A climatically representative analysis of Hungarian *Ixodes ricinus* tick bacteriome

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ABSTRACT

Background: The microbial communities of disease vectors may represent a key feature in several biological functions, and thus deserve special attention in light of climate change and the consequent need for the development of novel control strategies. Nevertheless, vector-borne microbial networks are still poorly understood. Assessing the microbial interactions and climatic dependencies of vectors may contribute to better estimating pathogen transmission characteristics, public health risks and the urge for control steps.
Results: After the collection of *Ixodes ricinus* ticks from a climatically representative set of sampling points in Hungary and the next generation sequencing based acquisition of metagenomic shotgun sequencing datasets. Furthermore, reports of the core bacteriome constituting of higher agglomerated genus-level relative abundance read counts than 1% in at least one of the samples, along with and β-diversity and the statistically significant differences and correlations of the genus-level bacterial relative abundances were presented in various developmental stage tick groups. Besides these, the identified pathogenic bacteria were also listed. Genera constituting the core bacteriome (*Arsenophonus*, *Bacillus*, *Candidatus Midichloria*, *Curtobacterium*, *Cutibacterium*, *Mycobacteroides*, *Pseudomonas*, *Rhodococcus*, *Rickettsia*, *Sphingomonas*, *Staphylococcus*, *Stenotrophomonas* and *Wolbachia*) showed several significant relative abundance differences and correlations among developmental stages and at sampling points with different climatic conditions. Reads of pathogenic bacteria from the genera of *Anaplasma*, *Borrelia*, *Borreliella*, *Ehrlichia*, *Rickettsia* were associated with the developmental stage and the sampling geolocation of the host ticks.
Conclusions: The composition and correlations of vector-borne microbiota members showed significant alterations at sampling points with different temperature and precipitation histories and at the various developmental stages of the tick hosts. Our findings not only pave the way towards the understanding of tick-borne bacterial networks and interdependencies, but also raise light to the high potential for the presence of a possible biological tick control species, the tick parasitoid, *Ixodiphagus hookeri* based on related bacteriome patterns. The results of conscious tick microbiome assessment studies may contribute to the precision tick control strategies of the future.

Introduction

While almost all aspects of human life are influenced by the direct or indirect consequences of the Earth's climate system dynamics, several organisms, such as arthropods, are especially sensitive to climatic factors, given that they are ectothermic.[1] Such arthropods, e.g. mosquitoes, sand, blackflies, fleas, lice, tsetse flies, triatome bugs, and ticks are the vectors of several infectious diseases worldwide. Indeed, according to the latest estimations, around 80% of the human population worldwide is at risk of one or more vector-borne diseases (VBDs).[2] VBD burden is remarkably high in the tropical regions of the world, where clinical manifestations caused by vectored viruses, bacteria, or parasites underlie approximately 20% of all tropical infectious disease cases and at least 1 million deaths annually.[2] In the temperate regions of the world, the variety of vectors and VBDs is less diverse, several VBDs are only endemic to tropical and subtropical regions.[3] Due to global warming, these endemic countries may face several alterations in the vector-pathogen transmission cycles that are hardly predictable.[4] Furthermore, along with climate change, globalization, various socioeconomic mechanisms and sociodemographic factors, such as higher population densities associated with high urbanization rates may also indirectly increase the impact of vector-borne diseases on human and animal health.[5,6] At the same time, the current absence of numerous arthropod vectors and the expectations for the earlier occurrence time and elevating severity of climate change toward the poles[7] is likely to place temperate countries, and as

such, the nations of Europe, in a higher risk of emergence and re-emergence of certain VBDs.[4] Presently, ticks, mosquitoes and sandflies are the most significant vectors occurring in Europe, with ticks accounting the widest geographic distribution range.[8] In the perspective of response to changing climate, ticks and dipteran vectors appear to be different.[4] While both above-mentioned vector groups are predicted to change the area of habitat in response to climate warming and altered rainfall patterns, the exact mechanisms of the geographic shifts appear to show variations. Dipteran populations are associated with rapid responses to short-term weather and climate changes, while ticks are affected by spatiotemporal means in climate rather than by climate variability. According to these findings, changes of the risks associated to dipteran-borne diseases (TBDs) may be of both long-term and short-term nature, while TBDs seem to be barely characterized by interannual variations and rather respond to long-term modifications.[4] Considering the long-term representative potential of tick populations, and the fact, that Hungary, a landlocked country with temperate climate situated in Central Europe hosts relatively many, namely at least 27 hard tick species (Ixodidae)[9]), the understanding of TBDs has a high local public health significance. As part of the VectorNet project the European Centre of Disease Prevention and Control (ECDC) currently monitors seven tick species in Europe that may ransfer infectious diseases to humans or animals (ECDC), out of which all hard ticks (*I. ricinus*, *I. persulcatus*, *D. reticulatus*, *H. marginatum*, *R. bursa*, *R. sanguineus*) are present in Hungary.[9] The primary vector of the most prevalent TBDs in Europe, tick-borne encephalitis (TBE) and of Lyme borreliosis (LB), is *I. ricinus*. While the incidence rate reports of these TBDs do not show consistent global trends,[10,11] European *I. ricinus* populations themselves do. As a supposed consequence of climate change, *I. ricinus* has been reported to appear both at extremes of altitude and latitude apart from its prior range and its distribution also shifted northerly within the European continent.[8,12,13] Similarly, the geographic distribution range of LB has also expanded, especially towards greater altitudes and latitudes.[10,11] Taking climatic factors into consideration, a shift towards a more thermophilic tick fauna has already been described in Hungary as well.[9] Yet, in-depth studies accounting climate-dependent, tick microbiome traits have not been carried out within this Central European country. Since the microbiota of eukaryotes is interconected with several biological functions and thus, has a great influence on the host, the knowledge of climate-related tick microbiota alterations can be a public highlight.[14,15] Apart from the fact, that the members of the tick microbiota are highly interconnected, and as such, certain bacterial co-occurrences may influence tick-born pathogens (TBPs) of high public health significance, bacteria can confer multiple detrimental, neutral, or beneficial effects to the tick hosts as well. These effects may underlie various adaptation mechanisms of ticks, including nutritional, developmental, reproductional and immune defense factors.[15–18] Since the tick bacteriota includes several climate-dependent, environmental members, bacterial interaction patterns may also be defined by climatic factors.[19] Nevertheless, ecological traits causing microbial variations in tick species, including *I. ricinus*, that transmits the widest range of pathogens have been subject to relatively few research projects.[19] Our study focuses on defining the microbiome variations of *I. ricinus* ticks from a set of climatically representative geolocations from Hungary, assessing bacterial correlations and deliberations on possible parasitoid detection markers.

Materials and Methods

Sampling design and sample collection

As the study's main goal was to understand the natural bacteriome differences in *I. ricinus*, we have designed the sampling to be representative to Hungary. For this purpose, a feasible way was to identify sampling points representative of climatic conditions.[20] In Hungary, there are 175 local administrative units (LAU 1), for each of these units, we have calculated the 10-year average of the yearly growing degree days (GDD) with base 10°C and the yearly total precipitation. Meteorology data for period 2008-2017 was gathered from the ERA-Interim reanalysis data repository[21] by the spatial resolution of 0.125°. For both environmental variables, two-two categories were defined: cooler-warmer and less-more for GDD and precipitation, respectively. Regarding GDD the lower two quartiles were classified as cooler and the upper two quartiles as warmer. For precipitation, the yearly mean below the country-wide median was assumed as less, above the median as more. Each LAU 1 was categorised by its own climatic variables (Fig 1). By stratified spatial random sampling,[22,23] twenty local administrative units were chosen as sampling areas. The strata's sample size was proportional to the stratifying GDD and precipitation categories' country-wide frequency in order to be representative. All data management and analysis were performed in the R environment.[24] Within each of the 20 selected LAUs, a sampling forest edge was identified. Between 23/3/2019 and 20/5/2019 questing ticks were collected by flagging and dragging. In the laboratory ticks from the frozen samples were classified taxonomically and 10 nymphs and 10 females of *I. ricinus* were selected per sampling sites. Since we could not collect at least 10 females and at least 10 nymphs at three sampling sites, samples collected at the remained 17 sampling sites were included in the sequencing and further analyses. Before DNA extraction the ticks were washed twice by 99.8% alcohol.

