Human pathogen co-occurrence in *Ixodes ricinus* ticks: effects of landscape topography, climatic factors and microbiota interactions

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Abstract

The factors shaping microbial communities within organisms are still poorly understood. Besides ecological factors and host characteristics, direct interactions among microbes may shape the occurrence of microbes and the structure of communities. In the past it has been difficult to disentangle if patterns of microbial co-occurrence are due to facilitation or competition effects, or shaped by shared ecological preferences (i.e., environmental filtering). Here we use a joint species distribution model to characterize the bacterial microbiota composition of an important human disease vector, the sheep tick *Ixodes ricinus*, along ecological gradients in the Swiss Alps, and to test for facilitation or competition effects among human pathogens and tick endosymbionts. We identify a number of ecological variables that significantly predicted the diversity of tick microbial community and the occurrence of specific tick endosymbionts and human pathogens. However, ecological associations were generally microbe-specific rather than universal. We also found evidence for significant microbe interactions, in particular widespread facilitation among pathogens, which promotes pathogen co-infection within ticks, as well as competition between the tick endosymbiont *Spiroplasma* and a number of human pathogens. These findings highlight that direct interactions among microbes can affect the vector competence of ticks and thereby tick-borne disease dynamics.

Keywords: disease ecology, tick-borne pathogens, species distribution modelling, community composition, *Borrelia burgdorferi*, Lyme disease, facilitation, microbiome, endosymbionts
Introduction

Microbial communities within organisms consist of symbionts, commensals, mutualists and pathogens that co-occur simultaneously and potentially influence each other [1–3]. These microbial communities may be shaped by a range of factors and processes, including the environment, host and microbe genetics and the occurrence and abundance of other microbial species. For example, certain microbial species might tolerate only certain biotic or abiotic conditions, which makes it more likely that species with similar requirements co-occur within a host (‘environmental filtering’ [4]). Similarly, the host’s immune system can influence colonization success of microbes [5], with cross-immunity preventing the colonization of different microbes with similar antigenic properties [6]. Finally, direct interactions among microbes might affect colonization, or replication success after colonization, through competition or facilitation processes. Competition may occur when different microbes use the same, limited resources within a host [7], whereas facilitation may occur directly through the production of public goods [8] or indirectly through the modification of the host’s physiology [9] or immune defense [10].

Ticks are important vectors for a range of zoonotic pathogens, including Borrelia spp., the causative agents of Lyme borreliosis [11] and Rickettsia sp., the causative agents of, among others, spotted fever and typhus [12]. Recent studies have found that pathogen co-infection is common in ticks [13–16]. Yet, the causes of pathogen co-occurrence remain poorly understood. Gaining insights into the factors that shape microbial communities in ticks in general, and the co-occurrence of human pathogens in particular, is of relevance from a fundamental ecological perspective, but also for public health, and it might inform tick-borne disease control programs.

Ticks have complex lifecycles, and require two or three bloodmeals to reach sexual maturity. They thus come into contact with several vertebrate hosts species throughout their life [17]. The composition of the host community might thus be a major determinant of the microbial community in ticks as different hosts might harbor different microbial (including pathogen) species [18, 19].
Host communities in turn are likely influenced by a range of biotic and abiotic factors such as vegetation structure, food abundance, elevation, temperature or rainfall. In addition, these biotic and abiotic factors might influence tick microbial communities directly, through effects on tick physiology or activity patterns [20].

In addition, there is some evidence that facilitation and competition processes shape the microbial community of ticks [21, 22]. For example, pathogenic *Rickettsia* species prevent co-infection with other *Rickettsia* species in *Dermacentor variabilis* ticks [23], whereas the presence of *Francisella* sp. endosymbionts increases the colonization success of pathogenic *Francisella novicida* in *D. andersoni* ticks [24]. Facilitation has also been suggested to promote co-infection with different *Borrelia afzelii* strains in *Ixodes ricinus* ticks [15]. Most strikingly, dysbiosed *I. scapularis* ticks (i.e., ticks with low microbiotal diversity) have a defective peritrophic matrix which decreases the colonization success of *B. burgdorferi* s.s., suggesting that the pathogen requires the presence of an intact microbiota to be able to infect ticks [25]. Thus, the microbial community may have a crucial impact on vector competence of ticks and thereby on disease dynamics.

