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A bacteriome survey of *Ixodes ricinus* in Hungary

Ixodes ricinus kullancs bakteriomfelmérése Magyarországon

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Abstract

Background

Ixodes ricinus, the castor bean tick, has long been a prominent subject of discussion in Europe, serving as a critical vector for transmitting significant diseases such as Tick-borne encephalitis (TBE) and Lyme borreliosis (LB). As a result, various studies about *I. ricinus* have been published. *I. ricinus* is a poikilothermic organism dependent on climatic factors. However, In-depth studies about the intricate relationship between *I. ricinus* and climatic factors have been lacking within Central European countries. The main goal of this study was to understand natural bacteriome differences in *I. ricinus* dependent on life stage based on climate variation representative for Hungary.

Method

By stratified spatial random sampling, seventeen local administrative units (LAUs) were selected from 175 LAUs. We divided sampling sites based on yearly growing degree days (GDD) - “cooler” and “warmer” - and total yearly precipitation – “less” or “more”- for each of the years in each LAU. Ten nymphs and ten females of *I. ricinus* were collected by flagging and dragging method per each LAU. Illumina NextSeq platform was used to sequence the fragmented DNA made by the NEBNext Ultra II DNA Library Prep Kit for Illumina.

Result

By analyzing the bacterial composition, we found significant core bacteriome differences between female and nymph samples. No significant difference in core bacteriome was seen between either female samples of GDD or precipitation categories. Similarly, there was no significant difference between groups within nymphs. Moreover, considering the core bacteriome of females and nymphs, the genera of *Arsenophonus, Bacillus, Candidatus* Midichloria*, Rhodococcus, Sphingomonas, Staphylococcus, and Wolbachia* showed significant differences. In female samples, we found *Curtobacterium, Pseudomonas*, and *Sphingomonas* showing differences according to GDD groups. There was no significant difference in abundance in precipitation groups in females. In nymph, *Curtobacterium* showed a significant difference according to GDD groups and *Bacillus* and *Curtobacterium* according to precipitation groups.

Absztrakt

Háttér

Az Ixodes ricinus, a, vagyis a közönséges kullancs, régóta nagy jelentőséggel bír Európában, mivel több fontos betegség mellett például a Tick-borne encephalitis (TBE) és a Lyme-kór (LB) vektora is. Ennek okán számos tanulmány jelent meg az I. ricinusról. Az I. ricinus poikilotherm élőlény, vagyis az időjárási tényezőktől függően változik a testhője. Mindazonáltal, a közép-európai országokban az I. ricinus mikrobiomja és az időjárási tényezők közötti kapcsolat mélyreható tanulmányozására eddig nem volt példa. A kutatás fő célja az volt, hogy felmérjük az I. ricinus bakteriomjának életstádiumtól függő változásait a magyarországi klimatikus viszonyokat is figyelembe véve.

Módszer

Véletlenszerű mintavételezés segítségével 175 helyi közigazgatási egységből (LAU) tizenhétet választottunk ki. Az éves hőmérsékletemelkedési foknapok (GDD) és az éves összes csapadék alapján felosztottuk a mintavételezési helyeket - "hidegebb" és "melegebb" -, valamint "csapadékosabb" vagy "kevésbé csapadékos" - a LAU-kra. Minden LAU-ban tíz- tíz I. ricinus nimfat és nőstényt gyűjtöttünk dragging and flagging (zászlózás) módszerrel. Aszekvenálás Illumina NextSeq platformon, Illumina NEBNext Ultra II DNA könyvtár készítő készlettel segítségével történt.

Eredmények

A baktériális összetétel elemzése során jelentős magbakteriom különbségeket találtunk a nőstény és nimfa minták között. Nem találtunk jelentős különbséget a nőstény minták magbakteriomjában sem a GDD, sem a csapadék kategóriák viszonylatában. Hasonlóképpen, nem volt jelentős különbség a nimfák között sem. A magbakteriom tekintetében a nőstények és nimfák között *Arsenophonus*, *Bacillus, Candidatus* Midichloria, *Rhodococcus, Sphingomonas, Staphylococcus* és *Wolbachia nemzetségek* mutattak szignifikáns különbségeket. A nőstény mintákban a *Curtobacterium, Pseudomonas* és *Sphingomonas, nemzetségekben szignifikáns* különbségeket találtunk a GDD csoportok szerint. A csapadék csoportokban nem volt jelentős különbség a baktériumok relatív abundanciájában a nőstényeknél. A nimfák esetében *Curtobacterium* nemzetség szignifikáns különbséget mutatott a GDD csoportok szerint, míg *Bacillus* és *Curtobacterium* a csapadék csoportok szerint.

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1. Introduction

Ticks, parasitic arachnids, have gained significant attention due to their role in transmitting various hazardous pathogens to humans and animals (Anderson and Magnarelli 2008). WHO states that approximately 80% of people in the world are at risk of tick-borne diseases (TBDs) in their lives (Organization 2017). *Ixodes ricinus,* which belongs to the *Ixodidae* family, transmits the most prevalent TBDs, such as Tick-borne encephalitis (TBE) and Lyme borreliosis in Europe (O'Connell et al. 1998; Gritsun et al. 2003). Thus, exploring the microbiome of ticks has been an interesting topic of significance in importance of public health (Carpi et al. 2011a; Rizzoli et al. 2014). With the help of next-generation sequencing (NGS) and metagenomics, comprehensive insights into the tick microbiome have been provided, introducing complex microorganisms residing within the tick (Metzker 2010). Before the onset of NGS, the traditional culturing method and Sanger sequencing technology, developed in 1977 by Frederick Sanger, were prominent (Sanger et al. 1977). However, those approaches had limitations. Both required time, labor, and cost, which constrained the scope and pace of genetic research (Liu et al. 2012; Grada and Weinbrecht 2013; Behjati and Tarpey 2013). In the 21st century, NGS technology was made to overcome this limitation by employing massively multi-parallel or deep sequencing DNA fragments, enabling the analysis of millions or more of DNA molecules in a single run (Grada and Weinbrecht 2013).

Ixodes ricinus microbiome can be influenced by a variety of factors: the development stage of the tick (Batool et al. 2021a; Namina et al. 2023), sex differences (Van Treuren et al. 2015), the specific anatomical part of the tick sampled, such as salivary gland or midgut (Wiesinger et al. 2023), or by the geographical location, in order words environmental differences (Van Treuren et al. 2015; Batool et al. 2021a; Grandi et al. 2023).

In this study, we focused on tick microbiome changes based on climatic environmental factors. We compared the bacteriome of *I. ricinus* female tick and nymph samples collected based on yearly growing degree days and total yearly precipitation based on a climatically representative survey in Hungary. The presented work is based on our accepted publication paper (DOI: 10.1128/spectrum.01243-23) in Microbiology Spectrum (Tóth et al. 2022).

2. Literature review

2.1. Next-generation Sequencing (NGS)

NGS allows high-throughput analysis of organisms, and microbial communities associated with various organisms and environments by sequencing the nucleic acid content of organisms (Liu et al. 2012). In tick-bacteriome research, NGS has played a significant role in discovering the complex interactions between ticks, their associated microorganisms, and the environmental factors that influence these interactions (Greay et al. 2018). It provided faster, results and higher genomic data yield to the researchers than previous Sanger sequencing technology - the first-generation sequencing platform (Liu et al. 2012).