DNA extraction and metagenomics library preparation

For the DNA isolation the blackPREP Tick DNA/RNA Kit (Analytik Jena GmbH) was used. Isolated total metagenome DNA was used for library preparation. In vitro fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550

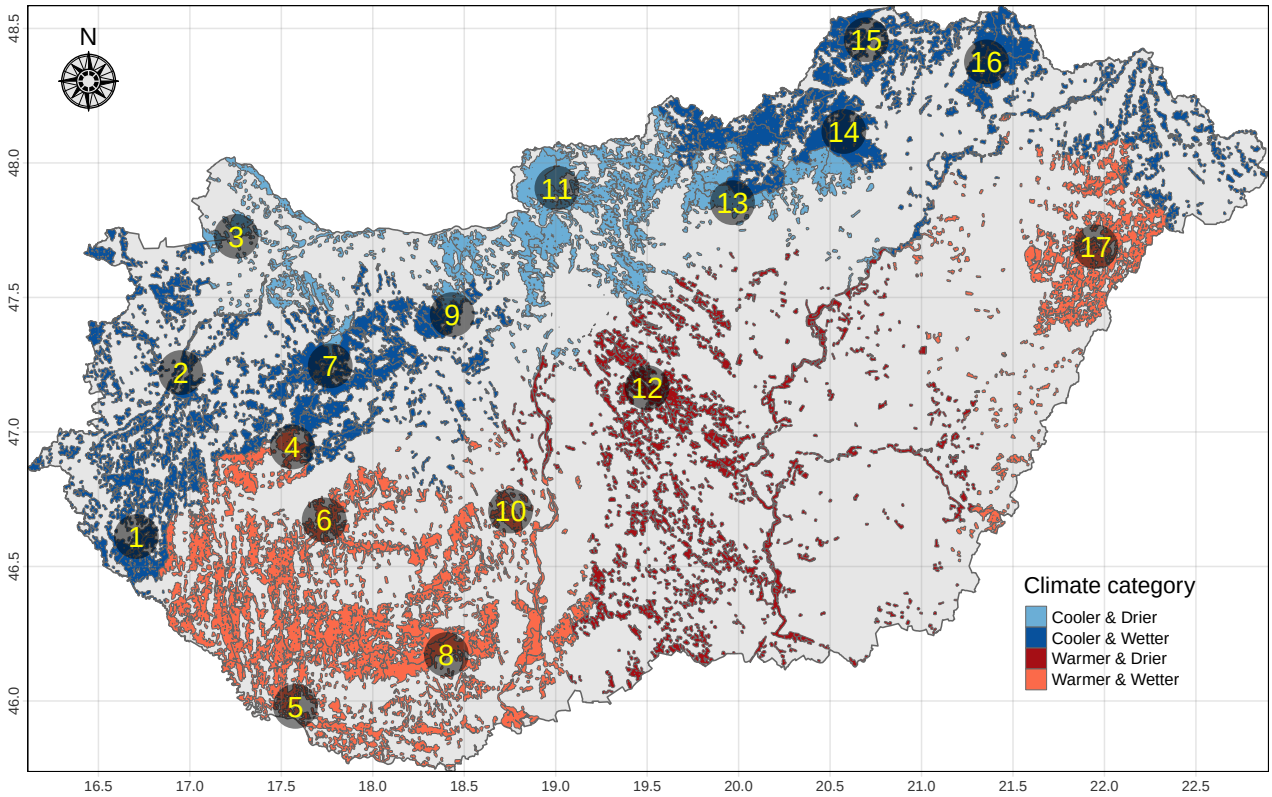

Figure 1. Climate category spatial pattern and sampling points. The forests coloured by climatic categories based on growing degree days (GDD) and precipitation of the period 2008-2017.

High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

Bioinformatic analysis

After merging the paired-end reads by PEAR[25] quality-based quality based filtering and trimming was performed by TrimGalore (v.0.6.6, `https://github.com/FelixKrueger/TrimGalore`), setting 20 as a quality threshold, retaining reads longer than 50 bp only. The remaining reads after deduplication by VSEARCH[26] were taxonomically classified using Kraken2 (k=35)[27] with a database created (26/03/2022) from the NCBI RefSeq complete archaeal, bacterial, viral genomes. For taxon assignment the `-confidence 0.5` parameter was used to obtain more precise hits. Core bacteria was defined as the relative abundance of agglomerated counts on genus-level above 1% in at least one of the samples. The taxon classification data was managed in R[24] using functions of package phyloseq,[28] microbiome[29] and metacoder.[30] The preprocessed reads were assembled to contigs by MEGAHIT (v1.2.9)[31] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. The assembly-generated contigs that were classified to a pathogen bacteria genus by Kraken2 we reclassified by BLAST[32] on the representative prokaryotic genomes (downloaded on 16/6/2022). For each contig, the longest and the smallest e-value hit were kept and reported.

Statistical analysis

The within-subject (α) diversity was assessed using the numbers of observed species (richness) and the Inverse Simpson's Index (evenness). These indices were calculated in 1000 iterations of rarefied OTU tables with a sequencing depth of 158. The average over the iterations was taken for each sample. The α-diversity expressed by Inverse Simpson's Index was compared between the conditions using linear models. Comparing the female and nymph samples collected, a mixed-effect model was applied to manage the repeated measure by sampling site as a random factor.

The between-subject (β) diversity was assessed by UniFrac distance[33] based on the relative abundances of bacteria species. Using this measure, principal coordinate analysis (PCoA) ordination was applied to visualise the samples' dissimilarity. To

examine statistically whether the bacterial species composition differed by strata PERMANOVA (Permutational Multivariate Analysis of Variance[34]) was performed using vegan package[35] in R.[24]

The abundance differences in core bacteriome between groups were analysed by a negative binomial generalised model of DESeq2 package[36] in R.[24] This approach was applied following the recommendation of Weiss et al.[37] None of the compared groups had more than 20 samples, and their average library size ratio was less than 10. According to the multiple comparisons, FDR-adjusted p value less than 0.05 was considered significant. The relationship of the bacteria species relative abundances was quantified by the SparCC correlation coefficient.[38,39] The statistical tests were two-sided.

Results

After the basic demography of the samples we present the α-diversity of the full bacteriome. From the analysis of the core bacteriome we report the species that are part of it, the β-diversity, the differences and the correlations of the genera level expressed relative abundances. Finally the pathogen bacteria findings are listed.

Among the ticks collected at the sampling sites, the median proportion of nymphs was 76.52% (IQR: 19.33), females 15.32% (IQR: 8.58) and males 7.91% (IQR: 11.47).

The numbers of observed species and the Inverse Simpson's Index α-diversity metrics by strata are shown in Fig 2. Alpha diversity showed no significant difference between groups in either comparison. For the comparison of females and nymphs, the p-value was 0.138. Within females, the p-values obtained from comparing groups from colder and warmer environments and from drier and wetter environments were p=0.562 and p=0.577, respectively. While within nymphs p=0.174 and p=0.309 respectively.

