Bacterial microbiota composition of *Ixodes ricinus* ticks: the role of environmental variation, tick characteristics and microbial interactions

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Ecological factors, host characteristics and / or interactions among microbes may all shape the occurrence of microbes and the structure of microbial communities within organisms. In the past, disentangling these factors and determining their relative importance in shaping within-host microbiota communities has been hampered by analytical limitations to account for (dis)similar environmental preferences ('environmental filtering'). Here we used a joint species distribution modelling (JSDM) approach to characterize the bacterial microbiota of one of the most important disease vectors in Europe, the sheep tick *Ixodes ricinus,* along ecological gradients in the Swiss Alps. Although our study captured extensive environmental variation along elevational clines, the explanatory power of such large-scale ecological factors was comparably weak, suggesting that tick-specific traits and behaviours, microhabitat and -climate experienced by ticks, and interactions among microbes play an important role in shaping tick microbial communities. Indeed, when accounting for shared environmental preferences, evidence for significant patterns of positive or negative co-occurrence among microbes was found, which is indicative of competition or facilitation processes. Signals of facilitation were observed primarily among human pathogens, leading to co-infection within ticks, whereas signals of competition were observed between the tick endosymbiont *Spiroplasma* and human pathogens. These findings highlight the important role of small-scale ecological variation and microbe-microbe interactions in shaping tick microbial communities and the dynamics of tick-borne disease.

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Abstract

Ecological factors, host characteristics and / or interactions among microbes may all shape the occurrence of microbes and the structure of microbial communities within organisms. In the past, disentangling these factors and determining their relative importance in shaping within-host microbiota communities has been hampered by analytical limitations to account for (dis)similar environmental preferences ('environmental filtering'). Here we used a joint species distribution modelling (JSDM) approach to characterize the bacterial microbiota of one of the most important disease vectors in Europe, the sheep tick *Ixodes ricinus,* along ecological gradients in the Swiss Alps. Although our study captured extensive environmental variation along elevational clines, the explanatory power of such large-scale ecological factors was comparably weak, suggesting that tick-specific traits and behaviours, microhabitat and -climate experienced by ticks, and interactions among microbes play an important role in shaping tick microbial communities. Indeed, when accounting for shared environmental preferences, evidence for significant patterns of positive or negative co-occurrence among microbes was found, which is indicative of competition or facilitation processes. Signals of facilitation were observed primarily among human pathogens, leading to co-infection within ticks, whereas signals of competition were observed between the tick endosymbiont *Spiroplasma* and human pathogens. These findings highlight the important role of small-scale ecological variation and microbe-microbe interactions in shaping tick microbial communities and the dynamics of tick-borne disease.

Keywords: tick-borne pathogens, species distribution modelling, community composition, Borrelia burgdorferi, Lyme disease

Introduction

Microbial communities within organisms consist of symbionts, commensals, mutualists and pathogens that co-occur simultaneously and potentially influence each other (Petney and Andrews 1998; Rigaud et al. 2010; Sofonea et al. 2015). 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 (Adair and Douglas 2017). For example, certain microbial species might tolerate only specific abiotic conditions, which makes it more likely that species with similar requirements co-occur within a host ('environmental filtering', Dallas and Presley, (2014)). Similarly, the host's immune system can influence colonization success of microbes (Hawley and Altizer 2011), with cross-immunity preventing the colonization of different microbes with similar antigenic properties (Durand et al. 2015). Furthermore, mutualistic interactions between hosts and microbes can influence the structure of bacterial communities within host individuals (Chu and Mazmanian 2013; Lee et al. 2013). 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 (Lello et al. 2004), whereas facilitation may occur directly through the production of public goods (West and Buckling 2003) or indirectly through the modification of the host's physiology (Abraham et al. 2017) or immune defense (Rodríguez et al. 1999).

Ixodes ricinus is the most common tick species in Europe and an important vector for a range of human, domestic animal and wildlife pathogens (Medlock et al. 2013). Its distribution and abundance are strongly influenced by environmental conditions, in particular temperature and humidity (Cortinas et al. 2002; Gatewood et al. 2009). Previous studies that characterized the

bacterial community composition of *I. ricinus* ticks have found mostly environmental and free-living bacteria but also several endosymbionts and human, domestic animal or wildlife pathogens, including *Borrelia* (Mannelli et al. 2012), *Rickettsia* (Venclikova et al. 2014), *Anaplasma* (Jahfari et al. 2014) and *Candidatus* Neoehrlichia (Kawahara et al. 2004).

