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Sympatric *Lepus* spp. in the central Italian Alps host significantly different gut microbiotas

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Sympatric *Lepus* spp. in the central Italian Alps host significantly different gut microbiotas

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Abstract

The mountain hare (*Lepus timidus*) is an arctic-alpine species with relictual populations in the Italian Alps, typically occurring at elevations above 2000 m a.s.l. This species is threatened by habitat loss and fragmentation, and declining snow cover due to climate warming. Moreover, as treelines shift upward, the European brown hare (*L. europaeus*) is expanding its distribution into areas previously dominated by the mountain hare, potentially leading to resource competition, and loss of local adaptation through hybridization and inter-specific gene flow. In particular, the consequences of sympatry on diversity and composition of prokaryote and fungal communities of the gut microbiota, which are critical to individual health, are currently unknown.

Here, we compared the gut microbiota of these two hare species in an area of overlap in the central Alps by analysing fresh faecal pellets collected from Val Mazia/Matschertal, Italy along an elevational gradient (1000 to 2500 m a.s.l.). For the first time, we describe the prokaryote diversity and composition of *L. timidus*, and the fungal gut communities (mycobiota) of both *Lepus* species. Species identity was confirmed for 95 samples via mtDNA barcoding, while gut microbiota richness and composition were investigated using amplicon sequencing, targeting the V3-V4 region of the prokaryote 16S rRNA gene and fungal ITS2 regions.

Distinct prokaryote and fungal communities were observed for each species, even where their distributions overlap, indicating differences in their functional diversity. Interestingly, for both *Lepus* species, elevation influenced fungal but not prokaryote diversity. Therefore, sympatry appears to have had minimal impact on gut microbiota composition of either species thus far. Given the expected upward range shift of *L. europaeus* under climate warming and its continued restocking for hunting, our findings provide an important baseline for assessing the health and adaptability of *L. timidus* as well as the effectiveness of conservation efforts aimed at protecting this species. However, expanding this research to other areas of sympatry will be essential to understand if gut microbiota composition is indicative of *L. timidus* conservation status across its range.

Keywords: *Lepus europaeus, Lepus timidus*, metataxonomy, mtDNA, 16S rRNA gene, ITS2

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Introduction

One of the most dramatic and rapid impacts of climate warming in the Alps is the shifting of vegetational zones to higher elevations [1, 2, 3], accompanied by upward distributional changes of animal species as they track their preferred habitats [4, 5, 6]. An emblematic example, estimated from 30 years of hunting bag data [7], is the mountain hare (*Lepus timidus*), a species generally associated with boreal zones above the treeline [8, 9]. However, results in [7] also indicated that the European brown hare (*Lepus europaeus*), a steppe-adapted species [10], is also shifting upward even more rapidly, which may lead to a reduction in mountain hare habitat. In addition, sympatry of the two hare species could result in their hybridization, as already reported by several authors for northern European as well as Alpine populations (e.g. Fennoscandia: [11]; Swiss Alps: [12]; western Italian Alps: [13] and references therein). These risk factors could threaten the long-term persistence of the mountain hare and its unique genetic pool in Alpine habitats.

Interspecific hybridization and range shifts (associated with dietary changes) could also lead to the loss of local adaptation, for example by altering the gut flora, which in hybrids can be intermediate between the two parental lineages (e.g. equids: [14]; suids: [15]). It is now well-recognized that an intact gut microbiota (including prokaryotes, fungi, and viruses) is essential for immune and metabolic function, as well as for development of the neuroendocrine system [16, 17, 18], impacting host health and resilience. It has been suggested that the presence of specific microorganisms in the gut may be important for host fitness and should be considered for the conservation and management of wild animal species [19, 20]. While host phylogeny has a strong influence on gut flora for many mammals, with various authors reporting species-specific compositions [21, 22], extrinsic factors such as habitat preference and diet are also expected to have an impact on gut flora alpha and beta diversities [23, 24].

Since the European brown hare is rapidly colonizing higher elevations in the Alps due to climate warming, increasing sympatry with the mountain hare is inevitable. Thus, defining gut microbiota of the two hare species is useful for understanding the potential consequences of their interaction. Here, for the first time, we describe and compare the gut microbiota composition of the two *Lepus* species in an area of sympatry in Val Mazia/Matschertal, Province of Bolzano, Italy using MiSeq amplicon sequencing of the 16S rRNA and ITS2 genes. This research aims to understand how host species and elevation influences the microbiota of these lagomorphs. Although the prokaryote gut microbiota of the European brown hare has previously been reported by Stalder et al. [25], no similar data exist for that of the mountain hare. In addition, the mycobiota of both species has not been studied to date, despite its potential relevance for host health [26]. Importantly, no studies have yet examined the gut microbiota of sympatric populations.

