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Comparative analysis of bacterial microbiomes in *Ixodes ricinus* female ticks from military training areas in Poland

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Abstract

Introduction and Objective. Ticks, especially *Ixodes ricinus*, are known vectors of multiple pathogens affecting human and animal health. Monitoring tick microbiomes, particularly in areas of military activity, is essential to understand the epidemiological threats they pose. This study investigates the microbiomes of *I. ricinus* ticks collected from military areas in Poland using next-generation sequencing (NGS).

Materials and Method. *Ixodes ricinus* ticks were collected in spring and autumn from military training grounds using the flagging method. After segregation (by stage and gender), DNA was isolated, libraries were prepared, and sequencing was performer. Data quality was assessed with fastQC. Pathogens were identified using Kraken2. The data was further analyzed using Bracken's classification methodology.

Results. Metagenomic analysis of *Ixodes ricinus* ticks revealed a diverse bacterial community composed of symbionts, environmental taxa, and potential pathogens. Core endosymbionts were consistently detected across all samples, while medically relevant genera, such as Borrelia, Rickettsia, Ehrlichia and Bartonella, were also identified. The results highlight both the complexity of the tick microbiome and its potential importance for human and animal health.

Conclusions. The study provides a preliminary overview of the microbiome of adult *Ixodes ricinus* ticks from Polish military training areas. Core endosymbionts were consistently detected, while variation in less abundant taxa suggests environmental influences. The presence of potential pathogens highlights the need for broader studies, and underlines the relevance of metagenomic approaches for public health and military medicine.

Key words

epidemiology, Borrelia, Ixodes ricinus, Rickettsia, One Health, next-generation sequencing, tick-borne pathogens, Tick microbiome, Ehrlichia, military areas

INTRODUCTION

Ticks have been around in much the same form for approximately 200 million years, and are among the oldest and most successful groups of arthropods [1]. Tick-borne diseases, long known but historically underappreciated, are increasingly recognized as significant threats to public health [2]. Assessing the microbial diversity in arthropod vectors of medical importance is crucial for monitoring endemic infections and surveillance of newly-emerging zoonotic pathogens. It also helps us to understand the bacteria associated with the host [3, 4]. In addition to pathogenic microorganisms, ticks also contain a diverse group of commensal and mutualistic microorganisms, acquired through blood meal, vertically or from the environment [3]. Assemblages of microorganisms that colonize various niches in the host body are called the microbiome. The latest definition of the microbiome is the complete community of microorganisms, metabolites and structural elements of microorganisms, and environmental conditions occurring in a specific place [5]. Knowledge about the common bacterial communities in the tick microbiome is key to the subsequent identification and description of endosymbiotic bacterial species, as well as bacterial species that are symbiotic or competitive with pathogens. Endosymbionts are described as bacteria essential to the development of the host, which can influence the viability and fitness of the host and therefore the transmission of pathogens [6].

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Unlike pathogens, the biology and impact of symbionts on ticks remain largely unexplored and, in fact, are often neglected. The first research on the microbiota of ticks was published in 2011 [7]. Research on material from *Rhipicephalus microplus* ticks feeding on cattle showed that their microbiota includes many types of bacteria, which can be traced back to either the host or the environment [7]. Since then, an increasing number of studies have used next-generation sequencing technologies to characterize the composition of the tick microbiota, which allows for a broader illustration of the diversity of its components [8]. In addition to bacteria, the microbiota also includes protists, nematodes, fungi and viruses [9]. In addition to endosymbionts and commensals, ticks harbour many pathogenic microorganisms of medical and veterinary importance, including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, Spotted Fever Group Rickettsia, among others [10]. The bacteria inhabiting the intestines of *I. ricinus* may influence its role as a vector, for example by modulating the colonization of pathogens, such as *B. burgdorferi* or *A. phagocytophilum* in tick tissues [9, 10]. These scientific discoveries may be the basis for creating new tools to combat the spread of vector-borne diseases by modulating the qualitative and quantitative composition of the tick microbiota [11].