Differences in the bacterial community structure and composition of ticks across habitats (Estrada-Peña et al. 2018), geographical sites (Carpi et al. 2011), and tick life stages and sexes (Carpi et al. 2011; Vayssier-Taussat et al. 2013) have been documented. Large-scale biotic or abiotic factors such as vegetation structure, elevation, temperature or rainfall may influence tick microbial communities directly, or indirectly through effects on tick physiology or activity patterns (van Treuren et al. 2015) or via influencing the distribution and abundance of tick hosts species (Randolph et al. 1999; MacDonald et al. 2017). Small-scale and/or tick-specific effects on microbial communities may be explained by microhabitat or microclimatic conditions experienced by individual ticks (Gern et al. 2008), individual tick behavior or genetics (Hawlena et al. 2013), direct biotic interactions among microbes (Moutailler et al. 2016) or parallel acquisition from a host during a bloodmeal (Andersson et al. 2014; Belli et al. 2017; Swei and Kwan 2017).

Currently, the relative importance of these factors in shaping tick microbial communities is not well understood, which hampers progress in our understanding of the processes shaping microbial communities in nature and predicting the occurrence of specific microbes (e.g., human pathogens). Elevational gradients are excellently suited to quantify the importance of large-scale ecological variation in shaping tick bacterial microbiota because they cover a large range of environmental conditions within a small geographical area. Furthermore, including replicated

transects along gradients allow us to quantify the robustness of ecological associations within sites and along elevational clines on tick microbial communities.

Ixodes ticks are commonly found to be co-infected with several (human, domestic animal and/or wildlife) pathogens (Andersson et al. 2013; Michelet et al. 2014; Diuk-Wasser et al. 2016; Moutailler et al. 2016). Currently, it is unknown whether these co-infection patterns are caused by similar environmental preferences of pathogens, parallel acquisition from host communities or direct microbe-microbe interactions within ticks. Yet, previous studies suggest that the latter process, (i.e., facilitation and competition processes among microbes) may play a role in shaping microbial communities (Haine 2008; Bonnet et al. 2017). For example, it has been found that pathogenic *Rickettsia* species prevent co-infection with other *Rickettsia* species in *Dermacentor variabilis* ticks (Macaluso et al. 2002), whereas the presence of *Francisella* sp. endosymbionts increases the colonization success of pathogenic *Francisella novicida* in *D. andersoni* ticks (Gall et al. 2016). Facilitation has also been suggested to promote co-infection with different *Borrelia afzelii* strains in *Ixodes ricinus* ticks (Andersson et al. 2013). Most strikingly, dysbiosis in *I. scapularis* ticks (i.e., ticks with low microbiotal diversity) leads to 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 invade ticks (Narasimhan et al. 2014). Thus, the microbial community may have a crucial impact on vector competence of ticks and thereby on disease dynamics.

Yet, as outlined above, co-occurrence of microbes can be due to environmental filtering or direct microbial interactions, and distinguishing between these processes is non-trivial. Indeed, previous studies that have documented pathogen co-occurrence in ticks have not accounted for potential confounding variables such as shared ecological requirements, and are thus limited in

101 their ability to differentiate between co-occurrences due to shared environmental niches, and co-
102 occurrence shaped by facilitation or competition among microbes.

103 To address these gaps, we exploited the substantial environmental heterogeneity along replicated
104 elevational gradients in the Swiss Alps to quantify the relative importance of environmental
105 factors, tick characteristics and direct microbial interactions in influencing the structure of
106 bacterial communities in *I. ricinus* ticks in general, and the (co-)occurrence of pathogens in
107 particular, using a combination of 16S sequencing and joint species distribution modelling
108 (JSDM) (Ovaskainen et al. 2015; Warton et al. 2015). By taking shared environmental
109 preferences into account, JSDMs allows to identify residual co-occurrence patterns among
110 microbes that can result from unaccounted environmental effects or direct microbial interactions.
111 However, the correct spatial scale with regards to the focal biological processes is of importance,
112 as well as the type of the hypothesized biotic interaction (Araújo and Rozenfeld 2014; Zurell et
113 al. 2018) when interpreting JSDM patterns (Dormann et al. 2018).

114 Specifically, we ask (i) how do large-scale abiotic factors and small scale tick-level variables
115 affect tick microbiota composition, (ii) which large-scale abiotic and small-scale tick-level
116 variables predict pathogen occurrence, and (iii) are there patterns of non-random microbial co-
117 occurrence that cannot be explained by environmental responses and might be due to
118 unmeasured variables, such as microbial interactions.

119

120 **Materials and methods**

121

122 *Tick sampling*

123 Questing *Ixodes ricinus* ticks were collected at three locations in the Swiss Alps (Kanton
124 Graubünden). At each location, one site at low (630 - 732 m above sea level, masl), one at
125 medium (1 094 – 1 138 masl) and one at high (1 454 – 1 673 masl) elevation were identified
126 (Fig. 1, Table 1, N = 9 sampling sites). At each site, questing ticks were sampled thrice, once in
127 June, once in July, and once in August 2014 by dragging a white blanket (1 m x 1 m) over the
128 ground vegetation as described previously (Lemoine et al. 2018). Ticks were collected from the
129 blanket and stored in 95% ethanol. Tick species, sex and life stage were verified by
130 morphological features following (Hillyard 1996) using a stereomicroscope.

