Small scale geographical variation of three-way relationships between gut microbiota, helminth assemblages and bacterial infections in wild rodent populations

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Abstract

Background

Despite its central role in host fitness, the gut microbiota may differ greatly between individuals. This variability is often mediated by environmental or host factors such as diet, genetics, and infections. Recently, a particular attention has been given to the interactions between gut bacteriome and helminths, as these latter could affect host susceptibility to other infections. Further studies are still required to better understand the three-way interactions between gut bacteriome, helminths and other parasites, especially because previous findings have been very variable, even for comparable host-parasite systems.

Methods

In our study, we used the V4 region of the 16S rRNA gene to assess the variability of gut bacteriome diversity and composition in wild populations of a small mammal, the bank vole *Myodes glareolus*. Four sites were sampled at a fine geographical scale (100 km) along a North-South transect in Eastern France. We applied analyses and concepts of community and microbial ecology to evaluate the interactions between the gut bacteriome, the gastro-intestinal helminths and the pathogenic bacteria detected in the spleen.

Results

Regarding the gut bacteriome composition and diversity among bank voles, we identified important variations that were mainly explained by sampling localities and reflected the North/South sampling transect. In addition, we detected two main enterotypes, that might correspond to contrasted diets. We found geographic variations of the gut bacteriome richness and Firmicutes/Bacteroidetes ratio, both correlating positively with body mass index. We found positive correlations between the specific richness of the gut bacteriome and of the helminth community, as well as between the composition of these two communities, even when accounting for the influence of geographical distance. For the pathogenic bacteria community, no broad patterns have been detected. The helminths *Aonchothecha murissylvatici, Heligmosomum mixtum* and the bacteria *Bartonella* sp were the main taxa associated with the whole gut bacteriome composition. Besides, changes in relative abundance of particular gut bacteriome taxa were specifically associated with other helminths (*Mastophorus muris*, *Catenotaenia henttoneni*, *Paranoplocephala omphaloides* and *Trichuris arvicolae*) or pathogenic bacteria. Especially, infections with *Neoehrlichia mikurensis*, *Orientia* sp or *Rickettsia* sp were associated with lower relative abundance of the family

Erysipelotrichaceae (Firmicutes), while coinfections with higher number of bacterial infections were associated with lower relative abundance of a Bacteroidales family (Bacteroidetes).

Conclusions

These results emphasize complex interlinkages between gut bacteriome and infections in wild animal populations. They remain difficult to generalize due to the strong impact of environment, even at fine geographical scales, on these interactions. Abiotic features, as well as small mammal community composition and within host parasite coinfections, should now be considered to better understand the spatial variations observed in the relationships between gut bacteriome, gastro-intestinal helminths and bacterial infections.

Keywords : bank voles, co-infections, interactions, microbial community ecology, zoonoses

Introduction

Vertebrate gut microbiome plays key roles in host fitness, through functions including, among others, nutrient acquisition, immunity and defence against infectious exogenous agents (hereafter called 'parasites' and including micro- and macroparasites) or proliferating indigenous organisms (Belkaid & Hand, 2014; Kamada *et al.*, 2013; Round & Mazmanian, 2009). Nonetheless, the gut microbiome may differ greatly in natural environments between individuals, populations and species (Vujkovic-Cvijin *et al.*, 2020). This variability reflects hosts intrinsic factors (notably phylogeny, genetics or vertical transmission from mother to offspring) as well as extrinsic features (acquisition of microorganisms from the environment, potentially through diet Ley *et al.*, 2008; Moran *et al.*, 2019). It might also reveal disruption of host-associated gut microbiome (termed "dysbiosis") caused by environmental factors, among which are anthropogenic pressures (e.g., chemical exposures, Rosenfeld, 2017) or parasite infections (Trevelline *et al.*, 2019).

Today, it is obvious that understanding the relationships between gut microbiome and parasite transmission is crucial regarding their potential impacts on human and animal health (Clemente *et al.*, 2012). To this extent, vertebrate microbiome studies have placed particular emphasis on the gut bacterial microbiota (called hereafter 'gut bacteriome') and their interactions with gastro-intestinal helminth parasites. On one hand, the gut bacteriome may act as an innate immune barrier to intestinal infections and influence the colonisation and growth of eukaryotic parasites, including helminths, through competitive metabolic interactions or induction of host immune responses (Leung *et al.*, 2018). On the other hand, helminth infections may also affect, directly or indirectly through similar ecological and/or evolutionary processes, the composition of the gut bacteriome, via physical contact, competition for resources or host immunoregulation (see Kreisinger *et al.*, 2015). Helminths and the gut bacteriome interactions may thus lead to positive and negative associations (Loke & Lim, 2015), with potentially local but also systemic physiological changes affecting host health. For example, helminth infections can lead to malnutrition and weight loss, through the dysfunction of microbial metabolism that could result from negative impacts on fermentative gut bacteria (Leung *et al.*, 2018). Besides, some helminth infections favour gut bacteria that produce short-chain fatty acids from dietary fiber. These metabolites circulate throughout the body and are important regulators of host physiology (glucose and fat metabolism) and immune system (Honda & Littman, 2016; Kim, 2021). Interactions between these helminths and gut bacteria may here increase the host anti-inflammatory and regulatory T cell suppressor responses, what may in turn affect host susceptibility to other infections as well as the outcomes of infections (Glendinning *et al.*, 2014).

Besides, the gut microbiota may also influence microparasite infections, through their immune function against pathogenic bacteria colonization and their role in maintaining the intestinal epithelium integrity (Khosravi & Mazmanian, 2013). However, up to now, the systemic impact of gut bacteriome on microparasite infections and vice-versa, represents a fundamental knowledge gap in wild animal populations (Pascoe *et al.*, 2017; Rolhion & Chassaing, 2016).

Moreover, the three-way interactions between host's gut bacteriome, gastro-intestinal helminths and microparasites have been scarcely investigated in a single system, despite clearly becoming pivotal in disease ecology. Yet, the growing interest on gut bacteriome/parasitism relationships in recent literature (Johnson *et al.*, 2015) highlights the critical need for further empirical works, given the relatively low concordance of findings between previous studies – even for comparable host-parasite systems. Up to now, most of the research on this topic have been conducted on model species in laboratory settings. Although experiments under controlled conditions may help emphasizing general patterns and deciphering the mechanisms underlying these interactions between gut bacteriome and parasites in vertebrates (Pascoe *et al.*, 2017), they also have inherent limitations. On the one hand, they only included a restricted number of targeted parasites (usually helminths and/or microparasites), omitting the potential effects of species interactions between and within parasite communities at the intra-host level (Telfer *et al.*, 2010). Yet, co-infections are now recognized to be the rule in most hosts in natural environments, with wild animals that may carry simultaneously a large number of parasites (Hoarau *et al.*, 2020). On the other hand, they are intrinsically unable to include – and then capture the complexity of – the environmental conditions as drivers of the composition of gut bacteriome and of the exposure or sensibility to these latter (Adair & Douglas, 2017). From there, studies in natural contexts therefore deserve strong consideration because these extrinsic factors may impact deeply the relationships between gut bacteriome and parasitism.

Here, we strived to bridge these gaps by assessing the variability of gut bacteriome diversity and composition in wild populations of the bank vole *Myodes glareolus*, a small mammal reservoir of a large number of zoonotic agents (e.g., Abbate *et al.*, In_revision). We studied the relationships between its gut bacteriome, parasite infracommunities – focusing on gastro-intestinal helminths and pathogenic bacterial infections –, and host and environmental factors that may either influence or indicate the health status (e.g., proxies such as the body mass index (BMI)). The current study therefore addressed two main questions: (1) how is the structure (composition and diversity) of the gut bacteriome influenced by host and environmental factors? (2) does the structure of gut bacteriome reflect interactions with gastro-intestinal helminth and pathogenic bacterial

communities? For this purpose, we *(i)* characterized the composition of each intra-host community (gut bacteriome, pathogenic bacteria, gastro-intestinal helminths) across bank vole populations, *(ii)* investigated the alpha and beta diversity of each intra-host community, and the pairwise relationships between these communities for each diversity metrics, and *(iii)* assessed if the structure of gut bacteriome was mediated either by factors similar to those influencing the structure of gastro-intestinal helminths and pathogenic bacteria community, or by potential interactions between intra-host communities.

Material and methods

Sample collection

Bank voles (*Myodes glareolus*) were trapped between June and September 2014 in forests located in four French localities (Table 1, Figure S1) distributed along a North-South transect in Eastern France, and separated by 40 to 120 km from one another. The standardized trapping protocol used here was described in details in Dubois et al. (2018). Previous studies have revealed that these populations exhibit some variability in bank vole susceptibility to zoonotic viral infections and immune gene expression (Dubois *et al.*, 2017; Dubois *et al.*, 2018).

Rodents were euthanized using isofluorane and cervical dislocation, as recommended by Mills (1995). Morphological measures were taken. Age groups (juveniles and adults) were defined according to body mass and sexual maturity. This latter was inferred using testes length and position, and seminal vesicle development for males, or uterus size for females. Body condition was estimated using the body mass index (BMI = weight/length^2). The digestive tract and the spleen were removed and stored respectively in 96% ethanol and RNA later solution (-20°C).

Ethical statements: Animal capture and handling have been conducted according to the French and European regulations on care and protection of laboratory animals (French Law 2001-486 issued on June 6, 2001 and Directive 2010/63/EU issued on September 22, 2010). The CBGP laboratory has approval (D-34-169-003) from the Departmental Direction of Population Protection (DDPP, Hérault, France), for the sampling of rodents and the storage and use of their tissues.

Table 1- Prevalence of potentially pathogenic bacteria and gastro-intestinal helminths for each sampling locality. *N* is the number of bank voles analysed. N_T represents the number of individuals with data available for the three intra-host communities (gut bacteriome, pathogenic bacteria and gastro-intestinal helminths). N_{GB}, N_{PB} and N_{GIH} respectively represent the number of individuals with data available for each of these intra-host communities. '*Uninfected'* corresponds to the number of uninfected bank voles for a given intra-host community. '*Co-infection'* corresponds to the number of bank voles infected with at least two parasites of a given intra-host community. The color gradient illustrates variations in prevalence (0% = light red to 100% = dark red).

