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

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Abstract

1. The effects of systemic pathogens on gut microbiota of wild animals are poorly understood. Furthermore, coinfections are the norm in nature, yet most studies of pathogen-microbiota interactions focus on effects of single pathogen infections on gut microbiota.
2. We examined the effects of four systemic pathogens (bacteria *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato, apicomplexan protozoa *Babesia microti*, and Puumala orthohantavirus) and coinfections among them on the (bacterial) gut microbiota of wild bank voles (*Myodes glareolus*).
3. We hypothesized that: (1) the effects of coinfection on gut microbiota generally differ from those of a single pathogen infection, (2) systemic pathogens have individual (*i.e*., distinct) associations with gut microbiota, which are modified by coinfection, and (3) the effects of coinfection (compared with those of single infection) are idiosyncratic (*i.e.*, pathogen-specific).
4. The gut microbiota of coinfected bank voles differed from that of single pathogen infected individuals, though, as predicted, the effects of coinfections were unique for each pathogen. After accounting for coinfections, only Puumala orthohantavirus was associated with higher α-diversity, however, all pathogens affected gut microbiota ß-diversity in a pathogen-specific way, affecting both rare and abundant gut bacteria.
5. Our results showed that the effects of systemic pathogens on host's gut microbiota vary depending on the pathogen species, resulting in idiosyncratic signatures of coinfection. Furthermore, our results emphasize that neglecting the impact of coinfections can mask patterns of pathogen-microbiota associations.

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

Introduction

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

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

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

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

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

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

Methods and materials

Animal capture and sampling

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

Microbiota DNA extraction and sequencing data processing

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

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

Pathogen screening

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

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

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

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

Statistical analyses

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

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

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

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

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

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

Results

Infection prevalence in wild bank voles

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

Association between gut microbiota and coinfection status irrespective of pathogen identity

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

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

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

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

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

Infection by Ap was associated with shifts in both community composition and structure (*e.g.,* w-UniFrac *p*=0.03, R^2=0.02), and higher community dispersion (Fig. 2A). Bm associated with marginal shifts in community composition (*e.g.*, JI, *p*=0.09, R^2=0.006) but not structure; and lower community dispersion (*e.g.*, JI, *p*=0.02, Fig. S3A). Bbsl was associated with lower community dispersion (*e.g.*, Fig. 3A). While PUUV was associated with shifts in community composition (*e.g.*, JI, *p*=0.023 R^2=0.006). Full results for each pathogen and metric are provided in Table S9.

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

Association between gut microbiota and coinfection status of individual pathogens

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

Deterministic shifts in community composition and structure associated with Ap infection were 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 q=0.048, R^2=0.06). Similarly, higher group dispersion in Ap-S animals was counteracted by coinfection (Fig. 2B). Considering coinfection status removed the deterministic shift in composition associated with Bm and PUUV infection. The lower community dispersion in Bm-S animals was reversed by coinfection (Fig. S3B), likewise, the increased dispersion associated with PUUV-S was predominantly reduced by coinfection (Fig. S4).

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

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

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

Discussion

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

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

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

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

Pathogen-specific effects of systemic pathogens on gut microbiota

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

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

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

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

Overlooking coinfection can impact the conclusions about pathogen-microbiota associations

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

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

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

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

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

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

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

Authors' contributions

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

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Data availability statement

Raw reads are available from NCBI Sequence Read Archive, BioProject number PRJNA702897. All 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	*Anaplasma phagocytophilum*	*Babesia microti*	*Borrelia burgdorferi* 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

Table 2.

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

	Anaplasma phagocytophilum		*Babesia microti*		*Borrelia burgdorferi* 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

Figure captions

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

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

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