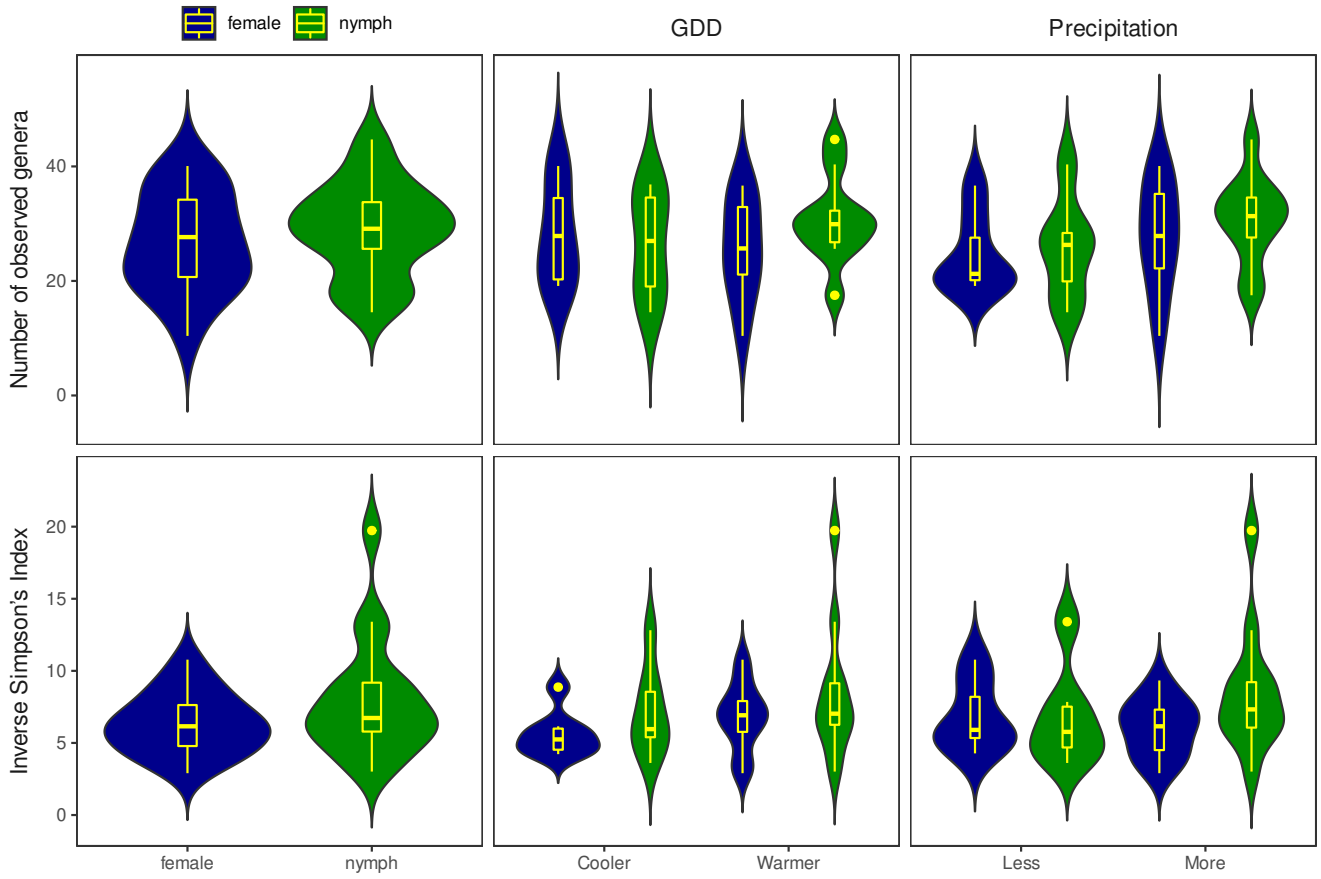

Figure 2. Richness and evenness of *Ixodes ricinus* bacteriome by sample groups. The numbers of observed species (richness) and the Inverse Simpson's Index (evenness) as α-diversity metrics are presented as a violin and box plot combination. These indices were calculated in 1000 iterations of rarefied OTU tables with a sequencing depth of 158. The average over the iterations was taken for each sampling site. The violin plot shows the probability density, while the box plot marks the outliers, median and the IQR.

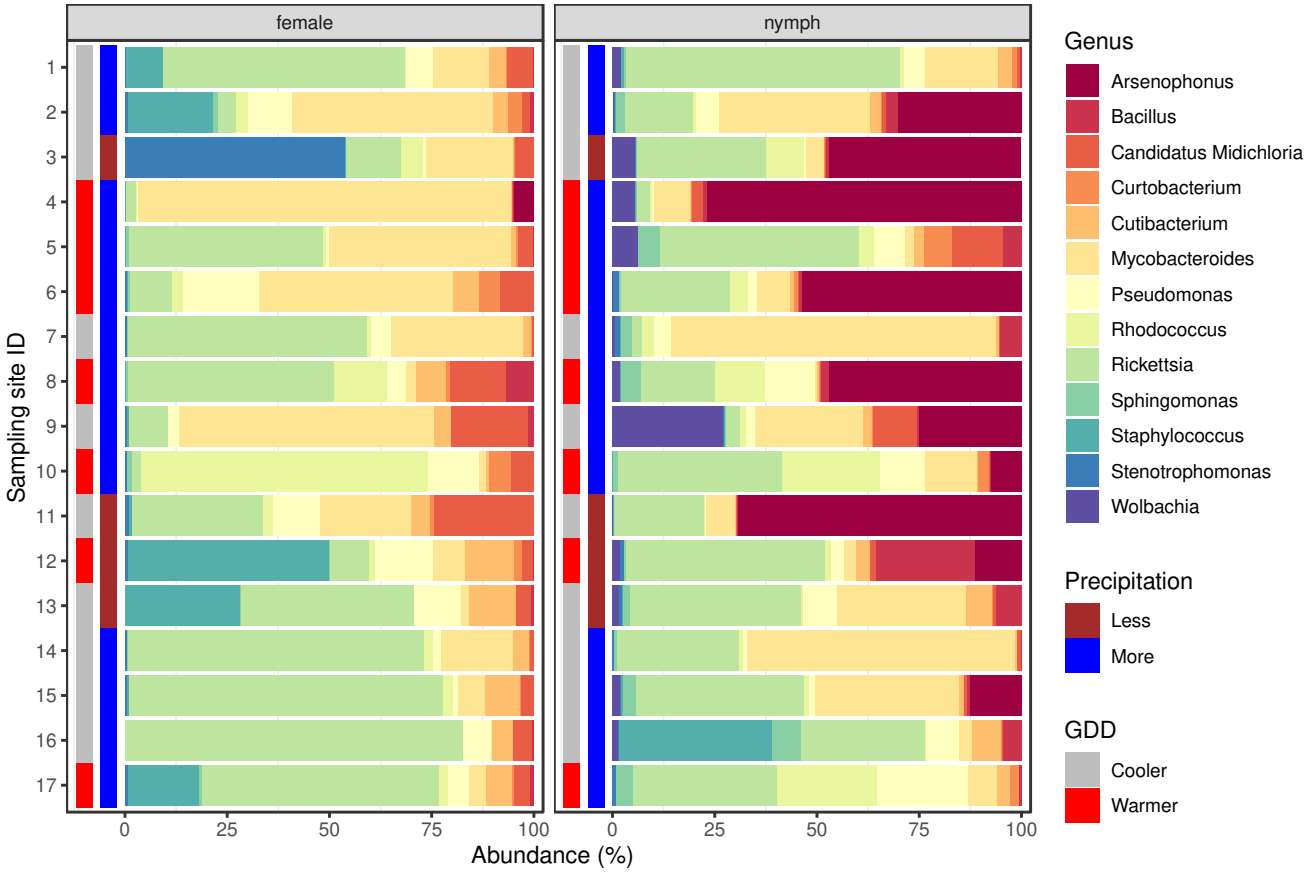

Figure 3. Core bacteriome composition of *Ixodes ricinus* samples The relative abundance is plotted for the females and nymphs. Besides the bacterial genera of the core bacteriome, the environmental condition (growing degree-day (GDD) and precipitation) categories of sampling places are also marked.

Core bacteriome

The core bacteriome is composed of the genera shown in Figure 3. The following species were detected in these genera: *Arsenophonus endosymbiont of Aphis craccivora*, *A. endosymbiont of Apis mellifera*, *A. nasoniae*. *B. frigoritolerans*, *B. cereus*, *B. mycoides*, *Bacillus* sp. 7D3, *B. thuringiensis*, *B. wiedmannii*. *Candidatus Midichloria mitochondrii*. *Curtobacterium flaccumfaciens*, *Curtobacterium* sp. 24E2, *Curtobacterium* sp. BH-2-1-1, *Curtobacterium* sp. C1, *Curtobacterium* sp. TC1. *Cutibacterium acnes*, *C. avidum*, *C. granulosum*, *C. modestum*. *Mycobacteroides [Mycobacterium] stephanolepidis*, *M. abscessus*, *M. chelonae*, *M. immunogenum*, *M. salmoniphilum*, *M. saopaulense*. *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*, *Pseudomonas 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*. *Rhodococcus 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*. *Sphingomonas 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*. *Staphylococcus arlettae*, *S. aureus*, *S. auricularis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. pasteuri*, *S. saccharolyticus*, *S. warneri*. *Stenotrophomonas 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. *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*.

The dissimilarity of the samples' core bacteriome genus profiles (β-diversity) is visualised by PCoA ordination (Fig 4), based on weighted UniFrac distance. By PERMANOVA analysis of bacteria genus composition significant (p=0.005) difference was found between the samples from females and nymphs. The core bacteriome of female samples showed no significant distance between either GDD (p=0.444) or precipitation (p=0.244) categories. Similarly, there was no significant difference between groups within nymphs (GDD p=0.108, precipitation p=0.722).

