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1 **Idiosyncratic effects of coinfection on the association between systemic pathogens and the gut**
2 **microbiota of a wild rodent, the bank vole (*Myodes glareolus*)**

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20 **Abstract**

21 1. The effects of systemic pathogens on gut microbiota of wild animals are poorly understood.

22 Furthermore, coinfections are the norm in nature, yet most studies of pathogen-microbiota
23 interactions focus on effects of single pathogen infections on gut microbiota.

24 2. We examined the effects of four systemic pathogens (bacteria *Anaplasma phagocytophilum* and
25 *Borrelia burgdorferi* sensu lato, apicomplexan protozoa *Babesia microti*, and Puumala
26 orthohantavirus) and coinfections among them on the (bacterial) gut microbiota of wild bank voles
27 (*Myodes glareolus*).

28 3. We hypothesized that: (1) the effects of coinfection on gut microbiota generally differ from those
29 of a single pathogen infection, (2) systemic pathogens have individual (*i.e.*, distinct) associations
30 with gut microbiota, which are modified by coinfection, and (3) the effects of coinfection
31 (compared with those of single infection) are idiosyncratic (*i.e.*, pathogen-specific).

32 4. The gut microbiota of coinfecting bank voles differed from that of single pathogen infected
33 individuals, though, as predicted, the effects of coinfections were unique for each pathogen. After
34 accounting for coinfections, only Puumala orthohantavirus was associated with higher α -diversity,
35 however, all pathogens affected gut microbiota β -diversity in a pathogen-specific way, affecting
36 both rare and abundant gut bacteria.

37 5. Our results showed that the effects of systemic pathogens on host's gut microbiota vary depending
38 on the pathogen species, resulting in idiosyncratic signatures of coinfection. Furthermore, our
39 results emphasize that neglecting the impact of coinfections can mask patterns of pathogen-
40 microbiota associations.

41 **Keywords:** Apicomplexa, bacteria, coinfection, disease ecology, gut microbiota, host-pathogen
42 interactions, pathogens, virus

43 **Introduction**

44 Vertebrates harbour diverse communities of commensal microbes in their gastrointestinal tract,
45 collectively called the gut microbiota, that provide essential services to their host (Lee & Hase, 2014).
46 For example, gut microbiota provides the host with important metabolites from otherwise
47 undigestible food (Morrison & Preston, 2016), and is involved in crosstalk with the host's immune
48 system (Zheng et al., 2020), hindering colonization by gastrointestinal pathogens (Buffie & Pamer,
49 2013), and training the host's immune system (Pickard et al., 2017). As the gut microbiota is an
50 essential part of host's physiology and health (McFall-Ngai et al., 2013) there is much interest to
51 identify factors associated with changes in gut microbiota.

52 Both, pathogens and gut microbiota have complex interactions with the host's immune system
53 (Vonaesch et al., 2018). Thus it is perhaps unsurprising, that infection by pathogens can affect gut
54 microbiota α -diversity (within-sample diversity) and β -diversity (between-sample diversity) (Libertucci
55 & Young, 2019), e.g., increasing the inter-individual variation of microbiota β -diversity (the so-called
56 Anna Karenina principle (Zaneveld et al., 2017, see Methods)). The specific effects on gut microbiota
57 depend on the pathogen and host species, for example, the gastrointestinal coccidian parasite
58 *Eimeria sp.* is associated with increased α -diversity of gut microbiota in rufous mouse lemur
59 (*Microcebus rufus*) (Avelo & Norberg, 2018), while Adenovirus infection was associated deterministic
60 shifts in β -diversity, and decreased inter-individual variation of the gut microbiota of the grey-brown
61 mouse lemur (*Microcebus griseorufus*) (Wasimuddin et al., 2019). Furthermore, gut microbiota may

62 itself influence an individual's susceptibility to infections (Fleischer et al., 2022; Kubinak et al., 2015;
63 Libertucci & Young, 2019), potentially affecting disease dynamics, for example, by affecting pathogen
64 shedding (Murray et al., 2020). Given the potential consequences of pathogen-microbiota interactions
65 for host health and disease dynamics, it is crucial to determine how pathogen infections affect wild
66 animal gut microbiota.

67 Most studies on host microbiota-pathogen associations typically focus on the effects of pathogens
68 inhabiting the same environment as microbiota, for example, skin microbiota and fungal skin infection
69 (Lemieux-Labonté et al., 2020) or gut microbiota and gastrointestinal parasites (Avelo & Norberg,
70 2018; Kreisinger et al., 2015). However, numerous pathogens occur in the blood or affect multiple
71 tissues (so-called systemic pathogens). Despite the zoonotic threat posed by many of the systemic
72 pathogens (Han et al., 2016), the relationship between host's gut microbiota and systemic pathogens
73 has received far less attention than local pathogen-microbiota associations.