2.1.1. Type of NGS

There are several NGS platforms nowadays. These platforms are used in various applications, such as Single-gene sequencing, whole-genome sequencing, shotgun sequencing, etc. (Kim et al. 2013). Among them, the most commonly used NGS in microbiomes is 16S rRNA sequencing. From the second-generation sequencing platforms, the most widely used platforms nowadays are – Illumina (2006), and Ion Torrent Sequencing platforms (2011), and for third-generation sequencing - Pacific Biosciences (PacBio.2010), and Oxford Nanopore Technologies platforms (2014) (Pervez et al. 2022). These platforms have distinct sequencing technologies and methodologies, which gives researchers the flexibility to select the platforms that best align with their specific research requirements. The core principle of those platforms has some similarities, and a variety of literature deals with the detailed principle and application of those platforms (Metzker 2010; Liu et al. 2012; Yohe and Thyagarajan 2017; Pervez et al. 2022). In this literature review, the advantages and disadvantages of the most common platform is delivered briefly.

Illumina Sequencing platforms provide different device options for the researchers: NovaSeq, NextSeq, Hiseq, MiSeq, MiniSeq, and iSeq (Illumina Inc). *https://www.illumina.com/systems/sequencing-platforms.html.* Illumina platforms produce large numbers of reads with a relatively low cost and high accuracy (>99%). This platform produces short reads with a read length of 100-300bp (Gilles et al. 2011; Degnan and Ochman 2012). Artifacts (contamination, amplification, and sequencing errors) can be produced in the Illumina platform that could lead to interpretation errors (Degnan and Ochman 2012).

The Ion Torrent sequencing platforms have seven types of applications. The advantages are low cost, fast processing, and small instrument size (Liu et al. 2012). It is also a short read platform that, for homopolymers, has relatively low accuracy (Quail et al. 2012).

The third-generation sequencing platforms introduced Single-Molecule Sequencing (SMS) and addressed some limitations currently observed in second-generation sequencing (2^{nd} GS) platforms. The limitation of 2^{nd} GS was, among others, because of PCR amplification, which produced sequencing errors (Pervez et al. 2022). Among the SMS, PacBio developed a single-molecule real-time (SMRT) sequencing method. It synthesizes parallelized single-molecule DNA sequences (Oulas et al. 2015; Pervez et al. 2022). The advantages of 3^{rd} GS are longer read lengths, high speed, single molecule sequencing, error profiles, and reduced composition bias. However, despite longer read lengths, PacBio still has limitations, high error rates (10-15%), and relatively expensive machines. (Quail et al. 2012; Carneiro et al. 2012; Teeling and Glockner 2012; Oulas et al. 2015) The Nanopore sequencing platform has another advantage in that it is cheap, and the machine is portable. The disadvantage is the same as with PacBio (Timp et al. 2010).

Notably, the Nanopore PromethION platform stands out as the fastest sequencing platform, which is commonly employed in many projects. Ion Torrent and Illumina are preferred for projects where high-quality results are essential. However, it is worth noting that they produce shorter read lengths, which can pose challenges for accurate NGS data analysis (Carneiro et al. 2012; Pervez et al. 2022).

type	Read length (average)	Maximum reads per run	Data production per day	Run time(hours)	Reference
Miseq (Illumina)	150 bp	25 million	7.5 Gb	4-24	*https://www.illumina.com/systems/sequencing-platforms.html*
(GeneXus) Ion torrent	Max. 200 bp	12-15 million	15 Gb	2.5	https://www.thermofisher.com/kr/ko/home/brands/ion-torrent.html
Oxford Nanopore	Max. 4 Mb (10-100 kb)		20 Gb	72	https://nanoporetech.com/ https://www.pacb.com/
PacBio	15-20 kb	4 million	3.1 Gb	30	https://www.pacb.com/

Table 1. Comparison of read length, maximum reads per run, Data production per day, and run time on commonly used NGS platforms.

2.1.2. Limitations

NGS has indeed brought a revolution in genomics and molecular biology. However, like any technological advancement, it has its own set of limitations. Here are some of the limitations found in NGS.

NGS is highly sensitive to sample contamination; even trace amounts of foreign DNA/RNA can distort the results. Careful handling of the sample during preparation or in sequencing is required to prevent reduced specificity by contamination and non-significant organism detection (Liu et al. 2021; Miller and Chiu 2022).

Even though it is faster than traditional Sanger sequencing technology, it is still expensive. It costs more than thousands or even hundreds of thousands of dollars to buy the machine, and

consumables are needed for the sample library preparation as well (Grada and Weinbrecht 2013). Furthermore, personal expertise is required to interpret the results (Behjati and Tarpey 2013).

Despite advancements in sequencing technologies, NGS platforms can still introduce errors during the sequencing process. Errors could lead sequences to misinterpretation, which could result in identifying of novel species that do not exist (Medinger et al. 2010; Liu et al. 2021).

Homologous and repetitive regions, as well as regions with extreme GC content, can pose challenges in the accurate assembly of complex genomes and identification of structural variations. For instance, pseudogenes, clinically relevant genes, pose difficulties in interpretation by current NGS platforms (Mandelker et al. 2014; Yohe and Thyagarajan 2017).

GC composition bias is a phenomenon observed in Illumina sequencing data. It is the relationship between fragment count and GC composition. GC composition bias is particularly prominent in regions with very high or deficient GC composition. This can result in the underrepresentation of specific sequences in the sequencing results, affecting the accuracy and completeness of the data (Benjamini and Speed 2012; Carneiro et al. 2012).

It is important to be aware of these limitations when designing NGS experiments and interpreting the results. Ongoing research and technology advancements are working to address these challenges, but they remain important considerations for researchers utilizing NGS technologies.

2.2. Metagenomics

The advent of Next-generation sequencing (NGS) technologies has revolutionized the field of metagenomics - a discipline devoted to analyzing the total genome content of samples (Thomas et al. 2012). Metagenomics bypasses the need for culturing microbial samples in isolation. This breakthrough permits researchers to access microbial information that was previously unattainable through pure culturing techniques. Metagenomics examines the collective genetic material in environmental samples rather than isolated ones. (Oulas et al. 2015). This approach has exposed the concealed diversity of microorganisms, empowering researchers to investigate the composition, function, and interactions within microbial ecosystems (Kunin et al. 2008; Thomas et al. 2012).

2.2.1. Steps in metagenomics analysis

Metagenomics analysis involves a series of critical steps, beginning with collecting samples. Ensuring the optimal collection of samples is crucial for the success of a metagenomics project. Various samples, including soil, water, feces, or other habitats with the desired microbial populations, can be collected. Size selection techniques, such as fractionating filtration or selective lysis, minimize contamination or reduce the broad range of collected genes (Thomas et al. 2012). However, this approach can introduce a bias in the community composition. For instance, tiny or particularly involved microbes that could play vital roles within the ecosystem in the sample could be filtered and overlooked (Teeling and Glockner 2012). This situation underlines the need for a balanced approach when utilizing the size selection technique during metagenomics sample collection. The next consideration is the timing of sample collection, especially when detecting pathogens. Some pathogens may only be present briefly during a disease, making their detection challenging (Miller and Chiu 2022). The last consideration is that collecting samples with sufficient biomass is essential. Inadequate samples can yield small quantities of DNA that may be insufficient for library construction. However, for low-abundance sample cases, enrichment methods can be used. For example, fluorescence-activated cell sorting or density gradient centrifugation. However, those enrichment methods have limitations. Since they might introduce chimeras and sequence bias (Thomas et al. 2012; Teeling and Glockner 2012). So again, like size selection, the researchers must consider overall pros and cons and decide whether amplification is needed.