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Materials and methods

Study area and sample collection

Single fresh pellets of *Lepus* spp. were collected at least 20 m apart, from 12 sampling areas, three from each of four elevations (1000, 1500, 2000 and 2500 m a.s.l.) in July 2019 and 2020 in the Vinschgau Valley LTSER, South Tyrol, Italy (site code LTER_EU_IT_097, 46.6928°, 1.6157°; https://deims.org/11696de6-0ab9-4c94-a06b-7ce40f56c964; Fig. 1) as part of the EUREGIO project MICROVALU. Freshly deposited faecal pellets were identified by their shiny (mucous smeared) external surface. Fieldworkers wore N95 masks and used sterile gloves and tweezers to collect the pellets, which were placed in certified sterile DNA/DNAse-free 15 ml tubes (Sarstedt, Germany) and stored at -20°C for up to 24h before being transferred to the Animal, Environmental and Antique DNA Platform at the Fondazione E. Mach (FEM), where they were archived at -80°C until further processing. Sample details are provided in the Supplementary Tab. S1.

Meteorological data were collected on site using Campbell Scientific CR1000/CR1000X loggers and provided by Zandonai et al. [27], including daily average temperature in the three weeks prior to sampling and sum of precipitation of the entire vegetation period (May 1st to Sept 30th). Temperature was recorded with three loggers per elevation, while precipitation was recorded by one meteorological station at each elevation. Species richness was estimated using a square plot with a grid of 100 cm^2 (10x10 cm) units to calculate the number of plant species in each elevational zone, while plant richness was obtained from Hilpold et al. [28]. Botanical data of the 2500 m a.s.l. sites was then added to the previously published elevation surveys.

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DNA extraction

Under a sterile Class II biological safety cabinet (BSL2, Telstar, Spain) and using sterile DNA/DNase-free equipment and consumables, 50 mg of faecal material were taken from the centre of each pellet to avoid the outer layer that had potentially been in contact with soil. Total DNA was extracted from each 50 mg subsample of a single faecal pellet with the NucleoSpin Soil mini kit (Macherey-Nagel, Germany) using the lysis buffer SL1 in combination with 50 µl of Buffer SX, as suggested by Praeg, Pauli, and Illmer [29]. Each sample was co-extracted with a 0.15 dose of ZymoBIOMICS™ Spike-in Control I (High Microbial Load; EuroClone, Milan, Italy), before the initial lysis step following Galla et al. [30], unless otherwise stated (Tab. S1). This mock community was added as an in situ positive control to validate the accuracy and reliability of sequencing and bioinformatics pipeline. Negative DNA extraction controls were included up to the sequencing step to monitor environmental and reagent contaminations. The purity and quantity of all DNA extracts

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were assessed by checking the UV/VIS spectra of each extract with a Spark multimode microplate reader (Tecan, Switzerland). Following quantification, all DNA extracts were diluted to a final concentration of 3 ng/µl using nuclease-free water and subsequently used for species identification and amplicon sequencing.

Host species identification

To determine the origin of each pellet (*L. europaeus* or *L. timidus*), a ~500 bp-fragment of the mitochondrial DNA D-loop region was amplified using GoTaq DNA polymerase by Promega with primers (H) GTTGCTGGTTTCACGGAGGTAG and (L) TCCTACCATCAGCACCCAAAGC' previously designed by Wilkinson & Chapman [31]. Cycling conditions consisted of an initial denaturation step at 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 45 s, with a final extension step at 72 °C for 5 min. Negative PCR controls (PCR-grade water instead of DNA template) were included to check for contamination. Amplified DNA was purified using ExoProStarTM 1-Step (Cytiva, USA) and sequenced at the FEM Sequencing and Genotyping Platform with both primers H and L using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reaction products were further purified with the Dynabeads Sequencing Clean-Up Kit (ThermoFisher Scientific) and subsequently analyzed on an ABI 3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA), 96 capillaries (50 cm) and POP07 Performance Optimized Polymer (Applied Biosystems). The sequences were processed with Sequencher software (Gene Codes Corporation-USA) to delete any automatic base assignment errors. BLASTn was used to identify sequences with ≥99% identity as defined using the Standard Nucleotide method. Moreover, D-loop sequences were trimmed to a 334 bp region shared by all sequences as well as 147 D-loop sequences deposited in NCBI by Melo-Ferreira et al. [32], Pecchioli et al. (unpublished PhD thesis) [33] and Zachos et al. [34] and aligned with MEGA v. 10.0.5 (option Muscle; [35]). A Neighbor-Joining phylogenetic tree was constructed in MEGA, using the Phylogeny option, based on pairwise genetic distance.