Most of what we know about tick microbial diversity and composition comes from 16S rRNA gene sequencing [8]. The development of NGS technology has enabled higher-resolution sequencing, advancing understanding of their microbiota's composition and uncovering unexpected diversity in these arthropods [8]. Perhaps the most remarkable recent observation is the crucial role of symbiotic interactions in tick biology [3]. Symbionts, i.e. microorganisms involved in close and long-term interactions with tick hosts, are incredibly diverse: at least 10 different types of vertically inherited bacteria have been described in these arthropods over the last decade [12]. Three of these symbionts are found only in ticks: *Coxiella*-LE endosymbionts, which have been reported from at least two-thirds of tick species, Candidatus *Midichloria mitochondrii*, which inhabits the mitochondria of some tick species, and *Francisella*-LE endosymbionts, which have so far been described in only a few tick species [3]. The seven remaining symbiont genera (*Wolbachia, Cardinium, Arsenophonus, Spiroplasma, Rickettsia, Rickettsiella,* and *Lariskella*) occur at varying frequencies in other arthropod species, including several well-studied insects [2]. In tick species that are repeatedly colonized, symbionts form communities that can reach high levels of complexity. Indeed, at least six distinct genera of maternally inherited symbionts (*Midichloria, Spiroplasma, Coxiella*-LE, *Rickettsia, Wolbachia* and *Rickettsiella*) coexist in *I. ricinus* tick populations [2].

The aim of the study is to determine the microbiota of *I. ricinus* ticks in 9 adult females caught in 2023 in Polish military areas, which made it possible to learn about the epidemiological threat posed by ticks to soldiers staying in these areas.

MATERIALS AND METHOD

Trapping ticks. The tick collection sites were selected due to the intensity of training military troops in the Orzysz, Ustka and Drawsko Pomorskie area on a given training ground, and the favourable environment in each location (lightly wooded places with high relative humidity).

Ticks were caught in the spring and autumn months using the flagging method. For this purpose, white cotton pieces of canvas were used and slowly dragged over the vegetation in a specific area, usually of 100 m^2. The collection was made twice a year in the same places. In order to estimate the probability of a soldier acquiring a tick-borne disease, ticks were also caught in areas where no activities were carried out, and there was little or no human presence, the so-called blank test.

The caught ticks were placed in Eppendorf tubes. In order to maintain appropriate humidity in the samples during their transport to the laboratory, a blade of grass was placed inside. After delivery to the laboratory, the ticks were kept in the refrigerator stored at -20 until segregation, depending on the collection site, developmental stage, and gender, which were determined using a microscope. Each tick (nymph or female) was given an individual number. In 2023, 78 females and 333 nymphs were collected. Each of the ticks received an individual assignment (Q number), which was subsequently applied. Ticks Q678, Q679, Q704 were collected in Drawsko Pomorskie, while Q744, Q745, Q739, Q742 and Q738 were collected in Orzysz. A single sample, Q1233, was collected in Ustka. Such marking makes further analysis easier and allows for correlating microbiota sites with their collection locations.

Tick processing. Ticks were homogenized using a sterile needle tip under a laminar flow hood to prevent contamination. The genetic material was isolated using the AllPrep QIAcube kit (QIAGEN, Hilden, Germany) according to the protocol, and manually using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). After isolation, the eluted DNA was tested and then frozen at -20 °C for later analysis. After isolation, the quantity of eluted DNA was assessed using a Quantus™ Fluorometer with the QuantiFluor® dsDNA System, and the purity was verified using a Nanodrop Lite Spectrophotometer at 260/280 nm. DNA samples that met the quality criteria were selected for sequencing. In total, 9 DNA aliquots from adult female *I. ricinus* were used for further metagenomic analysis.

