ* gene and 5S-23S rDNA IGS)	NE	Slovakia	[51]
		B. miyamotoi + *B. afzelii*	NT	NT	0.12				
	Larvae	*B. burgdorferi s.l.* + *R. helvetica*	NT	NT	4.5	Realtime PCR (*flaB* and *opsA* genes)	E	The Netherlands	[103]
		R. helvetica + *Ca.* N. mikurensis	NT	NT	0.7				
		A. phagocytophilum + *R. helvetica*	NT	NT	0.7				
	Nymphs	*B. burgdorferi s.l.* + *R. helvetica*	NT	NT	9.6				
		B.burgdorferi s.l. + *Ca.* N. mikurensis	NT	NT	3.5				
		B. burgdorferi s.l. + *A. phagocytophilum*	NT	NT	3.5				
		R. helvetica + *Ca.* N. mikurensis	NT	NT	1.9				
		A. phagocytophilum + *R. helvetica*	NT	NT	1.5				
		B. burgdorferi s.l. + *B. miyamotoi*	NT	NT	0.2				
		B. miyamotoi + *Ca.* N. mikurensis	NT	NT	0.2				
		B. burgdorferi + *R. helvetica*+ *Ca.* N. mikurensis	NT	NT	1.3				
		B. burgdorferi s.l. + *R. helvetica* + *A. phagocytophilum*	NT	NT	0.6				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
	Nymphs/adults	*B. garinii* + *B. afzelii*	NT	NT	4.3	Realtime Microfluidic PCR + PCR [*gltA* (*Bartonella-Rickettsia* spp.), 23S rRNA-*rpoB-fla-ospA-glpQ* (*Borrelia* spp.), *groEL* (*Candidatus* Neoehrlichia mikurensis)]	NE	Romania	[20]
	Nymphs/adults	*B. garinii* + *B. lusitaniae*	NT	NT	3.0				
	Nymphs	*B. garinii* + *B. spielmanii*	NT	NT	0.7				
	Nymphs	*B. afzelii* + *B. bissettii*	NT	NT	0.2				
	Nymphs	*B. afzelii* + *B. lusitaniae*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. valaisiana*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *B. valaisiana*	NT	NT	0.9				
	Nymphs	*B. garinii* + *B. afzelii* + *B. lusitaniae*	NT	NT	0.2				
	Adults	*B. garinii* + *B. afzelii* + *B. spielmanii*	NT	NT	1.3				
	Adults	*B. garinii* + *B. valaisiana* + *B. lusitaniae*	NT	NT	1.3				
	Nymphs	*B. garinii* + *R. monacensis*	NT	NT	0.4				
	Nymphs/adults	*B. valaisiana* + *Bartonella spp.*	NT	NT	0.4				
	Nymphs	*B.afzelii* + *Ca.* N. mikurensis	NT	NT	0.4				
	Nymphs	*B. valaisiana* + *Ca.* N. mikurensis	NT	NT	0.4				
	Adults	*B. valaisiana* + *R. monacensis*	NT	NT	1.3				
	Nymphs	*B. valaisiana* + *Rickettsia spp.*	NT	NT	0.2				
	Nymphs	*B. afzelii* + *Rickettsia spp.*	NT	NT	0.2				
	Nymphs	*B. miyamotoi* + *Ca. N. mikurensis*	NT	NT	0.2				
	Nymphs	*B. miyamotoi* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs	*B. garinii* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs	*B. spielmanii* + *A. phagocytophilum*	NT	NT	0.2				
	Nymphs	*B. garinii* + *Rickettsia spp.*	NT	NT	0.2				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
	Nymphs	*B. garinii* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. afzelii* + *R. helvetica*	NT	NT	0.2				
	Nymphs	*Borrelia spp.* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*Borrelia spp.* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs/ adults	*B. garinii* + *B. afzelii* + *Rickettsia spp.*	NT	NT	0.6				
	Nymphs/ adults	*B. garinii* + *B. lusitaniae* + *Rickettsia spp.*	NT	NT	0.4				
	Nymphs	*B. garinii* + *B. afzelii* + *R. monacensis*	NT	NT	0.4				