74 Comparably, most studies on pathogen-microbiota interactions focus on infections by a single
75 pathogen, though coinfections are common in nature (Hoarau et al., 2020; Stutz et al., 2018; Telfer et
76 al., 2010). Coinfecting pathogens can affect host's health and immune response (Clerc et al., 2019;
77 Djokic et al., 2019). In addition, pathogen interactions can affect disease dynamics, for example,
78 reduced gastrointestinal nematode burden increased the prevalence of Sin Nombre orthohantavirus
79 in two species of rodents (Sweeny et al., 2020). Few studies have addressed the effects of coinfecting
80 pathogens on host's gut microbiota, especially in wild animals. Though their results indicate that
81 coinfection may affect pathogen-microbiota interactions. For instance, coinfection with
82 gastrointestinal nematodes and bovine tuberculosis (*Mycobacterium bovis*) modified the changes in
83 α -diversity and affected the abundance of several bacterial taxa of African buffalo (*Syncerus caffer*)

84 gut microbiota (Sabey et al., 2021). The pathogen-specific interactions with host's gut microbiota
85 could be masked if coinfections are not accounted for, thus potentially leading to spurious
86 conclusions on pathogen-microbiota associations (Sabey et al., 2021; Schmid et al., 2022).

87 Our overarching aim was to quantify systemic pathogen-microbiota associations in wild bank voles
88 (*Myodes glareolus*). These widely distributed rodents are hosts to many zoonotic pathogens (Abbate
89 et al., 2021). The four pathogens of our study (bacteria *Anaplasma phagocytophilum* and *Borrelia*
90 *burgdorferi* sensu lato, apicomplexan protozoan *Babesia microti* and Puumala orthohantavirus) are
91 common in bank voles (Cayol, Jääskeläinen, et al., 2018; Kallio et al., 2014; Voutilainen et al., 2016),
92 making the bank vole an excellent wildlife model species to study associations between single or
93 multiple pathogen infections and gut microbiota. While these pathogens typically cause
94 asymptomatic infections and apparently have limited effects on animal health and fitness (see
95 Methods), complex interactions between these pathogens have been described in rodents (Djokic et
96 al., 2019; Holden et al., 2005; Telfer et al., 2010).

97 We hypothesize that (1) the effects of coinfection on gut microbiota generally differ from those of a
98 single pathogen, and the differences are evident even irrespective of pathogen's identity. However,
99 given the specificity of pathogen's associations with gut microbiota and host's immunity, we
100 hypothesize that (2) associations between each pathogen and gut microbiota are, in fact, unique and
101 are modified by coinfection. Moreover, we adopt a recently proposed framework (Schmid et al.,
102 2022) to define the effects of coinfection on gut microbiota. Specifically, we test whether the
103 apparent impacts of a coinfection on gut microbiota relative to a single infection are (a) antagonistic
104 (counteracting those of a single-pathogen infection, (b) neutral (similar), or (c) synergistic (if the
105 coinfection exacerbates the effects of a single pathogen infection). Thus, we hypothesize (3) the

106 effects of coinfection compared to those of a single infection (antagonistic, neutral, synergistic) are
107 idiosyncratic (dependent on the pathogen identity), such that analyses of any pathogen-microbiota
108 associations will be modified by unrecognised coinfections.

109 **Methods and materials**

110 **Animal capture and sampling**

111 We trapped bank voles in August 2018 from two locations in Finland: Kemi-Tornio and Harjavalta (Fig.
112 S1) using live traps for two consecutive nights. Trapped animals were immediately euthanized using
113 cervical dislocation, transferred onto dry ice, and stored at -80°C until dissecting. Bank vole ($n=230$)
114 weight, sex, and gravidity status were determined during dissection (Table S1). Organs, ear biopsies,
115 and faeces (from the distal 2 cm of the colon) were stored at -80°C. All animal procedures followed
116 the Finnish Act on the Use of Animals for Experimental Purposes, approved by the Finnish Animal
117 Experiment Board (ESAVI-3981-2018). Additional information on animal sampling is available in
118 electronic supplementary material (ESM1).

119 **Microbiota DNA extraction and sequencing data processing**

120 We used PowerFecal DNA Kit (Qiagen, Germany) to extract total DNA from faeces. Samples ($n=200$,
121 excluding samples with small DNA yield) were sequenced on Illumina MiSeq (250 bp paired-end
122 reads) using 515F/806R primers (Caporaso et al., 2011) targeting the V4 region of 16S rRNA. Full
123 details on sequencing data processing are provided in ESM1 and Brila et al., 2021. Briefly, data were
124 denoised using DADA2 (Callahan et al., 2016) plugin in QIIME2 v.2019.10 (Bolyen et al., 2019) and
125 taxonomy was assigned to amplicon sequence variants (ASVs) based on SILVA v.132 database (Yilmaz
126 et al., 2014). We removed low-abundance ASVs (<10 reads in the entire data set), and ASVs not

127 assigned to a bacterial phylum or that were classified as mitochondria or chloroplasts. After
128 rarefaction (27,000 reads/sample) the final dataset consisted of 192 samples (one was below
129 rarefaction threshold and seven samples failed sequencing), representing 3,648 ASVs and 5,184,000
130 reads. Rarefied dataset was used in analyses unless stated otherwise.