131

132 *Environmental variables*

133 For each sampling site, we compiled information on large-scale, site-level ecological variables
134 by obtaining data on elevation, slope and aspect using DHM25, land use data from swissTLM3D
135 (both from Federal Office of Topography swisstopo) and data on temperature and precipitation
136 from Landscape Dynamics (Swiss Federal Research Institute for Water, Snow and Landscape
137 Research WSL and Federal Office of Meteorology and Climatology MeteoSwiss, (Thornton et
138 al. 1997)). Data on *I. ricinus* abundance and the abundance of a key tick host, the bank vole
139 (*Myodes glareolus*), as well as the ratio of bank vole to other rodents at our sampling sites were
140 obtained from Cornetti et al. (2018). Details on the different variables and a justification why
141 they were included to characterise large-scale ecological conditions is provided in the
142 Supplementary Material.

143

144 *Tick DNA isolation and quantification of neutral genetic diversity*

145 The number of analysed ticks per site is presented in Table 1. Although we aimed to include
146 similar numbers of *I. ricinus* ticks from each sampling site and month, variation in the number of
147 ticks per site (Table 1) was unavoidable because of variation in tick abundance across sites
148 (Lemoine et al. 2018). To avoid contamination, we performed DNA isolation and amplifications
149 in a laminar flow cabinet. Each tick was washed thrice with sterile water before sterilizing it with
150 3% hydrogen peroxide. Ticks were then cut in half with a sterilized blade to facilitate DNA
151 isolation. DNA was extracted using DNeasy Blood & Tissue kit (Qiagen; Hilden, Germany).

152 Host genetics may affect pathogen and endosymbiont colonisation and replication success
153 (Archie and Ezenwa 2011). In order to quantify individual and population-level genetic diversity,
154 we genotyped ticks at 11 microsatellite markers in two multiplexed amplifications (see
155 Supplementary Material for details). Not all markers were successfully amplified in all samples,
156 but none of the samples contained more than two failed markers. We used package *poppr*
157 (Kamvar et al. 2014) in R 3.4.1 (Team 2013) to test for linkage disequilibrium and deviation
158 from Hardy-Weinberg equilibrium. Individual observed heterozygosity was determined for each
159 tick as a proportion of heterozygous markers to all successfully amplified markers. Expected
160 population level heterozygosity was determined with *poppr*. The former was used as a tick-level
161 explanatory variable (together with tick sex and life stage), the latter was used as a site-level
162 explanatory variable.

163

164 *Tick microbiota sequencing*

165 We characterized tick bacterial community composition by sequencing the hypervariable V4
166 region of the 16S rRNA (16S) gene. Negative controls (extraction reagent blank, N=2 and PCR

167 controls, N=3) were processed alongside the tick samples. Sequencing libraries were prepared
168 following the Earth Microbiome 16S Illumina Amplicon protocol, using the primers 515FB and
169 806RB (Carey et al. 2013) (see Supplementary Material for details). Samples and negative
170 controls were randomized across two plates. The libraries were sequenced on Illumina MiSeq at
171 the Functional Genomic Center Zurich with a target length of 250 bp following the
172 manufacturer's protocol. The obtained sequence data were analyzed following the *mothur*
173 pipeline with MiSeq standard operation procedures (Kozich et al. 2013). Sequences have been
174 deposited to the Sequence Read Archive under BioProject PRJNA506875. The complete
175 metadata of the samples and their matching sequence accession numbers have been submitted to
176 FigShare (doi: 10.6084/m9.figshare.7380767).

177 As we are not able to assess whether individual OTUs are resident or not, and we do not know
178 their transmission routes, a special focus of our analysis was on tick endosymbionts and tick-
179 borne human, domestic animal or wildlife pathogens (Table 2), which are obligate residents. This
180 approach does not mean that the other OTUs would not have a substantial effects on ticks and
181 other tick symbionts. Identification of endosymbionts and pathogens is described in the
182 Supplementary Material.

183

184 *Joint species distribution modelling of microbiota composition*

185 Only samples with > 500 reads and OTUs which were present in at least two samples were
186 included in the analyses (Table 1). As the most common OTU, the intra-mitochondrial
187 endosymbiont *Candidatus* Midichloria (Lo et al. 2006), was present in all samples, it was not

188 included in the modelling. For the occurrence matrix, an OTU was determined to be present in a
189 tick if >5 reads were identified in a sample (following Aivelo and Norberg 2017).