	Nymphs	*B. valaisiana* + *B. spielmanii* + *R. monacensis*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. valaisiana* + *R. helvetica*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *A. phagocytophilum*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *Ca. N. mikurensis*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. valaisiana* + *Ca. N. mikurensis*	NT	NT	0.2				
	Nymphs	*B. garinii* + *R. helvetica* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs	*B. valaisiana* + *R. monacensis* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. valaisiana* + *Rickettsia spp.* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*Borrelia spp.* + *R. monacensis* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *B. lusitaniae* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. burgdorferi s.l.* + *B. miyamotoi*	NT	NT	0.4	Realtime PCR (*glpQ* gene)	NE	The Netherlands	[55]

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
I. frontalis	Adults	*B. burgdorferi s.l.* + *A. phagocytophilum*	NT	NT	18.2	Realtime PCR (*flaB* and *ospA*)		The Netherlands	[103]
	Larvae	*B. valaisiana* + *B. turdi*	NT	NT	2.5	Nested PCR (*flaB*, 5S and 23S rRNA IGS)	E	Spain	[56]
I. holocyclus and I. tasmani	Adults	—	*Trypanosoma . irwini, T.gilletti, T. copemani and T. vegrandis*	NT	27,3 and 12,2	NGS (18SrRNA)	E	Australia	[53]
I. scapularis	Nymphs/ adults	*B. burgdorferi s.l.* + *A. phagocytophilum*	NT	NT	1.8	Realtime PCR [23S (*Borrelia*), tubulin (*Babesia*), *msp2* (*Anaplasma*)]	E	USA	[49]
		B. burgdorferi s.l.	*B. microti*	NT	1				
		A. phagocytophilum	*B. microti*	NT	0.4				
		B. burgdorferi s.l. + *A. phagocytophilum*	*B. microti*	NT	0.3				
I. persulcatus	Nymphs/ adults	*B. garinii* + *SFG Rickettsia*	NT	NT	16.2	Nested PCR	NE	China	[47]
		B. burgdorferi + *A. phagocytophilum*	NT	NT	4.9				
		SFG *Rickettsia* + *A. phagocytophilum*	NT	NT	2.9				
		B. burgdorferi + *A. phagocytophilum* + SFG *Rickettsia*	NT	NT	2.5				
	Adults	*B. burgdorferi s.l.*	NT	TBEV	1.6	Realtime PCR (*gltA* and *ompA*)	E	Russia	[52]
		B. burgdorferi s.l. + *Ehrlichia chaffeensis*	NT	NT	1.6				
		B. burgdorferi s.l. + *A. phagocytophilum*	NT	NT	1.6				
Dermacentor marginatus	Adults	*R. raoultii*	NT	TBEV	4.2				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
D. reticulatus	Adults	*A. phagocytophilum*	NT	TBEV	0.32	PCR [*gltA (Rickettsia* spp.), *fla* (*B. burgdoferi* s. l.), B1 fragment (*T. gondii*), 18S rRNA gene (*Babesia* spp.)]	NE	Poland	[54]
		R. raoultii	NT	TBEV	4.26				
		B. burgdorferi s.l.	NT	TBEV	0.16				
		A. phagocytophilum + *R. raoultii*	NT	NT	0.63				
		R. raoultii + *B. burgdorferi s. l.*	NT	NT	1.1				
		R. raoultii	*Babesia spp.*	NT	0.47				
		R. raoultii	*Toxoplasma gondii*	NT	0.95				
			Babesia spp. + *Toxoplasma gondii*	NT	0.16				
			Toxoplasma gondii	TBEV	0.45				
Haemaphysalis longicornis	Nymphs/ adults	*A. capra*	NT	SFTSV		PCR (*Pmy* gene)	NE	China	[50]
Rhipicephalus sanguineus	Adults	*E. canis*	*H. canis* + *L. infantum chagasi*	NT	28.6	PCR [16S rRNA (*Anaplasma*), 18S rRNA (*Babesia*), 16S rRNA (*Ehrlichia*), 18S rRNA (*Hepatozoon*), kinetoplast DNA (*Leishmania*)]	E	Brazil	[43]

NT, not tested; TBEV, tick-borne encephalitis virus; SFTSV, severe fever with thrombocytopenia syndrome virus.

Table A1.