131 **Pathogen screening**

132 We determined the status (positive/negative) of four pathogens. Puumala orthohantavirus (PUUV) is
133 a directly and indirectly (through contaminated bedding) (Kallio et al., 2006) transmitted RNA virus.
134 PUUV reservoir hosts are bank voles (Brummer-Korvenkontio et al., 1980), in which the infection is
135 asymptomatic (Bernshtein et al., 1999) with limited effects on host health, though lower over-winter
136 survival has been reported (Kallio et al., 2007). In humans, PUUV causes haemorrhagic fever with
137 renal syndrome (Olsson et al., 2010). To detect PUUV antibodies, we rinsed the heart of each bank
138 vole in 200 µl of sterile PBS (Voutilainen et al., 2012) and screened these samples using
139 immunofluorescence assay (Kallio-Kokko et al., 2006). As PUUV infection and shedding are lifelong
140 (Voutilainen et al., 2015), all seropositive voles were considered infected.

141 *Anaplasma phagocytophilum* (Ap) are intracellular bacteria that infect host's granulocytes, primarily
142 neutrophils (Rikihisa, 2010). In rodents, Ap usually causes short infection (Foley et al., 2004) with
143 transient cytopenias (Johns et al., 2009), although long-term persistence in several species can occur
144 (Rar et al., 2020). In humans, Ap is the causative agent of human granulocytic anaplasmosis (Bakken &
145 Dumler, 2015). Babesiosis causing apicomplexan protozoa *Babesia microti* (Bm) infect erythrocytes
146 (Chauvin et al., 2009) and cause persistent, asymptomatic infection in rodent hosts (Sherlock et al.,
147 2013; Taylor et al., 2018). Ap and Bm are tick-borne pathogens, in Finland primarily transmitted by
148 *Ixodes trianguliceps* (Kallio et al., 2014). *Borrelia burgdorferi* sensu lato (Bbsl) are spirochete bacteria,

149 transmitted by *I. ricinus* in our study region (Cayol, Jääskeläinen, et al., 2018) that cause tick-borne
150 Lyme disease in humans (Cook, 2014), while in rodents the infection is usually asymptomatic, with
151 limited health effects (Cayol, Giermek, et al., 2018; Zhong et al., 2019).

152 We used spleen samples to detect Ap and Bm and ear biopsy samples to detect the presence of Bbsl.
153 DNA from spleens was extracted using DNeasy Blood & Tissue kit (Qiagen, Germany) and from ear
154 biopsies following method by Laird et al., 1991. The presence or absence of Ap, Bm, and Bbsl was
155 assessed using qPCR-based assays (ESM1).

156 **Statistical analyses**

157 To examine whether differences in gut microbiota of single infected *versus* coinfecting animals are
158 evident irrespective of pathogen identity we categorised animals into three groups: “N”- no infection,
159 “S”- infected by a single pathogen, and “C”- coinfecting animals. Then we tested whether each
160 pathogen associates with specific changes in gut microbiota, using the status of each infection (P-
161 positive, N-negative), irrespective of coinfection status. Last, we examined how coinfection status
162 affects the previously found associations between gut microbiota and specific pathogens, by
163 categorising animals into coinfection groups for each pathogen (*e.g.*, Ap-N, Ap-S and Ap-C),
164 irrespective of the specific coinfections (Table S1). Furthermore, we used a framework proposed by
165 Schmid et al., 2022 to compare the effects of coinfections on gut microbiota to those of a single
166 infection. The framework defines “synergistic” effects of coinfection as those that exacerbate changes
167 in the gut microbiota. When coinfection counteracts the effects of a single pathogen infection (*e.g.*, so
168 that the microbiota becomes more like that of uninfected animals) the effects of coinfection are
169 described as antagonistic. Effects of coinfection are neutral if they do not differ from those of a single-
170 pathogen infection (Schmid et al., 2022).

171 We calculated three metrics of α -diversity: Shannon's diversity index and (ASV) richness using
172 phyloseq v.1.40.0 (McMurdie & Holmes, 2013), and Faith's phylogenetic diversity using picante v.1.8.2
173 (Kembel et al., 2010). As Shannon's diversity index models showed non-normality of residuals, we
174 used bestNormalize v.1.8.3 (Peterson, 2021) to find the best transformation of the response variable,
175 and, consequently, Yeo-Johnson transformed Shannon's index was used in all analyses. For each
176 metric, we used linear mixed models (LMMs) fitted using lmerTest v.3.1-3 (Kuznetsova et al., 2017)
177 with degrees of freedom and p-values calculated according to Satterthwaite's method and conditional
178 and marginal R^2 calculated using MuMIn v.1.47.1 (Bartoń, 2022).

179 We quantified microbiota β -diversity using four metrics: Jaccard index (JI) and unweighted UniFrac
180 distance (u-UniFrac) were used to characterize community composition (presence/absence of ASVs),
181 while Bray-Curtis dissimilarity (BCD) and weighted UniFrac distance (w-UniFrac) were used to
182 characterize community structure (presence of ASVs weighted by their abundance). UniFrac distances
183 use phylogenetic tree branch length and thus account for phylogenetic distance between samples
184 (Lozupone et al., 2007). BCD and JI were calculated using phyloseq and u-/w-UniFrac were calculated
185 using rbiom v.1.0.3 (Smith, 2021).