Once samples are collected, the following critical step in metagenomics analysis is DNA extraction. This process isolates the genetic material from the microbial community within the sample. Extracted DNA serves as a representation of all microbial cells present in the sample. This DNA must possess enough high-quality nucleic acids to enable successful library construction. Modern metagenomics benefits from advanced extraction methods that enhance the efficiency and accuracy of this critical step (Burke et al. 2009; Devi et al. 2015; 2023).

Following DNA extraction, the extracted genetic material undergoes a series of steps to prepare a library compatible with high-throughput sequence analysis (Miller and Chiu 2022). This library preparation involves several processes: DNA fragmentation, repairing and end-polishing of fragmented DNA, and ligating platform-specific adaptors (van Dijk et al. 2014). Currently,

Illumina sequences are most frequently used in bacterial genome and metagenome sequencing because of their high throughput capacity. As to that, lots of library preparation kits are introduced for Illumina sequencing. However, each kit has its limitations. Nextera XT kit has a strong bias in low GC regions, while the TruSeq nano kit has a strong bias in strong GC content, and the Nextera DNA Flex Library Prep Kit did not introduce strong GC content (Sato et al. 2019).

Following the completion of library preparation, the subsequent stage involves the generation of millions of short DNA sequences through various NGS platforms, as previously mentioned. The choice of the NGS platform depends on factors such as sequencing depth, read length, and specific research goals.

Once the NGS sequencing data is acquired, the crucial phase of bioinformatics analysis commences. This analysis is crucial for deriving meaningful insights from the vast sequence of information obtained. The analysis workflow typically encompasses the following key steps: The short-sequenced DNA reads are processed and assembled to longer contigs (Kunin et al. 2008). Then, quality-based filtering and trimming are performed. Then, data deduplication, alignment to a pathogen database, taxonomic classification, and genome mapping (Oulas et al. 2015; Miller and Chiu 2022).

The collaborative application of these bioinformatics techniques transforms raw NGS data into a comprehensive understanding of the genetic composition, diversity, and potential functions of the microbial community within the sampled environment. These metagenomics techniques have introduced a revolution in microbial ecology, which will be further explored in the subsequent section.

2.2.2. Current usage of metagenomics

The utilization of the 16S rRNA sequence has revealed that 99.8% of microbes existing in numerous environments remain resistant to traditional culturing methods (Streit and Schmitz 2004; Kodzius and Gojobori 2015). With its distinct advantage of not relying on pure cultivation, metagenomics has introduced a new era of microbial investigation by expanding the spectrum of microbial exploration.

The Human gut microbiome has 100 more genes than the human host genome. Due to the difficulty of culturing most gut microbial species, finding pathogenesis and microbial roles in the gut was challenging in the past. With the help of NGS and bioinformatics analysis technique, gut microbial interaction and their roles were investigated (Gao et al. 2021). Like gut microbiome, metagenomics gave a pathway to diagnose a disease that was previously hard to diagnose by conventional methods (e.g., meningitis, sepsis, endocarditis) (Miller and Chiu 2022). So, metagenomics contributed to understanding the human microbiome, identifying disease-associated microbes, and investigating microbial roles in health and disease.

Furthermore, metagenomics gave pathways to find novel genes and interactions within microbial environments that were previously challenging to access. This is especially evident in extreme environments such as the deep ocean and deserts, where conventional sampling and culturing approaches face substantial limitations (Fancello et al. 2013; Kodzius and Gojobori 2015). With its ability to directly analyze genetic material from samples, metagenomics has transcended these limitations and provided novel insights.

Consequently, with the help of these metagenomics characteristics, researchers were able to delve deeply into the microbiome of *I.ricinus* microbiome, uncovering various viruses, bacteria, tick parasites, fungi, and more that had previously gone undetected (Moutailler et al. 2016). Moreover, metagenomics facilitated exploring the interaction between tick microbiomes and environmental factors, such as humidity, and temperature, ultimately leading to a profound understanding of tick ecology.

2.3. *Ixodes ricinus*

Ixodes ricinus, a member of the *Ixodidiae* family, is commonly called the castor bean tick. *I. ricinus* has gained importance in Europe for transmitting hazardous Tick-borne diseases to animals and humans. *I. ricinus* incidence and geographical range increased over the years (Jones et al. 2008). The reasons can be the tick's ecology, climate change, and host association (Alkishe et al. 2017; Cull et al. 2018; Kahl and Gray 2023).

2.3.1. *Ixodes ricinus* ecology

Its life cycle comprises four stages: egg, larva, nymph, and adult. Among these, larva, nymph,

and adult stages are called active stages, which comprise only about 1% of its life cycle (Kahl and Gray 2023). They need blood in the larva and nymph stages for development and in female adults for reproduction (Anderson and Magnarelli 2008). After the tick takes blood for its purpose, it will drop off from the host. Then, it will be either in the developmental stage, in the diapause stage, or in the reproductive stage for laying. (Kahl and Gray 2023). The diapause stage is when the tick prepares for unfavorable conditions, likely in late summer and autumn. By this tick lifestyle, we can assume that when the tick is in its adult stage, it will bypass three hosts during its lifetime (Balashov 1972). So, *Ixodes ricinus,* a three-host tick, could further transmit pathogens of one host to another host (Anderson and Magnarelli 2008).

Tick has a relatively slower feeding process than other arthropods. For larvae and nymphs, the feeding days are an average of 2.5 to 8 days, and for adults, 5 to 12 feeding days. This slow feeding process is because of the cuticle production during its feeding process (Anderson and Magnarelli 2008). In a female tick, when the tick is fully fed, it gains an average of 100 to 120 times body mass (Anderson and Magnarelli 2008), larva 10 to 20 times, and Nymph 15 to 40 times body mass (Kahl and Gray 2023). So, to compromise the body burst, it needs to produce a cuticle, and because of that, the duration of blood intake is slow. The slow feeding process allows the tick to travel to a further distance with the help of the host, increasing the incidence of the ticks even between the continents. (Anderson and Magnarelli 2008)

2.3.2. *Ixodes ricinus* relation with climate change

Ixodes ricinus is sensitive and dependent on environmental and climatic factors. This is because *I. ricinus* is a poikilotherm organism, which cannot regulate body temperature internally. Its temperature fluctuates based on external temperature changes (Huey and Berrigan 2001). The tick's unique lifestyle also manifests the tick's environmental dependence. *I. ricinus* lifestyle is divided into two phases: host phase, and off-host phase. During the host phase, the tick consumes blood but also manages water elimination with the help of salivary glands to maintain nutrition balance, osmotic equilibrium, and ionic balance (Buczek 1999; Anderson and Magnarelli 2008; Kahl and Gray 2023). When off-host phase, the tick does not take blood. So, to maintain water equilibrium, the tick counterbalances water loss by absorbing water vapor from the atmosphere. In this period, the tick usually needs 80% or more relative humidity for survival (Kahl 1989; Anderson and Magnarelli 2008). So, the scarcity of *I. ricinus* in the

mediterranean region (southern part of the EU) is not surprising. (Gray et al. 2021) Ruiz-Fons and colleagues manifested the nymph's positive correlation between abundance and moisture variables (Ruiz-Fons et al. 2012a). Like humidity, a tick necessitates a minimum temperature of 7°C for the development stage (Campbell 1948). This manifests that in cold winter conditions, particularly in parts of the northern EU, the tick population remains low (Campbell 1948). Ruiz-Fons and colleagues also manifested that larva abundance is positively related to increased mean annual temperature and negatively related to increased frost days (Ruiz-Fons et al. 2012b). *Ixodes ricinus* commonly appears in northern, western, eastern, and central Europe, but has less appearance in the southern part of Europe nowadays (Kahl and Gray 2023). Most species are found in the tropics and subtropics and lower latitudes (Anderson and Magnarelli 2008). However, due to climate changes and global warming, the tick population has shifted into further southern and northern parts of the EU (Jaenson et al. 2012; Alkishe et al. 2017).