190 We used a JSDM framework called Hierarchical Modelling of Species Communities (HMSC,
191 Ovaskainen et al. 2017a) to examine how environmental variables correlate with pathogen and
192 tick endosymbiont occurrence in ticks, and whether there are non-random residual associations
193 among different OTUs and/or oligotypes, implying direct facilitation or competition effects
194 among microbes. This approach combines information on environmental covariates, bacterial
195 species traits, spatiotemporal context and sampling design to explain the presence or absence of
196 OTUs (Fig. S2). The associations among OTUs are captured with the latent part of the
197 framework, modelling the residual variance after accounting for the effects of the environment
198 with latent variables. The estimates for these latent variables can be then translated into residual
199 correlations among response variables, i.e. OTUs and/or oligotypes. These correlations thus
200 reflect (dis)associations which cannot be explained by shared responses to the environment.

201 We compiled occurrence matrices for OTUs for each individual tick as a response variable. For
202 each sampling unit, i.e. a row in our response variable matrix, we included information on the
203 identity of the sampling unit (tick ID), its location, sampling site (for which we included also the
204 spatial structure as coordinates) and month, describing the study design. To reach a better
205 resolution within specific OTUs, we analyzed known human, domestic animal or wildlife
206 pathogens, tick endosymbionts and their close relatives within the 100 most common OTUs with
207 oligotyping pipeline (Eren et al. 2014). Oligotyping uses all the sequences, which form an OTU,
208 and performs Shannon Entropy Analysis to regroup sequences based on within-OTU variation.
209 This results in higher-resolution grouping than OTUs as the different oligotypes might differ
210 only by a single nucleotide (Eren et al. 2014). We used the standard operation procedures of the

211 oligotyping pipeline software (http://oligotyping.org; Eren et al. 2013). We labelled the resulting
212 oligotypes through BLAST search (Camacho et al. 2009). For some species, such as *Rickettsia*
213 spp., the V4 region of 16S might not have enough resolution (Greay et al. 2018), and thus, the
214 labels should not be considered as definite identifications.

215 Including a large number of explanatory variables in statistical models is inherently challenging.
216 To reduce the number of variables, while maintaining their information value, we used two
217 variable sets in the model: a) a set of full-effect explanatory variables, and b) explanatory
218 variables under variable selection (Ovaskainen et al. 2017b). The full-effect variable set included
219 an intercept, two tick-level variables (tick sex or life stage and individual heterozygosity) and
220 two site-level variables (tick abundance and elevation of the sampling site). Additionally, we
221 included information whether a specific OTU is an endosymbiont and/or a human, domestic
222 animal or wildlife pathogen (Abrego et al. 2016). This allowed us to test if endosymbionts and/or
223 pathogens respond differentially to environmental conditions than other OTUs. The set of
224 explanatory variables under variable selection included additional information on the
225 environmental conditions of the sites (namely the number of days above 7 °C during the year,
226 monthly precipitation, mean monthly temperature, forest coverage, slope, aspect, bank vole
227 abundance, the proportion of voles to other rodents and expected tick heterozygosity) (Table S1).
228 We considered all parameter estimates, including associations among bacterial OTUs, having
229 strong statistical support and thus being ’significant‘ if the 90% central credible interval of the
230 parameter did not overlap with zero (see Supplementary Material for additional model details).
231 The model was run in Matlab R2017 (The MathWorks; Natick, MA, USA).

232

233 **Results**

234

235 Ixodes ricinus *microbiota composition*

236 We 16S sequenced the bacterial community of 92 *Ixodes ricinus* ticks which resulted in 13 214
237 477 reads. No amplification was observed in the five negative controls (i.e., their sequencing did
238 not result in any reads) and one tick was sequenced twice. After contig assembly and quality
239 control 1 656 287 reads were retained. Most of the discarded reads were either shorter than 250
240 bp or chimeras. There was a median of 1 562 quality-controlled reads per sample, with an
241 interquartile range of 6 319. 82 samples with more than 500 reads per sample, a plateauing
242 accumulation curve and a Good's coverage estimator ≥0.95 were included in the subsequent
243 analyses (Fig. S1). In total, 5 181 bacterial OTUs were identified. The median number of OTUs
244 when rarified to 500 reads per sample was 89 OTUs, with a 95% confidence interval of 78.3 -
245 98.5 OTUs.

246 Six OTUs were present in at least 90% of the samples: *Ca.* Midichloria (Otu0001),
247 *Sphingomonas* (Otu0002, 0006 and 0007*)*, *Pseudomonas* (Otu0011) and *Delftia* (Otu0012).
248 Together, they represented 50.2 % of all reads. We used oligotyping to further divide OTU0031
249 '*Rickettsia*' into two oligotypes labelled as '*R. helvetica*' and '*R. monacensis*', and OTU0086
250 '*Borrelia*' into four oligotypes labelled as '*B. afzelii*', '*B. valaisian*a' and '*B. garinii*' and '*B.*
251 *miyamotoi*'. 635 OTUs and oligotypes were used in subsequent analyses, including 14
252 endosymbionts and / or human, domestic animal or wildlife pathogens (Table 2).