186 As β -diversity reflects community-wide differences between individuals, the effects on β -diversity are
187 most often characterized as either deterministic or stochastic. Deterministic effects, seen as a change
188 in the location of the group centroid, indicate a group-wide shift to a different microbiota community
189 configuration. Stochastic changes are seen as increase in inter-individual variation (dispersion) within
190 a group and have been termed the Anna Karenina principle (AKP, Zaneveld et al., 2017), indicating
191 that microbiota of each individual responds uniquely. Deterministic shifts in β -diversity were tested

192 using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, using
193 adonis2 in vegan v.2.6-2 (Anderson, 2001), calculating the marginal effects of terms included in the
194 models. Pairwise differences between groups were calculated using pairwiseAdonis v.0.4 (Arbizu,
195 2017) with FDR control using Benjamini-Hochberg adjustment. The differences in community
196 dispersion were calculated using betadisper with bias adjustment for small sample size followed by
197 permutest in vegan. Metrics of community composition are qualitative and provide insight into
198 contribution of rare ASVs, while metrics of community structure are quantitative and emphasize the
199 influence of abundant ASVs (Lozupone et al., 2007). Thus, to aid interpretation of the results, we
200 classified ASVs based on their average relative abundance across all samples. ASVs with average
201 relative abundance <0.001% were classified as “rare” ($n=1,816$), those with average relative
202 abundance >0.05% as “abundant” ($n=239$), and remaining ASVs were classified as “intermediate”
203 ($n=1,593$) (Jiao et al., 2017; Pan et al., 2022) (Table S2). Additionally, differentially abundant ASVs and
204 genera were identified using ANCOM-BC v.1.6.2 (Lin & Peddada, 2020) using the unrarefied data.

205 As host traits and environmental heterogeneity can affect gut microbiota (Heitlinger et al., 2017;
206 Mallott et al., 2020), we included host’s sex/reproductive status (male, gravid female, non-gravid
207 female), location, and previously assigned metal pollution group (Brila et al., 2021) as covariates in all
208 microbiota analyses. Correlations between pollution group and variables of interest were assessed
209 using Pearson’s χ^2 test followed by calculation of Cramér’s V with bias correction. As only weak
210 correlations with BBsl and PUUV were detected ($p=0.02$ and 0.03 , Cramér’s $V=0.15$ and 0.17 ,
211 respectively), pollution group was included in all models. All statistical analyses were done in R v.4.2.1
212 (R Core Team, 2022), and R packages ggplot2 v.3.3.6 (Wickham, 2016), ggvenn v.0.1.9 (Yan, 2021) and
213 patchwork v. 1.1.2 (Pedersen, 2022) were used for data visualization.

214 **Results**

215 **Infection prevalence in wild bank voles**

216 Of the 192 animals, 89 (46%) had no infection, 67 (35%) had one infection, and 36 (19%) were
217 coinfecting. We identified nine different coinfections (Fig. 1), with one animal infected by all four
218 pathogens. The pathogen-specific prevalence was: Ap 15% (95% CI: 10-20%, $n=28$), Bm 26% (20-32%,
219 $n=49$), Bbsl 24% (18-30%, $n=47$), and PUUV 13% (8-18%, $n=25$). The prevalence of individual
220 pathogens confirms previous reports (Cayol, Jääskeläinen, et al., 2018; Kallio et al., 2014; Olsson et al.,
221 2010) that these pathogens are endemic and widespread in bank voles in Finland. Additional details
222 on pathogen prevalence are shown in Table S3.

223 **Association between gut microbiota and coinfection status irrespective of pathogen identity**

224 Animals with a single infection (S) had slightly higher phylogenetic diversity than animals with no
225 infections (N) ($q=0.09$, $R^2=0.02$), but no differences were detected between S and C (coinfecting) or
226 between N and C animals, or between any of the groups based on Shannon's diversity index of ASV
227 richness (Table S4).

228 Coinfection status had weak associations with gut microbiota β -diversity (Fig. S2, Table S5). We
229 identified shifts in community composition between N and C animals (JI, $q=0.024$, $R^2=0.011$; u-
230 UniFrac, $q=0.045$, $R^2=0.013$), and marginally significant shift between S and C animals (JI, $q=0.057$,
231 $R^2=0.012$). We found marginal and weak shifts in community structure based on BCD between N and S
232 animals ($q=0.07$, $R^2=0.012$) and S and C animals ($q=0.07$, $R^2=0.015$). Additionally, N animals had
233 marginally higher group dispersion than S animals (u-UniFrac, $p=0.098$), while S animals had higher
234 dispersion than C animals (BCD, $p=0.034$).

235 Consistent with differences between N, S and C animals in β -diversity, we identified more DA ASVs
236 between N and C animals ($n=530$) than between N and S animals ($n=382$), with 434 ASVs DA between
237 S and C animals (Table S6). With similar pattern at the genus level (N-S $n=9$; N-C $n=14$) except no
238 genera were DA between S and C animals (Table S7).