179

	Total	Sampling localities			
		Mont-sous-Vaudrey	Chaux-des-Crotenay	Chatillon	Cormaranche
Total					
N_T	124	22	34	36	32
Gut bacteriome					
N_{Gb}	161	37	42	41	41
Pathogenic bacteria					
N_{Pb}	138	22	34	38	37
Uninfected$_{Pb}$	11	3	1	3	4
Co-infection$_{Pb}$	67	10	21	16	20
Bartonella sp	0.73	0.80	0.81	0.69	0.66
Mycoplasma haemomuris	0.36	0.28	0.39	0.36	0.39
Anaplasma phagocytophilum	0.14	0.04	0.33	0.08	0.11
Neoehrlichia mikurensis	0.13	0.16	0.00	0.08	0.29
Orientia tsutsugamushi	0.08	0.00	0.08	0.21	0.00
Rickettsia sp	0.05	0.00	0.08	0.05	0.05
Spiroplasma sp	0.04	0.04	0.00	0.00	0.11
Borrelia miyamotoi	0.02	0.04	0.00	0.03	0.03
Borreliella afzelii	0.02	0.08	0.00	0.00	0.03
Mycoplasma coccoides	0.01	0.04	0.00	0.00	0.00
Gastro-intestinal helminths					
N_{Gih}	153	37	42	39	35
Uninfected$_{Gih}$	29	16	4	6	3
Co-infection$_{Gih}$	64	2	22	13	27
Heligmosomoides glareoli	0.48	0.49	0.36	0.54	0.54
Heligmosomum mixtum	0.41	0.00	0.76	0.00	0.86
Aonchotheca murissylvatici	0.35	0.03	0.26	0.59	0.54
Catenotaenia henttoneni	0.10	0.03	0.19	0.10	0.06
Paranoplocephala omphaloides	0.06	0.00	0.14	0.00	0.09
Arostrilepis horrida	0.03	0.00	0.00	0.00	0.14
Mastophorus muris	0.01	0.05	0.00	0.00	0.00
Trichuris arvicolae	0.01	0.03	0.00	0.00	0.00

180

181 **Characterisation of intra-host bacterial and helminth communities**

182 Gut bacteriome

183 We first characterized the gut bacteriome of bank voles. We focused on the colon as rodent gut
184 microbiome exhibits the highest level of bacterial diversity in this part of the digestive tract (Suzuki
185 & Nachman, 2016). DNA was extracted from a 5 mm piece of colon (taken about 1 cm far from the
186 caecum) of each bank vole using the ZymoBiomics 96 DNA Kit (Zymo) following the
187 manufacturer's instructions. We amplified a 251-bp portion of the V4 region of the 16S rRNA gene
188 (16S-V4F [GTGCCAGCMGCCGCGGTAA] and 16S-V4R
189 [GGACTACHVGGGTWTCTAATCC]), following Kozich et al. (2013) and as described in Galan
190 et al. (2016). Samples were multiplexed using dual-indexes (index i5 in the forward primer and
191 index i7 in the reverse primer). Negative controls for extraction (whole reagents without DNA), for
192 PCR (PCR mix without DNA), and for indexing (wells without reagents corresponding to particular
193 dual-indexes combinations). All DNA extractions were analysed twice using two independent
194 technical replicates of amplicon libraries. PCR products were pooled, migrated and excised on a
195 low melting agarose gel (1.25%) then purified using the NucleoSpin Gel and PCR Clean-Up kit
196 (Macherey-Nagel) and quantified using the KAPA library quantification kit (KAPA Biosystems)
197 standardized to 4nM by qPCR spectrophotometry (assay). Sequencing was performed on a 251-bp
198 paired-end Illumina MiSeq run. The raw sequence reads (.fastq format) have been deposited in the
199 Zenodo Repository.
200 Sequence data were processed as described in Galan et al. (2016) using the pipelines implemented
201 in FROGS (Find Rapidly OTU with Galaxy Solution, Escudié *et al.*, 2018). Briefly, the paired-end
202 sequences were trimmed with CUTADAPT (Martin, 2011), merged with FLASH (MAGOC & SALZBERG,
203 2011), and clustered into operational taxonomy OTU units at 97% identity using the SWARM
204 algorithm (Mahe *et al.*, 2014) executed with aggregation parameter distance d=1 and a second pass
205 performed on the seeds of previous clusters with d=3. Putative chimeras were removed using
206 VSEARCH tools with de novo VUCHIME and the cross-validation method. Taxonomy was assigned
207 with BLASTN+ (Camacho *et al.*, 2009) using the SILVA SSU Ref NR 119 database as a reference
208 (http://www.arb175silva.de/projects/ssu-ref-nr/). Filtering for false positives was carried out as
209 proposed by Galan et al. (2016). In short, we discarded positive results associated with sequence
210 counts below two OTU-specific thresholds, which checked respectively for cross-contamination
211 between samples (using the negative controls for extraction and PCR) and incorrect assignment due
212 to the generation of mixed clusters on the flow cell during Illumina sequencing, using a false index-
213 pairing rate for each PCR product of 0.02%, based on estimates from Galan et al. (2016). For each
214 sample, only OTUs found in the two technical replicates were considered as positive, and OTUs
215 found in only one of the two replicates were removed. The number of sequences obtained for each
216 technical replicate from a sample were summed.

217 Lastly, we discarded OTUs and samples containing less than 500 reads in the dataset, as well as
218 OTUs considered to be contaminants, following (Salter *et al.*, 2014). OTUs number of reads were
219 finally normalised to proportional abundance within each rodent (McKnight *et al.*, 2019). Two
220 taxonomic ranks were considered for further analyses, phylum and family.
221 Further description and analyses of bacterial communities were performed using the PHYLOSEQ
222 package (McMurdie & Holmes, 2013). We looked for enterotypes, *i.e.* distinct community
223 composition types of gut bacteriome, as found in humans (Arumugam *et al.*, 2011; Holmes *et al.*,
224 2012), using the Dirichlet Multinomial Mixtures DMM (Morgan, 2021). We next analysed the
225 Firmicutes /Bacteroidetes (F/B) log-ratio, as it is often used as a proxy of health or metabolism in
226 humans and mice, see refs in (Lavrinienko *et al.*, 2018). We calculated this ratio with the
227 MICROBIOME package (Lahti & Shetty, 2017) and we tested the influence of individual
228 characteristics (age class, gender, BMI) and localities on this log-ratio using generalized linear
229 models.
230
231 Pathogenic bacteria
232 We next described the presence/absence of pathogenic and zoonotic bacteria from the spleen of
233 each bank vole. This lymphoid organ filters microbial cells in mammals and as such, enables to
234 recover recent infections (Abbate *et al.*, In_revision; Diagne *et al.*, 2017). Molecular protocols,
235 bioinformatics pipelines and data filtering were similar to those described above (gut bacteriome),
236 except for the DNA extraction from splenic tissue using DNeasy 96 Tissue Kit (Qiagen). The
237 potential pathogenicity of each bacterial OTU was assessed based on published literature and on the
238 Gideon database (https://www.gideononline.com/). Opportunistic pathogens (*i.e.* commensal agents
239 in healthy hosts, that become pathogenic when the balance of the immune system is disrupted) were
240 discarded from the dataset. Only the information of the presence / absence of pathogenic/zoonotic
241 OTUs was considered.
242
243 Gastro-intestinal helminths
244 For each bank vole, helminths were carefully extracted and counted from the different sections of
245 the digestive tract (stomach, small intestine, large intestine and caecum), and classified by
246 morphotype then stored in 95% ethanol for further accurate identification. The latter was based on
247 unambiguous morphological criteria using conventional microscopy and generalist identification
248 keys or specific literature when available (Anderson *et al.*, 2009; Khalil *et al.*, 1994; Ribas Salvador
249 *et al.*, 2011).
250

Statistical analyses

All statistical analyses were implemented in R v4.0.3 (team, 2020). For more convenience, gut bacteriome, pathogenic bacteria and gastro-intestinal helminths were further described as 'intra-host communities'.

Assessment of the alpha diversity within intra-host communities

First, we characterized the alpha diversity, *i.e.* the diversity within intra-host communities (the gut bacteriome, pathogenic bacteria and gastro-intestinal helminth communities) using two metrics, the specific richness (*i.e.* number of taxa within the host individual) and the Shannon index as recommended in (Haegeman *et al.*, 2013).

Second, we tested the influence of rodent characteristics (age class, gender, BMI), localities and the two-ways interactions between these factors, on the alpha diversity of the three intra-host communities using generalized linear models (GLMs). We considered a negative binomial error distribution for the specific richness and a gamma distribution for the Shannon index. Best model selection was performed considering models with all possible combinations of factors and the DREDGE function of the MUMIN package. The best model was selected using the Akaike information criterion corrected for small sample size AICc, (Johnson & Omland, 2004). We assessed the effect of each factor in the best model with the ΔAICc index. When the factor locality was significant, Tukey's post-hoc tests were applied to evaluate pairwise differences between localities, using the MULTCOMP package (Hothorn *et al.*, 2008). Residuals were checked to graphically to ensure that all assumptions regarding normality, independence and the homogeneity of variance were satisfied.

Third, we analysed whether the alpha diversity of each intra-host community was influenced by the alpha diversity of the two other ones. We performed GLMs and followed a similar model selection process than the one explained above.

Assessment of the beta diversity of intra-host communities

We analysed the beta diversity, *i.e.,* the dissimilarity between host individuals in their intra-host communities (gut and pathogenic bacteria, and helminth communities) composition, using Bray-Curtis distances. We considered the relative abundance of OTUs for the gut bacteriome (phylum and family), the occurrence (presence/absence) of pathogenic bacteria and the abundance of gastro-intestinal helminths.

To assess the impact of the spatial dimension on beta diversity, we performed Mantel tests using Pearson correlation (10,000 permutations) and evaluated the influence of geographic distances on the dissimilarities in intra-host gut, pathogenic bacterial and helminth communities.

We then analysed the factors shaping the dissimilarities in intra-host community composition using several functions of the VEGAN package (Oksanen *et al.*, 2020). Rodents that were not infected (pathogenic bacteria or helminths) could not be included in these analyses. Distance-based redundancy analyses (db-RDA) were performed to analyse the effect of individual explanatory factors (age class, gender, BMI) and sampling localities on dissimilarities in intra-host community composition, using the CAPSCALE function, followed by permutational multivariate analyses of variance (PERMANOVA). We selected the best model, *i.e.*, the most parsimonious one, using the ORDIR2STEP function (*P*-value adjusted and R^2 adjusted). For each factor, we evaluated the intra-group dispersion using the BETADISPER function as PERMANOVA analyses are sensitive to differences in dispersion among groups.