Coinfections reported in the literature in the last 4 years.

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
I. ricinus	Adults	*Rickettsiella*, *Rickettsia*, *Midichloria*, *Paenibacillus*, *Borrelia*, *Lactococcus*, *Ralstonia*	Ion torrent [16S [V1–V2])	Australia	[104]
	Nymphs	*Borrelia*, *Escherichia*, *Rickettsia*, *Candidatus Neoehrlichia*, *Wolbachia*, *Methylobacterium*, *Mycobacterium*, *Phyllobacterium*, *Sphingomonas*, *Hymenobacter*, *Pseudomonas*, *Williamsia*	454 pyrosequencing [16S (V6)]	Italy	[88]
	Adults	*Borrelia*, *Escherichia*, *Rickettsia*, *Candidatus Neoehrlichia*, *Methylobacterium*, *Mycobacterium*, *Phyllobacterium*, *Sphingomonas*, *Hymenobacter*, *Pseudomonas*, *Williamsia*			
	Nymphs	*Anaplasma*, *Coxiella*, *Ehrlichia*, *Borrelia*, *Rickettsia*, *Bartonella*, *Francisella*	Hiseq (bacteria)	France	[92]
	Adults				
	Adults	*Borrelia*, *Ehrlichia*, *Ca midichloria*, *Spiroplasma*, *Anaplasma*, *NeoEhrlichia*	RNA seq (bacteria)	Czech Republic	[94]
	Adults	*Borrelia*, *Lactobacillus*, *Streptococcus*, *Ureaplasma*, *Grimontia*, *Bacillus*, *Luteimonas*, *Vibrio*, *Rickettsia*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
I. persulcatus	Adults	*Proteus*, *Acinetobacter*, *Rickettsia*, *Pseudomonas*	MiSeq [16S (V4)]	China	[50]
	Adults	*Rickettsia*, *Spiroplasma*, *Coxiella*	454 pyrosequencing [16S (V1–V3)]	Japan	[106]
	Adults	*Pseudomonas*, *Sphingomonas*, *Acidovorax*	MiSeq [16S (V3–V5)]	Russia	[107]
	Adults	*Pseudomonas*, *Enterobacter*, *Serratia*, *Stenotrophomonas*, *Achromobacter*	Hiseq (bacteria)	China	[93]
	Adults	*Chlamydophila*, *Ureaplasma*, *Streptococcus*, *Helicobacter*, *Campylobacter*, *Prochlorococcus*, *Borrelia*, *Mycoplasma*, *Clostridium*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
I. scapularis	Adults	*Rickettsia*, *Brevibacillus*	454 pyrosequencing [16S (V1–V3)]	America	[108]
	Adults	*Rickettsia*, *Francisella*	454 pyrosequencing [16S (V1–V3)]	America	[109]
	Nymphs	*Rickettsia*, *Sphingomonas*, *Rhizobium*	MiSeq [16S (V3–V4)]	America	[90]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
	Adults	*Rickettsia*, *Wolbachia*, *Sphingomonas*, *Methylobacterium*, *Pseudomonas*			
	Nymphs	*Rickettsia*, *Acidovorax*, *Novosphingobium*, *Aquabacterium*	454 pyrosequencing [16S (V2)]	America	[84]
	Adults				
	Nymphs	*Acinetobacter*, *Rickettsia*, *Lysinibacillus*, *Corynebacterium*, *Staphylococcus*, *Enterococcus*, *Delftia*	MiSeq [16S (V4)]	America	[85]
	Adults				
I. affinis	Adults	*Rickettsia (>70%)*, *Methylobacterium*, *Borrelia*	MiSeq-454 pyrosequencing [16S (V1–V3; V4)]	America	[97]
I. holocyclus	Adults	*Wolbachia*, *Sphingobacterium*, *Hymenobacter*, *Friedmaniella*, *Nocardioides*, *Streptomyces*, *Paenibacillus*, *Clostridium*	Ion Torrent [16S [V1–V2])	Australia	[104]
	Nymphs	*Propionibacterium*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Ca. Midichloria*, *Ralstonia*	MiSeq [16S (V1–V2)]		[105]