Previous studies on the bacterial community of *I. ricinus* ticks have found approximately 100 OTUs (operational taxonomic units; [26]) per tick [27]. Most of them are environmental and free-living bacteria but also several endosymbionts and human pathogens were found [28, 29]. Differences in the bacterial community structure of ticks across habitats [30], geographical sites [28] and tick life stages and sexes [28, 31] have been documented. Yet, the processes that shape these patterns remain poorly understood [32]. In particular, whereas previous studies have investigated the potential interactions among microbes in ticks, they have not accounted for potential confounding variables such as shared ecological requirements, and are thus limited in their ability to differentiate between accidental co-occurrences due to shared environmental niches, and co-occurrence caused by direct interactions (such as facilitation or competition) among microbes. To address this gap, we use a
joint species distribution modelling (JSDM) approach to disentangle environmental effects on tick microbiota composition from direct microbial interactions [33, 34].

*I. ricinus* is the most common tick species in Europe and its distribution and abundance are strongly influenced by environmental conditions, in particular temperature and humidity [35, 36]. *I. ricinus* harbours a range of human pathogens, including several genospecies of *Borrelia* [37], *Rickettsia* sp. [38], *Anaplasma* sp. [39] and *Candidatus* Neoehrlichia [40]. Spatial variation in pathogen occurrence and prevalence is often pronounced [41]. For some pathogens, such as *B. afzelii*, previous studies have identified a range of ecological factors such as elevation and its associated abiotic factors [42, 43] or vegetation type and its associated effects on host communities [44], which contribute to this spatial variation. For other pathogens, however, the ecological drivers of distribution and prevalence remain poorly understood and little is known about the role of other microbes in influencing pathogen occurrence in *I. ricinus* ticks.

In our study, we exploited the environmental heterogeneity along replicated elevational gradients in the Swiss Alps to quantify the relative importance of environmental factors, tick characteristics and direct microbial interactions in influencing the structure of bacterial communities in *I. ricinus* ticks in general, and the occurrence of human pathogens in particular, using a combination of 16S sequencing and JSDM [45, 46]. Specifically, we ask (i) how do abiotic factors and tick-related variables affect tick microbiota composition, (ii) which abiotic and tick-related variables predict pathogen occurrence, and (iii) whether there are patterns of non-random microbial co-occurrence that cannot be explained by environmental similarities and are thus likely caused by direct facilitation or competition among microbes.
Materials and methods

Tick sampling

Questing *Ixodes ricinus* ticks were collected at three locations in the Swiss Alps (Kanton Graubünden). At each location, one site at low (630 - 732 m above sea level), one at medium (1 094 – 1 138 masl) and one at high (1 454 – 1 673 masl) elevation were identified (Figure 1, Table 1, N = 9 sampling sites). At each site, questing ticks were sampled thrice, once in June, once in July, and once in August 2014 by dragging a white blanket (1 m x 1 m) over the ground vegetation as described previously [47]. Ticks were collected from the blanket and stored in 95% ethanol. Tick species and life stage were verified using a stereomicroscope.

Environmental variables

For each sampling site, we compiled information on elevation, slope and aspect using DHM25 and land use data from swissTLM3D (both from Federal Office of Topography swisstopo) and information on temperature and precipitation provided by Landscape Dynamics (Swiss Federal Research Institute for Water, Snow and Landscape Research WSL and Federal Office of Meteorology and Climatology MeteoSwiss. [48]). Information on *I. ricinus* abundance and the abundance of a key tick host, the bank vole (*Myodes glareolus*), as well as the ratio of bank vole to other rodents at our sampling sites were obtained from [43]. Details on and justification for variables is provided in the Supplementary Material.

Tick DNA isolation and quantification of neutral genetic diversity

We randomly selected *I. ricinus* ticks from each sampling site for analysis (Table 1). Variation in the number of ticks per site was due to variation in tick abundance [47]. To avoid environmental
contamination, we performed DNA isolation and amplifications in a laminar flow cabinet. Each tick was washed thrice with sterile water before sterilizing it with 3% hydrogen peroxide. Ticks were then cut in half with a sterilized blade to facilitate DNA isolation. DNA was extracted using DNeasy Blood & Tissue kit (Qiagen; Hilden, Germany).

In order to quantify individual and population-level genetic diversity, we genotyped ticks at 11 microsatellite markers in two multiplexed amplifications (see Supplementary Material for details). Not all markers were successfully amplified in all samples, but none of the samples contained more than two failed markers. We used package ‘poppr’ [49] in R 3.4.1 [50] to test for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium. Individual observed heterozygosity was determined for each tick as a proportion of heterozygous markers to all successfully amplified markers. Expected population level heterozygosity was determined with ‘poppr’.