2.3.3. Ixodes ricinus host spectrum

I. ricinus has a remarkably shallow degree of host specificity, which means it can access a variety of hosts over 300 species and cause zoonotic disease (Gray et al. 2021). Its host specificity is different by its stage. For example, larvae feed blood primarily on rodents, nymphs on reptiles or medium-sized mammals and birds, and adult females on medium-sized and large mammals (Richter et al. 1991; Hofmeester et al. 2016). The animals that have host association with tick abundance can be ungulates such as deer (Hofmeester et al. 2017), and cattle (Boyard et al. 2007). It is because large mammals are the tick's reproductive host, an increased population of large animals near the *I. ricinus* habitat would increase the success rate of female ticks to lay eggs. Other host associations can be found in small mammals, birds, and reptiles. They can be reservoirs of *Borrelia burgdorferi* sensu lato (Piesman and Gern 2004). The increased population rate of those animals would likely increase the incidence rate of Borreliosis near the tick's collection site.

2.3.4. Ixodes ricinus microbiome

Inside the tick microbiome, various microorganisms coexist: non-pathogenic microorganisms like endosymbionts, commensal, mutualistic and hazardous pathogens, and tick parasites (Hodosi et al. 2022). Endosymbionts are bacteria that play an essential role in the tick's development during the off-host phase, which are transmitted vertically and detected in the

larva of the tick. (Krawczyk et al. 2022; Wiesinger et al. 2023) Collected endosymbionts in *I. ricinus* are *Candidatus* Midichloria mitochondrill(CMM), *Rickettsiella*, and *Spiroplasma* (Wiesinger et al. 2023). CMM is a distinctive endosymbiont exclusively found in *I. ricinus* (Papa et al. 2017). In addition to Endosymbionts, vast arrays of pathogens are also found within ticks: *Borrelia burgdorferi* sensu lato, TBEV-Eur, *Anaplasma phagocytophylum*, *Bartonella* ssp, *Rickettsia* ssp, *Babesia* ssp, *Francisella tularensis* etc. (Rizzoli et al. 2014; Hodosi et al. 2022). Lyme disease and Babesiosis are transmitted to the next development stage of the tick. This is called trans-stadial transmission (Anderson and Magnarelli 2008).

These pathogens can be attained from the environment or on the host's skin during blood–feeding (Narasimhan et al. 2017). Consequently, it is reasonable to assume that the pathogens detected in the individual ticks may vary based on environmental factors.

3. Materials and Methods

3.1. Sampling design and sample collection

The study's main goal was to understand the Ixodes ricinus bacteriome based on life stage and climatic factors in Hungary. We decided on the sampling area by using Hungary's local administrative units (LAU 1). In total 175 local administrative units, we measured each unit's average yearly growing degree days (GDD) – "cooler" and "warmer"- and total yearly precipitation – "less" or "more"- every year. The lower two quartiles of GDD were classified as "cooler", and the upper two quartiles of GDD were classified as "warmer". For precipitation, the country-wide median was the criteria – "less" meant lower precipitation than the median, and "more" meant higher precipitation than the median. Meteorological data from 2008 to 2017 was obtained from the ERA-interim reanalysis data repository (Dee et al. 2011), with a spatial resolution of 0.125. From the 175 local administrative units, we chose twenty local administrative units for the sampling area by stratified spatial random sampling (Stevens and Olsen 2004). The sample size for each stratum was proportionate to the country-wide frequency of GDD and precipitation to ensure representativeness. R environment was used in all data management and analysis (R Core Team 2022). We collected the ticks by flagging and dragging method in the forest edge between March 23, 2019, and May 20, 2019, in each of the twenty local administrative units. Ten nymphs and ten adult females of *I. ricinus* was the minimal criterion for this study, but we could not collect the minimum criteria number of ticks from three sampling sites. Hence, seventeen local administrative units were used in the analysis (Figure 1). After collecting, we froze the samples and classified them taxonomically in the laboratory (BSL2). We washed the ticks twice with 99.8% alcohol.

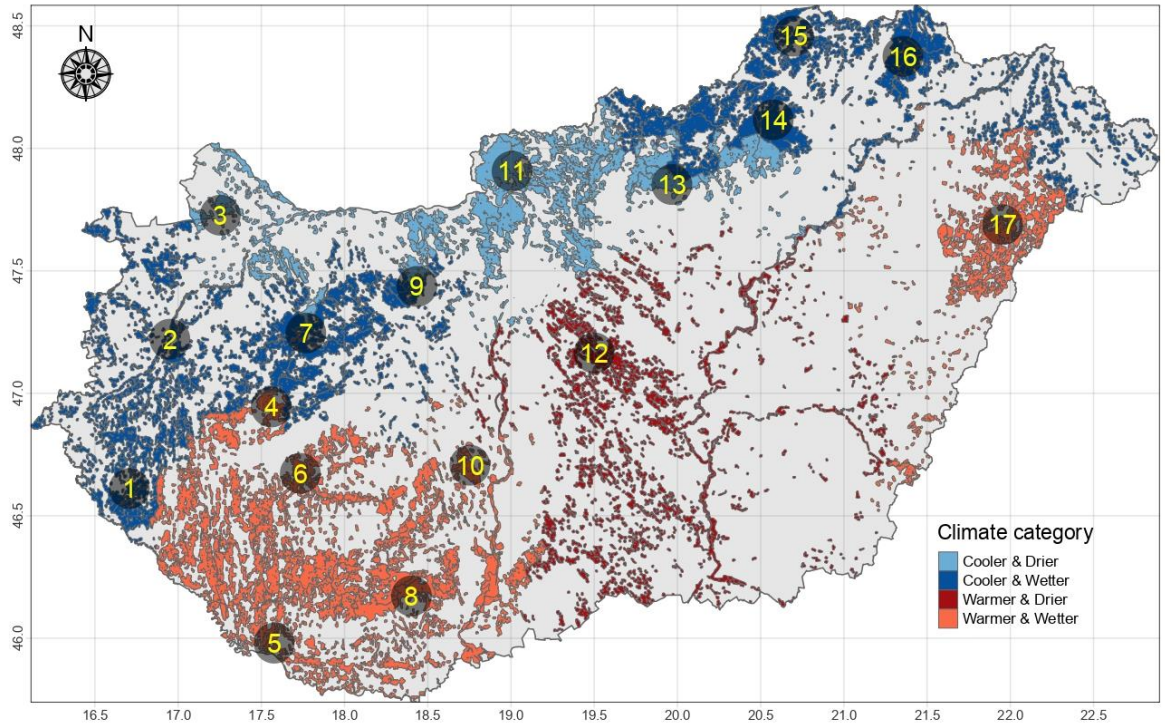

Figure 1. The yearly growing degree days (GDD) and precipitation rate over 2008-2017 in seventeen local representative units in Hungary. Figure 1 is based on our accepted publication paper (DOI: 10.1128/spectrum.01243-23) in Microbiology Spectrum (Tóth et al. 2022).