Characterization of V3-V4 16S rRNA gene and ITS2 faecal communities

The amplification of the V3-V4 regions of the prokaryote 16S rRNA gene was performed using the KAPA HiFi HS ReadyMix (Roche) in a 25 µl reaction volume containing 1X KAPA HiFi HS ReadyMix Buffer, 0.3 µM each of primers 341F_ILL (CCTACGGGNGGCWGCAG) and 805R-2_ILL (GACTACNVGGGTWTCTAATCC) modified with Illumina overhang adapters [36, 37] (https://support.illumina.com/documents/documentation/chemistry_documentation/16s) and 9 ng of DNA. The thermal profile for 16S V3-V4 amplification reactions was: 3 min at 95 °C, followed by

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31 cycles of 30 sec at 95 °C, 30 sec at 55 °C, 30 sec at 72 °C, and a single final extension step of 5 minutes at 72 °C. The amplification of the fungal rRNA Internal Transcribed Spacer ITS2 was performed in a 25µl reaction volume containing 1X FastStart High Fidelity Reaction Buffer (Roche Applied Science), 0.4 µM each of primers gITS7 (Illumina_forward_overhang-GTGARTCATCGARTCTTTG) and ITS4 (Illumina_reverse_overhang-TCCTCCGCTTATTGATATGC) [38, 39], 200 µM each dNTPs, 9 ng of DNA, and 1.5 U of FastStart High Fidelity Enzyme Blend (Roche Applied Science, Germany). The thermal profile for ITS2 amplification reactions was 3 min at 95 °C, followed by 35 cycles of 45 sec at 95 °C, 45 sec at 50 °C, 45 sec at 72 °C, and a single final extension step of 5 minutes at 72 °C. All amplifications were performed on a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems, USA). Non-template controls were included in each amplification reaction. Amplicons were visualized by high-resolution capillary electrophoresis using the QIAxcel Advanced System (Qiagen, Hilden, Germany). Quantification of individual amplicon libraries, normalization at equimolar concentrations, pooling of indexed libraries and high throughput sequencing by Illumina technology were performed at the FEM Sequencing and Genotyping Platform. Libraries were sequenced on an Illumina MiSeq platform using Standard Flow Cells (PE300), targeting a minimum sequencing depth of 30,000 reads per amplicon.

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Amplicon sequencing data analysis

Bioinformatic analyses were performed in R v. 4.3.1. DADA2 v. 3.14 [40] was used for sequence quality inspection, primer trimming, chimera removal and ASVs taxonomy assignment following the standard operating procedure. Taxonomic classification of 16S rRNA gene and ITS2 ASVs was performed using Silva v. 138.1 [41] and UNITE v. 9.0 [42] reference databases, respectively. Multiple sequence alignment of 16S rRNA gene ASVs was done with msa v. 1.32.0 [43] using ClustalW and default parameters. With the Phangorn package v. 2.11.1 [44], a phylogenetic tree was constructed with 16S rRNA gene ASVs, which was then used for further analysis.

Sequencing results of negative controls from both DNA extraction (N=12) and PCR amplification (N=13) were used to identify potential contaminants using decontam v. 1.20.0 [45] with the prevalence method. All mock community-related ASVs were removed from the dataset before performing subsequent analyses. Statistical analyses of 16S rRNA gene and ITS2 ASVs were performed using phyloseq v. 1.50.0 [46] and microeco v. 4.3.1 [47]. Images were generated using ggplot2 v. 3.3.5 [48], using RColorBrewer v. 1.1-2 [49] and viridis v. 0.6.5 [50].

The impact of species and elevation on taxonomic composition (alpha and beta diversity) of faecal microbiota were investigated by focusing on elevations where the two species live in sympatry (Tab.

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S1). *L. timidus* and *L. europaeus* datasets were then analysed separately to assess intraspecific variation in faecal microbiota across all elevations where they were sampled.

Taxonomic profiling of 16S rRNA and ITS2 regions

Taxonomic classification of 16S rRNA gene and ITS2 ASVs was estimated on non-rarefied data and expressed as relative abundances. Visualizations were generated using microeco, focusing on the 10 most abundant phyla and 16 most abundant classes and families. The relative abundance of prokaryote and fungal taxa was compared between the two species with LEfSE [51].

Alpha and beta diversity estimation

To avoid bias in richness estimates due to different sequencing depths, alpha diversity indices Chao1, inverse Simpson, Shannon and Faith's Phylogenetic Diversity (PD; this latter index for prokaryotes only) were estimated using 16S rRNA gene and ITS2 datasets rarefied to 10,564 and 10,804 ASVs per sample, respectively. Alpha diversity estimates were compared between *Lepus* species and elevations using Wilcoxon rank-sum tests, with microeco. Venn diagrams representing the number of ASVs shared between *L. timidus* and *L. europaeus* were generated using the rarefied data, microeco. Beta diversity estimates were computed on non-rarefied ASV abundances normalized as relative abundances using Bray-Curtis, unweighted Unifrac (for 16S rRNA gene only) and Jaccard (for ITS2 only) indices. Ordination based on distance/dissimilarity matrices was done using non-metric multidimensional scaling (NMDS). Multivariate homogeneity of group dispersions, was tested using betadisper with Vegan v. 2.6.4 [52]. To compare beta-diversity estimates between groups, permutational multivariate ANOVA (PERMANOVA), and pairwise-PERMANOVA were performed with 999 permutations using the function adonis2 implemented in microeco. The same package was used for the analysis of similarities (ANOSIM). Pairwise-PERMANOVA p-values were adjusted using the false discovery rate (FDR) method to control for multiple testing.