	Adults	*Propionibacterium*, *Mycobacterium*, *Corynebacterium*, *Streptococcus*, *Ca. Midichloria*, *Ralstonia*			
I. ovatus	Adults	*Spiroplasma*, *Coxiella*, *Ehrlichia*, *Rickettsia*, *Leptotrichia*	454 pyrosequencing [16S (V1–V3)]	Japan	[106]
	Adults	*Rickettsia*, *Ureaplasma*, *Mycoplasma*, *Clostridium*, *Ehrlichia*, *Helicobacter*, *Francisella*, *Borrelia*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
I. pacificus	Nymphs	*Rickettsia*, *Methylobacterium*, *Flavobacterium*, *Sphingomonas*	MiSeq (16S)	America	[110]
	Adults	*Rickettsia*, *Methylobacterium*			
I. pavlovskyi	Adults	*Acinetobacter*, *Rickettsia*, *Chryseobacterium*, *Escherichia*, *Janthinobacterium*	MiSeq [16S (V3–V5)]	Russia	[107]
Amblyoma americanum	Nymphs	*Rickettsia*, *Coxiella*, *Borrelia*, *Wolbachia*, *Midichloria*, *Ehrlichia*, *Pseudomonas*	454 pyrosequencing [16S (V1–V3)]	America	[111]
	Adults	*Rickettsia*, *Coxiella*, *Borrelia*, *Wolbachia*, *Midichloria*, *Ehrlichia*, *Pseudomonas*			
	Nymphs	*Rickettsia*, *Coxiella*			[112]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
	Adults	*Rickettsia*, *Midichloria*, *Coxiella*, *Ehrlichia*, *Sphingomonas*			
	Adults	*Coxiella*, *Brevibacterium*, *Rickettsia*, *Staphylococcus*	MiSeq [16S(V3–V4)]		[113]
	Adults	*Hymenobacter*, *Flavobacterium*, *Rickettsia*, *Methylobacterium*, *Ehrlichia*, *Burkholderia*, *Anaplasma*	MiSeq [16S(V1–V4)]		[114]
	Adults	*Coxiella*, *Rickettsia*, *Arsenophonus*, *Pseudomonas*, *Acinetobacter*	?? [16S (V1–V9)]		[96]
A. longirostre; A. nodosum, *A. maculatum*, *H. juxtakochi*	Adults	*Lactococcus*, *Raoultella*, *Wolbachia*, *Francisella*, *Propionibacterium*, *Ewingella*, *Elizabethkingia*, *Rickettsia*, *Massilia*, *Methylobacterium.*	454 pyrosequencing [16S (V1–V3)]	America	[115]
A. maculatum	Adults	*Francisella*, *Propionibacterium*, *Rickettsia*, *Pseudomonas*, *Corynebacterium*, *Escherichia*,	454 pyrosequencing [16S (V1–V3)]	America	[74]
A. testudinarium	Nymphs Adults	*Pseudoalteromonas*, *Rickettsia*, *Synechococcus*, *Wigglesworthia*, *Clostridium*, *Orientia*, *Bordetella*, *Bacillus*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
A. triguttatum	Adults	*Francisella*, *Rickettsia*, *Flavobacterium*, *Pedobacter*, *Ralstonia*, *Mycobacterium*	MiSeq [16S (V1–V2)]	Australia	[105]
A. tuberculatum	Adults	*Rickettsia*, *Francisella*, *Dietzia*, *Arthrobacter*, *Acinetobacter*	454 pyrosequencing [16S (V1–V3)]	America	[116]
D. andersoni	Adults	*Francisella*, *Rickettsia*, *Arsenophonus*	Pacific Bioscience (PacBio, Menlo Park, USA) [16S (V1-V9)]	America	[117]
	Adults	*Arsenophonus*, *Acinetobacter*, *Francisella*, *Rickettsia*	454 pyrosequencing [16S (V4)]	America	[118]
D. marginatus	Adults	*Flavobacterium*, *Rickettsia*, *Curvibacter*, *Acidovorax*, *Shigella*	454 pyrosequencing [16S (V1–V3)]	Turkey	[119]
D. occidentalis	Adults	*Rickettsia*, *Francisella*, *Sphingomonas*, *Methylobacterium Hymenobacter*	MiSeq [16S (V4)]	America	[120]