3.2. DNA extraction and metagenomics library preparation

DNA extraction of the collected ticks was performed using a Qiagen DNeasy® Blood & Tissue Kit (Zhang et al. 2014) (Qiagen, Hilden, Germany). For DNA isolation, the blackPREP Tick DNA/RNA Kit (Analytik Jena GmbH) was applied. The extracted total DNA was then utilized for library preparation. Using the NEBNext Ultra II DNA Library Prep Kit for Illumina, total DNA was fragmented. TG NextSeq 500/550 High Output Kit v2 (300 cycles) generated paired-end fragments on the Illumina NextSeq sequencer. Base-calling was performed with Bbcl2fastq software (v2.17.1.14, Illumina).

3.3. Bioinformatic analysis

We used PEAR(Zhang et al. 2014) to integrate the paired-end reads. We conducted quality-based filtering and trimming using TrimGalore (v.0.6.6, https://github.com/FelixKrueger/TrimGalore), with a quality threshold fixed at 20 and kept the

reads that were longer than 50 bp. We used VSEARCH (Rognes et al. 2016) to deduplicate the reads. We applied Kraken2 (k=35)(Wood et al. 2019) for taxonomic classification, utilizing a database from the NCBI RefSeq complete archaeal, bacterial, and viral genomes as of March 26, 2022. To enhance the accuracy of taxon assignment, the parameter `–confidence 0.5` was used. In the genus which has over 1% relative abundance in the collected samples, at least one of the samples was determined as core bacteria. We utilized R (R Core Team 2022) to handle taxon classification data, making use of functions from the phyloseq package (McMurdie and Holmes 2013), microbiome (Lahti 2012), and metacoder (Foster et al. 2017). MEGAHIT (v1.2.9)(Li et al. 2015) subjected pre-processed reads to contigs with default settings. We also applied Kraken2 for the taxonomic classification of the contigs. Then, we reassigned the classified Kraken2 using BLAST (Lozupone and Knight 2005) (NCBI Resource Coordinators 2016) against representative prokaryotic genomes (downloaded on June 16, 2022).
For each contig, we retained and reported the hit with the longest alignment and the hit with the smallest e-value.

4. Results

4.1. Collected core bacteriome species

Arsenophonus: *Arsenophonus* endosymbiont of *Aphis craccivora*, *A.* endosymbiont of *Apis mellifera*, *A. nasoniae.* In our study, we found *A. nasonie* climatic abundance difference in the nymph. In warmer and rainier areas, nymphs carried more *A. nasonie* than ones from warmer areas.

Bacillus: *B. frigoritolerans*, *B. cereus, B. mycoides, Bacillus* sp. 7D3, *B. thuringiensis, B. wiedmannii. Bacillus* sp. exhibited significantly higher levels of abundance in nymphs from wetter areas.

Candidatus Midichloria mitochondrii. In our study, adults had a higher abundance rate than nymphs. However, the overall count of the bacterium was low.

Curtobacterium: *Curtobacterium flaccumfaciens, Curtobacterium* sp. 24E2, *Curtobacterium* sp. BH-2-1-1, *Curtobacterium* sp. C1, *Curtobacterium* sp. TC1. In our study, *Curtobacterium* showed a higher abundance rate in warmer areas both in adult females and nymphs, and nymphs were shown to prefer little precipitation areas. It showed a slightly higher abundance in female

adults than in nymphs.

Cutibacterium: *C. acnes, C. avidum, C. granulosum, C. modestum.* In our study, female adult ticks had a slightly higher abundance than nymphs.

Mycobacteroides [*Mycobacterium*]: *M. stephanolepidis, M. abscessus, M. chelonae, M. immunogenum, M. salmoniphilum, M. saopaulense.* In our study, female adult ticks had a slightly higher abundance than nymphs.

Pseudomonas: *P. aeruginosa, P. alcaligenes, P. antarctica, P. azotoformans, P. brenneri, P. cichorii, P. congelans, P. eucalypticola, P. extremorientalis, P. fluorescens, P. frederiksbergensis, P. graminis, P. lurida, P. moraviensis, P. orientalis, P. poae, P. prosekii, P. putida, P. qingdaonensis, P. rhizosphaerae, P. soli, Pseudomonas* sp. 15A4, *Pseudomonas* sp. CIP-10, *Pseudomonas* sp. DG56-2, *Pseudomonas* sp. HN2, *Pseudomonas* sp. HN8-3, *Pseudomonas* sp. LBUM920, *Pseudomonas* sp. NS1(2017), *Pseudomonas* sp. OE 28.3, *Pseudomonas* sp. S49, *P. stutzeri, P. synxantha, P. syringae, P. tensinigenes, P. trivialis, P. umsongensis. Pseudomonas* in adults showed higher abundance rates in higher GDD areas, while no significant differences were shown in nymphs. It showed a slightly higher abundance in female adults than in nymphs.

Rhodococcus: *R. erythropolis, R. fascians, R. globerulus, R. koreensis, R. opacus, R. qingshengii, Rhodococcus* sp. AQ5-07, *Rhodococcus* sp. B7740, *Rhodococcus* sp. H-CA8f, *Rhodococcus* sp. MTM3W5.2, *Rhodococcus* sp. P1Y, *Rhodococcus* sp. PBTS 1, *Rhodococcus* sp. YL-1. *R. amblyommatis, R. asiatica, R. bellii, R. conorii, R.* endosymbiont of *Ixodes scapularis*, *R. helvetica, R. monacensis, R. parkeri, R. rhipicephali.* In our study, *Rhodococcus* had a higher abundance rate in adults than in nymphs.

Sphingomonas: *S. aliaeris, S. alpina, S. insulae, S. koreensis, S. melonis, S. paucimobilis, S. sanguinis, S. sanxanigenens, Sphingomonas* sp. AAP5, *Sphingomonas* sp. FARSPH, *Sphingomonas* sp. HMP9, *Sphingomonas* sp. LK11, *Sphingomonas* sp. LM7, *Sphingomonas* sp. NIC1, *Sphingomonas* sp. PAMC26645, *S. taxi.* Nymphs showed a higher abundance rate than female adults in our study.

Staphylococcus: *S. arlettae, S. aureus, S. auricularis, S. capitis, S. epidermidis, S. haemolyticus, S. hominis, S. pasteuri, S. saccharolyticus, S. warneri.* In our study, a higher abundance rate was seen in adult females than in nymphs.

Stenotrophomonas: *S. acidaminiphila, S. indicatrix, S. maltophilia, S. rhizophila, Stenotrophomonas* sp. 169*, Stenotrophomonas* sp. DR822, *Stenotrophomonas* sp. LM091, *Stenotrophomonas* sp. NA06056, *Stenotrophomonas* sp. SI-NJAU-1, *Stenotrophomonas* sp. SXG-1. In our study, female adult ticks had a slightly higher abundance than nymphs.

Wolbachia: *Wolbachia* endosymbiont of *Anopheles demeilloni, W.* endosymbiont of *Ceratosolen solmsi*, *W.* endosymbiont of *Chrysomya megacephala*, *W*. endosymbiont of *Corcyra cephalonica*, *W*. endosymbiont of *Delia radicum*, *W.* endosymbiont of *Drosophila simulans*, *W*. endosymbiont of *Wiebesia pumilae*, *W. pipientis*. *Wolbachia* ssp. had a higher abundance rate in nymphs than adults and had a slightly higher abundance rate in warmer and rainier areas.

4.2. Pathogens found in the samples.

As the result of the taxon classification with Kraken 2, the following pathogenic bacteria were classified:

Anaplasma phagocytophilum was found in nymphs (sample sites: 3, 7)

Reads from *Borrelia coriaceae* were identified in females (sample site: 1). *B. miyamotoi* was found in females (sample sites: 2, 16) and nymphs (sample sites: 1, 10). *Borreliella garinii* was found in nymphs (sample site: 11). *B. valaisiana* was found in nymphs (sample sites: 8, 11). *B. afzelii* was found in nymphs (sample site: 2).