Library preparation. The entire genome was analyzed using short-read sequencing on the Illumina platform. DNA extracted from ticks was measured using a QuantusTM Fluorometer with the QuantiFluor® dsDNA System. The concentration of the samples was verified by measuring absorbance at 260/280 nm. Samples with a coefficient below 1.8 or above 2.2 were not used. DNA libraries were prepared using the Illumina DNA Preparation Kit as per the Illumina DNA Prep Reference Guide, which involved tagmentation, post-tagmentation clean-up, amplification of tagmented DNA, and library clean-up.

The quantity of DNA libraries was measured using the QuantusTM Fluorometer with the QuantiFluor® dsDNA System. The length of DNA libraries was estimated using the Agilent 4200 TapeStation with the High Sensitivity D1000 ScreenTape Assay. The prepared DNA library was loaded onto the Illumina flow cell, and sequencing was performed on the Illumina MiSeq using the MiSeq Sequencing Kit v3 600 cycles. The library was spiked with 5% PhiX Control v3 for quality control.

Metagenomic analysis. Data quality was carefully assessed using FastQC. Adapters were removed, low-quality reads were filtered out, and duplicate reads were removed to ensure the highest data quality for subsequent analysis. After preprocessing, a second round of quality analysis was conducted using FastQC. The genetic data were then analyzed to identify tick-borne pathogens using Kraken2, a tool for classifying DNA sequences [13]. The database used was named Standard-16 with a database size of 16 GB. The data was further analyzed using Bracken's classification methodology [14]. Taxonomy databases were utilized to map the sequence reads, and the results were presented in an Excel file. This report provided information on the classification of sequences at different taxonomic levels (such as species, genera, and families) and included the percentage of individual taxa in the sample.

RESULTS

The results of using short-read sequencing using the Illumina platform were analyzed. An appropriate cut-off point of 0.001 (0.1%) was chosen to indicate the significance of bacterial species sequences. This abundance threshold is commonly used to detect rare and low-abundance microorganisms that may still play a role in pathogen transmission and microbial ecology [3].

Species analysis of the microbiome of 9 adult female *I. ricinus* ticks confirmed the microbiome composition reported in scientific publications, including endosymbionts and pathogens presented in Fig. 1, adapted from Bonnet et al. (2017), is an illustrative example of the bacterial diversity that may occur in tick microbiotas [15].

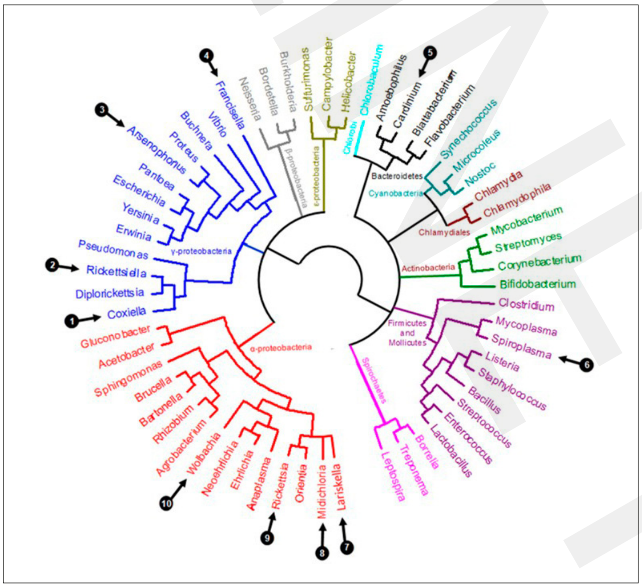

Figure 1. Composition of the bacterial microbiome of *I. ricinus* ticks. The numbers indicate the endosymbiont genera of the tick [3]

The taxonomic composition obtained from shotgun metagenomic sequencing (samples Q1233, Q406, Q411, Q414, Q415, Q678, Q679, Q704, Q738) in the current study, showed strong similarities to previously published tick-associated microbiota profiles [3] (Fig. 1).

A total of 62 microorganisms, with varying prevalence, were detected in 9 samples. Metagenomic analysis of *I. ricinus* females revealed bacterial taxa that could be grouped into 4 main categories.