D. reticulatus	Adults	*Francisella*, *Rickettsia*, *Acinetobacter*, *Acidovoraxi Chryseobacterium*	MiSeq [16S (V3–V5)]	Russia	[107]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
D. silvarum	Adults	*Pseudomonas*, *Coxiella*, *Rickettsia*, *Acinetobacter*	454 pyrosequencing [16S (V3–V4)]	China	[121]
D. variabilis	Adults	*Francisella*, *Brevibacillus*, *Arsenophonus*, *Stenotrophomonas*, *Mycobacterium*, *Rickettsia*	454 pyrosequencing [16S (V1–V3)]	America	[108]
	Adults	*Francisella*, *Arsenophonus*	454 pyrosequencing [16S (V1–V3)]	America	[109]
Haemaphysalis bancrofti	Nymphs	*Flavobacterium*, *Pedobacter*, *Propionibacterium*, *Rickettsia*, *Francisella*, *Pseudomonas*, *Stenotrophomonas*	MiSeq [16S (V1–V2)]	Australia	[105]
	Adults	*Francisella*, *Pseudomonas*, *Stenotrophomonas*, *Delfia*, *Ralstonia*, *Rickettsia*, *Sphingomonas*, *Agrobacterium*, *Flavobacterium*, *Pedobacter*, *Propionibacterium*, *Kineococcusi Mycobacterium*			
H. bispinosa	Nymphs / Adults	*Coxiella*, *Rickettsia*, *Bacillus*, *Mycobacterium*, *Sphingomonas*, *Pseudomonas*	Ion Torrent [16S [V6])	Malaysia	[122]
H. flava	Adults	*Coxiella*	454 pyrosequencing [16S (V1–V3)]	Japan	[106]
H. formosensis	Nymphs / Adults	*Chlamydophila*, *Streptococcus*, *Chlamydia*, *Helicobacter*, *Prochlorococcus*, *Campylobacter*, *Bacillus*, *Clostridium*, *Borrelia*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
H. hystricis	Nymphs / Adults	*Coxiella*, *Rickettsia*, *Bacillus*, *Mycobacterium*, *Sphingomonas*, *Pseudomonas*	Ion Torrent [16S [V6])	Malaysia	[122]
H. longicornis	Nymphs	*Mycobacterium*, *Propionibacterium*, *Flavobacterium*, *Pedobacter*, *Staphylococcus*, *Steptococcus*, *Agrobacterium*, *Rasltonia*, *Delfia*, *Coxiella*, *Pseudomonas*, *Francisella*, *Stenotrophomonas*	MiSeq [16S (V1–V2)]	Australia	[105]
	Adults	*Mycobacterium*, *Flavobacterium*, *Coxiella*, *Francisella*			
	Nymphs / Adults	*Lactobacillus*, *Salmonella*, *Grimontia*, *Providencia*, *Coxiella*, *Cyanothece*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Acinetobacter*, *Mycoplasma*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
H. wellingtoni	Nymphs	*Coxiella*, *Rickettsia*, *Bacillus*, *Mycobacterium*, *Sphingomonas*, *Pseudomonas*	Ion Torrent [16S (V6)]	Malaysia	[122]
	Adults				
R. annulatus	Adults	*Flavobacterium*, *Curvibacter*, *Acidovorax*, *Stenotrophomonas*, *Shigella*, *Variovorax*	454 pyrosequencing [16S (V1–V3)]	Turkey	[119]
R. microplus	Adults	*Achromobacter*, *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Coxiella*	454 pyrosequencing [16S (V1–V3)]	America	[91]
R. sanguineus	Nymphs	*Rickettsia*	MiSeq [16S (V5–V6)]	France	[123]
	Adults	*Rickettsia*, *Coxiella*, *Bacillus*, *Acinetobacter*			
	Adults	*Coxiella*, *Bacillus*			
	Adults	*Coxiella*, *Bacillus*		Russia	
R. turanicus	Adults	*Propionibacter*, *Bacteroides*, *Ralstonia*, *Serratia*, *Pseudomonas*	454 pyrosequencing [16S (V4–V6)]	Israel	[89]

Table A2.
NGS studies and tick microbiota composition reported in the literature.

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