Abundance differences

The abundance differences (log2 median fold change, Log2FC) of groups per taxon comparison are summarized in Figure 5. Comparing females and nymphs the following genera showed significant (adjusted p<0.05) differences in abundance: *Arsenophonus*, *Bacillus*, *Candidatus Midichloria*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*, *Wolbachia*. In the female samples, according to GDD, the following were found differentiating: *Curtobacterium*, *Pseudomonas*, *Sphingomonas*. There was no genus with a significant difference between precipitation categories in females. In the nymphs, *Curtobacterium* showed a significant variation between GDD groups and *Bacillus* and *Curtobacterium* between precipitation levels.

Abundance correlations

Correlation (Fig 6) analysis for all samples showed a significant (p<0.05) negative correlation between *Arsenophonus* and *Cutibacterium*, *Staphylococcus*; *Bacillus* and *Candidatus Midichloria*, *Stenotrophomonas*; *Candidatus Midichloria* and *Sphingomonas*; *Mycobacteroides* and *Pseudomonas*; *Sphingomonas* and *Stenotrophomonas*. Using all samples there was a significant positive correlation between the following genuses *Arsenophonus* and *Bacillus*, *Wolbachia*; *Bacillus* and *Wolbachia*; *Candidatus Midichloria* and *Cutibacterium*, *Staphylococcus*; *Curtobacterium* and *Pseudomonas*; *Cutibacterium* and *Pseudomonas*, *Rickettsia*, *Staphylococcus*; *Pseudomonas* and *Staphylococcus*.

In the female samples, a significant negative correlation was observed between *Bacillus* and *Stenotrophomonas*; *Curtobacterium* and *Rickettsia*. following genuses *Arsenophonus* and *Rhodococcus*; *Cutibacterium* and *Rickettsia*, *Staphylococcus*.

Significant negative correlations between nymphs were found for the following *Pseudomonas* and *Wolbachia*; *Rhodococcus* and *Staphylococcus*. Significant positive correlations were found in nymphs between *Curtobacterium* and *Pseudomonas*; *Cutibacterium* and *Staphylococcus*; *Pseudomonas* and *Sphingomonas*.

Significant negative correlation in females from warmer environments with *Bacillus* and *Rickettsia*; *Candidatus Midichloria* and *Sphingomonas*; *Curtobacterium* and *Rickettsia*; *Cutibacterium* and *Wolbachia*; *Pseudomonas* and *Rickettsia*, *Stenotrophomonas*. In the same group, a positive significant correlation was found between pairs *Arsenophonus* and *Mycobacteroides*; *Candidatus Midichloria* and *Rhodococcus*; *Cutibacterium* and *Staphylococcus*; *Mycobacteroides* and *Rhodococcus*; *Rhodococcus* and *Sphingomonas*.

In females from cooler environments, significant negative correlation between *Bacillus* and *Rhodococcus*, *Stenotrophomonas*; *Curtobacterium* and *Cutibacterium*; *Pseudomonas* and *Stenotrophomonas*; *Staphylococcus* and *Stenotrophomonas*. In the same group, a positive significant correlation was found between pairs between *Bacillus* and *Wolbachia*; *Cutibacterium* and *Rickettsia*; *Mycobacteroides* and *Stenotrophomonas*; *Rhodococcus* and *Stenotrophomonas*.

In females from a wetter environment, significant negative correlation between *Curtobacterium* and *Cutibacterium*, *Rickettsia*; *Cutibacterium* and *Rickettsia*. No positive significant correlation was found in the same group.

Significant negative correlation in females from drier environments between *Curtobacterium* and *Rickettsia*; *Cutibacterium* and *Mycobacteroides*; *Mycobacteroides* and *Pseudomonas*. No positive significant correlation was found in the same group.

Significant negative correlation in nymphs from warmer environments for *Arsenophonus* and *Staphylococcus*; *Candidatus Midichloria* and *Wolbachia*. In the same group, a positive significant correlation was found for pairs between *Candidatus Midichloria* and *Rhodococcus*; *Cutibacterium* and *Stenotrophomonas*; *Pseudomonas* and *Rhodococcus*, *Sphingomonas*; *Rhodococcus* and *Sphingomonas*.

Significant negative correlation in nymphs from cooler environments between *Arsenophonus* and *Pseudomonas*; *Bacillus* and *Sphingomonas*; *Sphingomonas* and *Wolbachia*. In the same group, a positive significant correlation was found between pairs *Arsenophonus* and *Wolbachia*; *Curtobacterium* and *Pseudomonas*; *Cutibacterium* and Rhodococcium.

In nymphs from a wetter environment, significant negative correlation between *Rhodococcus* and *Wolbachia*. In the same group, a positive significant correlation was found for *Curtobacterium* and *Rhodococcus*; *Cutibacterium* and *Staphylococcus*.

No significant negative correlation was found in nymphs from drier environments. In the same group, we found a positive significant correlation between *Arsenophonus* and *Rickettsia*; *Cutibacterium* and *Pseudomonas*, *Staphylococcus*.

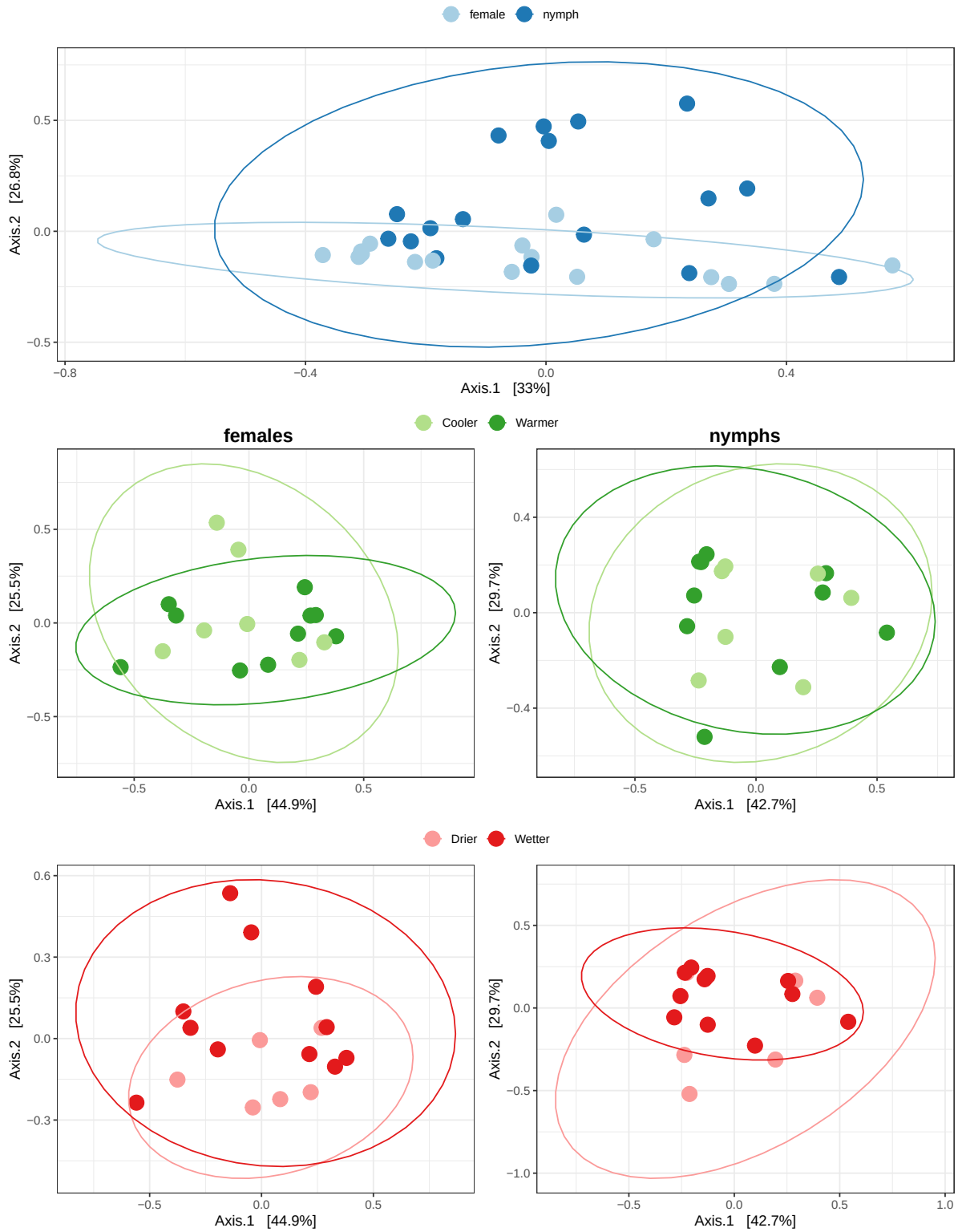

Figure 4. Principal coordinate analysis (PCoA) plots of β-diversity estimated based on the core bacteriome of *Ixodes ricinus* samples.