Endosymbionts. Across all samples, endosymbiotic bacteria were dominant, particularly Candidatus *M. mitochondrii* (15.16%, 9/9) and the *Rickettsia endosymbiont of Ixodes scapularis* (38.42%, 9/9). In addition, *Wolbachia endosymbiont of Corcyra cephalonica* (0.25%, 3/9) was also detected.

Potential pathogens. Sequences were assigned to taxa, including bacteria of medical importance, such as *Borreliella afzelii*, *B. bavariensis*, *B. spielmanii*, *B. valaisiana* (all present in single samples), *Rickettsia helvetica* (13.74%, 2/9), *R. amblyommatis*, *R. asiatica*, *R. rickettsii*, *Ehrlichia muris* (4.58%, 1/9), *Ehrlichia sp.* (0.08%, 1/9), *Bartonella krasnovii* (0.47%, 1/9), *Brucella sp. 1315* (3.94%, 6/9), *Clostridium botulinum* (0.34%, 5/9), *Mycobacterium avium* (0.17%, 1/9), *Salmonella enterica* (0.60%, 6/9), *Staphylococcus aureus* (0.33%, 4/9), and *Vibrio anguillarum* (4.37%, 9/9).

Environmental and commensal bacteria. This group included taxa frequently detected in several samples, which may represent the natural environmental diversity associated with tick habitats. Examples included *Acinetobacter* spp., *Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., *Micromonospora* sp., *Nocardia wallacei*, *Mycolicibacterium litorale*, *Methylobacterium nodulans*, *Inhella inkyongensis*, *Luteibacter aegosomaticola* and *Rhizobium leguminosarum*.

Probable contaminants. Some detected taxa, due to their low frequency, atypical ecological associations, or well-known links to laboratory environments, are more likely to represent potential contamination rather than genuine components of the tick microbiome. These include *Escherichia coli* (1.09%, 9/9), *Escherichia fergusonii*, *Klebsiella pneumoniae*, *Proteus terrae*, *Staphylococcus cohnii*, as well as *Shewanella sp.* and *Thermus sp.*, which may have originated from environmental or laboratory contamination. Additionally, 86.36% of the tick samples carried at least one of the detected bacterial microorganisms.

All samples were also tested for biodiversity. A beta assessment of biodiversity was performed using the Bray-Curtis factor. Results are presented on a plot PCoA Figure 4 and a heatmap Figure 2. They show diversity in the bacteriological microbiome between groups of samples based on the Bray-Curtis factor.

The microbiota of female *I. ricinus* ticks collected from military areas were analyzed, in which both similarities and differences were observed. Core microbiota components included endosymbionts, such as Candidatus *M. mitochondrii* and *Rickettsia* spp., which were present in all samples examined. Comparison analysis shows that samples from Drawsko Pomorskie (Q678, Q679, Q704) exhibited the highest level of similarity and had a stable microbiota composition – Bray-Curtis matrix 0.15 ± 0.05. Tick samples from Orzysz (Q744, Q745, Q739, Q742, Q738) displayed a more intermediate profile of differences, with higher abundances of taxa like *Paenibacillus* sp. and *Brucella* sp. – Bray-Curtis matrix 0.32 ± 0.08. However, the tick sample from Ustka (Q1233), Bray-Curtis matrix 0.32 ± 0.08, was the most dissimilar, showing increased levels of environmental