**Tick microbiota sequencing**

We quantified tick bacterial community composition by sequencing the hypervariable V3-V4 region of the 16S gene. Negative controls (N=5) were processed alongside the tick samples. Sequencing libraries were prepared following the Earth Microbiome 16S Illumina Amplicon protocol, using the primers 515FB and 806RB[51] (see Supplementary Material for details). Samples and negative controls were randomized across two plates. The libraries were sequenced on Illumina MiSeq at the Functional Genomic Center Zurich with a target length of 250 bp following the manufacturer’s protocol. The obtained sequence data were analyzed following the *mothur* pipeline with MiSeq standard operation procedures [52].
Sequences have been deposited to the Sequence Read Archive under BioProject PRJNA50687. The complete metadata of the samples and their matching sequence accession numbers have been submitted to FigShare (doi: 10.6084/m9.figshare.7380767).

There has been a debate whether ticks have a stable microbiota [53], mirroring the wider debate on how common resident microbiota is in arthropod hosts [54]. Thus, in addition to analyses of overall microbiota diversity and composition, a special focus of our analysis was on tick endosymbionts and tick-borne human pathogens (Table 2), which are obligate residents.

**Tick microbiota diversity**

Only samples with > 500 amplicons and OTUs which were present in at least two samples were included in the analyses. We removed the most common OTU, the intra-mitochondrial endosymbiont *Candidatus* Midichloria [55], as it was present in all samples and compromised 19.3% of all amplicons. After exclusion of Midichloria, we rarified the samples to lowest sample size to account for variation in amplicon numbers.

Bacterial alpha diversity (inverse Simpson index; [56]) and beta diversity (Bray-Curtis dissimilarity index, which takes shared species proportions into account when calculating dissimilarities; [57]) was calculated with the R package ‘vegan’ [58]. We ran linear mixed models with the package ‘lme4’ [59] to test for associations between bacterial alpha diversity and elevation, tick life stage, sex and sampling month, with full interactions. Site was included as a random effect in the model.

We performed both permutational ANOVA with dissimilarity matrices and analysis of multivariate homogeneity of group dispersion using the package ‘vegan’ to test for association between measures of beta diversity and elevation, tick life stage, sex, site and sampling month.

For each model, non-significant factors were removed from the model starting with the least significant term. In addition, we used a model selection approach based on Akaike’s Information
Criterion [60] and model fit with conditional $R^2$ [61] in the package ‘piecewiseSEM’ [62] to test which combination of factors best describes variation in tick bacterial diversity.

*Joint species distribution modelling of microbiota composition*

We used a framework for JSDM called Hierarchical Modelling of Species Communities (HMSC, [45]) to test how environmental variables correlate with human pathogen and tick endosymbiont occurrence and whether there are non-random residual associations among different OTUs and/or oligotypes, implying direct facilitation or competition effects among microbes.

This approach combines information on environmental covariates, bacterial species traits, spatiotemporal context and sampling design to explain the presence or absence of OTUs (Figure S3). The associations among OTUs are modelled with the latent part of the framework, using the residual variance after accounting for the effects of the environment. Correlations among OTUs in this analysis thus reflect (dis)associations which cannot be explained by shared responses to the environment.

We compiled occurrence matrices for OTUs for each individual tick as a response variable with the same sample exclusion criteria as for the diversity analyses. An OTU was determined to be present in a tick if >5 non-rarefied amplicons were identified in a sample. For each sampling unit, i.e. a row in our response variable matrix, we included information on the identity of the sampling unit (tick ID), its location, sampling site and month, describing the study design.

To reach a better resolution within specific OTUs, we analyzed known human pathogens, tick endosymbionts and their close relatives within the 100 most common OTUs with oligotyping pipeline [63]. Oligotyping uses all the sequences, which form an OTU, and performs Shannon Entropy Analysis to regroup sequences based on within-OTU variation. This results in higher-
resolution grouping than OTUs as the different oligotypes might differ only by a single nucleotide [63]. We used the standard operation procedures of the oligotyping pipeline software [64].