253

254 *Tick microbiota variance partitioning*

255 Variance partitioning revealed that most of the variation in tick microbiota composition
256 explained by our model related to tick ID: for the hundred most common OTUs, tick ID
257 accounted for 64.1% of the variation explained by the model. Fixed effects (e.g., tick life stage,
258 elevation; see Table S1) accounted for 12.5% (tick-level: 7.3%, site-level: 5.2%) and spatial and
259 temporal random effects (i.e., location, site and month) explained 23.3% (Fig. 2). This suggests
260 that there is extensive tick-level variation which cannot be accounted for by tick-specific
261 characteristics included in our model (i.e., sex, life stage, genetic diversity) or site-level
262 environmental factors. The pattern differed slightly for endosymbionts and human, domestic
263 animal or wildlife pathogens: while tick ID was still the most important variable explaining
264 49.9%, fixed effects explained 31.8% (tick-level: 17.5%, site-level: 14.3%) and spatial and
265 temporal random effects explained 18.3% of the total variation explained by the model, when
266 averaged over all pathogens and endosymbionts (Fig. 2). Thus, tick- and site-level fixed effects
267 explained a larger proportion of the variation in the occurrence of obligate resident pathogens
268 and endosymbionts than the occurrence of other (potentially non-resident) OTUs.

269

270 *Tick-specific and environmental factors related to OTU occurrence*

271 The occurrence of tick endosymbionts and pathogens was strongly associated with specific
272 explanatory variables, yet associations were typically microbe-specific rather than universal
273 (Table 3). The two most important variables explaining the presence or absence of tick
274 endosymbionts and human, domestic animal or wildlife pathogens were tick sex and elevation of
275 the sampling site: adult female ticks were less likely to harbour the endosymbionts *Spiroplasma*,
276 *Rickettsiella*, *Lariskella* and *Rickettsia* spp. (Table 3), and ticks at higher elevations had higher
277 probability to harbour *R. helvetica* and *R. monacensis*, but were less probable to harbour *B.*

278 *garinii* (Table 3). Slope and aspect were also significant predictors of tick endosymbionts and
279 pathogen occurrence, with ticks from sites facing northwards having a higher probability of
280 harbouring *Spiroplasma* and *B. afzelii*, and ticks from sites on steeper slopes having a higher
281 probability of harbouring *Rickettsia sp*. (Table 3). Furthermore, a higher tick abundance was
282 associated with a higher probability of *Rickettsiella* and *Ca.* Neoehrlichia occurrence (Table 3).
283 Relationships between tick life stage, mean temperature, the number of days > 7 °C or forest
284 cover and the occurrence of specific OTUs were not strongly statistically supported.

285 The effect sizes of strongly statistically supported associations varied substantially (Fig. S4a-i).
286 For example, threefold increase in vole abundance corresponded to less than one percentage
287 point decrease of *R. monacensis* prevalence (Fig. S4b), whereas a threefold increase in tick
288 abundance corresponded to a threefold increase in *Neoehrlichia* prevalence from 8% to 27%
289 (Fig. S4e).

290

291 *Patterns of microbial association within ticks*

292 Numerous bacterial OTUs were either significantly more or less likely to co-occur within a tick
293 than expected by chance after accounting for shared environmental preferences (Fig. 3a; Table
294 S2). At the level of the individual tick, the occurrence of the tick endosymbiont *Spiroplasma* was
295 negatively associated with the occurrence of the endosymbiont *Lariskella* and several tick-borne
296 pathogens, namely *Rickettsia* sp., *Ca*. Neoehrlichia and *B. miyamotoi* (Fig. 3a). Associations
297 among pathogens, if they occurred, were all positive (Fig. 3a), suggesting that ticks are more
298 likely to be co-infected with several human, domestic animal or wildlife pathogens
299 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*. At the level of the sampling site, significant associations were sparser. *Spiroplasma* was more likely to co-occur with *Lariskella* and *Rickettsiella* across sites, whereas the latter two were less likely to co-occur across sites than expected by chance after accounting for shared environmental preferences (Fig. 3b). At the level of month or location, there were no significant associations.

Discussion

We used a JSDM framework to quantify the relative importance of large scale, site-level environmental variables, tick-level characteristics and interactions among microbes in shaping tick microbiota composition along elevational gradients in the Swiss Alps. We show that although our study captured extensive environmental variation, with sampling sites spanning across an elevational gradient from 630 – 1 580 masl, and a large number of ecological variables was considered in our models, the explanatory power of such large-scale ecological factors was comparably weak. In contrast, individual tick ID explained over 60% of the variation in microbiota composition. This substantial microbiota variation across individual ticks may be partly explained by some of the bacteria present in ticks being non-resident (i.e., bacteria that were by chance obtained from the environment through the mouth, the anal pore or spiracles or during blood-feeding; Horner-Devine and Bohannan 2006; Engel and Moran 2013; Zolnik et al. 2016, 2018; Ross et al. 2018). Indeed, there has been a debate whether ticks have a stable microbiota (Ross et al. 2018), mirroring the wider debate on how common resident microbiota is in arthropod hosts (Hammer et al. 2017).