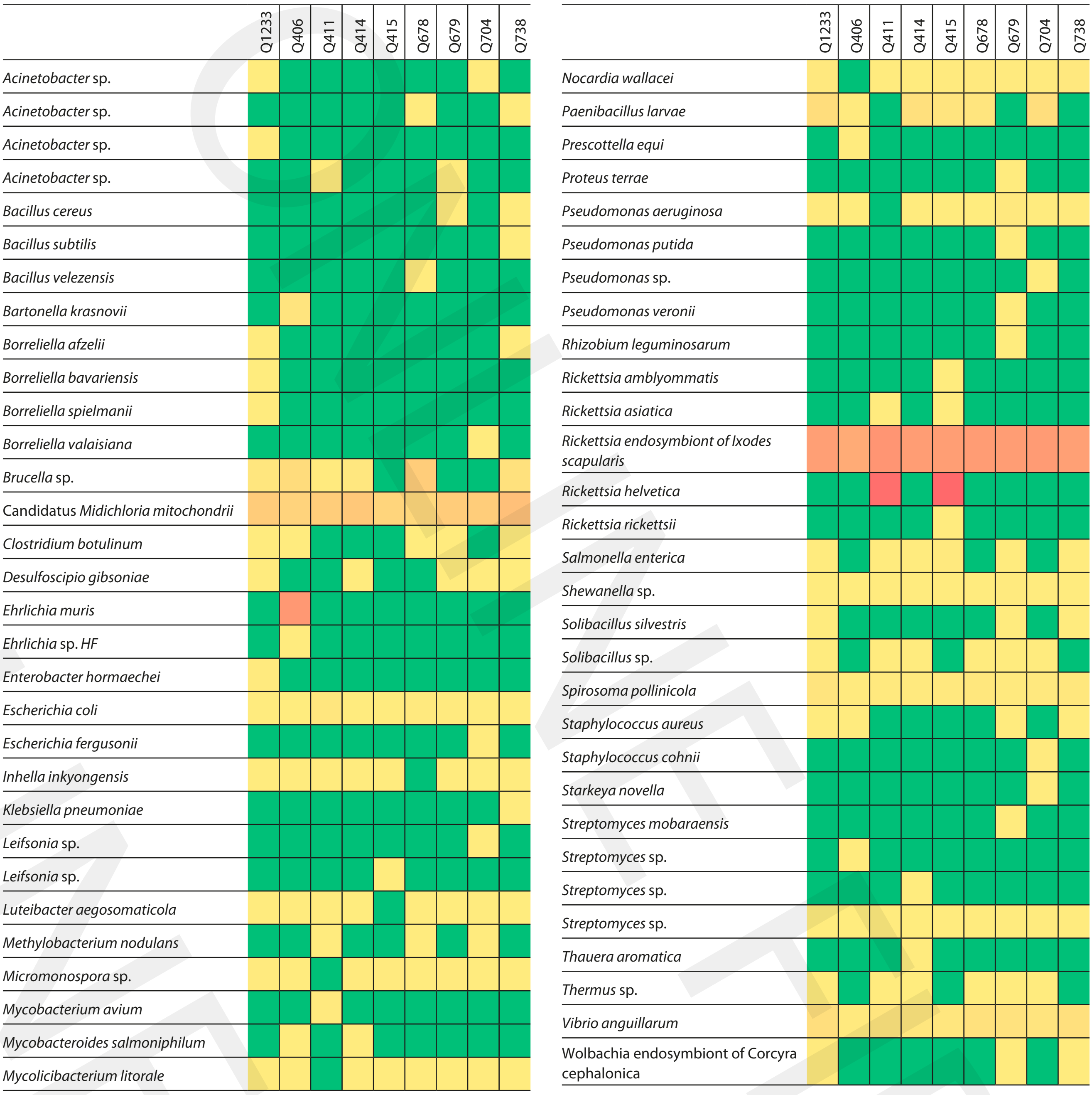

Figure 2. Heat map showing the presence of bacterial species sequences isolated from the whole-genome DNA of *I. ricinus* (summary). The Table uses a 0.1% (0.001) cut-off point. Taxa were assigned based on shotgun metagenomic sequencing. Colours indicate relative abundance levels: green – no reads, yellow – low abundance, orange – medium abundance, red – high abundance. Taxa are shown at the genus or species level

bacteria such as *Pseudomonas* sp. and *Streptomyces* sp., as well as sequences from the taxon *Bartonella* sp. – Bray-Curtis matrix 0.48 ± 0.06. These findings were confirmed by PCoA analysis, which showed that samples from the same location tended to cluster together.