Including a large number of explanatory variables in statistical models is inherently challenging. To reduce the number of variables, while maintaining their information value, we used two variable sets in the model: a) a set of full-effect explanatory variables, and b) explanatory variables under variable selection [46]. The full-effect variable set included an intercept, two individual-tick level variables (tick sex or life stage and individual heterozygosity) and two site-level variables (tick abundance and elevation of the sampling site). Additionally, we included information whether a specific OTU is an endosymbiont and/or a pathogen as traits [65]. This allowed us to test if endosymbionts and/or pathogen respond differentially to environmental conditions than other OTUs. The set of explanatory variables under variable selection included additional information on the environmental conditions of the sites (namely the number of days above 7 C° during the year, monthly precipitation, mean monthly temperature, forest coverage, slope, aspect, bank vole abundance, the proportion of voles to other rodents and expected tick heterozygosity) (Table S1).

We considered all parameter estimates, including associations among bacterial OTUs, having strong statistical support if the 90% central credible interval of the parameter did not overlap with zero [following 73]. See Supplementary Material for additional model details.

Although JSDM is a powerful approach to model microbial community structure, it has a number of limitations. First, it assumes that interactions among microbes are similar across environments [but see 66]. This is not necessarily the case as both abiotic and biotic factors may shape microbial interactions [68]. Second, the model assumes that the explanatory variables affect the microbial community composition (or rather, the presence or absence of individual OTU), but not vice versa. However, this is a valid assumption for most environmental (e.g. elevation and temperature) and tick-related variables (e.g. tick sex, life stage) included in our models. Thirdly, covariation among variables poses a problem to any modelling approach. Our model is built on two distinct variable
sets to account for such covariation: the full variable set includes elevation, whereas the variables
with the strongest covariation (i.e., temperature and precipitation) are included in the variable
selection set.

Results

Ixodes ricinus microbiota composition

We 16S sequenced the bacterial community of 92 Ixodes ricinus ticks which resulted in 13 214 477
amplicons. No amplification was observed in the negative controls. After contig assembly and
quality control 1 656 287 sequences were retained. There was a median of 1 562 quality-controlled
amplicons per sample, with an interquartile range of 6 319. After exclusion of Ca. Midichloria, 82
samples with more than 340 amplicons per sample and a Good’s coverage estimator \( \geq 0.95 \) were
included in the subsequent analyses. In total, 5 181 bacterial OTUs were identified. The median
number of OTUs per rarified sample was 89 OTUs, with a 95% confidence interval of 78.3 - 98.5
OTUs.

Six OTUs were present in at least 90% of the samples: Ca. Midichloria (Otu0001), Sphingomonas
(Otu0002, 0006 and 0007), Pseudomonas (Otu0011) and Delftia (Otu0012). Together, they
represented 50.2% of all amplicons. We used oligotyping to further divide OTU0031 ‘Rickettsia’
into two oligotypes labelled as ‘R. helvetica’ and ‘R. monacensis’, and OTU0086 ‘Borrelia’ into
four oligotypes labelled as ‘B. afzelii’, ‘B. valaisiana’ and ‘B. garinii’ and ‘B. miyamotoi’. After
excluding rare OTUs, we used 635 OTUs and oligotypes in subsequent analyses, including 14
endosymbionts and / or human pathogens (Table 2).

Ixodes ricinus microbiota diversity
We observed a significant association between tick microbiota alpha diversity and elevation with lower bacterial diversity observed at higher elevations ($F_{1,78}=3.98, p = 0.05$, conditional $R^2 = 0.07$, Figure 2). No other environmental variables were significantly associated with tick microbiota alpha diversity (Table S2).

The analysis of tick microbiota beta diversity revealed a significant interaction effect between tick life stages/sex and elevation, indicating that differences in tick microbiota beta diversity across tick life stages/sex are shaped by elevation (pseudo-$F_1 = 1.59$, $p = 0.01$, $R^2 = 0.03$, Figure S1; Table S3). For other variables no significant association with microbial beta diversity was observed (Table S3). There was a significantly larger group dispersion at lower elevation sites ($F_{8,73} = 19.25$, $p < 0.001$, Figure S2) suggesting that among-individual variation in bacterial community composition is higher at lower elevation. For other variables, no significant heterogeneity in bacterial community composition was observed (Table S4).