239 **Association between gut microbiota and individual pathogens, irrespective of coinfection status**

240 Of the four pathogens tested, only PUUV (Table S8) was associated with marginally higher Shannon's
241 diversity index ($p=0.06$, $R^2=0.016$) and ASV richness ($p=0.08$, $R^2=0.014$) and significantly higher Faith's
242 phylogenetic diversity ($p=0.03$, $R^2=0.021$).

243 Infection by Ap was associated with shifts in both community composition and structure (*e.g.*, w -
244 UniFrac $p=0.03$, $R^2=0.02$), and higher community dispersion (Fig. 2A). Bm associated with marginal
245 shifts in community composition (*e.g.*, JI, $p=0.09$, $R^2=0.006$) but not structure; and lower community
246 dispersion (*e.g.*, JI, $p=0.02$, Fig. S3A). Bbsl was associated with lower community dispersion (*e.g.*, Fig.
247 3A). While PUUV was associated with shifts in community composition (*e.g.*, JI, $p=0.023$ $R^2=0.006$).

248 Full results for each pathogen and metric are provided in Table S9.

249 While we identified many DA ASVs associated with each pathogen ($n=674-1028$, Table 1), 442 (35% of
250 the total number of DA ASVs) of them were DA for all pathogens (Tables S10-13). Similarly, of the 34
251 DA genera, six were DA for all pathogens (Tables S14-17). Most taxa DA between all pathogens based
252 on both ASV and genus level analysis belonged to families *Ruminococcaceae* and *Lachnospiraceae*
253 (Tables S10-17).

254 **Association between gut microbiota and coinfection status of individual pathogens**

255 Accounting for coinfections revealed that the marginal increase in Shannon's diversity was driven by
256 coinfecting animals (PUUV_{N-C} (PUUV-N compared to PUUV-C animals): $q=0.047$, $R^2=0.026$), while only
257 single-infected animals had marginally higher phylogenetic diversity (PUUV_{N-S}: $q=0.08$, $R^2=0.03$, Table
258 S18).

259 Deterministic shifts in community composition and structure associated with Ap infection were
260 counteracted by coinfection (*e.g.*, JI, Fig. 2B and u-UniFrac, Ap_{N-S}: $q=0.021$, $R^2=0.01$, Ap_{S-C}: and
261 $q=0.048$, $R^2=0.06$). Similarly, higher group dispersion in Ap-S animals was counteracted by coinfection
262 (Fig. 2B). Considering coinfection status removed the deterministic shift in composition associated
263 with Bm and PUUV infection. The lower community dispersion in Bm-S animals was reversed by
264 coinfection (Fig. S3B), likewise, the increased dispersion associated with PUUV-S was predominantly
265 reduced by coinfection (Fig. S4).

266 Contrasting the effects of Bm and PUUV described above, considering coinfections revealed
267 deterministic shifts in community composition associated with Bbsl. Antagonistic effects indicated by
268 u-UniFrac (Bbsl_{N-S}: $q=0.024$, $R^2=0.01$) contrasting the synergistic effects indicated by JI (Fig. 3B) suggest
269 effects on phylogenetically related, rare taxa. Indeed, though 27% Bbsl-N, 22% Bbsl-S, and 13% Bbsl-C
270 ASVs were unique to the respective group, the majority of unique ASVs (~81-85%) being rare (Table
271 S3), most of the ASVs (~68-78%) unique to each group were from three families – *Lachnospiraceae*,
272 *Ruminococcaceae*, and *Muribaculaceae*. Furthermore, Bbsl was also associated with lower community
273 dispersion with neutral effects of coinfection on the rare (Fig. 3B), and synergistic effects on the

274 abundant microbiota members (Table S19). For detailed results on each pathogen and metric see
275 Tables S18-19.

276 We found a higher number of DA ASVs when coinfection is considered *versus* when ignored for all
277 pathogens (Table 1, Tables S10-17). Out of 81 DA genera, 28 were DA across all pathogens, 16 of
278 which were DA across all pathogens between both N-S and N-C animals (Fig. S5). Only 15 genera were
279 DA abundant in only one pathogen and one infection status comparison. For example, an uncultured
280 member of genus *Barnesiella* had a strong negative association with a single PUUV infection, but not
281 with PUUV coinfection or any other pathogen. Similar to analyses when coinfection status was
282 ignored, the two families most represented in DA analysis were *Lachnospiraceae* and
283 *Ruminococcaceae* (Fig. S5).

284 **Discussion**

285 Infections by intestinal pathogens are often associated with changes in the gut microbiota of wild
286 animals. However, the effects of systemic pathogens, and crucially, coinfections on host microbiota
287 are poorly understood. Here, we quantified the association between four systemic pathogens and the
288 gut microbiota of their reservoir host, the bank vole. We found that each of the four systemic
289 pathogens associated with specific changes in gut microbiota. Likewise, the effects of coinfection on
290 any pathogen-microbiota associations were pathogen-specific.