Impact of environmental variables on beta diversity

Significant differences in daily temperature, pre-sampling total precipitation and plant species richness between elevations were inspected using a Wilcox rank-sum test (data not shown). Autocorrelation among environmental variables was assessed using Spearman's Rank-Order Correlation using microeco. For both 16S rRNA gene and ITS2 datasets, Mantel tests employing Spearman rank order and Pearson correlation coefficients were used to examine associations between environmental parameters and beta diversity estimates. Spearman rank-order correlations were

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calculated to evaluate relationships between environmental parameters and the abundance of each 16S rRNA and ITS2 ASVs, also using microeco.

Characterization of prokaryotic functional diversity

Prokaryote functional diversity was inspected using the PICRUSt2 pipeline v. 2.5.3 [53] implemented on Galaxy (https://usegalaxy.eu/) with default settings. Estimated MetaCyc pathway abundances were normalized to relative abundances. Alpha diversity (Shannon index) and beta diversity (Bray-Curtis dissimilarity) were estimated from normalized MetaCyc pathway abundances using microeco. Differences in MetaCyc pathway abundances between *Lepus* species and elevations was tested using Wilcoxon rank-sum text with FDR p-value adjustment. Differential pathways were defined by a Log_2FC cutoff of ≥1 and an adjusted p-value of ≤0.01. Plots were generated with ggplot2. A heatmap clustering samples and pathways based on MetaCyc pathways abundances was generated using ClustVis [54] with default parameters and further formatted using GIMP v. 2.10.18 [55]).

Results

Lepus species identification

Of 108 total fresh faecal pellets, 95 provided reliable mtDNA D-loop results, identifying 72 *L. europaeus* and 23 *L. timidus* samples. As expected, *L. timidus* was restricted to higher elevations, with nine and 14 samples found at 2000 m and 2500 m a.s.l., respectively. In contrast, *L. europaeus* ranged across the study area, with 27, 26, 16 and 3 samples collected at 1000, 1500, 2000, and 2500 m a.s.l., respectively (Fig. 1).

Taxonomic composition of *L. timidus* and *L. europaeus* faecal microbiota

The profiling of *L. europaeus* and *L. timidus* faecal microbiota identified 6145 ASVs for the prokaryote 16S rRNA gene and 2219 ASVs for the fungal ITS2 region. Of these, 22 and 16 ASVs were identified by decontam as potential contaminants and removed from the 16S rRNA gene and ITS2 datasets, respectively. Additionally, 24 ASVs were classified as belonging to the two taxa composing the mock community: *Allobacillus* spp. (7 ASVs) and *Imtechella* spp. (17 ASVs); all of these ASVs were also removed from the dataset before statistical analyses. The overall median relative abundance of these mock community-related ASVs was 3.2% (ranging from 0.2% to 15%) and was comparable between *Lepus* species and elevations (Kruskal-Wallis, p-value: 0.3487). Following rarefaction, 16S rRNA gene and ITS2 datasets accounted for 4877 and 1514 ASVs, respectively (Fig. S1a-b). Rarefaction resulted in the loss of seven 16S rRNA gene (5 *L. europaeus* and 2 *L. timidus*) and five ITS2 libraries (three *L. europaeus* and two *L. timidus*).

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Both species were characterized by private ASVs accounting for approximately 82% of 16S rRNA and 12% ITS2 sequence reads. In contrast, only about 6% of prokaryote and 9% of fungal ASVs were shared by faecal samples of both species. Private *L. europaeus* prokaryote ASVs prevailed in the gut community which consisted of about 70% of sequenced reads. In contrast, dominant fungal ASVs were shared, accounting for approximately 84% of generated sequence reads (Fig. S1a-b). Restricting the analysis to elevations where the two species were sympatric produced results consistent with those above, with approximately 45% of prokaryote ASVs found only in *L. europaeus* samples, 35% of prokaryote ASVs uniquely found in *L. timidus* samples (Fig. S1c) and only a small fraction of prokaryote ASVs found in both species with relatively high abundance (e.g. 10 ASVs detected in both species and elevations accounted for 10% of sequence reads). Regarding fungi, most ASVs were shared by both species at all elevations (Fig. S1d).