**Tick microbiota community modelling**

**Tick microbiota variance partitioning**

Variance partitioning revealed that most of the variation in tick microbiota composition was explained by tick ID: for the hundred most common OTUs, tick ID accounted for 83.5% of the variation explained by the JSDM model. Fixed effects accounted for 9.3% and other random effects (location, site and month) explained 7.2% (Figure 3). This suggests that there is extensive among-individual variation which cannot be accounted for by environmental variation. Notably, the situation differed for endosymbionts or pathogens: while tick ID was still the most important variable, fixed effects explained 21.9% and random effects explained 11.2% of the total variation explained by the model, when averaged over all pathogens and endosymbionts (Figure 3).
Tick-specific and environmental factors associated with OTU occurrence

The occurrence of some OTUs was strongly predicted by specific explanatory variables (Table 3).

Slope, for example, was a strong predictor of the occurrence of a number of endosymbionts and pathogens with a higher probability of *R. helvetica*, *Rickettsia* sp., *Anaplasma* and *B. afzelii* and a lower probability of *Ca. Neoehrlichia* occurrence on steeper slopes. Females were less likely to harbour the endosymbionts *Spiroplasma*, *Lariskella* and *Rickettsia* spp. (Table 3) and ticks at higher elevations had higher probability to harbour *R. helvetica* and *R. monacensis*, but were less probable to harbour *B. garinii* (Table 3). Ticks from sites facing northwards had a higher probability of harbouring *Spiroplasma* and *B. afzelii*, but a lower probability to harbor *Anaplasma* (Table 3).

Higher tick density was associated with a higher probability of *Rickettsiella* and *Ca. Neoehrlichia* occurrence (Table 3), while more extensive forest coverage was associated with a higher probability of *R. helvetica* and *B. afzelii* occurrence (Table 3). Associations between tick life stage, temperature, the number of days > 7 C°, precipitation or relative vole abundance and the occurrence of specific OTUs were not strongly statistically supported.

The effect sizes of strongly statistically supported associations varied substantially (Figure S5a-i).

For example, threefold increase in vole abundance corresponded to one percentage point decrease of *R. monacensis* prevalence (Figure S5b), whereas a threefold increase in tick abundance corresponded to a twofold increase in *Neoehrlichia* prevalence from 20% to 40% (Figure S5e).

Microbial facilitation or competition effects within ticks

Numerous bacterial OTUs were either significantly more or less likely to co-occur within a tick than expected by chance after accounting for shared environmental preferences (Figure 4; Table S5). The occurrence of the tick endosymbiont *Spiroplasma* was negatively associated with the occurrence of the endosymbiont *Lariskella* and several tick-borne human pathogens, namely *Anaplasma* sp., *Ca. Neoehrlichia* and *B. miyamotoi* (Figure 4). Associations among pathogens, if
they occurred, were all positive (Figure 4), suggesting that ticks are more likely to be co-infected with several human pathogens simultaneously than expected by chance or based on shared environmental preferences. *Borrelia* oligotypes showed positive co-occurrence patterns among each other, except for *B. miyamotoi*, which was not associated with other *Borrelia* sp., but negatively with *Spiroplasma* and positively with *Lariskella*. As our approach allowed us to control for shared environmental preferences, the significant negative or positive associations among endosymbionts and pathogens can be considered as indications of competition and facilitation.

**Discussion**

Using a Joint Species Distribution Modelling approach, our study provides evidence that a range of tick endosymbionts and human pathogens are more or less likely to co-occur within *I. ricinus* ticks than expected by chance or due to shared environmental preferences. Although the co-occurrence of microbes within ticks has been documented before, both in *I. ricinus* [16, 69] and other tick species [70, 71], previous studies did not control for shared environmental preferences (i.e., environmental filtering) and were therefore unable to disentangle shared responses to the environment from direct microbe-microbe interactions. Our study reveals that when accounting for shared environmental preferences, associations among tick endosymbionts and human pathogens are mostly positive, suggesting that facilitation processes significantly contribute to endosymbiont-pathogen co-occurrence as well as pathogen co-infections within ticks. Such facilitation processes will affect the structure and dynamics of microbial communities [9, 71]. At the same time, the resulting co-occurrence of pathogens within ticks has implications for the severity, diagnosis, treatment and control of tick-borne diseases.

Although associations among microbes were mostly positive, there were negative associations between the tick endosymbiont *Spiroplasma* sp. and several human pathogens, which is indicative
of competition. Similar protective effects have been previously described in Drosophila sp., where Spiroplasma sp. is associated with a decreased probability of nematode and parasitoids infections [72, 73]. Such competition effects among tick endosymbionts and human pathogens might open up new and exciting avenues for the control of tick-borne pathogens in areas with high disease incidence.