Ehrlichia muris-related reads were found in female 12 and nymph 14 samples.

Reads originating from the genus *Rickettsia* were found in all samples: *R. amblyommatis* in females (sample site: 5) and nymphs (sample site: 6). *R. asiatica* in females (1, 7, 12) and nymphs (1, 2, 10, 15). *R. bellii* in females (5) and nymphs (6, 10). *R. conorii* in females (8). *R. helvetica* in females (1, 3-5, 7-10, 13-17) and nymphs (1-6, 8, 10, 11, 13-17). *R. monacensis* in females (3, 5, 8) and nymphs (1, 6, 10, 12). *R. parkeri* in nymphs (10). *R. rhipicephali* in females (5, 8) and nymphs (1, 6, 10).

We did not detect species from the genera *Bartonella, Coxiella, and Francisella*.

The outcome of the taxon classification of assembly-generated contigs based on the BLAST

(NCBI Resource Coordinators 2016) is as follows:

Candidatus Odyssella thessalonicensis L13 was found in females (sample site: 5). *Candidatus* Rickettsia colombianensi in females (5, 8, 15) and nymphs (4, 6, 10, 13).

Orientia tsutsugamushi in females (5) and nymphs (10).

Rickettsia: *R. akari* in nymphs (6, 10). *R. asembonensis* in females (5, 8, 17) and nymphs (6, 8, 10, 13). *R. asiatica* in females (3, 4, 5, 7, 8, 14, 15, 16, 17) and nymphs (1, 2, 3, 4, 6, 8, 10, 11, 13, 14, 15, 16, 17). *R. australis* in females (5, 8, 15) and nymphs (3, 4, 6, 10, 11, 14). *R. bellii* in females (8) and nymphs (1, 6, 10). *R. canadensis* in females (3, 5, 7, 8, 14, 17) and nymphs (1, 3, 6, 10, 11, 15). *R. conorii* in females (5) and nymphs (6, 10, 15). *R. felis* in females (1, 5, 7, 8, 13, 15, 17) and nymphs (1, 3, 4, 6, 10, 11, 14, 15). *R. fournieri* in females (4, 5, 8, 15) and nymphs (1, 4, 5, 6, 10, 11, 15). *R. gravesii* in females (5, 8) and nymphs (3, 6, 10). *R. helvetica* in females (1, 3, 4, 5, 7, 8, 13, 14, 15, 17) and nymphs (1, 2, 3, 6, 8, 10, 11, 13, 14, 15, 16). *R. honei* in females (5, 7, 8, 14, 17) and nymphs (3, 6, 10). *R. hoogstraalii* in females (4, 5, 8, 15, 17) and nymphs (1, 3, 6, 10, 15). *R. japonica* in females (5) and nymphs (6, 10). *R. monacensis* in females (5, 8, 15) and nymphs (1, 4, 6, 10). *R. prowazekii* in females (5, 8, 14, 15) and nymphs (1, 6, 10, 11). *R. rhipicephali* in females (1, 3, 5, 8) and nymphs (1, 3, 6, 10, 11). *R. rickettsii* in females (5) and nymphs (6, 10). *R. sibirica* in females (5) and nymphs (6). *R. slovaca* in females (1) and nymphs (6, 10, 15). *Rickettsia* sp. *MEAM1* in females (5, 7, 8, 15) and nymphs (6, 10, 11). *R. tamurae* in females (4, 5, 7, 8, 15, 17) and nymphs (1, 4, 6, 10, 13, 14, 15). *R. tillamookensis* in females (4, 5, 17) and nymphs (1, 2, 3, 6, 10, 11, 15). *R. typhi* in females (8) and nymphs (4, 6, 10).

Spiroplasma endosymbiont of Danaus chrysippus in females (13).

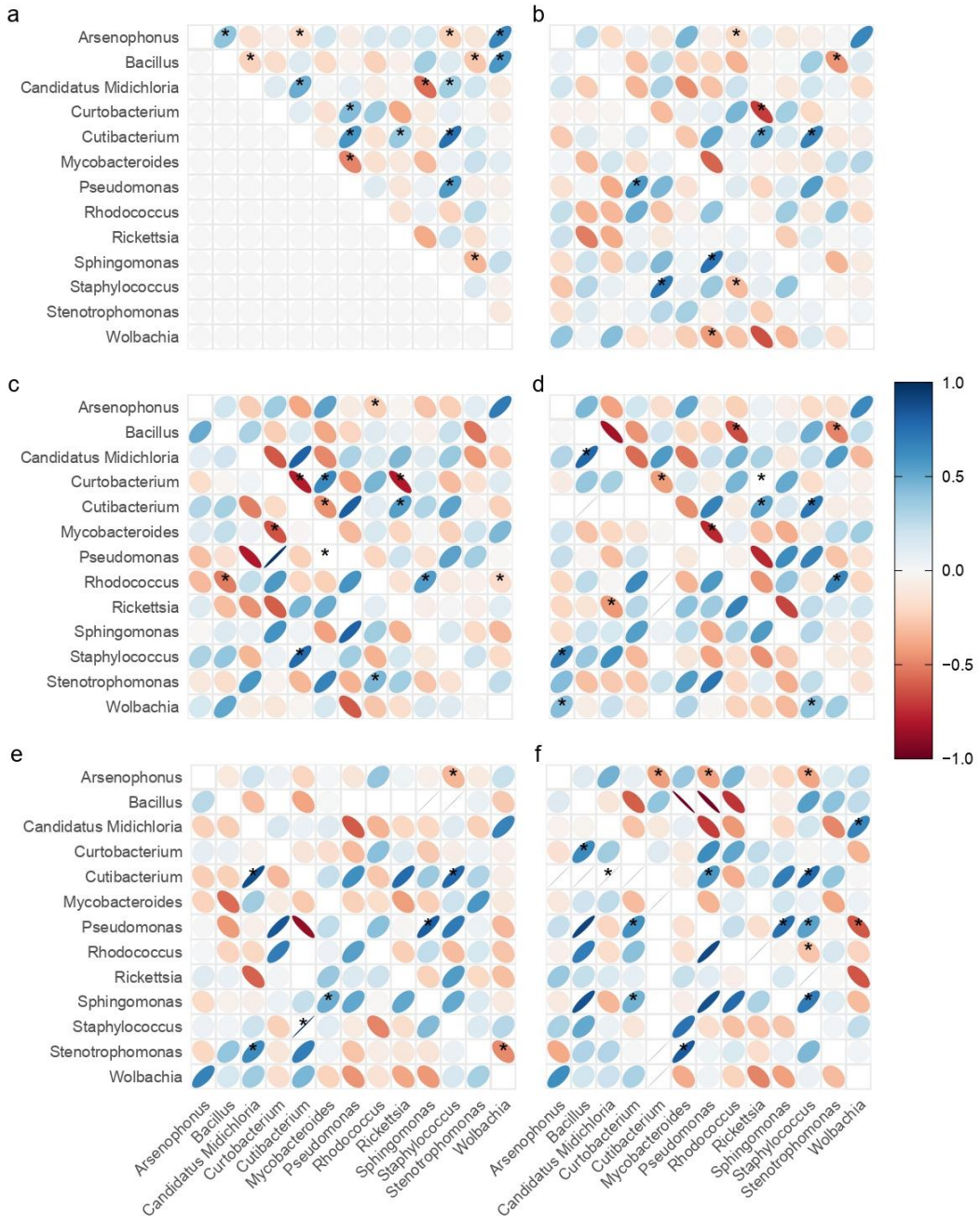

Figure 2. The correlation among all ticks is presented in subfigure a. In subfigure b, the lower half represents nymphs, and the upper half represents females. Subfigure c displays the correlations, where the lower triangle represents females from cooler environments, and the upper one corresponds to females from warmer environments. Subfigure d displays the correlations, where the lower triangle represents females from drier environments, and the upper one corresponds to females from wetter environments. Subfigure e displays the correlations, where the lower triangle represents nymphs from cooler environments, and the

upper one corresponds to nymphs from warmer environments. Subfigure f displays the correlations, where the lower triangle represents nymphs from drier environments, and the upper one corresponds to nymphs from wetter environments. Significant ($p<0.05$) relationships are indicated by *. Figure 2 is based on our accepted publication paper (DOI: 10.1128/spectrum.01243-23) in Microbiology Spectrum (Tóth et al. 2022).