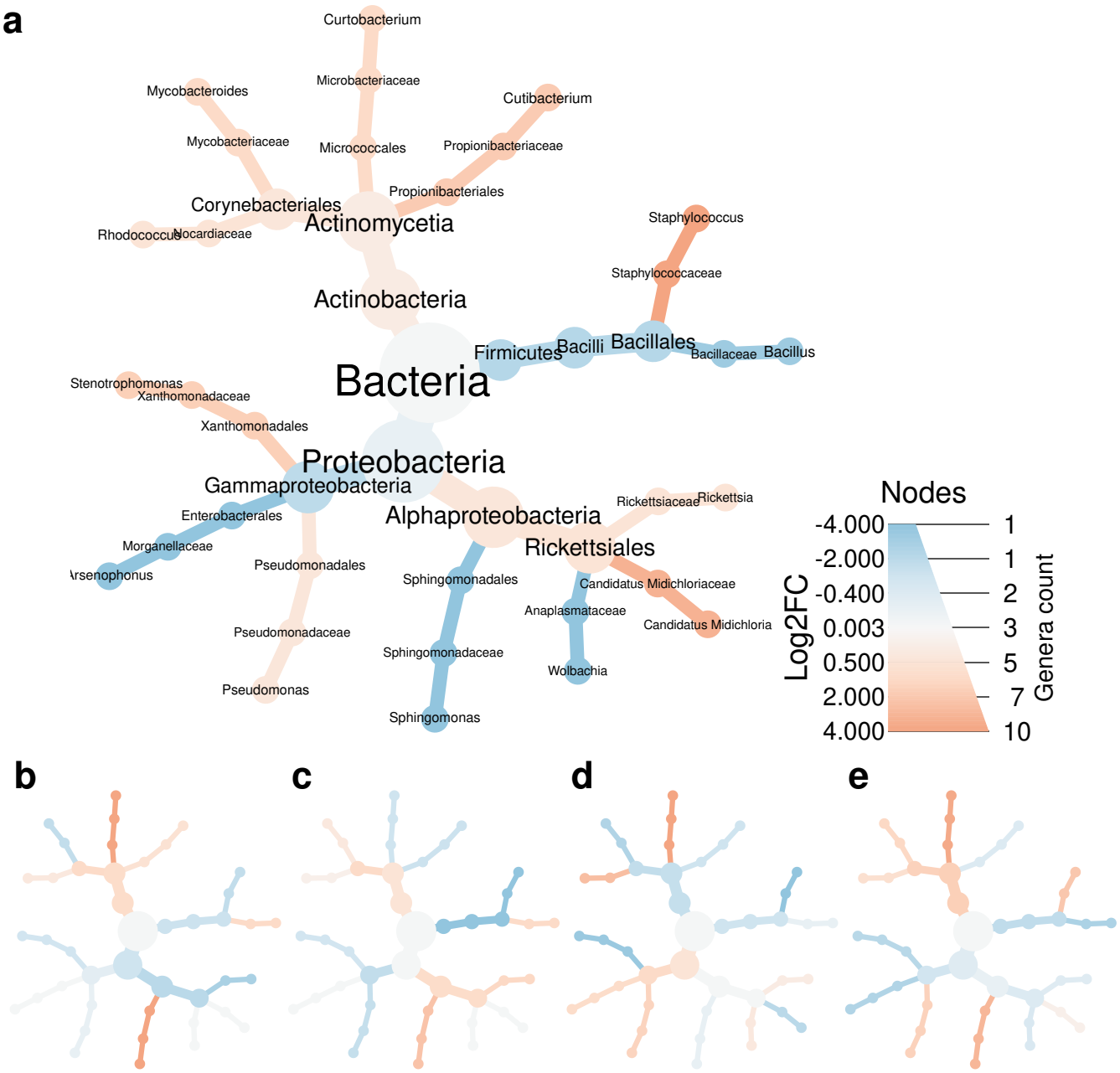

Figure 5. Core bacteriome abundance fold changes by taxonomic ranks. The colours represent the log2 fold change (Log2FC) of the median abundances of the compared groups. The subfigure **a** shows the ratio of the abundances in females comparing nymphs as reference. Figure **b** compares female samples from warmer areas to cooler ones. While figure **c** compares samples from drier areas to those from wetter areas among females. Comparisons of GDD groups in nymphs are shown in figure **d**, while comparisons of precipitation groups are shown in figure **e**.

Pathogens

Taxon classification of short reads with Kraken2 resulted in hits for the following pathogenic bacteria. *Anaplasma phagocytophilum* was found in nymphs from sampling sites 3 and 7. Reads from *Borrelia coriaceae* were identified in females collected from sampling site 1. *B. miyamotoi* was found in females from sampling sites 2 and 16 and in nymphs collected from sampling sites 1 and 10. *Borreliella garinii* was at sampling site 11 in the nymphs. *B. valaisiana* was found in nymphs collected at