As this study focused on the variability of the gut bacteriome, we also used DESEQ2 package (Love *et al.*, 2014), that is designed to perform differential analysis of high-dimensional count data, to identify the changes in bacteria taxa (phyla or families) that best explained gut bacteriome dissimilarities between individuals and localities. We performed GLMs with negative binomial error (NBINOMWALDTEST method) and significant differences were obtained after Benjamini & Hochberg corrections. They were visualised using the METACODER package (Foster *et al.*, 2017).

Interactions between intra-host gut bacteria, pathogenic bacteria and gastro-intestinal helminth communities

We applied partial Mantel tests using MULTI.MANTEL (phytools package Revell, 2012) to analyse the correlation between two matrices of dissimilarities (corresponding to two different communities), while controlling for the effect of a third dissimilarity matrix (third community). Then, we used db-RDA to analyse more deeply the relationships between the gut bacteriome and the pathogenic (bacteria and helminths) communities. We included the alpha diversity indices (richness specific and Shannon index) and the infectious status (presence / abundance) of pathogens with prevalence greater than 10% in at least one locality as explanatory variables in these analyses. We selected the best model using the ORDIR2STEP method. As described above, we used DESEQ2 to determine the phyla and families of the gut bacteriome whose relative abundances changed with significant explanatory variables.

Results

317

318 A total of 186 bank voles were trapped during the fieldwork campaign over the four targeted
319 localities. For technical reasons (e.g., poor sample preservation, missing data), we could describe
320 the three intra-host communities for 124 rodents only.

321

322 **Composition of the intra host communities**

323 Gut bacteriome

324 Once the filtration steps were applied, the gut bacteriome dataset included 161 bank voles. We
325 detected 10 phyla and 61 families of bacteria. At the phylum level, we found six predominant taxa
326 that represented 99% of the gut bacteria relative abundance (Figures S2): *Firmicutes* (41% of
327 reads), *Proteobacteria* (20% of reads), *Epsilonbacteraeota* (12% of reads), *Actinobacteria* (12% of
328 reads), *Bacteroidetes* (11% of reads) and *Spirochaetes* (3% of reads). At the family level, 11
329 families represented 93% of the relative abundance of the gut bacteriome (Figure 1A):
330 *Lactobacillaceae* (21% of reads), *Lachnospiraceae* (11% of reads), *Ruminococcaceae* (4% of
331 reads), *Clostridiales* (3% of reads) of the *Firmicutes* phylum, *Desulfovibrionaceae* (*Proteobacteria*
332 phylum, 19% of reads), *Eggerthellaceae* (*Actinobacteria* phylum, 12% of reads), *Muribaculaceae*
333 and *Bacteroidales F1* (*Bacteroidetes* phylum, 8% and 1% of reads respectively), *Helicobacteraceae*
334 (*Epsilonbacteraeota,* 12% of reads) and *Spirochaetaceae* (*Spirochaetes* phylum, 3% of reads).
335 We distinguished two enterotypes from the DMM approach. One (enterotype 1) was mainly
336 composed of the families *Helicobacteraceae*, *Lachnospiraceae*, *Muribaculaceae and*
337 *Desulfovibrionaceae*, while the other (enterotype 2) mainly included *Lactobacillaceae*,
338 *Desulfovibrionaceae* and *Eggerthellaceae* (Figure 1B).
339 Lastly, we found that the *Firmicutes / Bateroidetes* ratio varied significantly between localities
340 (Figure 1C). Overall, northern localities exhibited lower F/B ratio than southern ones, with all
341 pairwise comparisons being significant except Chatillon *versus* Chaux-des-Crotenay. Individual
342 characteristics did not influence this ratio (Table S1).

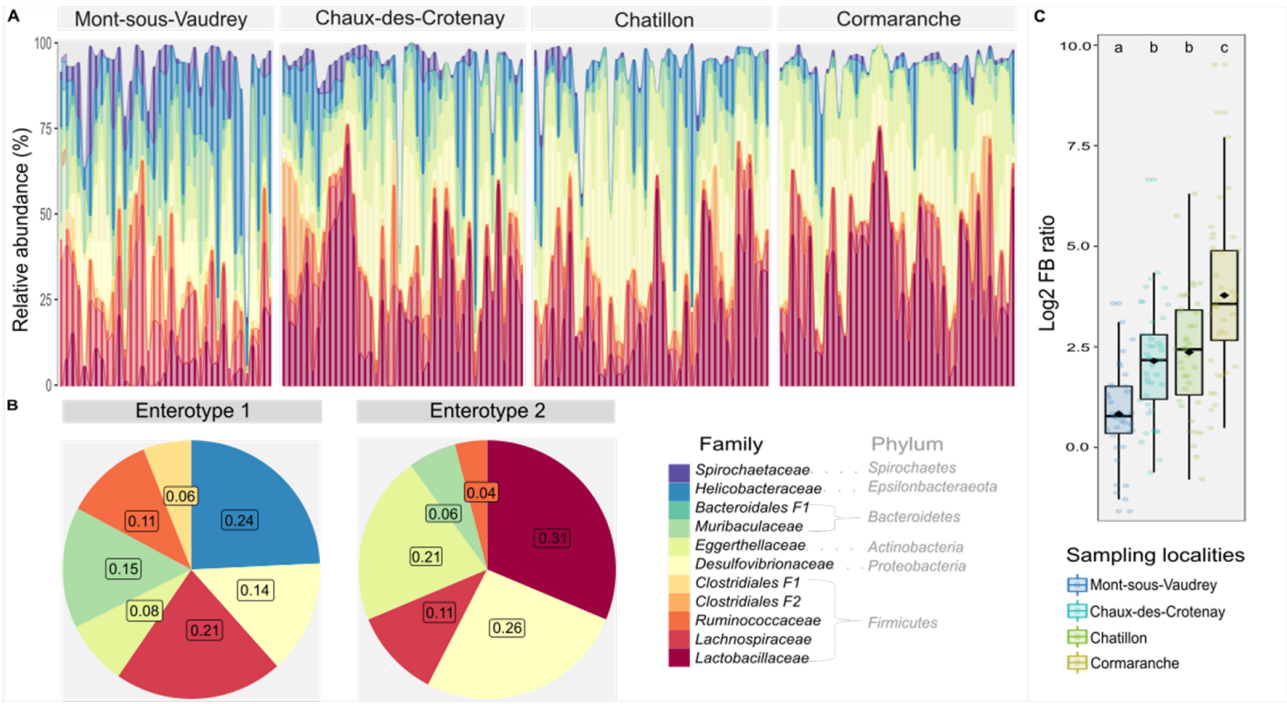

Figure 1- Composition of the intestinal bacteriome. A) The Bar plot shows the individual variations of 11 bacterial families (F= Unknown family) belonging to 6 phyla and representing 93% of the total composition. Individuals (bars) are grouped by sampling localities, which are ordered from north to south. Each color represents a taxon. B) The composition of the two enterotypes identified using Dirichlet multinomial mixtures (DMMs), at family rank, is shown. Bacterial families are represented using the same colors as in A. and C) the ratio (Firmicutes / Bacteroidetes) is shown for each sampling locality. Box and whisker plots represent the median and interquartile values. Black dots correspond to the mean value, and colored dots correspond to individuals. Different letters indicate statistically significant differences at $P < 0.05$, with pairwise Tukey post hoc adjustments.

Pathogenic bacteria

Once the filtration steps were applied, the dataset corresponding to potentially pathogenic bacteria included 138 *M. glareolus*. Ten genus/species were considered as potentially pathogens. Four of them had prevalence levels greater than 10%: *Bartonella* sp *(73%), Mycoplasma haemomuris (36%), Anaplasma phagocytophilum (14%), Candidatus Neoehrlichia mikurensis (13%)* (Table 1). Most *M. glareolus* individuals were infected with at least one bacteria species (92%), and about half bank voles (49%) were infected with two or more bacteria species (Table 1).

Gastro-intestinal helminths

We detected eight species of gastro-intestinal helminths among the 153 *M. glareolus* analysed. Four helminth species exhibited prevalence levels higher than 10%: the nematodes *Heligmosomoides glareoli* (prevalence: 48%), *Heligmosomum mixtum* (prevalence: 41%) and the cestodes

365 *Aonchotheca murissylvatici* (prevalence: 35%) and *Catenotaenia henttoneni* (prevalence: 10%)
366 (Table 1). Note that the prevalence of *Paranoplocephala omphaloides* and *Arostrilepis horrida*
367 were greater than 10% in Chaux-des-Crotenay and Cormaranche. Most bank voles (85%) were
368 infected with at least one helminth species and almost half (42%) were infected with at least two
369 different helminth species (Table 1).
370
371 **Factors influencing the alpha diversity within intra-host communities**
372 Gut bacteriome
373 At the phylum level, none of the factors significantly explained the variations of specific richness of
374 the gut bacteria community between bank voles (Table S2A; Figure S3A). When considering
375 Shannon index, we found that the sampling locality had a significant effect on the alpha diversity of
376 the gut bacteriome (ΔAICc = 8.70, R^2_{GLM} = 0.09, Table S2A; Figure S3B). After post-hoc
377 corrections, Mont-sous-Vaudrey and Cormaranche were the only localities that differed
378 significantly from the others (P_{adj} < 0.001). Mont-sous-Vaudrey showed higher levels of diversity
379 than Cormaranche (β = -0.19; 95% CI = [-0.29, -0.10]). The other localities also exhibited lower
380 levels of alpha diversity than Mont-sous-Vaudrey (Chaux-des-Crotenay: β = -0.12; 95% CI = [-
381 0.22, -0.03]; P = 0.01; P_{adj} = 0.05; Chatillon: β = -0.11; 95% CI = [-0.21, -0.01]; P = 0.02; P_{adj} =
382 0.10) but these differences were not significant after correcting for multiple tests.
383 At the family level, the sampling locality had a significant effect, whatever the alpha diversity
384 metrics used (Specific richness: ΔAICc = 14.28; R^2_{GLM} = 0.21; Figure 2A; Shannon index: ΔAICc =
385 7.65; R^2_{GLM} = 0.08; Figure 2B; Table S2B). The locality Cormaranche exhibited a higher specific
386 richness than all other localities (Tukey post hoc test: Mont-sous-Vaudrey: β = 0.22; 95% CI =
387 [0.10, 0.30]; $P_{adj} < 10^{-3}$, Chaux-des-Crotenay: β = 0.19; $P_{adj} < 10^{-3}$ and Chatillon: β =0.15; P_{adj} = 1.7
388 x 10^{-3}) but a lower level of diversity than Mont-sous-Vaudrey when correcting for rare taxa (Tukey
389 post hoc test, Shannon index: β = -0.76; 95% CI = [-1.2; -0.4]; P_{adj} = 2 x 10^{-3}, Figure 2B). Body
390 condition (BMI) was also found to have a significant effect, but only when considering specific
391 richness (ΔAICc=1.99; β = 0.18; 95% CI = [0.1, 0.3], P = 4 x 10^{-3}) – with higher values of BMI
392 associated with increasing species richness. All these results are detailed in Table S2B and Figure
393 S4A,D,G).
394
395 Pathogenic bacteria
396 Host gender was the only factor that significantly influenced variations in alpha diversity regardless
397 of the index considered (Specific richness: ΔAICc = 0.32; R^2_{GLM} =0.04; Shannon index: ΔAICc =
398 3.4; R^2_{GLM} =0.05; Figure 2C; Figure S4B,E,H). Males had a higher diversity of pathogenic bacteria

than females (Specific richness: $\beta = 0.22$; 95% CI = [0.03,042]; $P = 0.02$; Shannon index: $\beta = 0.17$; 95% CI = [0.03,0.32]; $P=0.02$). All these results are detailed in Table S2C.