Although the aim of this study was to perform a comparison analysis of the microbiota, it is important to note that among the identified taxa, potential pathogens for human health were also found. This group includes bacterial genera such as *Borreliella*, *Rickettsia*, *Ehrlichia* and *Bartonella*. However, due to limitations of whole genome sequencing analysis and the possibility of misclassification because of the limited number of reads, some of the identifications need to be confirmed and verified using more sensitive and specific methods.

DISCUSSION

This study continues previous research and provides a more detailed metagenomic description of the microbiome of *I. ricinus* ticks collected in Poland [16]. The results confirm that the tick microbiome is a complex system composed of endosymbionts, potential pathogens, and a broad range of environmental bacteria. Genetic material isolated from 9 ticks was analyzed using short-read sequencing on the Illumina platform. A total of 62 microorganisms with different carrier rates were detected in 9 samples. Analysis of the material revealed genetic material from 62 microorganisms, 9 of which were considered particularly important for human health.

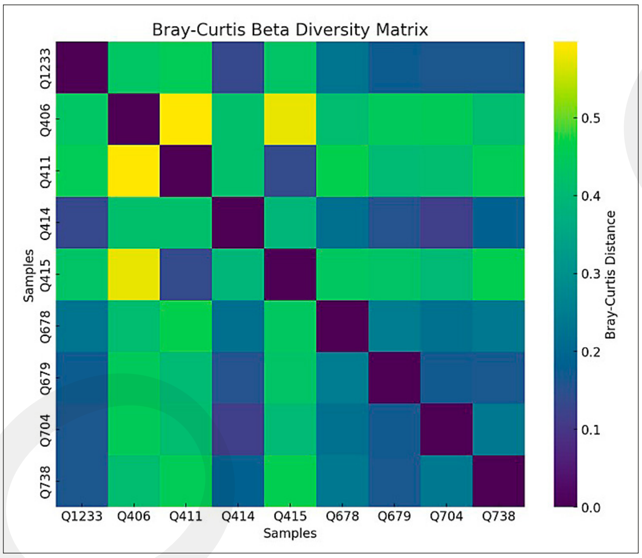

Figure 3. The Bray-Curtis beta diversity matrix for your samples. Each cell shows the distance (Bray-Curtis distance) between a pair of samples: Purple colours (close to 0) indicate samples with very similar species compositions. Light yellow colour (close to 0.5) indicates greater differences in species compositions between samples

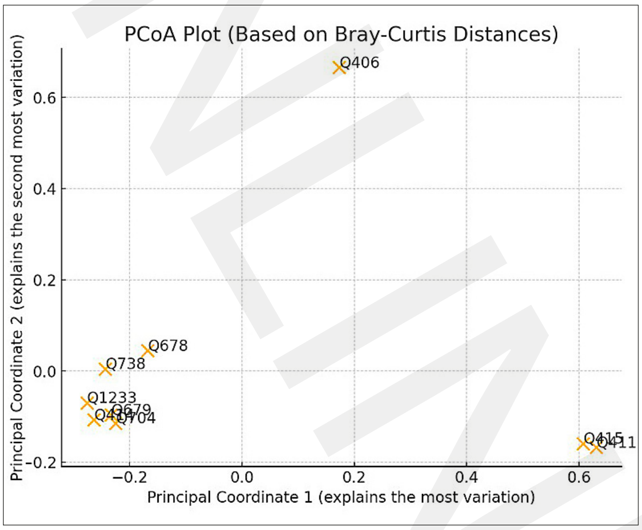

Figure 4. Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis distances between samples. The first principal coordinate explains the most significant amount of variation in the dataset, while the second principal coordinate explains the second-largest. Samples close to each other on the plot have similar species compositions, while those farther apart are more dissimilar.

So far, studies using PCR, nested PCR, or PCR-RFLP methods have detected a maximum of several pathogen species in these vectors. Using short-read sequencing, in the current study the research team detected a significantly higher number of pathogens in the collected ticks [17].