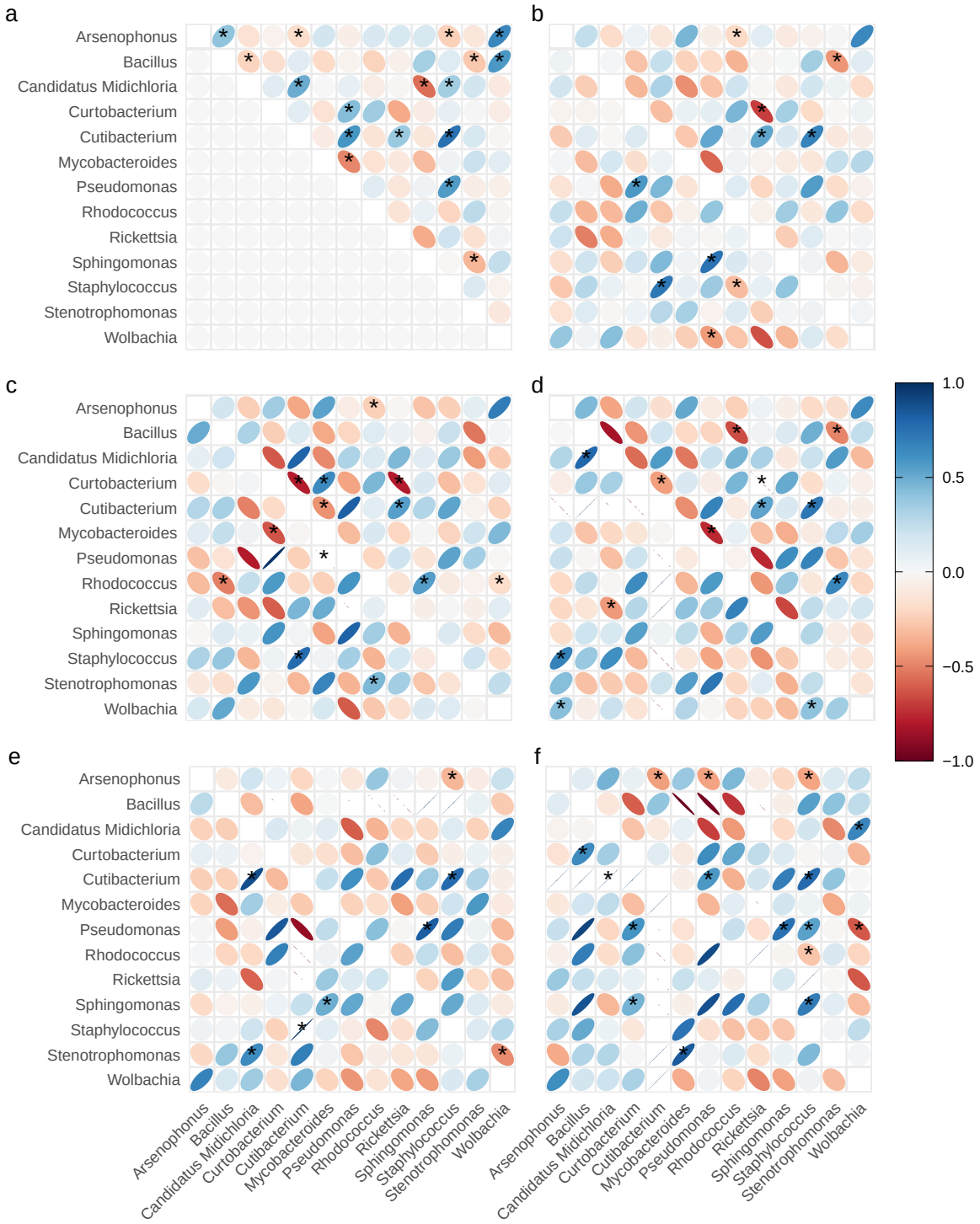

Figure 6. Abundance correlation plots. The correlation in all ticks is shown in Figure a). The lower half of figure b) is obtained in nymphs and the upper half in females. In figure c), the correlations in females from cooler environments are in the lower, and those from warmer environments are in the upper triangle. In figure d), the correlations for females from drier environments are in the lower triangle and those from the wetter environment in the upper one. In figure e), the correlations of nymphs from cooler environments are in the lower triangle and those from warmer environments in the upper triangle. In figure f), the correlations of the nymphs from drier environments are in the lower triangle, and those from a wetter environment are in the upper triangle. Significant ($p<0.05$) relationships are marked by *.

sampling site 8 and 11. *B. afzelii* was was found at the sampling site 2 in nymphs. *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). No species from the genera *Bartonella*, *Coxiella* and *Francisella* was found.

The result of the BLAST[32] based taxon classification of the assembly-generated contigs 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 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).

Discussion

Our shotgun sequencing-based microbiome analysis assesses an in-depth characterization of *I. ricinus* nymphs and adult females collected from a climatically representative set of sampling points from Hungary. Prior to this work, the life stage dependent microbiome study of *I. ricinus* ticks of geographical sampling units with a conscious consideration of climatic conditions has never been performed in Hungary. Our 'niche study' revealed a comprehensive picture of the bacterial diversity and associations in various host categories in *I. ricinus* ticks representing the climatic regions of Hungary.

While *I. ricinus* ticks had previously been linked with relatively higher alpha diversity scores than other common tick species,[40] no significant difference could have been observed among the nymph and adult female stages, or among warmer-colder and drier-wetter areas of origin. Interestingly, Carpi and colleagues found that the bacterial communities of geographically distant ticks of the same developmental stage differ more to those from the same regions.[41] Moreover, Batool and colleagues found that in Ukraine, that is a neighboring country to Hungary with an area of around 6.5 times as big, the alpha diversity analyses demonstrated differences in tick microbiota patterns of various administrative regions.[42] As of the dissimilarity of the samples' core bacteriome genus profiles (beta diversity), inter-regional comparison of developmental stages (females or nymphs) produced significant differences, while developmental stage-wise testing of climatic condition associated localization did not. Similarly, Batool and colleagues described that tick sex comparisons resulted in significant differences on various beta-diversity tests regardless of the area of origin.[42] While these results are interesting to compare, different study models and testing methods should be taken into account.

During the tick collection phase of the study, three categories of ticks were evaluated for metagenome sequencing: nymphs, adult females and adult males. Nevertheless, the sequencing of adult males was rejected as the number of males was relatively lower to nymphs and adult females at all sampling points. Sex ratio shifts by ticks are not uncommon.[43,44] The elucidation of the reason for the observed shift towards females in the adult life stage in our study would require further investigations, nonetheless, the presence of certain maternally inherited genera, namely *Arsenophonus*, *Rickettsia*, *Spiroplasma* and *Wolbachia* in the metagenomes is worth considering. These genera are described to induct parthenogenesis, feminize or kill males and thus, manipulate the reproduction of their host species towards the production of daughters.[45,46] Sex ratio skewness may be adaptive from the perspective of upper generation ticks, since it diminishes the competition of related males in tick-dense localities by reducing their numbers, while increases the numbers of related females that can be fertilized by fewer males as well.[47,48]

Besides the members of the core bacteriome of nymphs and adult females that are namely *Arsenophonus*, *Bacillus*, *Candidatus Midichloria*, *Curtobacterium*, *Cutibacterium*, *Mycobacteroides*, *Pseudomonas*, *Rhodococcus*, *Rickettsia*, *Sphingomonas*, *Staphylococcus*, *Stenotrophomonas* and *Wolbachia*, reads deriving from further bacterial genera with relatively lower abundance rates but high clinical relevance (pathogens), such as *Anaplasma*, *Bartonella*, *Borrelia*, *Borreliella* and *Ehrlichia* have also been detected. While *Anaplasma phagocytophylum*, cause of *Anaplasmosis* has previously been associated with the presence of a tick parasitoid, *Ixodiphagus hookeri*, based on its lifestyle and its mode of hunting,[49] its positive correlation with the

genera of *Arsenophonus* and *Wolbachia*, endosymbionts of *I. hookeri*, was not affirmed.[15] Within our study, the geolocations where *Anaplasma* sp. occurred did not match the detection points *I. hookeri* in Hungary described in a recent study.[50] Despite the high incidence rates of Lyme disease in Europe,[51] *Borreliella burgdorferi* itself was not detected in our samples, while other species of *Borrelia* and *Borreliella* were identified. Since the causative agent of Lyme disease is normally mentioned as *Borrelia burgdorferi* sensu lato,[52,53] referring to this species in the broad sense, involving other members of the genera as well, the presence of *Borreliella garinii*, *Borreliella valaisiana*, *Borreliella afzelii*, *Borrelia coriaceae* and *Borrelia miyamotoi* can also be associated with the common disease. *Bartonella* spp. and *Ehrlichia* spp. are pathogens, that are also often isolated in European settings.[54–56] The presence of pathogens may influence the composition of tick microbiota.[57]

Some genera constituting the core bacteriome, namely *Arsenophonus*, *Candidatus Midichloria*, *Rickettsia* and *Wolbachia* are believed to be maternally-inherited, or strongly tick-associated for direct or indirect reasons, while the members of *Bacillus*, *Curtobacterium*, *Cutibacterium*, *Mycobacteroides*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus* and *Stenotrophomonas* are related to soil, water, plants, skin or mucosa of vertebrates, thus may rather be acquired from the environment of the ticks.[15,15,42,58,59] The representatives of the environmental genera may either participate at the transient or long-term microbiota of ticks. Several environmental, tick gut or surface bacteria cause opportunistic infections in humans, especially in patients with immunocompromised history.[60,61] Even though, certain bacteria, such as cutibacteria and staphylococci may be considered as bacterial contaminants from the sample processing steps,[15] according to a recent study, the effects of a possible, minor level contamination are not noticeable in the overall relative bacterial abundance.[42] Nevertheless, it is possible, that some environmental genera were only present at the cuticle of the collected ticks regardless of the repeated laboratory washing steps with 99.8% alcohol.

Except for *Cutibacterium*, *Mycobacteroides*, *Rickettsia* and *Stenotrophomonas*, genera constituting the core bacteriome showed statistically significant alterations in the examined tick groups differing in life stage and climatic condition-associated geographical origins. This finding is not surprising, considering the fact that both the life stage and the season associated climatic conditions are very important in the composition of the tick bacteriome.[62,63]

The members of *Rickettsia* are maternally inherited or transstadially passed symbionts[64,65] that may cause tick-borne infections[64] and have been described to be a dominant in tick microbiomes in several studies,[41,66–70] However, high rickettsial abundance rates are not necessarily present in the ticks. Certain studies presented relatively low rickettsial genome fragment counts.[42] The reason for these alterations is that the appearance rates of *Rickettsia* spp. vary between the geographical populations of ticks. According to a recent study, the members of *Rickettsia* are more abundant in ticks from forests,[59,71] where ticks included in the present study also derived from. Considering that nymphal rickettsial counts were high in other studies as well,[66] while male ticks are normally associated with relatively less members of the genus *Rickettsia*,[66,67] the number of rickettsias seem to gradually decrease throughout the life of male ticks. This theory is in line with a study on microbiome changes during tick ontogeny.[72] On the other hand, certain rickettsial species are also associated with male killing, that may also cause their decreasing numbers associated to male adult ticks.[73] *R. helvetica* and *R. monacensis*, two species that have been identified in our samples as well, have been connected with the presence of adult *I. hookeri* wasps that are the parasitoids of ticks. The reason for the association may either be related to the role of the parasitoid wasps in the circulation of rickettsias among ticks or with digested bacterial DNA in the wasp body lumen.[74]

Even though, the members of Mycobacteroides are rather considered as either long-term or transient, environmentally derived residents of the tick microbiota, recent studies revealed that certain species might be able to multiplicate inside the host and be transovarially inherited.[58,75] Nevertheless, the relative abundance of the species seems dependent on the geographic region,[68] and in our studies a statistically non-significant shift could have been observed in the direction of cooler and dryer regions. Considering climatic tendencies, Thapa and colleagues reported higher mycobacterial genome fragment counts in ticks from Texas than from Massachusetts,[68] that shows a contrary mycobacterial temperature preference. The difference may be underlain by the different species-level *Mycobacterium* composition in the U.S. and in Europe. Since several *Mycobacterium* species may be pathogenic to humans or to animals, this finding may have a public health significance, mostly in light of climate change.