Gastro-intestinal helminths

We found a significant effect of the locality on the alpha diversity of the gastro-intestinal helminth community, whatever the metrics used (Specific richness: ΔAICc=24.53, $R^2_{GLM} = 0.30$; Shannon index: ΔAICc= 41.11; $R^2_{GLM} = 0.30$; Table S2D). When considering the specific richness, voles from Mont-sous-Vaudrey showed lower value than in all other localities (Tukey post hoc test, Figure 2D, Table S2D). In addition, bank voles from Chatillon had lower specific richness than Cormaranche (Tukey post hoc test, $\beta = 0.61$; $P_{adj} < 10^{-3}$, Figure 2D). When considering Shannon index, we found that bank voles from Cormaranche exhibited significantly higher levels compared with the other localities (Table S2D). Moreover, bank voles from Chaux–des-Crotenay exhibited higher levels of Shannon index than those from Mont-sous-Vaudrey ($\beta = 0.38$, $P_{adj} < 10^{-3}$).

We also found that the age of bank voles significantly influenced the specific richness of the helminth community, with adults being infected by a higher diversity of helminths than juveniles (Specific richness: ΔAICc=0.49; $\beta = 0.31$; 95% CI = [0.03, 0.61]; $P = 0.03$; Table S2D, Figure S4C,F,I).

Relationships between the alpha diversity of the three intra-host communities

Gut bacteriome as the dependant variable. For the species richness, a significant relationship was only found at the family level between the gut bacteriome and the helminth community (ΔAICc = 8.43; $P < 10^{-3}$), with a more diverse gut bacteriome associated with a greater number of helminth species infecting bank voles ($\beta = 0.07$; 95% CI = [0.03, 0.10]; $R^2 = 0.10$; Figure 2E; Table S2E,F). For the Shannon index, we did not find any effect between the alpha diversity estimates of each intra-host community, whatever the taxonomic rank considered (Figure 2E; Tables S2E,F).

Pathogenic bacteria as the dependant variable. For both species richness and Shannon index, we found a positive relationship with, respectively, the specific richness (ΔAICc = 1.02; $P < 10^{-3}$) (ΔAICc = 1.02; $P < 10^{-3;}$ Figure 2E) and the Shannon index (ΔAICc = 3.47; β=0.19; $P = 0.04$, R^2=0.02; Figure 2E; Table S2E,F) of the gastro-intestinal helminth community.

Gastro-intestinal helminths as the dependant variable. For the specific richness, we found a significant effect of the specific richness of the gut bacteriome (Family level only) and of pathogenic bacteria ($R^2 = 0.14$; gut bacteriome ΔAICc = 6.29; P =0.02; $\beta = 0.06$; pathogenic bacteria ΔAICc = 1.82; $P = 0.03$; $\beta = 0.17$; Figure 2E, Table S2F). For Shannon index, only the alpa

diversity of pathogenic bacteria remained significant (ΔAICc = 3.29; $\beta = 0.23$; $P = 0.02$; Figure 2E, Table S2F).

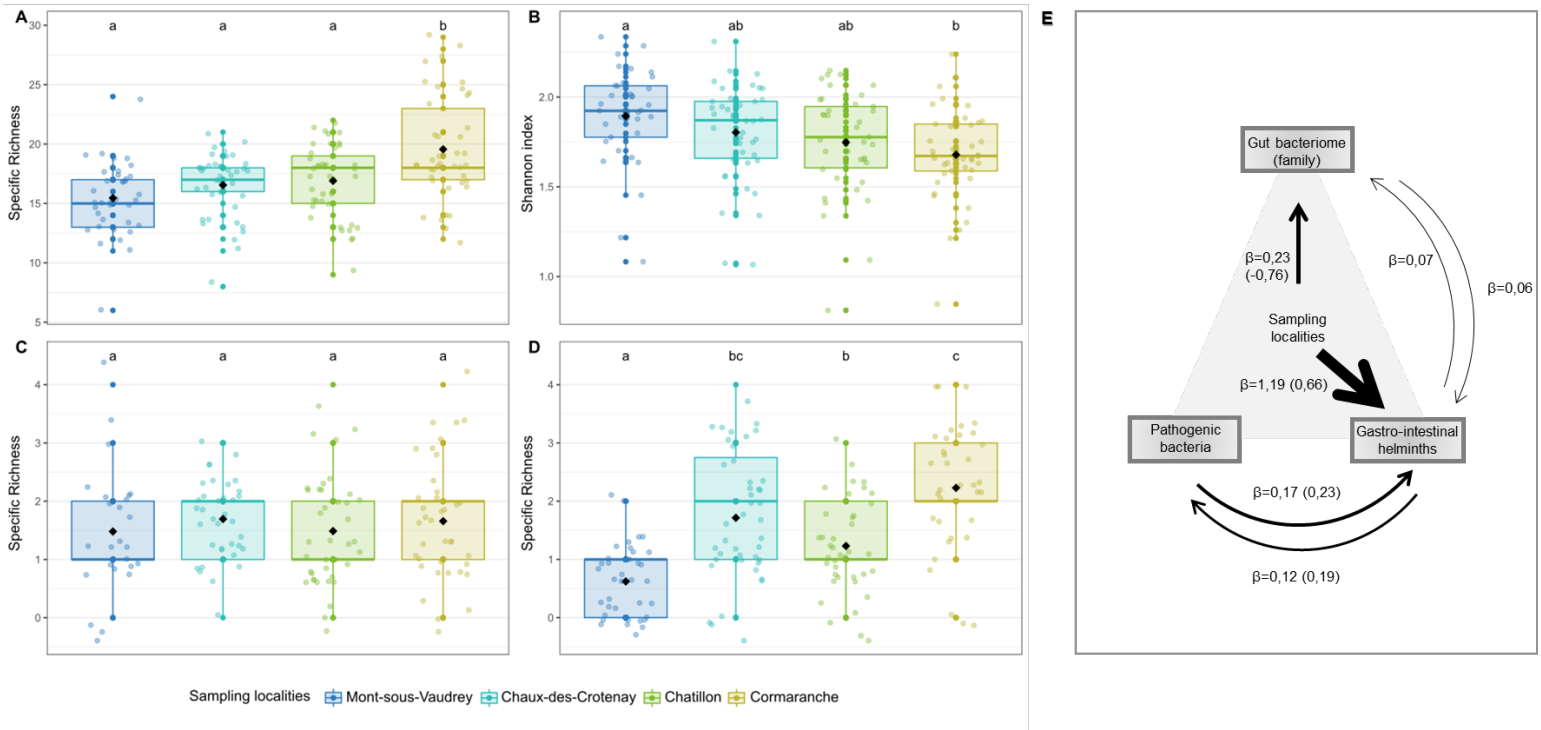

Figure 2- Variations of alpha diversity between localities, for the intestinal bacteria (family rank), pathogenic bacteria and gastro-intestinal helminth communities of bank voles. Alpha diversity is represented using A) the specific richness of the gut bacteriome, B) the Shannon index of the gut bacteriome, C) the specific richness of the pathogenic bacteria and D) of the gastro-intestinal helminths. Results are shown per locality, ordered from North to South. Each colored point represents an individual. Black points indicate the average alpha diversity per locality. Box-and-whisker plots represent the median and interquartile values. Different letters denote statistically significant differences at $P < 0.05$, with pairwise post-hoc Tukey adjustments. E) Diagram showing the relationships between the alpha diversity estimates of the three communities, while taking into account the influence of the sampling localities. The effect size and the direction of the relationship between communities are represented using an arrow, its width corresponding to estimate (β) x 10. B is given for the specific richness metrics, and for the Shannon index metrics (in brackets). Only significant effects are represented.

Factors influencing the beta diversity of intra-host communities

Gut bacteriome

Mantel tests revealed a significant positive relationship between the dissimilarities in gut bacteriome composition and the geographic distance, whatever the taxonomic rank considered (Phylum: $r = 0.19$; $P = 10^{-4}$; Family: $r = 0.24$; $P = 10^{-4}$).

At the phylum level, the distance-based redundancy analyses (db-RDA) revealed a significant effect of the sampling localities ($P = 1 \times 10^{-3}$) and host gender ($P = 8 \times 10^{-3}$) on gut microbiome composition. Age class and BMI had no significant effect (Table S3A). The best model explained 18% of the total variance, with most of the variance being explained by localities (locality: $R^2_{adj} = 0.16$; gender: $R^2_{adj} = 0.02$). The variables constrained on two dimensions explained 15.40% of the variance in phylum composition (CAP1 = 10.8%; CAP2 = 4.6%). The localities that contributed the

most to the CAP1 axis were Mont-sous-Vaudrey (negative values) and Cormaranche (positive values) (Figure 3A). The CAP2 axis discriminated the gut bacteriome of male bank voles from Chaux-des-Crotenay (negative values) and females from Chatillon (positive values). Similar results were obtained at the family level (locality: $P = 1 \times 10^{-3}$; $R^2_{adj} = 0.16$; gender: $P = 0.027$; $R^2_{adj} = 0.01$), with the CAP1 axis strongly discriminating Mont-sous-Vaudrey and Cormaranche localities (12.7% of the total variance, Figure 3B). However, these results have to be taken cautiously as significant differences of data dispersion were detected between localities (*betadisper,* Phylum: $P = 1 \times 10^{-3}$; Family: $P = 1 \times 10^{-3}$). The locality Cormaranche showed a lower dispersion compared to all other localities (Tukey multiple comparisons, Table S3A).