The taxonomic classification of prokaryote phyla and fungal classes found in *L. europaeus* and *L. timidus* faecal pellets is shown in Fig. 2. At all elevations, the faecal microbiota of *L. europaeus* was dominated by the prokaryote phyla Firmicutes (median: 63.3%, range: 0.28% - 80.8%) and Bacteroidota (median: 16.4%, range: undetected - 33.0%) followed, but with much lower percentages, by Actinobacteriota (median: 2.0%, range: undetected - 70.3%), Spirochaetota (median: 1.2%, range: undetected - 17.1%) and Patescibacteria (median: 1.0%, range: undetected - 30.5%). In addition, about 15% of the 72 samples collected across all four elevations were characterised by a notably high abundance of Proteobacteria (median: 0.7%, range: 0.1% - 88.0%) (Fig. 2a). In contrast, *L. timidus* faecal microbiota was dominated by Proteobacteria (median: 62.9%, range: 4.6% - 78.9%), Bacteroiodota (median: 22.3%, range: undetected - 29.2%) and, to a lesser extent, Actinobacteriota (median: 5.7%, range: 0.2% - 25.8%) and Acidobacteriota (median: 1.2%, range: undetected - 4.2%) (Fig. 2a). Firmicutes was detected in *L. timidus* faecal samples as well, but in lower percentages (median: 0.3%, range: 0.1% - 26.2%).

The faecal mycobiota of both species was characterized by a high relative abundance of Dothideomycetes (*L. europaeus* median: 51.8%, range: 6.3 - 87.9%; *L. timidus* median: 43.6%, range: 2.8 - 75.1%), Leotiomicetes (*L. europaeus* median: 15.5%, range: 1.0% - 74.9%; *L. timidus* median: 22.1%, range: 0.4% - 81.3%) and Sordariomycetes (*L. europaeus* median: 6.0%, range: undetected - 49.1%; *L. timidus* median: 0.9%, range: 0.1 - 20.3%). *L. europaeus* faecal samples also had non negligible occurrences of Pezizomycetes (median: 1.2%, range: undetected - 66.6%) and Microbotryomycetes (median: 0.1%, range: undetected - 40.9%) (Fig. 2b), whereas *L. timidus* faecal samples were notable for the abundance of Tremellomycetes (median: 14.9%, range: 0.1% - 53.4%), this latter class being particularly abundant in samples collected at 2500 m a.s.l. (Fig. 2b).

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The taxonomic composition of faecal microbiota was significantly different between the two study species where they were found to be sympatric (2000 and 2500 m a.s.l.). As shown in Fig. 3 and Tab. S2, *L. europaeus* faecal microbiota was significantly enriched with the following prokaryote phyla: Firmicutes (LEfSE; LDA = 5.47, adjusted p-value < 0.0001), Spirochaetota (LEfSE; LDA = 4.11, adjusted p-value < 0.0001), Verrucomicrobiota (LEfSE; LDA = 3.57, adjusted p-value < 0.0001). Instead, *L. timidus* faecal microbiota had significantly higher abundances of Proteobacteria (LEfSE; LDA = 5.42, adjusted p-value < 0.0001), Actinobacteriota (LEfSE; LDA = 4.05, adjusted p-value < 0.0001), Acidobacteriota (LEfSE; LDA = 3.93, adjusted p-value < 0.0001) and Myxococcota (LEfSE; LDA = 3.38, adjusted p-value < 0.001), among others (Fig. 3a, Fig. S2, Tab. S2).

We also found differences in relative abundance of fungal classes between the two host species at 2000 and 2500 m a.s.l., mainly related to taxa belonging to Ustilaginomycetes (phylum Basidiomycota; LEfSE; LDA = 4.00, adjusted p-value = 2.57E-3) and Pezizomycetes (phylum Ascomycota; LEfSE; LDA = 3.86, adjusted p-value = 4.13E-3), that were all more abundant in *L. europaeus* (Fig. 3, Tab. S2). Instead, the two Basidiomycota fungal classes Tremellomycetes (LEfSE; LDA = 4.93, adjusted p-value = 9.34E-4) and Cystobasidiomycetes (LEfSE; LDA = 3.67, adjusted p-value = 5.57E-3) had a higher relative abundance in *L. timidus* (Fig.3b, Fig. S2, Tab. S2).

Interspecific variation in diversity of *L. timidus* and *L. europaeus* faecal microbiota

Wilcoxon rank-sum tests indicated no significant differences in faecal microbiota richness (Chao1), diversity (Shannon, Inverse Simpson), or Faith's phylogenetic diversity (PD) between the two *Lepus* spp. species (Fig. S3), regardless of whether all four elevations were included or only the two where both species were found in sympatry (i.e. 2000m, 2500 m a.s.l.). However, NMDS based on beta diversity estimates highlighted a clear separation between the microbial communities of *L. europaeus* and *L. timidus* (Fig. 4a, c). Consistently, the PERMANOVA analysis performed on samples collected from both species at 2000 and 2500 m a.s.l. which indicated significant differences in prokaryote community composition (PERMANOVA: unweighted Unifrac: $R^2 = 0.20$, p-value = 0.001; Bray-Curtis: $R^2 = 0.27$, p-value = 0.001; Tab. 1). These findings were further corroborated by the lack of differences in group dispersions across species and elevations (betadisper; p-value > 0.05 in all cases except *L. timidus* at 2000 m a.s.l. vs *L. timidus* at 2500 m a.s.l.). The clear differentiation in faecal prokaryotic communities between *L. europaeus* and *L. timidus* at 2000 and 2500 m a.s.l. was further confirmed by ANOSIM (Bray-Curtis: R = 0.886, p-value = 0.001; unweighted Unifrac R = 0.675, p-value = 0.001).