However, also for endosymbionts and human, domestic animal or wildlife pathogens, which are obligate resident, tick ID accounted for half of the variation in occurrence, suggesting that microhabitat or -climatic conditions experienced by individual ticks, tick-specific traits and behaviors not included in our models, as well as microbial interactions within ticks such as facilitation and competition (Abraham et al. 2017; Gurfield et al. 2017), play a crucial role in shaping microbiota composition and the occurrence of endosymbionts and human or wildlife pathogens in *I. ricinus*. Focusing on such small-scale variables, rather than large-scale climatic or environmental factors as is usually done when modelling tick-borne pathogen prevalence (Norman et al. 2016; Rosà et al. 2018), is thus likely a more fruitful approach to advance our understanding of microbiota composition of natural populations as well as (tick-borne) disease dynamics.

Co-occurrence of human, domestic animal or wildlife pathogens in ticks has been documented previously, both in *I. ricinus* (Lommano et al. 2012; Michelet et al. 2014) and other tick species (Gurfield et al. 2017; Laaksonen et al. 2018). Yet, previous studies did not control for environmental filtering, which limited their ability to disentangle shared responses to the environment from direct microbe-microbe interactions. Our study revealed that when accounting for shared environmental preferences, associations among human or wildlife pathogens were often pronounced and mostly positive. These positive associations may result from direct facilitation among microbes or parallel colonization from co-infected tick hosts. Because our sampling unit was the whole tick, whereas bacteria inhabiting a tick can be situated in different organs, co-occurrence at the tick-level does not necessarily mean that there is direct interaction between co-occurring OTUs, although indirect interactions, via, e.g., host immune system, can still occur.

346

347 Within ticks, the significant positive associations among the Lyme disease-causing *Borrelia*
348 genospecies (*B. afzelii*, *B. garinii* and *B. valaisiana*) were particularly striking. This positive co-
349 occurrence is surprising because *B. garinii* and *B. valaisiana* are bird specialists (Hanincova et
350 al. 2003b; Comstedt et al. 2011), whereas *B. afzelii* is a rodent specialist (Hanincova et al.
351 2003a). Thus, the parallel colonization from co-infected tick hosts cannot explain this pattern.
352 Rather the positive co-occurrence is indicative of facilitation processes among *Borrelia*
353 genospecies, as has been suggested previously (Andersson et al. 2013). Such facilitation, and the
354 resulting co-infection of ticks with several *Borrelia* genospecies has implications for the severity,
355 diagnosis, treatment and control of Lyme disease. Finally, the co-occurrence of these different
356 *Borrelia* genospecies suggests that *I. ricinus* feeds on multiple, phylogenetically diverse host
357 species during its life cycle and does not show pathogen-mediated host specialization as has been
358 suggested previously (McCoy et al. 2005, 2013).

359 Although associations among microbes were mostly positive, there were negative associations
360 between the tick endosymbiont *Spiroplasma* and several human or wildlife pathogens, which
361 may be explained by competition. The most common infection route for *Spiroplasma* is maternal
362 (i.e., vertical) transmission (Herren and Lemaitre 2011), indicating that horizontal or
363 environmental transfer plays a minor role in its transmission. Protective effects of *Spiroplasma*
364 have been previously described in *Drosophila* spp., where *Spiroplasma* is associated with a
365 decreased probability of nematode and parasitoids infections (Xie et al. 2010; Jaenike et al.
366 2013). Although the exact mechanisms mediating *Spiroplasma*-induced competition effects are
367 currently unknown, this finding may stimulate further research into the potential of tick
368 endosymbionts to manage tick-borne pathogens.

In contrast to the numerous positive or negative associations among microbes at the tick-level, little statistical support for positive or negative microbial co-occurrence was found at the site-level, with the exception of the associations among three endosymbionts. Interestingly, the pattern of co-occurrence of *Spiroplasma* and *Lariskella* at the site-level was opposite from what was observed at the tick-level. It suggests that *Spiroplasma* and *Lariskella* are more likely to co-occur at the same sites but not within the same tick. Co-occurrence at the site-level can be due to an environmental variable not included in our model, for which the three OTUs had similar responses. It has also been suggested that negative associations generate checkerboard patterns of co-occurrence that can be captured at finer spatial scales but that are lost with increasing scales, but positive associations can be captured across scales (Araújo & Rozenfeld 2013).