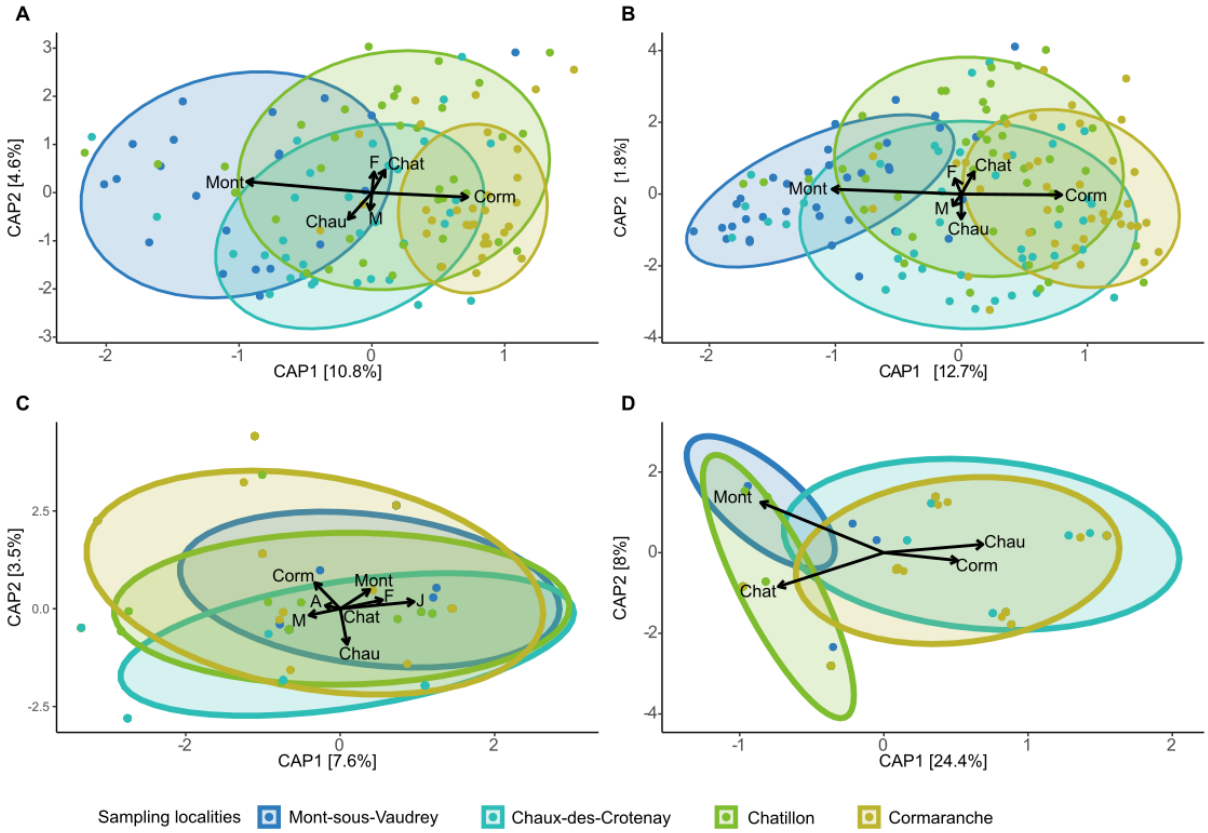

Figure 3- Distance-based redundancy analysis (db-RDA) of the intestinal bacteria (phylum (A) and family (B)), pathogenic bacteria (C) and gastro-intestinal helminth (D) communities, based on Bray–Curtis dissimilarities. Dots represent individuals. The colors and shapes of the dots are associated with different factors : Localities from North to South (Mont-sous-Vaudrey: Mont, Chaux-des-Crotenay: Chau, Chatillon: Chat and Cormaranche: Corm), age class (juvenils: J, adults: A) and gender (females: F and males: M). Significatif factors based on the ORDIR2STEP analysis are shown as arrows. Ellipses represent a 80% confidence interval around the centroid of the clusters, for each locality.

Significant differences in the relative abundance of specific taxa were detected at the phylum and family levels using DeSeq2 (Table 2; Table S3B). The main changes (Log2 fold values higher than 20) were detected between the northern (Mont-sous-Vaudrey) and southern localities. The northern population was involved in 75% of all significant pairwise differences (Log2 fold change in

composition > 10). The gut bacteriome of these bank voles includes less Clostridiales (one unknown family; Firmicutes), Bifidobacteriaceae (Actinobacteria) and Desulfovibrionales (two unknown families; Proteobacteria) than in all the three other southern populations. The gut bacteriome of bank voles from Cormaranche (South) is characterized by less Erysipelotrichaceae (Firmicutes), than in the three northern populations.

Table 2- Pairwise comparisons of the relative abundance of the gut bacteriome (phylum and family levels) between sampling localities. Mont = Mont-sous-Vaudrey, Chau = Chaux-des-Croteay, Chat = Chatillon, Corm = Cormaranche. The Log^2 fold value is indicated for significant changes in abundance between two localities. Blue and red colors respectively correspond to negative and positive values. Higher absolute changes in Log^2 fold are emphasized with darker colors. The notation "order_fx" or "class_fx" is used when there was no assignation at the family level with the SILVA database.

	Sampling localities					
	Mont vs Chau	Mont vs Chat	Mont vs Corm	Chau vs Chat	Chau vs Corm	Chat vs Corm
Epsilonbacteraeota						
All	0	1.91	3.64	0	2.18	0
Helicobacteraceae	0	0	1.91	0	0	0
Proteobacteria						
All	0	0	0	0	0	0
Rhizobiaceae	10.26	0	0	0	0	0
Desulfovibrionales_f3	−21.32	−26.19	−26.04	−4.8	−4.72	0
Desulfovibrionales_f1	−20.96	−21.26	−24.22	0	0	0
Desulfovibrionales_f2	−4.23	−5.58	−5.1	0	0	0
Desulfovibrionaceae	−1.3	−1.63	−1.54	0	0	0
Burkholderiaceae	0	0	0	0	0.67	0
Firmicutes						
All	0	0.76	0.97	0	0	0
Erysipelotrichaceae	0	0	19.57	0	21.81	25.96
Christensenellaceae	0	0	−3.04	0	0	0
Ruminococcaceae	0	0	1.48	0	1.5	1.08
Streptococcaceae	0	−5.57	0	−6.1	0	0
Clostridiaceae	0	0	−5.24	0	0	0
Lactobacillaceae	0	0	−1.81	0	0	0
Clostridiales	0	0	2.16	0	2.27	1.43
Clostridiales_f4	0	0	−3.29	0	0	0
Clostridiales_f2	−26.15	−26.31	−26.54	0	0	0
Tenericutes						
All	2.74	4.25	3.13	0	0	0
Mollicutes_f1	−3.43	0	−2.93	0	0	0
Patescibacteria						
All	0	−2.94	−2.83	0	0	0
Saccharimonadaceae	−5.82	−5.77	−5.45	0	0	0
Spirochaetes						
All	2.52	2.65	4.52	0	1.99	1.87
Spirochaetaceae	0	0	2.39	0	0	0
Actinobacteria						
All	0	0	−0.89	0	0	0
Bifidobacteriaceae	−20.6	−24.84	−23.21	0	0	0
Eggerthellaceae	0	−1.31	−2.42	0	−1.74	0
Bacteroidetes						
All	1.29	1.73	3.28	0	1.99	1.54
Bacteroidales_f4	0	0	0	0	0	−3.59
Muribaculaceae	0	0	1.74	0	1.93	1.64
Rikenellaceae	0	1.73	0	0	0	0
Marinifilaceae	−2.57	0	0	0	0	0

493 Besides, differences in the composition of the gut bacteriome between males and females bank
494 voles were driven by the phylum *Firmicutes*, with males exhibiting higher relative abundance of
495 this taxa than females (Table S3B).
496

497 Pathogenic bacteria
498 Mantel test indicated no significant relationship between the dissimilarities in the composition of
499 the pathogenic bacteria community and the geographical distances (r = 0.24; P = 0.34).
500 The db-RDA showed significant variations of the pathogenic bacteria community composition with
501 individual characteristics (age class: P =0.01; gender: $P = 1 \times 10^{-3}$) and localities ($P = 4 \times 10^{-3}$). The
502 best model explained 15% of the total variance, with all factors explaining only a small part of the
503 variance (locality: 5%; gender: 5%; age class: 3.5%). Data dispersion was similar between groups
504 (Table S3A). The db-RDA plot based on the two first axes only represented 11.1% of the total
505 variance (Figure 3C). CAP1 (7.6%) discriminated individuals mainly according to age class and
506 gender (male adults were opposed to juveniles and females), while CAP2 (3.5%) discriminated
507 Chaux-des-Crotenay from all other localities.
508

509 Gastro-intestinal helminths
510 Mantel test revealed a significant positive relationship between the dissimilarities in the
511 composition of the gastro-intestinal helminth community and the geographic distances (r = 0.16, P
512 $= 10^{-4}$).
513 The db-RDA emphasized a significant effect of the locality on the composition of the gastro-
514 intestinal helminth community (db-RDA, $P = 10^{-3}$; Table S3A). Individual characteristics (host
515 gender, age class and BMI) had no significant effect (P = 0.27, P = 0.43, P = 0.15, respectively).
516 Locality explained 41% of the total variance. The variables constrained on two dimensions
517 explained 32.4% of the variance (CAP1 = 24.4 % and CAP2 = 8%). CAP1 discriminated the
518 localities Mont-sous-Vaudrey and Chatillon from Chaux-des-Crotenay and Cormaranche, while
519 CAP2 discriminated Mont-sous-Vaudrey from Chatillon (Figure 3D). We found no significant
520 difference in the dispersion of data between localities (P = 0.06).
521

522 Interactions between intra-host communities' composition
523 Partial Mantel tests revealed a positive relationship between dissimilarities in gut bacteriome and
524 dissimilarities in gastro-intestinal helminth community composition (multiple Mantel test, phylum:
525 estimate = 0.18; P = 0.015; family: estimate = 0.22; P =4 x 10-3), but not with dissimilarities in
526 pathogenic bacteria community composition (multiple Mantel test: P = 0.14). After controlling for

geographic distances, dissimilarities in gut bacteriome composition remained significantly correlated with dissimilarities in helminth community composition at the family level only (partial Mantel test, phylum: $r = 0.03$, $P = 0.18$; family: $r = 0.06$, $P = 0.03$). Other results are detailed in Table S3C.