Cutibacterium and *Stenotrophomonas* are common environmental bacteria, that have been associated with ticks in several studies.[15,40,42] *Cutibacterium* is strongly associated with ticks in forests,[71] where our *I. ricinus* ticks derived from. *Stenotrophomonas maltophilia*, a species identified in our samples is an opportunistic pathogen, that is often isolated from the infections of immunocompromised individuals.[61]

The majority of the genera constituting the core bacteriome were found to demonstrate significant differences among various life stage and climatic condition associated tick host groups. By the abundance rates of specifically tick-associated genera, *Arsenophonus*, *Candidatus Midichloria* and *Wolbachia*, the significant differences among adult female ticks and nymphs were clearly explainable.

Arsenophonus, that showed significantly lower abundance rates in females than in nymphs, is a wide-spread, mainly insect-associated bacterial genus with a wide spectrum of either parasitic or symbiotic host relations.[59,76] While the high

number of *Arsenophonus*, more precisely *A. nasonie* associated reads could suggest the dominance of this genus in the nymph microbiota, its presence is associated to *I. hookeri*, a parasitoid wasp of ticks that is supposed to have a wide geographical distribution range around the world. *I. hookeri* oviposits to larval and nymphal hard tick hosts. Its eggs can only develop in engorging or engorged nymphs.[77,78] If the tick immune system-borne encapsulation of the *I. hookeri* eggs does not happen, the eggs hatch. Larvae start consuming the tick tissues, and thus cause the death of the host.[77] Furthermore, Bohacsova and colleagues found that *Arsenophonus nasoniae*, an endosymbiont of encyrtid wasps that have been identified in high numbers in our nymph samples, is only detectable in tick nymphs parasitized by the wasp.[74] According to these findings, nymphs harbouring *A. nasoniae* do not often reach the adult stage due to the parasitoid wasp, *I. hookeri*. Thus *A. nasoniae* deriving reads demolish from the adult tick population due to the death of nymph hosts. While the developmental stage dependence of *I. hookeri* associated *A. nasoniae* is beyond question, the median abundance of this bacterial genus also showed differences nymph groups of different climatical regions. Nymphs collected from warmer and rainier areas harbored more reads deriving from *Arsenophonus* spp. as ones collected from warmer areas. Nevertheless, as attempts have been made to use *I. hookeri* as a means of biological control of ticks for approximately 100 years,[78] the consideration of climatic conditions, as underlying causes for unstable control technique success rates, may improve current biological control methods. Moreover, *A. nasoniae* is described to be male-killing in several wasp species,[79] although in *I. hookeri* adult wasp males were also found to infected by *A. nasoniae*, although the emergence ratio of males and females was 1:3.6 in the infected populations.[74] Nevertheless, the presence of this bacterium may underlie decreased *I. hookeri* numbers at certain habitats or could even have contributed to insufficient abundance rates by biological tick control purposed, attempted mass releases of the parasitoid wasps in the past.[80,81] To assess the effects of *Arsenophonus*, and of the following genus, *Wolbachia*, on *I. hookeri*, and as a consequence, on the possible tick hosts of the parasitoid, further studies would be required.

The genus of *Wolbachia*, having a significant, strong abundance rate shift to nymphs, is reported to share several characteristics with *Arsenophonus* that affect tick life and potentially tick population size. One of the identified species, *Wolbachia pipientis*, is strongly related to the presence of *I. hookeri*.[82,83] Thus, the reason for the high number of wolbachial reads in nymphs may be the same as in case of the *Arsenophonus* genus. *Wolbachia* spp. are also described to selectively kill male hosts in some host insect species,[45,84] while in others it appears to be nonmale-killing.[85] To our knowledge, no studies exist on the the effect of *Wolbachia* spp. on *I. hookeri*. Furthermore, similarly to the case of *Arsenophonus*, warmer and rainier areas had a slight positive effect on the number of wolbachial reads in nymphs, although this effect was weaker than in case of *Arsenophonus*. Due to the nymphal loads of reads from *Arsenophonus* and *Wolbachia*, the prevalence of *I. hookeri* in the samples and at the sampling points in Hungary can be strongly hypothesized. Evidence for the presence of these parasitoid wasps has recently been discovered in Hungary.[50]

By *Candidatus Midichloria*, the strong, statistically significant difference that was observed for females among adult female and nymph ticks is in line with the results of other research groups and has an explanation in the scientific literature. Studies exploring the inter-sex microbiome differences of adult *I. ricinus* ticks[40,42,86,87] demonstrated much higher abundance rates of *Candidatus Midichloria* in females than in males independently of the regions of tick collection. The unique reason for this is that *Candidatus Midichloria mitochondrii* (CMM) invades the mitochondria of the cells within the ovaries of the female ticks. Despite the multiplication of the bacteria that consumes many ovarian mitochondria, the tick oocytes are supposed to develop normally. CMM is described to be vertically transferred to all eggs.[86,88] Even though, the nymphal sex ratio of ticks may not be exactly 1:1,[89] according to the available evidence, the presence of CMM does not result in sex ratio distortion in ticks.[86] At the same time, it has been observed that CMM is transferred to both male and female larvae, but later, during the nymph stage, its specialization occurs toward females.[87] Thus, a possible reason for the difference in CMM abundances among females and nymphs may be described by the fact that many nymphs are males. Furthermore, the multiplication of CMM appears to increase following engorgement.[87] According to this, the relatively low numbers of CMM might also be associated with the lack of engorgement among the nymphs that were collected for our study. Since tick collections occurred between the end of March and the middle of May, that represent the beginning of the activity period of nymphs,[90] information on the overall engorgement status based on CMM counts appears to be realistic.

By the environmentally derived bacterial groups (*Bacillus*, *Curtobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*), the explanation of statistically significant life stage- and climatic condition-wise differences in the bacterial composition appeared to be partly clear.

Bacilli inhabit a broad range of environments, ranging from soils to insect guts, and some species can be pathogens.[41] The finding, that the number of reads deriving from bacilli was significantly higher in nymphs than in adult females may originate from the choice to assess adult female ticks and exclude adult males from the study. While nymphs appeared at the study as a mixed population of males and females, the adult males collected at the sampling points are not represented due to their relatively low number compared to adult females. A study on the microbiome of *Rhipicephalus sanguineus* ticks describe a strong shift of *Bacillus* spp. towards the male tick population. Since our study only contained males in the nymph population, the observed shift may represent the male-relatedness of *Bacillus* spp. The nature of the relatedness of male

ticks and *Bacillus* spp. has not yet been characterized.[66] Nonetheless, *Bacillus* spp. are described not to be present in every tick microbiota,[68,91] and the detection rates of the members of this genus appear to show significant regional differences.[42] Within our study, reads deriving from *Bacilllus* spp. showed significantly higher abundances in nymphs from areas with more precipitation. Furthermore, Fernández-Ruvalcaba and colleagues reported that *Bacillus thuringiensis* strains significantly elevated mortality, and demolished oviposition and egg hatch among adult, pesticide-resistant *Rhipicephalus microplus* ticks.[92] Moreover, certain strains of *Bacillus wiedmannii* are known, while *Bacillus thuringiensis* strains are even commercialized as insecticids or nematicids for biological pest control,[93,94] although the entomopathogenic effect of environmental strains proved to be more present.[95] Both above-mentioned *Bacillus* species were present in our samples, that can decrease *I. ricinus* numbers, although potentially in both age groups. The key of biopesticide characteristics of these bacilli is the formation of functional crystaline *Cry* proteins that are specific toxins. Other entomopathogenic bacteria, such as *Bacillus cereus*, also present within our samples, are opportunistic, as they act in secondary, non-specific ways that are facilitated by certain weakening motives, such as the presence of *Cry* proteins.[94] Thus, the shift in *Bacillus* abundance towards nymphs rather appear to occur due to the sex determination of the adult ticks included in the study.