For the fungal community, NMDS clustering of *L. timidus* and *L. europaeus* faecal samples collected at sympatric elevations revealed distinct clusters associated with species (Fig. 4b, d). This

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was supported by PERMANOVA analyses, which highlighted significant differences in the fungal community composition between *L. timidus* and *L. europaeus* (PERMANOVA: Jaccard: $R^2 = 0.133$, p-value = 0.001; Bray-Curtis $R^2 = 0.240$, p-value = 0.001), as well as across the two considered elevations (PERMANOVA: Jaccard: $R^2 = 0.033$, p-value = 0.046; Bray-Curtis $R^2 = 0.052$, p-value = 0.007). A weaker but still significant species-by-elevation interaction was observed (Jaccard: $R^2 = 0.029$, p-value = 0.085; Bray-Curtis $R^2 = 0.041$, p-value = 0.022; Tab. 1). Additional support for species-specific differences in mycobiota at sympatric elevations was provided by ANOSIM (Bray-Curtis: R = 0.438, p-value = 0.001; Jaccard R = 0.517, p-value = 0.001).

Intraspecific variation in diversity along an elevational gradient

NMDS clustering and PERMANOVA analysis of *L. europaeus* samples across the entire elevational gradient (Fig. 5a, b; Tab. S3) provided limited evidence for an association between elevation and prokaryote community composition (PERMANOVA: Bray-Curtis: R^2: 0.064, p-value = 0.011; Unifrac: R^2: 0.053, p-value = 0.082). This result was also confirmed by the ANOSIM results which showed no significant differences across elevations (Bray-Curtis: R = 0.046, p-value = 0.075; unweighted Unifrac R = 0.032, p-value = 0.129). Conversely, the same ordination and statistical analysis of *L. europaeus* fungal communities revealed a clear association between elevation and composition of faecal mycobiota (PERMANOVA: Bray-Curtis $R^2 = 0.153$, p-value = 0.001. Jaccard $R^2 = 0.098$, p-value = 0.001; ANOSIM; Bray-Curtis: R = 0.244, p-value = 0.075; Jaccard: R = 0.3075, p-value = 0.129) (Tab. S3, Fig. S4b). Similarly, no association with elevation was detected for *L. timidus* prokaryote beta diversity estimates (PERMANOVA and ANOSIM: p-value > 0.05; Tab. S3; Fig. S4c). In contrast, mycobiota composition showed a significant association with elevation (PERMANOVA: Bray-Curtis $R^2 = 0.118$, p-value = 0.018. Jaccard $R^2 = 0.079$, p-value = 0.014; ANOSIM; Bray-Curtis: R = 0.339, p-value = 0.001; Jaccard: R = 0.360, p-value = 0.005; Tab. S3, Fig. S4d). Consistently, pairwise PERMANOVAs performed separately for *L. europaeus* and *L. timidus* revealed significant differences in fungal beta diversity between faecal samples collected at all elevation pairs, including those from neighbouring elevations (pairwise PERMANOVA: adjusted p-value < 0.05 for both species in all pairwise comparisons; Tab. S4). Conversely, no significant differences were observed in prokaryote communities across elevations for either species, including those between the most distant sites (pairwise PERMANOVA: adjusted p-value > 0.05 for both species in all pairwise comparisons; Tab. S4).

Influence of environmental factors on *Lepus* spp. microbial communities

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No significant correlations were detected between prokaryote diversity and temperature, pre-sampling precipitation, or plant richness for either *L. europaeus* or *L. timidus* (Mantel test: Spearman's ρ adjusted p-values > 0.01; Tab. S5). Instead, fungal diversity in faecal samples of *L. europaeus*, but not *L. timidus,* was significantly associated with all three environmental variables: temperature (Mantel test; Bray-Curtis: Spearman's ρ =0.288, adjusted p-values = 0.002; Jaccard: Spearman's ρ = 0.262, adjusted p-values = 0.001), pre-sampling total precipitation (Mantel test; Bray-Curtis: Spearman's ρ = 0.118, adjusted p-values = 0.006; Jaccard: Spearman's ρ = 0.255, adjusted p-values = 0.001) and plant richness (Mantel test; Bray-Curtis: Spearman's ρ = 0.123, adjusted p-values = 0.002; Jaccard: Spearman's ρ = 0.279, adjusted p-values = 0.001) (Tab. S5). Furthermore, several fungal ASVs were found to be correlated with pre-sampling precipitation (four ASVs, positively correlated), temperature (15 ASVs positively correlated; seven ASVs negatively correlated), and plant richness (five ASVs positively correlated; 17 ASVs negatively correlated) (Fig. 5e).

Functional diversity of prokaryote microbiota in *Lepus* spp.