The db-RDA and model selection procedures showed that the whole composition of the gut bacteriome was significantly associated with the presence / absence of three pathogens: *Aonchotheca murissylvatici* (ordistep db-RDA, Phylum: $P = 0.002$, $R^2_{adj} = 0.02$ and Family: $P = 0.002$, $R^2_{adj} = 0.04$), *Bartonella* sp (ordistep db-RDA, Phylum: $P = 0.022$, $R^2_{adj} = 0.02$; Family: $P = 0.04$, $R^2_{adj} = 0.01$) and *Heligmosomum mixtum* (ordistep db-RDA, Phylum: $P = 0.002$, $R^2_{adj} = 0.04$ and Family: $P = 0.002$, $R^2_{adj} = 0.06$). Neither the specific richness of pathogenic bacteria nor the specific richness of the gastro-intestinal helminth community had a significant effect on the gut bacteriome (Table S3D). The db-RDA triplots based on the two first axes only represented 9.5% at phylum level and 10.2% at family level of the total variance (Figure 4; Figure S5). It showed that individuals infected with *Aonchotheca murissylvatici* had more Proteobacteria and Actinobacteria, but less Spirochaetes, Bacteroidetes and Epsilonbacteraeota. It also revealed contrasted patterns for individuals either infected with *Bartonella* sp but not with *Heligmosomum mixtum*, or infected with *H. mixtum* and not *Bartonella* sp. The former had more Bacteroidetes while the latter had more Firmicutes.

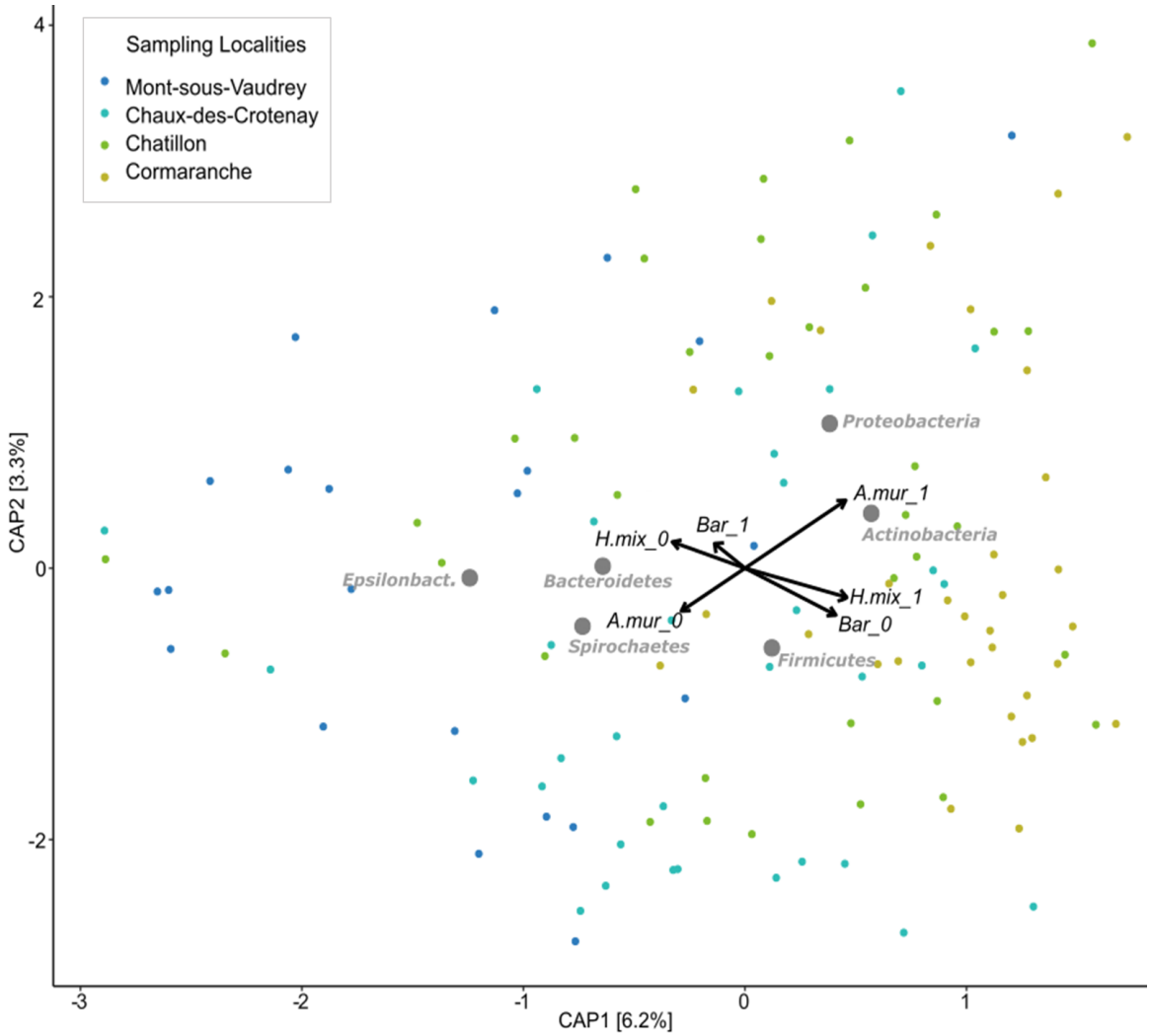

Figure 4- Relationships between the composition of the intestinal bacteria, pathogenic bacteria and gastro-intestinal helminth communities: db-RDA triplot shows the structure of the gut microbiome at the phylum level and the correlations with the pathogen communities. The arrows correspond to the significant explanatory variables. Each point corresponds to an individual, and the colors correspond to the different sampling localities. A.mur: *Aonchotheca murissylvatici*, Bar: *Bartonella* sp, H.mix: *Heligmosomum mixtum*.

Deseq2 analysis showed that infections with *A. murissylvatici* and *H. mixtum* were specifically associated with a slightly different relative abundance (Log2 fold changes did not exceed 3.5) of three main phyla (Epsilonbacteraeota, Spirochaetes and Bacteroidetes), and of Firmicutes and Patescibacteria for *A. murissylvatici* only (Table 3). Slight changes (mostly increases) in relative abundance of one specific family per phylum were also detected for *A. murissylvatici* infection (see details in Table S3E), and in a lesser extent for *H. mixtum*. It was the case for an undetermined family of Mollicutes (Tenericutes).

Moreover, this analysis revealed that higher levels of alpha diversity of the helminth community were associated with slightly lower relative abundance of the phylum Epsilonbacteraeota, Firmicutes, Spirochaetes and Bacteroidetes, of the family *Rhizobiaceae* (Proteobacteria) and with slightly higher relative abundance of the family *Eggerthellaceae* (Actinobacteria) and *Mollicutes* (Tenericutes) (Table 3; Table S3E).

Table 3- Changes in relative abundance of the gut bacteriome at the phylum ad family levels, with regard to infectious status (helminths and pathogenic bacteria) and alpha diversity. The Log^2 fold change in relative abundance is indicated for significant values only. Negative values are represented with blue colors, positive values with red colors. Higher absolute changes in Log^2 fold are emphasized with darker colors.

	Phylum	Family	log2FoldChange
GI helminths (Specific Richness)			
	Spirochaetes		−1.02
	Bacteroidetes		−0.55
	Epsilonbacteraeota		−0.87
	Firmicutes		−0.25
	Actinobacteria		0.27
	Actinobacteria	*Eggerthellaceae*	0.67
	Proteobacteria	*Rhizobiaceae*	−3.79
	Epsilonbacteraeota	*Helicobacteraceae*	−0.89
GI helminths (Shannon Index)			
	Bacteroidetes		−1.26
	Spirochaetes		−2.16
	Epsilonbacteraeota		−1.93
	Firmicutes		−0.5
	Actinobacteria	*Eggerthellaceae*	1.76
	Proteobacteria	*Rhizobiaceae*	−8.08
	Tenericutes	*Mollicutes_f1*	2.8
Aonchotheca murissylvatici			
	Spirochaetes		−2.78
	Epsilonbacteraeota		−1.95
	Firmicutes		−0.55
	Bacteroidetes		−0.93
	Patescibacteria		1.73
	Proteobacteria	*Desulfovibrionaceae*	1.13
	Tenericutes	*Mollicutes_f1*	3.07
	Patescibacteria	*Saccharimonadaceae*	3.03
	Spirochaetes	*Spirochaetaceae*	−1.75
	Actinobacteria	*Eggerthellaceae*	1.4
Heligmosomum mixtum			
	Bacteroidetes		−1.36
	Epsilonbacteraeota		−1.88
	Spirochaetes		−1.93
	Tenericutes	*Mollicutes1*	3.34
Mastophorus muris			
	Proteobacteria	*Desulfovibrionales3*	−29.98
	Firmicutes	*Clostridiales_f1*	−21.41
	Firmicutes	*Clostridiales_f2*	−23.73
Catenotaenia henttoneni			
	Proteobacteria	*Rhizobiaceae*	−24.71
	Proteobacteria	*Burkholderiaceae*	−23.96
	Firmicutes	*Erysipelotrichaceae*	−20.6
Paranoplocephala omphaloides			
	Firmicutes	*Clostridiales_f2*	−25.37
	Proteobacteria	*Desulfovibrionales_f3*	−23.74
	Proteobacteria	*Rhizobiaceae*	−24.86
	Proteobacteria	*Burkholderiaceae*	−22.69
	Firmicutes	*Erysipelotrichaceae*	−22.4
	Firmicutes	*Christensenellaceae*	−5.24
Trichuris muris			
	Firmicutes	*Christensenellaceae*	−20.69
	Firmicutes	*Clostridiales_f1*	−18.62
	Bacteroidetes	*Bacteroidales_f4*	−18.38
Pathogenic bacteria (Specific Richness)			
	Bacteroidetes	*Bacteroidales_f3*	−28.51
Neoehrlichia mikurensis			
	Bacteroidetes		−1.43
	Firmicutes	*Erysipelotrichaceae*	−23.42
	Firmicutes	*Clostridiales*	−2.72
	Bacteroidetes	*Muribaculaceae*	−1.76
Orientia tsutsugamushi			
	Bacteroidetes		−2.8
	Firmicutes	*Erysipelotrichaceae*	−22.95
	Proteobacteria	*Desulfomicrobiaceae*	−5.71
	Bacteroidetes	*Muribaculaceae*	−2.45
Mycoplasma haemomuris			
	Spirochaetes		−1.99
Anaplasma phagocytophilum			
	Firmicutes	*Ruminococcaceae*	−1.75
Rickettsia sp			
	Firmicutes	*Erysipelotrichaceae*	−21.81