Among the significant microbe-microbe interactions, the strong positive associations among the Lyme disease-causing Borrelia genospecies (B. afzelii, B. garinii and B. valaisiana) were particularly striking and indicative of facilitation (see also [15]). The co-occurrence of these Borrelia genospecies within ticks is surprising because B. garinii and B. valaisiana are bird specialists [74, 75], whereas B. afzelii is a rodent specialist [76]. It suggests that I. ricinus ticks typically feed on multiple, phylogenetically diverse host species during their life cycle and do not show pathogen-mediated host specialization as has been suggested previously [77, 78].

In addition to testing for facilitation and competition among microbes, we used a JSDM framework to quantify the role of tick-specific and environmental variables in explaining spatial variation in tick microbiota composition. Even though environmental variation across our sampling sites was large (spanning an elevational gradient from 630 – 1 580 masl) and a large number of ecological and tick-related variables was considered in our models, overall they accounted for only 9.3% of the variation in bacterial microbiota composition. This finding highlights that unexplained variation in microbiota composition across individual ticks is substantial, which is in line with the idea that at least a part of the bacteria present in ticks may be non-resident [53]. Nevertheless, this can also result from missing relevant environmental variables in our data. However, this conclusion is further supported by the finding that the explanatory power of tick-related and environmental variables was higher for tick endosymbionts and human pathogens, which are obligate resident.

Despite the large among-tick variation in microbiota composition, we found significant associations between specific environmental variables and OTUs. Particularly pronounced was the association...
between tick microbiota diversity and elevation. Across three independent elevational gradients we
found that alpha diversity was consistently higher at lower elevations. Although a decrease in plant
and animal diversity with increasing elevation is a widely documented pattern in ecology, it has
been suggested that the biogeographical patterns exhibited by bacteria may be fundamentally
different from those of plants and animals [79]. Our findings do not support this hypothesis but are
in line with the idea that microbiota diversity decreases with increasing distance from the host’s
environmental optimum, such as at a host’s range edge or during torpor [80]. Similarly, we
observed changes in microbial beta diversity with elevation, particularly in female ticks, and a
significantly larger group dispersion in microbial beta diversity at lower elevations. Together with
the decrease in tick microbiota alpha diversity with increasing elevation, it highlights that
suboptimal environmental conditions permit the occurrence of only a subset of bacterial OTUs in
ticks, leading to more similar, depleted microbiota at such sites. The consequences of low microbial
diversity for individual ticks and tick populations are currently unknown. Yet, given the lack of an
association between microbial alpha or beta diversity and the occurrence of human pathogens it is
unlikely to affect human disease risk.

We identified a range of tick-specific and environmental variables that significantly predicted the
occurrence of various tick endosymbionts and human pathogens. However, these associations were
typically OTU specific rather than universal, with the same variable being both positively and
negatively associated with the probability of occurrence of different OTUs. For example, B. garinii
was less likely to occur at higher elevations whereas R. helvetica and R. monascensis were more
likely to occur at higher elevations. Generally, the environmental factors shaping Rickettsia spp.
distribution are poorly understood, as is their range of host species [44, 81]. Yet, it has previously
been found that spotted fever incidence in humans, caused by R. ricketsii, is highest in suboptimal
tick habitats [82]. This is in line with our findings and suggests that Rickettsia spp. are more likely
to colonize ticks living under suboptimal conditions. The finding that B. garinii is less likely to
occur at higher elevations is in line with previous observations [42] and may be explained by
elevational clines in the diversity and/or abundance of birds, the natural hosts of *B. garinii* [74]. In
contrast, the occurrence of the mammal specialist *B. afzelii* was not associated with elevation,
potentially because elevational clines in mammal diversity and/or abundance are less pronounced
[83]. Indeed we did not observe an association between elevation and bank vole abundance in our
study (ANOVA: F_{1,8}=0.357, p = 0.57, R^2 = 0.05).