Despite the large among-tick variation in microbiota composition, we identified a range of environmental variables that significantly predicted the occurrence of specific tick endosymbionts and human, domestic animal or wildlife pathogens. However, the predictor variables as well as their effect were typically OTU-specific rather than universal. 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 (Halos et al. 2010; Eremeeva and Dasch 2015). Yet, it has previously been found that spotted fever incidence in humans, caused by *R. ricketsii*, is highest in areas or regions, where ticks are less common (Atkinson et al. 2013). This is in line with our findings and suggests that *Rickettsia* spp. are more likely to colonize ticks living under suboptimal conditions (e.g. at range edges).

The finding that *B. garinii* is less likely to occur at higher elevations is in line with previous observations (Jouda et al. 2004b; Cornetti et al. 2018) and may be explained by changes in

vegetation structure and associated changes in host communities (Halos et al. 2010), in particular changes in the diversity and/or abundance of birds, the natural hosts of *B. garinii* (Comstedt et al. 2011). In contrast, the occurrence of the mammal specialist *B. afzelii* was not related to elevation, potentially because elevational clines in mammal diversity and/or abundance are less pronounced (McCain 2005). Indeed, we did not observe an association between elevation and bank vole abundance across our study sites (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 human or wildlife pathogens, with the exception of precipitation correlating positively with the probability of *Rickettsiella* occurrence. This may be partly explained by the temperature and precipitation measures included in our models not fully capturing the microclimatic variation across sites and along elevational clines. Indeed, slope and aspect, which are important determinants of the topography, and thus microclimate (Bennie et al. 2008), were significant predictors of pathogen and endosymbiont occurrence. The probability of *Rickettsia* sp. occurrence was higher on steeper slopes. Furthermore, the probability of occurrence was higher on north-facing slopes for *B. afzelii* and *Spiroplasma* and higher on south-facing slopes for *Rickettsiella* (see also (Stuen et al. 2013)). Microclimatic conditions may affect microbial occurrence directly, or indirectly via affecting tick behavior or host community composition (Swei et al. 2011; Lawson et al. 2014). Furthermore, topography can affect population connectivity and dispersal in metapopulation networks (Swei and Kwan 2017).

Previous work has found that tick abundance is a strong predictor of *Borrelia* spp. prevalence, potentially because larger tick populations facilitate co-feeding transmission (Jouda et al. 2004a).

No relationship 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 (Keesing et al. 2006). Whereas for some tick-borne pathogens the vertebrate hosts are known or suspected (e.g. small mammals for *B. afzelii* (Hanincova et al. 2003a) and *Ca*. Neoehrlichia (Jahfari et al. 2012), birds for *B. garinii* and *B. valaisiana* (Hanincova et al. 2003b), both for *Anaplasma* (Keesing et al. 2012) and *R. helvetica* (Sprong et al. 2009)), for others the host species range is less well understood (e.g. *B. miyamotoi* ;Wagemakers et al. 2015). The bank vole is a common tick host at our study sites and their abundance was a significant negative predictor of *R. monacensis* and *R. helvetica* occurrence. Interestingly, bank voles are not known hosts for either (Burri et al. 2014). Most likely, the relation is thus indirect, explained by an unmeasured biotic or abiotic variable that correlates with bank vole abundance. No evidence was found that the proportion of bank voles to other rodents affects the prevalence of tick-borne pathogens.

A limitation of our sampling design is the uneven sample distribution across sites. We collected ticks up to the upper elevational limit of tick distribution, which leads to a large variation in environmental variables included in our models, but at the same times means that we have a limited number of samples from the high elevation sites. Yet, adequate model fit suggests that this uneven sample distribution did not compromise model performance. Furthermore, although JSDM is a powerful approach to model community structure, it has a number of limitations. First, it assumes that interactions among microbes are similar across environments (but see

Tikhonov et al. 2017). This is not necessarily the case as both environmental factors and tick host community may shape microbial interactions (Elliot et al. 2002). Second, the model assumes that the explanatory variables affect the microbial community composition (or rather, the presence or absence of individual OTUs), 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 explanatory variables poses a problem to any correlative modelling approach. Our model is built on two distinct variable sets to aid in handling 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. Fourthly, the inferred residual associations between focal taxa are assumed to be symmetrical. If there are asymmetric interactions (e.g., predator-prey-relationships), the sum outcome can be seen as either positive or negative correlation (Zurell et al. 2018). However, in our study, the expectation was facilitation or competition, which are symmetric positive or negative interactions, respectively. Thus, given sufficient signal, we expect that the focal interactions can be captured by our modelling approach.

Conclusions

Our study demonstrates that a JSDM framework can contribute to a better understanding of the factors shaping bacterial communities in natural populations as well as patterns of co-occurrence among microbes. Overall, our study highlights the role of small-scale, tick-level characteristics rather than large-scale ecological variation in shaping microbial communities of *I. ricinus*. We identified a number of ecological variables that predict the occurrence of specific tick endosymbionts and human, domestic animal or wildlife pathogens with strong statistical support,

but these effects were typically microbe-specific rather than universal. This highlights that environmental change can have different, even opposite effects on different human pathogens, and thus disease risk. Furthermore, by accounting for shared environmental preferences, our approach identified patterns of microbial co-occurrence that are consistent with microbe-microbe interactions, which result in pathogen co-infections within ticks, as well as competition between *Spiroplasma* and a number of human, domestic animal or wildlife 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(on next page)

Tick sampling sites in the Swiss Alps.