The Wilcoxon Rank-Sum on predicted metaCyc pathway abundances suggested there were significant differences in Shannon (functional) diversity between faecal samples of sympatric *L. timidus* and *L. europaeus*, with *L. timidus* showing higher Shannon estimates than *L. europaeus* (Fig. 6a). However, no intraspecific difference in functional diversity estimates was found between elevations (Fig. 6b). The clustering of faecal prokaryote communities using NMDS with beta diversity estimates (i.e. Bray-Curtis dissimilarity) on predicted metaCyc pathway abundances highlighted a clear separation between the two *Lepus* spp. species (PERMANOVA: Bray-Curtis R^2 = 0.498, p-value = 0.001; ANOSIM: Bray-Curtis R = 0.327, p-value = 0.001; Fig. 6c). The clustering of *Lepus* spp. samples based on pathway abundances (Fig. 6d) identified the species as the main clustering variable. Consistently, differential abundance testing comparing the predicted metaCyc pathway abundances across *Lepus* spp. species and elevations identified 75 pathways, all showing significant differences between *L. europaeus* at 2000 m a.s.l. and *L. timidus* at 2500 m a.s.l. (Fig. 6d, Tab. S7). Of note, 61 out of these 75 pathways displayed significant differences in abundance between *L. europaeus* and *L. timidus* at 2000 m a.s.l. as well. Additionally, the contrast between *L. europaeus* and *L. timidus* at 2500 m a.s.l. identified 24 metaCyc pathways with significant differences between the two species, the majority of which (70.8%) were also included in the contrast between *L. europaeus* at 2000 m a.s.l. and *L. timidus* at 2500 m a.s.l. Differential pathways were involved in different classes of biological processes (e.g. superclasses), with Cofactor, Carrier, and Vitamin Biosynthesis (18 pathways, 24% of differential pathways), Fatty Acid and Lipid Biosynthesis (7 pathways, 9% of differential pathways), Carbohydrate Degradation (7 pathways, 9% of differential

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pathways), Amino Acid Degradation (5 pathways, 7% of differential pathways), and Nucleoside and Nucleotide Degradation (5 pathways, 7% of differential pathways) being the most represented (Fig. 6d, Tab. S7). Of note, while most of these superclasses displayed a relatively balanced proportion of pathway abundances between the two species, all differential pathways within the superclasses Fatty Acid and Lipid Biosynthesis and Amino Acid Degradation were significantly enriched in *L. timidus* compared to *L. europaeus*.

Discussion

Gut microbiota diversity and composition are known to significantly impact animal health and survival [56, 57]; thus, investigating gut microbial communities in species potentially threatened by rapid biotic and abiotic environmental changes may enhance our understanding of their conservation status and extinction risk. In this study, we characterized the gut microbiota of the mountain (*L. timidus*) and European brown (*L. europaeus*) hares using field-collected faecal samples to investigate the consequences of climate change-driven sympatry on their gut prokaryote and fungal communities. Our data confirms the effectiveness of non-invasive samples for the monitoring of gut microbial diversity and composition in elusive species such as *Lepus* spp. Unexpectedly, despite overlapping in the upper 1000 m of their vertical distribution, our results demonstrated that *L. europaeus* and *L. timidus* have distinct faecal microbiota and mycobiota profiles. For the first time, we also identified a significant association between elevation and fungal diversity in both species.

The composition of *L. timidus* and *L. europaeus* faecal microbiotas are distinct

The prokaryote faecal microbiota of the two *Lepus* spp. were characterized by comparable alpha diversity estimates (Fig. S3), but marked differences in community structure (Fig. 4), with up to 27% of observed variation associated with host species (Tab. 1). Accordingly, prokaryote communities displayed distinct taxonomic profiles (Fig. 2, 3), with only a limited number of shared abundant ASVs (i.e. ~6% ASVs accounting for ~21% sequence reads; Fig. S1). *L. europaeus* faecal samples were primarily dominated by the phylum Firmicutes, and rich in Bacteroidota and Spirochaetota. Instead, the most abundant phylum detected in *L. timidus* was Proteobacteria, followed by Bacteroidota and Acidobacteriota. A dominance of Firmicutes and Bacteroidota has already been reported for *L. europaeus* faecal samples [22] as well as *L. granatensis* [58] and *L. americanus* [59]. Instead, a high relative abundance of Proteobacteria, as seen in *L. timidus,* was previously reported for *L. sinensis* [60].