Interestingly, temperature and precipitation, which vary strongly across elevational gradients
(average temperature and precipitation: high elevation sites: 11.8 °C and 17.8 mm per month; in
low sites: 16.5 °C and 12.1 mm per month), were not significant predictors of the occurrence of
endosymbionts or pathogens. This may be partly due to the temperature and precipitation measures
included in our models not fully capturing microclimatic variation across sites and along elevational
clines. Indeed, slope and aspect, which are important determinants of the topography, and thus
microclimate [84], were significant predictors of pathogen and endosymbiont occurrence. The
probability of pathogen and endosymbiont occurrence was higher on steeper slopes. Furthermore,
the probability of occurrence was higher on north-facing slopes for *B. afzelii* and *Spiroplasma* on
north-facing slopes and and higher on south-facing slopes for *Rickettsiella* and *Anaplasma* sp. (see
also [85]). Microclimatic conditions may affect microbial occurrence directly, or indirectly via
affecting tick behavior or host community composition [86, 87]. Furthermore, topography can
affect population connectivity and dispersal in metapopulation networks [88].

Host community composition and density are crucial for pathogen and endosymbiont occurrence
and dispersal [41]. Previous work has found that tick abundance is a strong predictor of *Borrelia*
spp. prevalence, potentially because larger tick populations facilitate co-feeding transmission [89].
No association between *Borrelia* spp occurrence and tick abundance was observed in our study.
However, both *Ca. Neoehrlichia* and *Rickettsiella* were more common at sites where ticks were
more abundant, suggesting that co-feeding transmission may also play a role in the life cycle of
these microbes.

Finally, differences in host competence can lead to dilution effects and thus affect the prevalence of
tick-borne pathogens [90]. Whereas for some tick-borne pathogens the vertebrate hosts are known
or suspected (e.g. small mammals for *B. afzelii* [76] and *Ca. Neoehrlichia* [91], birds for *B. garinii*
and *B. valaisiana* [75], both for *Anaplasma* [92] and *R. helvetica* [93]), for others the host species
range is less well understood (e.g. *B. miyamotoi* [94]). The bank vole is a common tick host at our
study sites and their abundance was a significant positive predictor of *B. valaisiana* and negative
predictor of *R. monacensis* occurrence. Interestingly, bank voles are known hosts for neither of
these microbes [95]. Most likely, the association is thus indirect, explained by a biotic or abiotic
variable that correlates with bank vole abundance. For example, bank voles avoid open habitats and
prefer woodlands and heterogeneous habitats [96, 97]. Overall, no evidence was found that the
proportion of bank voles to other rodents affects the prevalence of tick-borne pathogens.

In conclusion, our results show how we can jointly model tick-associated bacterial communities,
and taking into account of the environmental effects, tease apart patterns in the data indicating
facilitation and competition among microbes. We identified a number of ecological variables that
predict the occurrence of specific tick endosymbionts and human pathogens with strong statistical
support, but these effects were generally microbe-specific rather than universal. This highlights that
environmental change can have different, even opposite effects on the probability of occurrence of
different pathogens, and thus human disease risk. Furthermore, our modelling approach reveals
significant among-microbe interactions, suggesting in particular widespread facilitation among
pathogens, which promotes pathogen co-infection within ticks, as well as competition between
*Spiroplasma* and a number of human pathogens. The latter opens up new and exciting avenues for
the control and management of tick-borne diseases in regions with high human disease incidence.
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Table 1: Tick sampling sites in the Swiss Alps.

| Location | Site   | Coordinates | Elevation (masl) | Sequenced *Ixodes ricinus* ticks |
|----------|--------|-------------|-----------------|---------------------------------|
|          |        | North       | East            | nympha | males | females |
| 1        | Sagogn | 46.783      | 9.233           | 693    | 0     | 9       | 15      |
|          | Flims  | 46.827      | 9.280           | 1138   | 3     | 5       | 3       |
|          | Ruschein | 46.795     | 9.169           | 1454   | 0     | 1       | 1       |
| 2        | Rodels | 46.760      | 9.425           | 630    | 2     | 5       | 4       |
|          | Tomils | 46.772      | 9.453           | 1144   | 3     | 6       | 4       |
|          | Feldis | 46.789      | 9.453           | 1673   | 1     | 1       | 0       |
| 3        | Passug | 46.840      | 9.538           | 732    | 0     | 5       | 6       |
|          | Castiel| 46.826      | 9.569           | 1094   | 0     | 3       | 3       |
|          | Praden | 46.817      | 9.589           | 1582   | 1     | 0       | 1       |
Table 2: Common tick endosymbionts and/or putative human pathogens observed in I. ricinus ticks. See Supplementary Materials for information on OTU assignment.