291 **Lack of universal signal of coinfection in gut microbiota of wild bank voles**

292 While multiple studies have indicated that pathogens can affect the gut microbiota of their wildlife
293 host (Avelo & Norberg, 2018; Wasimuddin et al., 2019), very few of these have considered possible
294 effects of coinfection (Sabey et al., 2021; Schmid et al., 2022). Yet, coinfections are ubiquitous in

295 wildlife (Hoarau et al., 2020), and indeed nearly 19% of voles in our study were coinfecting (Fig. 1). It is
296 therefore important to understand whether coinfection may affect the association between a
297 pathogen and host-associated microbiota. We found that the differences between the effects of a
298 single pathogen infection versus coinfection were evident even if pathogen identity was ignored, thus
299 supporting our first hypothesis. Uniform effects of coinfection on gut microbiota, compared to single
300 pathogen infection, across different pathogens, could suggest that the additive pathogen burden and
301 thus energetic and physiological cost to the host drive the differences in gut microbiota. However, the
302 unique association between gut microbiota and each pathogen shows that a single, universal
303 signature of coinfection in gut microbiota is unlikely. Which is perhaps unsurprising, given the
304 complexity and specificity of host-pathogen interactions and the intricate interactions between
305 coinfecting pathogens.

306 **Pathogen-specific effects of systemic pathogens on gut microbiota**

307 Systemic pathogens had limited effects on gut microbiota α -diversity, as only PUUV was associated
308 with higher α -diversity. In this instance, the higher phylogenetic α -diversity may suggest any impacts
309 of PUUV on host's ability to control microbiota membership (*e.g.*, increased growth of dormant or
310 transient bacterial lineages) may be counteracted by coinfecting pathogens. Higher α -diversity has
311 been associated with stability (Flynn et al., 2011) and instability of gut microbiota (Coyte et al., 2015),
312 with contrasting effects on resistance against disturbances and pathogen invasions (Lozupone et al.,
313 2012; Reese & Dunn, 2018). As such, whether the slightly higher α -diversity can affect the microbiota
314 function and host health remains unknown.

315 Though infection by any systemic pathogen associated with changes in gut microbiota β -diversity, the
316 pattern of association depended on the pathogen's identity. Ap and Bbsl associated with both

317 deterministic and stochastic changes in gut microbiota. Although effects on both group centroid and
318 dispersion can be statistically confounded and therefore must be interpreted with caution (Anderson
319 & Walsh, 2013), these results may hint towards complex interactions between pathogens and gut
320 microbiota. While Ap and PUUV (Fig. 2B and S4, respectively) associated with AKP effects, Bm (Fig.
321 S3B) and Bbsl (Fig. 3B) were associated with anti-AKP effects. Such contrasting effects of pathogens
322 on gut microbiota β -diversity are common, as an analysis of 27 case studies on human microbiome
323 disease found evidence for AKP effects in approximately 50% of the studies and anti-AKP effects in
324 25% of cases (Ma, 2020). While AKP effects may suggest host's inability to control community
325 membership and an influx of opportunistic bacteria, anti-AKP effects may indicate a restricted
326 community membership, loss of (possibly important) taxa, or dominance of community by few taxa
327 (Zaneveld et al., 2017). Whether AKP or anti-AKP effects indicate change in services delivered by the
328 microbiota to the host would require further studies (*e.g.*, using metagenomic or metatranscriptomic
329 methods).

330 The observed variation in associations between the gut microbiota and the four pathogens supports
331 our second hypothesis on pathogen-specificity of associations and may arise due to differences in
332 each pathogen's interaction with host's immune system and effects on host's physiology, and health
333 (Johns et al., 2009; Sherlock et al., 2013; Taylor et al., 2018; Zhong et al., 2019).

334 **Overlooking coinfection can impact the conclusions about pathogen-microbiota associations**

335 Disregarding the underlying coinfection status of the host inflated the increase in phylogenetic
336 diversity of gut microbiota associated with PUUV infection and modified the associations of all
337 pathogens with β -diversity. The evident loss of deterministic shift, associated with PUUV and Bm after

338 accounting for coinfections, may be driven by the small number of single-infected animals compared
339 to positive animals (PUUV: 8 vs 25, Bm: 21 vs 49). The emergence of a deterministic shift in
340 composition associated with Bbsl infection suggests that coinfection may obscure effects of single
341 pathogen infections, while of the four pathogens, only Ap had the same association with β -diversity
342 when coinfection status was ignored (Table 2). Thus, further supporting our second hypothesis, our
343 data provides evidence that coinfections can modify the pathogen-microbiota associations in an
344 idiosyncratic (pathogen-specific) way. Given the high diversity of pathogens in wild animals (Han et
345 al., 2016), we suggest future studies on pathogen-microbiota associations account for possible
346 coinfections using data on pathogen prevalence in the specific species and region, to decrease the
347 likelihood of erroneous findings on pathogen-microbiota interactions.