5. Discussion

The purpose of our study was to discover *I. ricinus* bacteriome of nymphs and female adults. Using the shotgun sequencing analysis method, we collected in-depth tick bacteriomes based on their life stage and climatic features. This discussion is also based on the results collected from the manuscript, accepted for publication paper in Microbiology Spectrum, DOI: 10.1128/spectrum.01243-23. (Tóth et al. 2022)

Summing up the result, we found in our study that *Arsenophonus*, *Candidatus Midichloria,* and *Wolbachia* showed noticeable differences across life stages, and *Cutibacterium*, *Mycobacteroides*, *Rickettsia,* and *Stenotrophomonas* showed slight changes.

Arsenophonus genus is widely distributed and endosymbionts of many insects (Nováková et al. 2009). *Arsenophonus* genus is mainly detected in ticks after parasitoid infection, for instance, by *Ixodiphagus hookeri* (Bohacsova et al. 2016). In our study, nymphs showed a higher abundance rate of *Arsenophonus* than females. Subramanian and colleagues also found 20 *I. ricinus* harboring *A.nasoniae* genus out of a total of 80 ticks, and among 20 ticks, nymph counts were 19 (Subramanian et al. 2012). The reason can be seen in *I. hookeri* parasitoid infection on a tick. *I. hookeri* deposits its eggs on the larva and nymphal stage of *I. ricinus*, and only in fully engorged nymphs does the egg growth begin (Hu and Hyland 1998). Then, the hatched wasp larva will begin to eat the tissue of the nymph and the blood collected by the host nymph, eventually resulting in the nymph's death (DAVIS 1986). As a result, in parasitized nymph, development to further adult stage is limited.

Candidatus Midichloria mitochondrii (CMM) is the intracellular bacterium and the first bacterium that was found to inhabit the host mitochondria (Sassera et al. 2006). In our study, the female adult ticks had a higher abundance rate than nymphs. In other studies, the rate of bacterium found in females and nymphs was similar (Sassera et al. 2006; Subramanian et al.

2012). Because CMM is inherited vertically, the prevalence rate in eggs, nymphs, and female adults is 100% (Lo et al. 2006; Sassera et al. 2006). Till the larva stage, the distribution of bacterium is similar in both males and females. However, in the nymph stage, the prevalence is deviated to females (Lo et al. 2006; Sassera et al. 2008). Hence, our study's findings could be interpreted that the majority sex of collected nymph samples was male.

Similar to *Arsenophonus*, the prevalence of *Wolbachia* in *I. ricinus* had a relation with *Ixodiphagus hookeri* (Plantard et al. 2012). *Wolbachia* had a higher abundance rate in nymphs than adults for the same reason as *Arsenophonus.* The same result could be found in other studies (Subramanian et al. 2012). Because *Arsenophonus* and *Wolbachia* are not natural tick microbiota, the higher abundance rate of these bacteria indicates higher *I. hookeri* count in the collected sample areas. (Plantard et al. 2012; Bohacsova et al. 2016). The observed current *I. hookeri* count in Hungary can be seen in this study (Tóth et al. 2023). Because *I. hookeri* can be used as a biological method for tick control, detected *Arsenophonus* and *Wolbachia* in *I. ricinus* gives suitable parameters for biological agent detection.

Mycobacterium is commonly detected in *I. ricinus* concerning the environment, independent of the region and tick sex (Portillo et al. 2019). It was detected in other countries, including the UK (Palomar et al. 2019) and Italy (Carpi et al. 2011b). *Mycobacterium* is known to be obtained from the environmental contaminant (Lejal et al. 2021), for example, in the canine skin during tick feeding (Jang and Hirsh 2002), or by transovarial transmissions (Ferreira et al. 2018). In our study, females showed slightly higher abundance than nymphs. Given that *Mycobacterium* is primarily obtained from the environment, it's reasonably explained that adults carry a larger number of bacteria compared to nymphs throughout their lifespan.

In our study, we detected *Cutibacterium* and *Stenotrophomonas*, commonly detected in ticks dwelling in forests (Estrada-Peña et al. 2018). Like *Mycobacterium*, these bacteria are collected based on tick environment factors, such as water, plants, soils, and the skin of the host (Lejal et al. 2021). Hence, a slightly higher abundance rate in adult females could be explained as likely as *Mycobacterium*.

Bacterial groups that can be collected based on environment (*Bacillus*, *Curtobacterium*, *Pseudomonas*, *Rhodococcus*, *Staphylococcus, Sphingomonas*) showed climate and life stage abundance differences.

In our study, it was notable that reads originating from *Bacillus* sp. exhibited significantly higher abundance levels in nymphs from wetter areas. This phenomenon may be related to a positive shift between *Rhipicephalus sanguineus* male tick and *Bacillus* spp (René-Martellet et al. 2017). Because we could not collect enough male adult ticks, the only males were from the nymph samples. However, the prevalence ratio of *Bacillus* spp showed regional differences. Batool and colleagues found an abundance of *Bacillus* spp in both male and female ticks (Batool et al. 2021b), and in other studies, no *Bacillus* spp were detected in the tick microbiota (van Overbeek et al. 2008; Thapa et al. 2019).

In our study, *Curtobacterium* showed a higher abundance rate in warmer areas both in adult females and nymphs, and nymphs were shown to prefer little precipitation areas. Adult female ticks showed a slight increase in abundance rate than nymphs. However, in Ukraine, *Curtobacterium* showed a higher abundance rate, irrespective of the geographical location and the sex of the ticks (Batool et al. 2021b).

Pseudomonas in adults showed higher abundance rates in higher GDD areas, while no significant differences were shown in nymphs. Adult female ticks showed an abundance rate slightly more than nymphs. The reason can be in the *Pseudomonas* strain's biofilm-forming capacity. *Pseudomonas* strain's capacity of biofilm increases at warmer temperatures (Donnarumma et al. 2010; Kim et al. 2020). Hence, in the summer, adult ticks could easily host a higher abundance of the *Pseudomonas* genus from the biofilm through their lifespan than nymphs. We could find in other studies the difference in the result of *Pseudomonas* abundance rate with our study. Similar to *Curtobacterium, Pseudomonas* showed a high abundance rate regardless of geographical location and the sex of the ticks in Ukraine (Batool et al. 2021b). In other studies, male ticks showed a higher abundance rate than female ticks (Portillo et al. 2019).

In our study, *Rhodococcus* had a higher abundance rate in adults than in nymphs. This result can also be found in another study (René-Martellet et al. 2017). The reason can be because the *Rhodococcus* genus is an environmentally obtained bacteria, especially in plants. Through life, adult ticks will have more chances to come across this genus in the plants than nymphs.