570

571 Besides, the most important changes observed in the relative abundance of gut bacteriome families
572 (Log2 fold higher than 18) were specifically associated with all other helminth species:
573 *Mastophorus muris, Catenotaenia henttoneni, Paranoplocephala omphaloides* and *Trichuris*
574 *arvicolae*. These associations were all strongly negative and concerned a wide array of gut
575 bacteriome families (Table S3E), most of them belonging to the phylum Firmicutes
576 (Christensenellaceae, two undetermined families of Clostridiales and Erysipelotrichaceae),
577 Proteobacteria (Burkholderiaceae and an undetermined family of Desulfovibrionales) and
578 Bacteroidetes (an undetermined family of Bacteroidales).
579 Considering pathogenic bacteria, we found that higher levels of alpha diversity (specific richness)
580 of the pathogenic bacteria community was associated with lower relative abundance of an
581 undetermined Bacteroidales family (Bacteroidetes), and that *Neoehrlichia mikurensis, Orientia*
582 *tsutsugamushi* and *Rickettsia* sp infections were associated with strong decreases in relative
583 abundance of *Erysipelotrichaceae* (Firmicutes) (Table 3; Table S3E). Other associations between
584 bacterial infections and changes in relative abundance of specific gut bacteriome taxa were
585 detected, but with little size effect (log2 fold changes lower than 5).
586

587 **Discussion**

588 Understanding the complex interlinkages between host microbiome, host-pathogen interactions and
589 health in wild animal populations has become a key topic in disease ecology, with consequences for
590 population dynamics, zoonotic risk management or biodiversity conservation. Here, we *(i)* use a
591 combination of morphological and metabarcoding approaches to describe intra-host communities of
592 wild rodent populations at a small geographical scale, *(ii)* provide evidence of intricate
593 interrelationships between the gut bacteriome and communities of gastro-intestinal helminths and
594 pathogenic bacteria, and *(iii)* investigate the influence of host individual and spatial factors on these
595 three intra-host communities, in a concomitant way.
596

597 A diverse intra-host community

598 This study brings a new light to the gut bacteriome composition in wild bank voles, which are
599 important zoonotic reservoirs in Europe. Their gut microbiome has been mainly studied in the
600 context of exposure to radioactive pollutants (e.g., Lavrinienko (2018), but see Knowles et al.
601 (2019)). Although we detect similar phyla and families than these previous studies, our in-depth
602 data allow us to detect other taxa as well as differences in their relative abundance. These
603 differences likely depend on the nature of the samples considered (faecal, colon or caecum

604 samples), given there is strong variation of the gut bacteriome both along the rodent digestive tract
605 (e.g., in mice Anders *et al.*, 2021; Gu *et al.*, 2013) and between digestive tract and faeces (Anders *et*
606 *al.*, 2021) of a single host individual.
607 Besides, our results confirm that helminths infections are the rule in natural populations of bank
608 vole. Indeed, 85% of vole individuals are infected by at least one helminth. This high prevalence
609 level is consistent with studies conducted on wild rodent populations (e.g., for bank voles (Grzybek
610 *et al.*, 2015; Ribas Salvador *et al.*, 2011) or other rodent species (Behnke *et al.*, 1999; Vanden
611 Broecke *et al.*, 2021)). It likely reflects host tolerance to helminths - as a result of a balance between
612 infection costs and immunopathologies-, or the ability of helminths to manipulate the host immune
613 system (Babayan *et al.*, 2018).
614 Our findings also confirm the status of bank voles as hyper-reservoirs of zoonotic agents, with at
615 least six zoonotic bacteria being detected using the 16S V4 metabarcoding approach. This
616 complements previous studies that specifically triggered particular bacteria, including *Leptospira*
617 sp., tick-borne bacteria or *Francisella tularensis* (Fischer *et al.*, 2018; Jeske *et al.*, 2019; Perez *et*
618 *al.*, 2017).
619
620 Intrinsic and extrinsic factors influencing the gut bacteriome
621 We provide evidence of significant inter-individual variations in gut bacteriome composition.
622 Factors intrinsic to bank voles only weakly explain these variations. We find that host gender
623 slightly influences the composition of bank voles' gut bacteriome, males having 1.4 times more
624 Firmicutes than females. Age has no influence on gut bacteriome diversity neither composition, as
625 already found in wild rodents (Goertz *et al.*, 2019; Maurice *et al.*, 2015). However, our sampling
626 did not allow to fully address this issue, given most individuals were adults.
627 Besides, we show that this inter-individual variability of gut bacteriome composition can be
628 categorized in two compositionally different clusters equivalent to enterotypes (Arumugam *et al.*,
629 2011). Enterotypes have already been described in wild rodents (Goertz *et al.*, 2019; Li *et al.*,
630 2016), and they might reflect distinct ways of generating energy from substrates available in the
631 digestive tract, as well as differences in diet (Rinninella *et al.*, 2019; Wang *et al.*, 2014). In bank
632 voles, these enterotypes could be associated with different diets, one oriented toward seeds and
633 plants and another one toward insects and berries. Indeed, enterotype 1 is characterized by families
634 (namely, Helicobacteraceae, Lachnospiraceae and Muribaculaceae) that are involved in the
635 breakdown of carbohydrates, fermentation of plant saccharid and degradation of glycan (see refs in
636 Goertz *et al.*, 2019). These families are also predictive signals of a high-fat diet in mice (Bowerman
637 *et al.*, 2021; Rodriguez-Daza *et al.*, 2020). In the opposite, enterotype 2 is characterized by families

638 (namely, Lactobacillae and Eggerthellaceae) that can be involved in the digestion of fermented food
639 (e.g., rodent food store over winter), and insect skeleton (see refs in Maurice *et al.*, 2015) or in the
640 degradation of polyphenol (Rodriguez-Daza *et al.*, 2020). All these aliments have varying
641 nutritional and chemical composition and may be part of bank vole diet (Ecke *et al.*, 2018). The
642 fraction of these different types of resources in bank vole diet may vary with resource preference or
643 availabilities, reproductive status, sampling date and location (e.g., Maurice *et al.*, 2015).
644
645 Spatial variations in gut bacteriome and their potential causes
646 There are now many evidence that geographical location is likely to shape variations in gut
647 bacteriome composition between localities sampled and studies. Previous works have already
648 shown that the structure of rodent gut microbiome varied between localities at large spatial scales
649 due to biogeographic or genetic factors (Linnenbrink *et al.*, 2013). Geographic variability has also
650 been found at smaller spatial scales (e.g., few km Goertz *et al.*, 2019). Here, our results provide
651 significant evidence for spatial structure of gut bacteriome between bank vole populations that are
652 between 50 and 130 km away, with no obvious barrier to dispersal or gene flow (Dubois *et al.*,
653 2018).
654 We observe gradual changes in terms of gut bacteriome richness, evenness and
655 Firmicutes/Bacteroidetes ratio, between bank voles from the northern and southern populations.
656 Although the links between the diversity and functional capacity of the gut bacteriome are still not
657 fully understood (Worsley *et al.*, 2021), it is largely assumed that changes in diversity are associated
658 with shifts in metabolism (Reese & Dunn, 2018). Bank voles from southern populations exhibit
659 higher specific richness and lower evenness of the gut bacteriome, as well as lower dispersion of
660 gut bacteriome composition. They have higher levels of body condition and F/B ratio, that are
661 indicative of an optimisation of calorie intake and absorption, weight gain and fat storage (see refs
662 in Wolf *et al.*, 2021). Altogether, these results could suggest strong constraints on gut bacteriome
663 function to maximise energy extraction. The northern populations show the opposite patterns.
664 Lower BMI and lower levels of F/B ratio might reflect energy production and conversion, amino
665 acid transport and metabolism, while diversity patterns (higher evenness and lower specific richness
666 of the gut bacteriome) could suggest lower stochasticity and/or directional selection. Further studies
667 are required to investigate the ecological processes driving these changes in gut bacteriome.
668 We also show that gut microbiome dissimilarities increased with distance between populations,
669 marked differences being noticeable between the two more distant localities sampled, Mont-sous-
670 Vaudrey in the North and Cormaranche in the South. These geographic differences might be
671 explained by dietary variations, and variable proportions of plants, seeds, berries and insects in bank