348 **Effects of coinfection on pathogen-microbiota associations depend on the pathogen's identity**

349 While the effects of coinfection with Ap and with Bm on gut microbiota β -diversity were antagonistic,
350 the effects on Bbsl and PUUV were less clear. For example, effects of coinfection on Bbsl-microbiota
351 association were antagonistic, neutral, or synergistic depending on the β -diversity metric used. While
352 such complexity may seem ambiguous, it emphasizes the benefits of using multiple β -diversity metrics
353 (qualitative, quantitative, and those including phylogenetic information) to gain deeper insights into
354 which members of the gut microbiota are most affected by the specific pathogen. For example, using
355 findings of three β -diversity metrics showed that Bbsl may affect phylogenetically related, rare taxa
356 on a group-wide scale (deterministic effects), while also affecting rare and abundant taxa at individual
357 host level (anti-AKP effects). Thus, coinfection may counteract the effects of Bbsl on gut microbiota
358 via altered host's control of microbiota membership, and an influx of rare, possibly transient taxa,
359 while also affecting the abundant members of the community.

360 The pathogen-specificity of modifying effects of coinfection supports our third hypothesis and is likely
361 due to the complex interactions between pathogens. For example, interactions among Ap, Bbs1 and
362 Bm have been shown to affect disease severity and infection susceptibility in rodents (Djokic et al.,
363 2019; Holden et al., 2005; Telfer et al., 2010). While we were not able to examine specific
364 coinfections, due to high number of different coinfections relative to our sample size (Fig. 1), our
365 findings strengthen the emerging studies demonstrating confounding effects of coinfection on
366 pathogen-microbiota interactions (Sabey et al., 2021; Schmid et al., 2022). Furthermore, our findings
367 and the ubiquity of coinfections in wild animals encourage future studies to examine the effects of
368 specific coinfections on gut microbiota and potentially, host's health.

369 **Pathogens might be able to affect hosts through effects on host-associated microbiota**

370 High inter-individual variation of gut microbiota β -diversity is typical for wild animals, as it can be
371 affected by numerous host and environmental factors, such as variation host's diet (Maurice et al.,
372 2015), social interactions (Raulo et al., 2021) and anthropogenic disturbances (Fackelmann et al.,
373 2021). The 0.4-5.6% of variation in microbiota β -diversity explained by systemic pathogens exceeded
374 the variation explained by environmental variables examined in this study (Tables S5, S9, S19), and
375 the effect sizes were comparable to those of gastrointestinal pathogens (Martínez-Mota et al., 2021;
376 Vlčková et al., 2018). Therefore, the effects of systemic pathogens on β -diversity may be biologically
377 relevant, as these pathogens could affect the host not only directly, but potentially via effects on host
378 microbiota. For example, genera from two broadly represented families *Lachnospiraceae* and
379 *Ruminococcaceae*, that were DA for all pathogens (Tables S10-17, Fig. S5), are important for digestion
380 of plant material (Biddle et al., 2013). Furthermore, effects on rare taxa are unknown. Yet, emerging
381 evidence from gut microbiota studies suggests that loss of rare taxa can be detrimental to the host, as

382 they can potentially affect host fitness (Antwis et al., 2019) and might provide a reserve for
383 maintaining community function under changing environmental and host conditions (Jousset et al.,
384 2017). Given our limited understanding of the interaction between systemic immunity and gut
385 microbiota (Zheng et al., 2020), experimental studies are needed to unravel mechanisms behind
386 systemic pathogen-gut microbiota associations.

387 If systemic pathogens can affect host's physiology and health via effects on gut microbiota, gut
388 microbiota may play part in vicious circle of disease (Beldomenico & Begon, 2010). In fact, host
389 microbiota has been shown to affect disease progression and outcome and proposed as a fourth edge
390 in "disease pyramid" (an extension of disease triangle), to describe disease as a four-way interaction
391 between the host, host's microbiota, pathogen, and the environment host inhabits (Bernardo-Cravo
392 et al., 2020). Yet, little is known about this four-way association in wild animals and potential
393 consequences to host health and disease spread, though many wild animals are reservoirs of zoonotic
394 pathogens (Han et al., 2016). As studies so far have (a) predominantly examined interactions between
395 gut microbiota and gut parasites, (b) been done on laboratory animals and (c) examined effects of
396 single pathogen infections, we lack proper understanding of microbiota-pathogen interactions in the
397 context of infection heterogeneity (*e.g.*, number of concurrent infections, infection length and
398 sequence of infections) that is typical in wild animal populations. Thus, future research should aim to
399 consider gut microbiota when studying host-pathogen associations, and account for coinfections,
400 when examining pathogen-microbiota associations, enabling a fuller understanding of health and
401 disease in wild animal populations.

402 **Authors' contributions**

403 Ilze Brila, Eva Kallio, Tapio Mappes and Phillip Watts designed the research; Ilze Brila, Anton
404 Lavrinienko, and Eugene Tukalenko collected the samples, Ilze Brila completed the laboratory work,
405 analysed the data and wrote the original draft. All authors contributed to the methodology and final
406 version of the manuscript and approved it for publication.

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417 **Data availability statement**

418 Raw reads are available from NCBI Sequence Read Archive, BioProject number PRJNA702897. All
419 metadata is available in Table S1, and the code used in analysis is available at Figshare (Brila, 2022).