From the gram-positive bacteria collected in the tick, *Staphylococcus* is the most frequent genera (Egyed and Makrai 2014). *Staphylococcus* spp are environmentally related bacteria that can be seen in tick microbiota transiently or long-term (Portillo et al. 2019; Pérez-Valera et al.

2019). In our study, *Staphylococcus* showed a higher abundance rate in adult females than in nymphs. The reason is the same as *Rhodococcus*, adult ticks have more chance to come across this environmental bacterium than nymphs.

Unlike other environmentally related bacteria, the *Sphingomonas* genus, gained from a contaminated environment (Leys et al. 2004), showed a higher abundance rate in nymphs than female adult ticks. Namina and colleagues showed the same result in Latvia (Namina et al. 2023). However, there was different result as well (Zolnik et al. 2016). The reason can be because of individual tick microbial community interactions. There is a study about *I. scapularis* that in female tick gut, *Anaplasma phagocytophilum* could decrease the *Sphingomonas* genus count (Mazuecos et al. 2023). Further studies would be necessary to clarify the relationship between *Sphingomonas* and *I. ricinus.*

With the help of Kraken 2, some clinically essential pathogens were detected: *Anaplasma phagocytophilum, Borrelia, Ehrlichia,* and *Rickettsia. Anaplasma phagocytophilum* causes tick-borne fever (TBF) in domestic ruminants (Stuen et al. 2013). In our study, the *A. phagocytophilum* genus was solely detected in nymphs. However, in other studies, the prevalence rate among nymph, adult male, and adult female ticks was similar (Mysterud et al. 2013) or increased with the development stage (Wielinga et al. 2006). Further, the *A. phagocytophilum* genus was found to have a negative correlation with the *Borrelia burgdorferi* sensu lato complex (Gandy et al. 2022).

Borrelia burgdorferi, which causes Lyme disease in Europe, was not detected in our samples. Instead, other *Borrelia* sp. and *Borreliella* were detected (Marchant et al. 2017). The reason may be related to tick microbiota community interactions where a negative correlation can be seen between *A. phagocytophilum* and *Borrelia burgdorferi* (Gandy et al. 2022). Gandy and colleagues found in areas where *A. phagocytophilum* was detected frequently, *Borrelia burgdorferi* was detected less, and vice versa (Gandy et al. 2022). In the prevalence rate of *Borrelia*, nymphs showed a higher rate than female adult ticks (Wielinga et al. 2006).

Ehrlichia spp. are pathogens frequently found in female adult ticks (Schouls et al. 1999; Wielinga et al. 2006; Subramanian et al. 2012). These pathogens are primarily found in dune areas and forests (Wielinga et al. 2006).

Rickettsia genus is known to be arthropod-vectored pathogens or arthropod endosymbionts such as *Wolbachia* (Weinert et al. 2009). In our study, *Rickettsia* ssp. are the most commonly detected pathogens. Among *Rickettsia* ssp, *R. helvetica* was the most detected pathogen. The detected abundance rate of *Rickettsia* ssp. was different among many studies. Batool and colleagues found that *Rickettsia* was among the least abundant genera. (Batool et al. 2021b) while Carpi and colleagues found it among the most abundant genera (Carpi et al. 2011b). The reason is the varying quantities of *Rickettsia* detected depending on geographical locations. It has been found that ticks harbor more *Rickettsia* genera in forests (Estrada-Peña et al. 2018), or on steeper slopes (Aivelo et al. 2019). Our samples are also collected from the forest; thus, this explains the abundance of *Rickettsia* species in our samples.

We could not detect species from the genera *Bartonella, Coxiella, and Francisella*. Subramanian and colleagues similarly could not detect the genera *Bartonella, Coxiella* in ticks collected in Slovakia, but Řeháček and colleagues found *Coxiella burnetii* in *I. ricinus* collected from Slovakia (Řeháček et al. 1991; Subramanian et al. 2012). While in Lativa, *Francisella* genera were detected primarily in males and a small amount in nymphs.

6. Summary

With the growing interest in tick-borne diseases (TBD), *I. ricinus* has an essential role as a vector. Consequently, exploring the intricate microbiome of the tick and examining the interactions between microbial communities within the tick is a vital step in comprehensively analyzing *I. ricinus*. The traditional cultivation method posed limitations for an in-depth understanding of tick microbiome. However, advancements in Next-generation sequencing and Metagenomics techniques have further enabled the analysis of previously unknown microbiomes inside the tick.

We collected ten samples of each nymph and adult female tick from each of 17 randomly assigned local administrative units (LAU) in Hungary. By using Illumina NextSeq platforms, we could get reads from the tick microbiome. The taxon was classified using Kraken 2 and BLAST.

In the result, apart from *Bacillus, Wolbachia, Sphingomonas,* and *Arsenophonus* genus, other bacteria exhibited a slightly higher or higher abundance rate in female adult ticks. Some results aligned with other studies findings, but there were also discrepancies. The bacteria that showed discrepancy were *Bacillus, Curtobacterium, Pseudomonas, Sphingomonas, Anaplasma,* and *Rickettsia spp.* This indicates that the tick microbiome exhibits differences based on environmental and geographical factors (Carpi et al. 2011b) or due to microbial interactions (Gandy et al. 2022) or related to gender, and developmental stage (Hodosi et al. 2022).

In our study, the findings related to the *Sphingomonas* genus remained inadequately explained. In a similar context, the understanding of tick microbiota concerning interactions among microorganisms and tick physiology is still confined, resulting in numerous speculations. Hence, further research is needed to validate and expand upon the microbial differences and correlations associated with tick development and climate. Additionally, the ongoing advancement in metagenomics approaches is expected to continue driving progress in this domain.

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9. Statements

9.1. Supervisor Counter-Signature Form

I hereby confirm that I am familiar with the content of the thesis entitled A bacteriome survey of *Ixodes ricinus* in Hungary

written by Yun Haeun (student name) which I deem suitable for submission and defence.

Date: Budapest, 08 day November month 2023 year

Dr. Adrienn Gréta Tóth

Dr. Norbert Solymosi

Supervisor name and signature

Centre for Bioinformatics

Department

9.2. Thesis Progress-Report Form

UNIVERSITY OF VETERINARY MEDICINE, BUDAPEST
founded in 1787, EU-accredited since 1995
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Thesis progress report for veterinary students

Name of student: Yun Haeun

Neptun code of the student: CZL6YO

Name and title of the supervisor: Dr. Solymosi Norbert- Head of Department, assoc. prof., Dr. Adrienn Gréta Tóth - PhD student

Department: Centre for Bioinformatics

Thesis title: A bacteriome survey of *Ixodes ricinus* in Hungary

Consultation – 1st semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	3	12	Literature review	
2.	2022	3	31	Disccusion	
3.	2022	4	15	Collecting Reference about bacteria found in *Ixodes Ricinus*	
4.	2022	5	6	Collecting Reference for extra Bacteria identified	
5.	2022	7	11	Learning about Metagnomics	

Grade achieved at the end of the first semester: 5

Consultation – 2nd semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	8	16	Literature review requirments	
2.	2023	9	1	Literature review feedbacks	
3.	2023	10	1	Abstract, introduction feedbacks	
4.	2023	10	12	Literature review feedbacks	

5.	2023	10	25	Literature review feedbacks	
6.	2023	11	2	Final thesis feedbacks	

Grade achieved at the end of the second semester: 5

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

Dr. Tóth

Gödöllő, Varlet

……...

signature of the supervisor

Signature of the student: ……

Signature of the secretary of the department: ……

Date of handing the thesis in 2023.11.17