672 vole diet. For example, Erysipelotrichaceae are less represented in the South. These taxa are
673 involved in the metabolism of products of cellulolysis (Bermingham *et al.*, 2017; Kaakoush, 2015).
674 Their expansion may be promoted by plant sterols (Harris *et al.*, 2014) and may be negatively
675 correlated with protein content of the diet (Bermingham *et al.*, 2017).
676 Lastly, these differences in gut microbiome composition between the northern and southern
677 populations might also reflect physiological variations related to physiology, health, and potentially
678 to immunity. Clostridiales and Bifidobacteriaceae participate in the maintenance of intestinal
679 homeostasis, and in the regulation of inflammation or in the gut barrier function (Arboleya *et al.*,
680 2016; Hakansson & Molin, 2011; Lopetuso *et al.*, 2013), while specific taxa within
681 Erysipelotrichaceae may be correlated with inflammation or have immunogenic potential
682 (Kaakoush, 2015; Zhai *et al.*, 2019). Desulfovibrionales activities result in the production of H_2S,
683 that in turn, leads to damages of the gut barrier, production of endotoxins and pro-inflammatory
684 cytokines (Hu *et al.*, 2022). Our previous work revealed that bank voles from these southern
685 populations had lower basal level of *Tnf-a* (a pro-inflammatory cytokine) and higher level of *Mx2*
686 antiviral gene expression than those from these northern populations (Dubois *et al.*, 2018). Future
687 studies should assess the potential relationships between variations in gut bacteriome composition
688 and the capacities to regulate or mount immune responses and inflammation in these bank vole
689 populations.
690
691 Marked relationships between gut bacteriome and helminth communities
692 While we did not detect any relationship between pathogenic bacteria and gut bacteriome diversity
693 or composition, we found evidence for strong association between helminth community and gut
694 bacteriome. The former pattern might be explained by the fact that the pathogenic bacteria detected
695 here do not constitute a functional community, in particular because their ecological niche can be
696 very different. For example, hemotrophic *Mycoplasma* parasitizes erythrocytes (Alabi *et al.*, 2020)
697 while *Borrelia* disseminates through the bloodstream and/or lymphatic system to invade and
698 colonize various tissue (Zeidner *et al.*, 2001). The latter pattern may be interpreted under two
699 perspectives.
700 First, the strong positive associations between the diversity of helminth community and gut
701 bacteriome might corroborate the hypothesis and experimental evidence showing that helminths
702 have the capacity to maintain higher gut microbiota diversity and may represent gut homoeostasis
703 (Kreisinger *et al.*, 2015). Indeed, low-intensity, chronic helminth infections are commonly linked to
704 high microbial diversity and predominance of bacteria typically associated with gut health (Peachey
705 *et al.*, 2017). Nevertheless, this interpretation has to be taken cautiously as the diversity of both

communities was strongly influenced by the localities of sampling. The environment might therefore shape similarly gut bacteriome and helminth community diversity.

Second significant associations between helminth community and gut bacteriome composition - which remain significant even when potential geographic confounding effects were removed - may be linked to the fact that both communities reside in the same environmental niche (host intestines). From there, they likely experience similar selection pressure (e.g., host immune responses), with potentially strong interactions and reciprocal influence expected between them, which could shape their composition (Glendinning *et al.*, 2014). Unfortunately, the causal processes behind these gut microbiome and helminths interactions are complex, multifaceted and difficult to assess, in particular because experimental studies can only focus on single helminth infections– while interactions between/within community are the rule within the host organism. The field of microbiome research would thus benefit from taking into account the whole composition of gastro intestinal helminth community rather than single helminth infections only.

Three-way relationships within intra-host communities

The associations detected between the gut microbiome diversity or composition and the presence/absence of infections (helminths and bacteria) are of two types, and might surprisingly not always reflect gut homeostasis/dysbiosis.

First, some infections are significantly associated with the global composition of the gut bacteriome, but have only little impact on specific gut bacterial taxa. This pattern concerns *Heligmosomum mixtum*, *Bartonella* and *Aonchothecha murisylvatici*. Such associations could suggest long co-adaptation/coevolution between these taxa and the gut bacteriome. Some evidence suggests that *Bartonella* may be acting as a symbiont more than a pathogen (refs in Lei & Olival, 2014). Significant coevolutionary congruence has been found between *Bartonella* species and their rodent hosts, and *Bartonella* infections in rodents lead to an asymptomatic long lasting intra-erythrocytic bacteraemia (Deng *et al.*, 2012; Lei & Olival, 2014). Besides, *Heligmosomum* species have been found to have only relatively low pathogenic impact on voles (e.g., Janova *et al.*, 2010). The negative associations detected here between *Bartonella* and *Heligmosomum mixtum,* and their respective links with Bacteroidetes and Firmicutes, deserve further attention to decipher the potential direct and indirect interactions between these taxa, bank vole immunity and gut bacteriome. Lastly, too little is known on *Aonchotheca murisylvatici* and its impacts on rodent fitness to interpret the relationships observed between this helminth and gut bacteriome composition.

739 Second, other infections are strongly associated with large changes in the relative abundance of one
740 or few specific taxa from the gut bacteriome, but not with the global composition of this later.
741 These species-specific associations concern *Trichuris muris, Caetenotaenia henttoneni,*
742 *Paranoplocephala omphaloides* and *Mastophorus muris* for the helminths and *Neoehrlichia*
743 *mikurensis, Orientia tsutsugamushi, Mycoplasma haemomuris, Anaplasma phagocytophilum* and
744 *Rickettsia* for the bacteria.
745 The bacterial infections were associated with a lower relative abundance of Erysipelotrichaceae.
746 This is striking because observed changes in the gut microbiota during infection are rarely
747 consistent, even with respect to single pathogens (Sabey *et al.*, 2021). In humans,
748 Erysipelotrichaceae have been associated with a number of diseases such as HIV and norovirus
749 infections, inflammation-related intestinal disease and metabolic disorders (Kaakoush, 2015). No
750 study has yet investigated the relationships between Erysipelotrichaceae, gut dysbiosis and
751 infections in rodents. We also find that a larger number of bacterial infections is correlated with
752 lower relative abundance of an unknown family of Bacteroidales. These later have been shown to
753 promote epithelial barrier function and maintain epithelial homeostasis (Kuhn *et al.*, 2018). It would
754 be interesting to investigate whether this family could be an indicator of rodent health, lower
755 abundance reflecting individuals in poor health and more susceptible to opportunistic infections.
756 The species-specific associations between helminths infections and gut bacteria included
757 Proteobacteria (Desulfovibiolales, Burkholderiaceae), Firmicutes (Clostridiales,
758 Erysipelotrichaceae) and Bacteroidetes (Bacteroidales). High-intensity, acute helminth infections
759 may correlate with changes in hosts gut microbiota and gut dysbiosis, through direct and indirect
760 (immune or other processes such as malnutrition) interactions (Peachey *et al.*, 2017). Nevertheless,
761 up to now, the patterns of shifts in gut bacteriome associated with helminth infections remain hardly
762 predictable. Research that addressed this issue, using laboratory or wild animals, have provided
763 variable and sometimes even contradictory conclusions, even for single host-helminth models. A
764 potential explanation is that these infection-associated microbiota shifts could depend on the
765 presence of other helminths and the duration of infection (e.g. Sabey *et al.*, 2021). Local
766 interactions between helminths and between helminths and gut bacteria could mediate changes in
767 infection outcomes as well as the gut bacteria and helminth populations themselves (Glendinning *et*
768 *al.*, 2014).
769

770 **Conclusion**

771 Altogether, these results emphasize complex interlinkages between gut bacteriome, gastro-intestinal
772 helminths and bacterial infections in wild animal populations. We emphasize the strong impact of

environment, even at fine geographical scales, on these interactions. Shifts in diet or host genetics could mediate the spatial changes observed in gut bacteriome. However, the processes shaping gut bacteriome diversity and composition are many and complex, and further investigations are required to decipher the relative importance of drift, dispersal or selection on bank vole gut bacteriome in the populations studied here. Besides, we find a diverse array of associations between gut bacteriome and gastro-intestinal helminths or pathogenic bacteria, some being significant at the scale of the whole community and other being species-specific only. Whether these patterns reflect coadaptation, dysbiosis or indirect interactions with host immunity and coinfections should now be considered to better understand the spatial variations observed in the relationships between gut bacteriome and health.

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Conflict of interest disclosure

The authors declare that they have no financial conflict of interest with the content of this article. N. C. is one of the PCI Inf recommenders. B.R. is part of the managing board of PCI Inf.

Data and script availability

Raw data and scripts are available on zenodo: https://doi.org/10.5281/zenodo.6573141

Supplementary information

Supplementary Figure S1. Maps showing the sampling area (left) and localities (right) in France. Forests are indicated in green and water in blue. The four sampling localities are represented with a colored polygon. The arrow indicates the North.

807

808 **Supplementary Figure S2**. Composition of the gut bacteriome. The relative abundance of six
809 phyla representing 99% of the total composition is represented. Individuals are grouped by
810 sampling localities, which are ordered from North to South. (A) Bar graph shows individual
811 variation in phyla composition (phylum=color). (B) Box and whisker plots represent median and
812 interquartile values for each phylum. Black dots correspond to mean values, and colored dots
813 correspond to individuals.

814

815 **Supplementary Figure S3**. Variations of alpha diversity between localities, for the gut bacteriome
816 (phylum level), pathogenic bacteria and gastro-intestinal helminth communities. Alpha diversity is
817 represented using the specific richness of the gut bacteriome (A) or the Shannon index of the gut
818 bacteriome (B). Results are shown per localities, ordered from North to South. Each colored point
819 represents an individual. Black point indicates the average alpha diversity per locality. Box-and-
820 whisker plots represent the median and interquartile values. Different letters denote statistically
821 significant differences at $P < 0.05$, after pairwise post-hoc Tukey adjustments.

822

823 **Supplementary Figure S4**. Variations of alpha diversity with individual factors, for the gut
824 bacteriome (family level), pathogenic bacteria and gastro-intestinal helminths of bank voles. Alpha
825 diversity is estimated using the Shannon index (A and D) or the specific richness (G) of the gut
826 bacteriome. In all other graphs, the specific richness of the pathogenic bacteria and gastro-intestinal
827 helminths communities is used. In G, H, I, the blue line corresponds to the linear regression line.

828

829 **Supplementary Figure S5**. Relationships between the composition of the gut bacteriome,
830 pathogenic bacteria and gastro-intestinal helminth communities: A) db-RDA triplot shows the
831 structure of the gut microbiome at the family level and the correlations with the pathogen
832 communities. The arrows correspond to the significant explanatory variables. Each point
833 corresponds to an individual, and the colors correspond to the different sampling localities.

834

835 **Supplementary Table S1.** Variation of the Firmicutes/Bacteroidetes ratio with localities and
836 individual factors.

837

838 **Supplementary Table S2.** Alpha diversity metrics and statistics for the gut bacteriome, pathogenic
839 bacteria and helminth communities of bank voles.

840

Supplementary Table S3. Beta diversity metrics and statistics for the gut bacteriome, pathogenic bacteria and helminth communities of bank voles.

AUTHOR CONTRIBUTIONS

M.B.: Data curation; Formal analysis; Methodology; Writing – original draft

M.G.: Conceptualization; Data curation; Formal analysis; Methodology; Supervision; Writing – review and editing

A.D.: Conceptualization; Data curation

C.A.D.: Data curation; Writing – review and editing

P.M.: Conceptualization; Funding acquisition; Investigation; Supervision; Writing – review and editing

B.R.: Conceptualization; Methodology; Supervision; Writing – review and editing

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