1 Table 1: Tick sampling sites in the Swiss Alps.

Location	Site	Coordinates		Elevation	Succesfully sequenced *Ixodes ricinus* ticks		
		North	East	(masl)	nymphs	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

2

Table 2(on next page)

Common tick endosymbionts and/or putative human pathogens observed in *I. ricinus* ticks.

See Supplementary Materials for information on OTU assignment.

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* Neoehrlichia	both	22
Otu0088	*Borrelia afzelii*	pathogen	9
	B. miyamotoi	pathogen	10
	B. garinii	pathogen	6
	B. valaisiana	pathogen	2

Table 3(on next page)

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.

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 life stage (Nymph)	Tick abundance	Tick heterozygosity	Elevation	Tick population expected heterozygosity	Number of days > 7C°	Precipitation	Mean temperature	Forest cover	Slope	Aspect	Vole abundance	Vole/ other rodents ratio
Otu0003	*Spiroplasma*	−											+		−
Otu0005	*Rickettsiella*	−		+	−		−		+				−		
Otu0022	*Lariskella*	−			−										
Otu0031	*Rickettsia helvetica*					+								−	
	R. monacensis					+	−							−	
Otu0067	*Rickettsia sp.*	−										+			
Otu0076	*Anaplasma*														
Otu0086	*Ca.* Neoehrlichia			+											
Otu0088	*Borrelia afzelii*												+		
	B. miyamotoi														
	B. garinii					−									

B. valaisiana

6

Figure 1

Location of tick sampling sites in the Swiss Alps.

Different shapes (i.e., circle, square and triangle) represent the different locations and different colours represent elevation (white: low, grey: middle, black: high). Rivers and motorway are shown in black. Map data © 2019 Google, GeoBasis-DE/BKG.

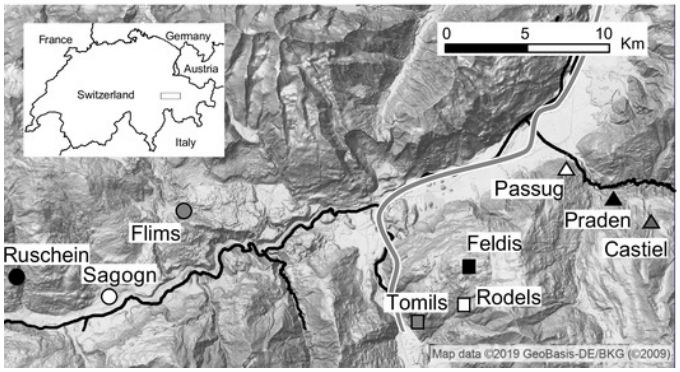

Figure 2

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 read 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-specific variables (life stage or sex, individual heterozygosity, abundance, expected population heterozygosity). See raw data in Figshare for information on OTU labels.

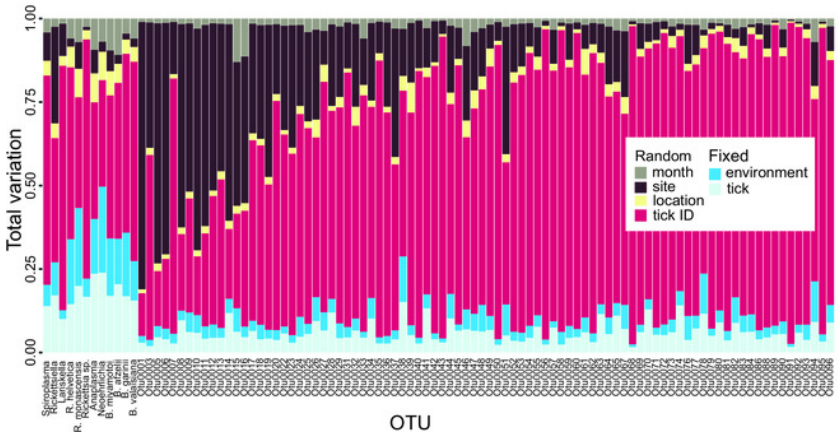
Total variation
0.00
0.25
0.50
0.75
1.00
OTU
Random
month
site
location
tick ID
Fixed
environment
tick

Figure 3

Residual association patterns among endosymbionts and human pathogens within ticks on a) individual tick-level and b) on site-level after accounting for shared environmental preference.

Red lines represent positive associations and blue lines negative associations. Only associations with strong statistical support (i.e., based on the 90% central credible interval) are presented. Darker colors indicate stronger associations.