Although, within our samples a significantly more rhodococcal reads derived from females than from nymphs, this result may be based on a simple factor. The dominating species, *Rhodococcus fascians* is a common bacterial phytopathogen that interacts with a broad array of plants, causing their malformation.[96] Older ticks may had had more opportunities for encounters with these environmental bacteria, than younger ones. Higher rhodococcal read counts in adults are in line with the findings of René-Martellet and colleagues.[66]

Association with *Staphylococcus* spp., that were significantly more abundant in adult females than in nymphs may rest on similar pillars. *Staphylococci* are common findings related to ticks[58,67] that often appear on skin and mucous membranes of the hosts of the ticks as well.[58]. Ticks carrying staphylococci may have had already engorged, and thus encountered these bacteria. Taking into consideration, that the number of engorged nymphs appeared to be relatively lower within our samples according to the reasons explained by the genus *Candidatus Midichloria*, less opportunities to encounter the members of this genus by nymphs is imaginable. Moreover, the relative abundance of *Staphylococcus* spp. also appears to be dependent on the region of tick collection[68] and, as described earlier, staphylococcal hits may also derive from contamination.[42]

The finding, that the members of another environmental bacterial genus, *Sphingomonas*[15] were identified with significantly higher abundance rates in nymphs than in adults is controversal to the hypotheses of *Rhodoccus* spp., *Staphylococcus* spp. and to the related findings of other authors.[72] Another finding was that among adult females the abundance rate of *Sphingomonas* spp. was significantly higher in warmer sampling areas than at cooler localities, while by nymphs, the temperature-wise difference was not relevant. Interestingly, another research group found that *Sphingomonas* spp. were much more abundant at adult ticks kept at 4 °C, then by those at 20 °C, 30 °C or at 37 °C. Moreover, according to their study, *Sphingomonas* was among the most abundant bacterial genera at 4 °C by males.[67] The reason for this discrepancy with our results on temperature-wise abundance may rest on the species-level *Sphingomonas* composition. Additionally, interactions with other bacterial groups may also underlie our findings. In any case, further studies would be required to elucidate the findings of *Sphingomonas* populations and confirm or invalidate our hypotheses.

Statistically significant, climatic condition-wise alterations in adult female and nymphal stage ticks only appeared in environmental bacterial genera, namely *Curtobacterium* and *Pseudomonas*. *Curtobacterium* spp. that appeared significantly more abundantly at warmer environments both by adult females and nymphs with a significant preference for little precipitation by nymphs, according to what the identified members of this genus appear to be strongly thermophilic. This finding may be relevant in light of climate change, mostly because a dominant species, *Curtobacterium flaccumfaciens* is a phytopathogenic bacteria with economic significance[97] and has also been isolated from a child with fatal septicemia.[98]

Interestingly, reads from the genus of *Pseudomonas* were detected with significantly greater abundances at areas with higher GDD in adult females, while temperatures appeared not to influence the relative abundance of *Pseudomonas* spp. in nymphs. The formation of biofilms might underlie this finding. Some *Pseudomonas* strains have better capacities of biofilm formation at warmer temperatures.[99,100] Thus, adult ticks from warmer environments, that have already survived at least on summer according to our knowledge on tick life cycle, may harbor more bacteria from the *Pseudomonas* genus, that managed to form biofilms and thus became more steadily present. Furthermore, according to a study, the egg wax composition of the cattle tick, *Rhipicephalus microplus* is able to inhibit the biofilm formation of *P. aeruginosa*.[101] Thus, certain mechanisms may influence the abundance of *Pseudomonas* species at earlier tick life stages. Nonetheless, even the exact composition of *Pseudomonas* strains should be examined to evaluate this hypothesis, as the genus is very versatile. At the same time, *Pseudomonas* spp. have been related to both nymphs and adults in other publications as well,[42] while certain studies report more stable *Pseudomonas* spp. appearance rates in males than in females.[40,68]

Naturally, all the above-mentioned bacterial groups share another factor, namely the interaction among the members of the bacterial community, that may be responsible for the microbial pattern assessed. In order to elucidate the possible influence of certain bacterial cooccurrences, the correlation analysis of the bacterial genera was performed in each development stage

and climatic category. Positive correlations among the taxa of microbial communities may be interpreted as the reflection of shared habitat or environmental condition preferences, cooperative activities, such as cross-feeding[102] or the representation of functional guilds performing complementary or similar functions.[103] In contrast, negative correlations may indicate competition for limiting resources, niche partitioning, inequivalent resistance to losses or active negative interactions.[102,103] In contrast to other studies,[15] the number of positive and negative correlations was rather equalized within our samples. All samples considered, *Arsenophonus* and *Wolbachia* correlated positively, that is assumed to have occurred due to the presence of *I. hookeri*. Interestingly, *Bacillus* spp. also showed positive correlations with the symbionts of *I. hookeri*. Furthermore, *Rickettsia* was observed in negative correlation with *Curtobacterium* spp. and *Cutibacterium* spp. in several development stages and climatic groups, that may indicate positive public health consequences and a possible step towards a future tick control tool. In contrast, *Candidatus Midichloria* spp., that were previously detected in positive correlation with *Rickettsia* spp.[15] showed no correlation in our samples. Interestingly, in line with another publication on the topic,[15] *Pseudomonas* and *Rickettsia* also correlated, albeit, the correlation was, in contrast to the above-mentioned study, negative in warmer environments. In opposition, *Bacillus* and *Rickettsia* showed negative correlations in warmer environments, that is harmonious with the finding of Lejal and colleagues.[15] Correlations among environmental bacteria of various climatic categories are likely to be based on their similar environmental preferences and on the previously mentioned interaction types. Additionally, further correlations among environmental and specifically tick-associated bacteria were observed. Due to the seasonal variability of environmental bacteria, the number of tick-borne, potentially pathogenic bacteria may increase or decrease correspondently with the taxa of environmental origins.[15] According to this, the presence or absence of certain environmental taxa, may reflect to the temporal dynamics of certain pathogens. The public health significance of this finding is particularly significant in light of climate change and potentially varying climatic conditions around the globe. Nevertheless, the interpretation of co-occurrence patterns and the nature of correlations require further studies, as abundance shifts are dependent on multiple factors. Thus, bacterial and parasitological interconnections are not exclusively responsible for the variations. Moreover, our results were obtained from the sequencing of entire tick individuals, and thus lack finer, e.g. organ scale considerations of the correlating taxa.

Conclusion

Here we reported the identification of the *I. ricinus* microbiome associated findings in adult females and nymphs collected from a climatically representative set of sampling points of a Central European country, Hungary. These results allowed us to show that (1) the *I. ricinus* microbiota is dependent on the temperature and precipitation history of the geolocation of sampling; (2) the *I. ricinus* microbiota is not stable in the developmental stages of the ticks; (3) based on the bacteriome patterns identified, the identified developmental stage-wise alterations may be associated with the presence of certain tick parasitoids, that exclude reaching the adult age; (4) tick-borne disease pathogens are widely distributed at the climatically representative sampling points. In the future, developmental stage- and climate-associated microbial differences and correlations identified in this ecosystem study could be confirmed with experimental approaches and complemented with further metagenome studies to achieve sufficient data volumes of tick microbial interrelatedness, and be able to exploit them as a promising resource for novel tick control strategies.

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Author contributions statement

NS takes responsibility for the integrity of the data and the accuracy of the data analysis. NS and RF conceived the concept of the study. EK, GM and MG performed sample collection and procedures. AGT, MP and NS participated in the bioinformatic and statistical analysis. AGT, HY, LM and NS participated in the drafting of the manuscript. AGT, GM and NS carried out the manuscript's critical revision for important intellectual content. All authors read and approved the final manuscript.

Additional information

Availability of data and material The short read data of samples are publicly available and accessible through the PRJNA828115 from the NCBI Sequence Read Archive (SRA).
Competing interests The authors declare that they have no competing interests.
Ethics approval and consent to participate Not applicable.
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