| OTU  | Label                | Human pathogen / tick endosymbiont | Occurrence (% of analyzed ticks) |
|------|----------------------|-------------------------------------|----------------------------------|
| Otu0001 | Midichloria           | endosymbiont                        | 100                              |
| Otu0003 | Spiroplasma          | endosymbiont                        | 41                               |
| Otu0005 | Rickettsiella        | endosymbiont                        | 63                               |
| Otu0021 | Lariskella           | endosymbiont                        | 49                               |
| Otu0031 | Rickettsia helvetica | both                                | 16                               |
|        | R. monacensis        | both                                | 6                                |
| Otu0067 | Rickettsia sp.       | both                                | 25                               |
| Otu0076 | Anaplasma            | both                                | 33                               |
| Otu0086 | Candidatus            | both                                | 22                               |
|        | Neoehrlichia          |                                     |                                  |
| Otu0088 | Borrelia afzelii     | pathogen                            | 9                                |
|        | B. miyamotii          | pathogen                            | 10                               |
|        | B. garinii            | pathogen                            | 6                                |
|        | B. valaisiana         | pathogen                            | 2                                |
Table 3. Associations between tick-specific and environmental variables and the occurrence of endosymbionts and human pathogens in *I. ricinus* ticks. A positive sign indicates that higher variable values are associated with a higher probability of OTU occurrence. A higher aspect value means that a site is facing northwards. Only associations with strong statistical support (based on the 90% central credible interval) are presented.

| Full variable set | Variable selection set |
|-------------------|------------------------|
| Tick sex (Female) | Tick population expected heterozygosity | Number of days > 7°C | Precipitation | Mean temperature | Forest cover | Slope | Aspect | Vole abundance | Vole/other rodents ratio |
| Tick life stage (Nymph) | | | | | | | | | |
| Tick abundance | | | | | | | | | |
| Tick heterozygosity | | | | | | | | | |
| Elevation | | | | | | | | | |

| OTU | Spiroplasma | Rickettsiella | Lariskella | Rickettsia helvetica | R. monacensis | Rickettsia sp. | Anaplasma | Ca. Neoehrlichia | Borrelia afzelii | B. miyamotoi | B. garinii | B. valaisiana |
|-----|-------------|--------------|------------|---------------------|---------------|------------|----------|----------------|--------------|--------------|------------|-------------|
| OTu0003 | − |  |  | + | − | + |  |  |  |  |  |  |
| OTu0005 |  | + |  |  |  |  |  |  |  |  |  |  |
| OTu0022 |  |  |  |  |  |  |  |  |  |  |  |  |
| OTu0031 |  |  |  | + | − | + | + |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| OTu0067 |  |  |  |  |  |  |  |  |  |  |  |  |
| OTu0076 |  |  |  |  |  |  |  |  |  |  |  |  |
| OTu0086 |  |  |  |  |  |  |  |  |  |  |  |  |
| OTu0088 |  |  |  |  |  |  |  |  |  |  |  |  |

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**Figure legends**

Figure 1: Location of tick sampling sites in the Swiss Alps. Different colours of circles (i.e., black, grey, white) represent the different locations. Rivers and motorway are shown in black. Modified from [98].

Figure 2: Association between bacterial alpha diversity (inverse Simpson index) and elevation at three locations in the Swiss Alps. Bacterial alpha diversity was consistently lower at higher elevations. Model predictions for each location are plotted as lines.

Figure 3: Tick microbial community variance partitioning for different fixed and random effects. The first three columns represent tick endosymbionts, the next three columns are OTUs which are both tick endosymbionts and human pathogens and the subsequent six columns represent human pathogens. The other columns represent the 88 most common OTUs found in *I. ricinus*, ordered by amplicon frequency. Month, sampling site, location and tick ID were included in the model as random effects, whereas fixed effects were divided into environmental (elevation, temperature, precipitation, forest coverage, slope, aspect, vole abundance and vole to other rodents ratio) and tick-related variables (life stage or sex, individual heterozygosity, abundance, expected population heterozygosity). See raw data in Figshare for information on OTU labels.

Figure 4: Facilitation or competition among endosymbionts and pathogens within ticks. Relationships after accounting for shared environmental preferences are shown. Red lines represent positive associations (i.e., facilitation) and blue lines negative associations (i.e., competition). Only associations with strong statistical support (i.e., based on the 90% central credible interval) are presented. Darker colors indicate stronger associations.