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680 **Table 1.**

681 Number of differentially abundant ASVs and genera per each pathogen both irrespective of the
682 coinfection status (N-negative, P-positive) and considering coinfection status (N-negative, S-single
683 infection, C-coinfection including this pathogen). Numbers in parentheses represent number of taxa
684 that have a positive (↑) or negative (↓) association with the (co)infection status when compared to
685 the baseline (N or S). Additionally, number of taxa differentially abundant in both S and C animals
686 compared to N animals (N-S and N-C) given, with the numbers of taxa showing the same association
687 shown in parentheses.

Comparison	<i>Anaplasma phagocytophilum</i>	<i>Babesia microti</i>	<i>Borrelia burgdorferi</i> sensu lato	Puumala orthohantavirus
ASV level				
N-P	992 (374↑, 618↓)	674 (263↑, 411↓)	784 (241↑, 543↓)	1028 (469↑, 559↓)
N-S	1237 (462↑, 775↓)	986 (392↑, 594↓)	1024 (292↑, 732↓)	1264 (495↑, 769↓)
N-C	1220 (467↑, 753↓)	900 (373↑, 527↓)	1028 (392↑, 636↓)	1155 (489↑, 666↓)
N-S and N-C	1150 (209↑, 476↓)	830 (149↑, 300↓)	910 (127↑, 416↓)	1117 (217↑, 430↓)
S-C	0	2 (1↑, 1↓)	1(1↓)	1(1↓)
Genus level				
N-P	28 (9↑, 19↓)	15 (2↑, 13↓)	20 (6↑, 14↓)	28 (14↑, 14↓)
N-S	59 (21↑, 38↓)	33 (9↑, 24↓)	34 (9↑, 25↓)	62 (35↑, 27↓)
N-C	50 (18↑, 32↓)	34 (12↑, 22↓)	35 (16↑, 19↓)	42 (22↑, 20↓)
N-S and N-C	45 (6↑, 16↓)	24 (3↑, 10↓)	29 (4↑, 11↓)	37 (13↑, 9↓)
S-C	1(1↓)	0	0	1(1↑)

688

689 **Table 2.**

690 Associations between pathogens and gut microbiota β -diversity irrespective of the coinfection status
 691 (N-negative, P-positive) and considering coinfection status (N-negative, S-single infection, C-
 692 coinfection including this pathogen). Community composition assessed using Jaccard index and
 693 unweighted UniFrac distance, and structure using Bray-Curtis dissimilarity and unweighted UniFrac
 694 distance. SIC – shifts in group centroid, CID – changes in group dispersion. Arrows indicate direction of
 695 changes. For coinfection the effects are compared to those of a single infection: A- antagonistic, N-
 696 neutral, M-mixed (if two β -diversity metrics show different patterns), S-synergistic.

	<i>Anaplasma phagocytophilum</i>		<i>Babesia microti</i>		<i>Borrelia burgdorferi</i> sensu lato		Puumala orthohantavirus	
Community composition								
Comparison	SIC	CID	SIC	CID	SIC	CID	SIC	CID
N-P	yes	↑	yes	↓	no	↓	yes	no
N-S-C	A	A	no	A	M	N	no	M
Community structure								
	SIC	CID	SIC	CID	SIC	CID	SIC	CID
N-P	yes	↑	no	no	no	↓	no	no
N-S-C	A	A	no	no	no	S	no	A

697

698 **Figure captions**

699 **Figure 1.** Venn diagram of infection and coinfection patterns in bank voles. The numbers within the
700 diagram represent the number of individuals infected or coinfecting with specific pathogens.

701 **Figure 2.** Principal coordinates analyses (PCoA) and nested dispersion boxplots of Jaccard index
702 visualizing association between *Anaplasma phagocytophilum* and community composition of gut
703 microbiota. A-irrespective of coinfection status, Ap is associated with a shift in community
704 composition ($p=0.01$, $R^2=0.01$) and higher community dispersion ($p=0.06$). B-considering coinfection
705 status, we found shifts in community composition between Ap-N and Ap-S ($q=0.01$, $R^2=0.01$) and
706 between Ap-S and Ap-C animals ($q=0.05$, $R^2=0.05$), furthermore, Ap-S animals had higher dispersion
707 than Ap-N ($p=0.02$) and Ap-C ($p=0.07$) animals. Each point represents an individual sample, large dots
708 represent respective group centroids in ordination plots, and the mean distance to group centroid in
709 boxplots.

710 **Figure 3.** Principal coordinates analyses (PCoA) and nested dispersion boxplots of Jaccard index
711 visualizing association between *Borrelia burgdorferi* sensu lato community and composition of gut
712 microbiota. A-irrespective of coinfection status, Bbsl is associated with lower community dispersion
713 ($p=0.07$). B-considering coinfection status, we found shifts in community composition between Bbsl-N
714 and Bbsl-S ($q=0.02$, $R^2=0.01$), Bbsl-N and Bbsl-C ($q=0.02$, $R^2=0.01$) and Bbsl-S and Bbsl-C animals
715 ($q=0.05$, $R^2=0.03$); furthermore, Bbsl-N animals had higher dispersion than Bbsl-S ($p=0.01$) and Bbsl-C
716 ($p=0.06$) animals. Each point represents an individual sample, large dots represent respective group
717 centroids in ordination plots, and the mean distance to group centroid in boxplots.