The obtained results are the first step in characterizing the microbiota of adult female *I. ricinus* ticks collected from military training areas in Poland. The study provides new data on the structure of the bacterial microbiota in female ticks and reveals the presence of core bacterial species and differences between samples from different localizations. Comparative analysis showed that samples from Drawsko Pomorskie (Q678, Q679, Q704) exhibited high similarity, forming a cluster dominated by endosymbionts such as Candidatus *M. mitochondrii* and *Rickettsia* sp. Tick females from Orzysz (Q744, Q745, Q739, Q742, Q738) exhibited variation among taxa, and a greater presence of bacteria such as *Paenibacillus* sp., *Brucella* sp., *Pseudomonas* sp., and *Streptomyces* sp.

The most distinct microbiota profile was observed in the Ustka (Q1233) sample, where the dominant environmental bacteria and sequences were assigned to the genus *Bartonella*. These findings are supported by both Bray–Curtis and PCoA analyses.

The core part of the identified microbiota consisted of endosymbionts – Candidatus *M. mitochondrii* and *Rickettsia* sp., which are present in all the analyzed samples. Their consistent presence confirms previous observations and suggests a key role in tick physiology. At the same time, the variable presence of taxa such as *Paenibacillus* sp., *Streptomyces* sp., or *Pseudomonas* sp. requires more careful interpretation. These taxa may represent environmental bacteria or contaminants.

It is important to note that only adult female ticks were analyzed, which was a limitation of the study. The life stages of *I. ricinus* include larvae, nymphs and males, each of which may have distinct microbiomes, therefore, further comparative analysis of all life stages is necessary. This approach will help us understand the structure and dynamics of this species' microbiota.

Potential pathogenic bacteria sequences, such as *Borreliella, Rickettsia, Ehrlichia*, and *Bartonella*, were detected. Given these findings, more sensitive and specific diagnostic methods should be employed for confirmation. These findings, however, should be interpreted with caution, as the small number of analyzed ticks (n = 9 females) and the cut-off threshold used may allow for the detection of rare taxa that could reflect either genuine low-level infection or environmental/laboratory contamination. In particular, bacteria such as *E. coli* and *S. aureus* are more likely contaminants than authentic members of the tick microbiome.

CONCLUSIONS

In conclusion, the presented study highlights the complexity of the *I. ricinus* microbiome in Poland, emphasizing the dominant role of endosymbionts and the occasional detection of pathogenic taxa. At the same time, it underscores the need for cautious interpretation and for further, more extensive research. The study also provides a preliminary characterization of the microbiome of adult *I. ricinus* females collected from 3 military training areas in Poland. Across all samples, core endosymbionts,such as Candidatus *M. mitochondrii* and *Rickettsia* endosymbionts, were consistently identified, while comparative analysis revealed differences in less abundant taxa between sampling sites [3]. These results indicate that both environmental factors and host-related variables may shape the tick microbiome.

Although sequences classified as taxa, including potentially pathogenic bacteria like *Borreliella, Rickettsia, Ehrlichia,* and *Bartonella,* were detected, their interpretation requires caution due to the limited size of the sample and the restriction to adult females. Therefore, the findings obtained should be considered a starting point for future research. Expanding such studies to include larvae, nymphs, and males, as well as larger numbers of specimens, will be necessary to better understand the structure and dynamics of *I. ricinus* microbiota across different environments.

Furthermore, much more advanced research will allow the identification of human-threatening pathogens vectored by ticks. Every year, an increasing number of cases of vector-borne diseases related to tick bites by *I. ricinus* are observed. It is essential to assess the level of exposure for soldiers training in military training locations. From a practical perspective, these insights are relevant for both public health and military medicine, as they can support preventive strategies in areas where tick exposure poses a significant risk.

The study also aligns with the broader One Health perspective, which emphasizes the interconnectedness of human, animal, and environmental health [18]. Metagenomic approaches, including high-throughput sequencing, may become essential tools for understanding vector biology and integrated surveillance of tick-borne pathogens.

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