The clear differentiation observed between the microbial communities of the two species, particularly at higher elevations where both species were present, suggests that any contaminants from environmental sources may have had only marginal effects on the diversity or composition of

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faecal bacterial communities. While Firmicutes, Bacteroidota and Proteobacteria are both very commonly found in the mammalian gut, the observed differences in prokaryote composition may reflect co-evolution of host species and associated microbiota after speciation and/or adaptation to different ecological niches [61]. For example, *L. europaeus* is primarily a steppe species (with the earliest fossil evidence in the Alpine region dating to approximately 8,000 years ago [62]), while *L. timidus* is a boreal-adapted species [63]. While there are no other studies addressing microbial diversity in sympatric Leporidae, the greater impact of phylogeny compared to other environmental factors, including diet, has been already documented in other mammals [64, 65, 66]. Therefore, despite their coexistence at higher elevations in our Alpine study area, in keeping with their evolutionary history, these species probably exploit distinct resources, with *L. europaeus* grazing on the high fat parts of weeds/grasses and various crop types richer in fats and proteins, as noted by Reichlin et al. [67] and Schai-Braun et al. [68]), while *L. timidus* may exploit significantly more ligneous plants and Ericaceae [69]. Future studies combining longitudinal vegetation surveys, diet metabarcoding, and metataxonomic investigations are needed to clarify the links existing between seasonal vegetation variation, diet and gut microbial diversity in the two species.

Although the effects of seasonal dietary variation over the course of the year on gut microbiota are still largely unknown for these species, the impact of dietary niche on the gut microbiota has been well-documented for many mammal species including humans [70], laboratory mice [71] and wild mammals (see review in: Ley et al. [58]; Alessandri et al. [72]). Additionally, the contrasting taxonomic composition of *L. europaeus* and *L. timidus* gut microbiota implies different strategies for energy resorption adopted by the two microbial communities [64, 73, 74]. In fact, our PICRUSt2 analysis supports the hypothesis that the two host-associated microbiotas have alternative biosynthetic and metabolic potential, as indicated by the differences in the number and relative abundance of predicted functions (Fig. 6). Although a high abundance of Ericaceae has been reported in the *L. timidus* diet [69], we found no differences in metabolic pathways associated with the degradation of phenolic compounds which are abundant in these plants. Interestingly, all differential pathways involved in fatty acid / lipid biosynthesis and amino acid degradation were significantly enriched in *L. timidus*, which may again reflect differences in diet between the two species [68, 69]. We also speculate that since host-mediated biosynthesis of fatty acids and lipids support metabolic processes that generate heat, thereby aiding in thermoregulation and overall survival in colder climates [75], microbial mediated biosynthesis of these molecules may help *L. timidus* to cope with the cold temperatures typically found in its natural home range. Overall, these results suggest that the composition of the gut microbiota may be associated with the adaptation of *L. timidus* to its boreal

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habitat. However, again, additional studies on the actual diet of the individuals sampled in this study are needed to confirm this hypothesis.

While the prevalence and relative abundance of Proteobacteria in *L. timidus* were consistent across faecal pellets, most *L. europaeus* samples were characterized by relatively low abundance of this phylum. However, 15% of investigated faecal samples displayed unexpectedly high abundances (e.g. > 80% of sequence reads) of Proteobacteria, mostly belonging to Enterobacteraceae, Erwiniaceae and Oxalobacteraceae (Fig. S2). Enterobacteriaceae is the most studied family of these three and includes both commensal bacteria contributing to the maintenance of the gut anaerobic environment, the production of secondary metabolites (e.g. vitamins) and the protection against gut pathogens, as well as opportunistic disease-causing pathogens [76, 77]. Although Enterobacteriaceae are commonly found in low abundance in the gut microbiota of healthy mammals, their proliferation in humans is also considered to be a biomarker of gut dysbiosis, which has been associated with several inflammatory bowel diseases [78]. Because only non-invasive faecal samples were collected, we do not know if the observed abundance of Proteobacteria in a limited, but not negligible, number of *L. europaeus* samples could be attributable to a state of inflammation of the host. Changes in microbiota composition or diet may also lead to favourable environmental conditions for Proteobacteria (e.g. variation in carbon sources or decreased hypoxia, among others [78]). This unusually high abundance of Proteobacteria should be further investigated, since a disrupted gut microbiota, or microbiota consisting of a number of potentially pathogenic taxa could impact the health of *Lepus* individuals. Furthermore, future studies should also plan detailed investigations targeting specific Enterobacteriaceae taxa as biomarkers, which could be useful for health monitoring of these species.

Similarly, both diversity and taxonomic composition of fungal taxa between *L. europaeus* and *L. timidus* diverged significantly, (Fig. 4), with up to 24% of observed variation, associated with host species (Tab. 1). The fungal classes Tremellomycetes and Cystobasidiomycetes were significantly enriched in mountain hare gut microbiota, while higher abundances of Ustilaginomycetes and Pezizomycetes were detected in the European brown hare. The class Tremellomycetes is a nutritionally heterogeneous group comprising saprotrophs, animal parasites, and fungicolous species previously isolated from various habitats including alpine soil [79], subalpine grasses [80], snow collected at high elevations [81] and the Antarctic Polar Plateau [82]. Due to the ability of some Tremellomycetes to survive in mountainous or cold habitats, we hypothesize that the high abundance of Tremellomycetes found in *L. timidus* resulted from these animals having more opportunities to feed on these fungi or have contact with them at higher elevations. However, this fungal class has also been linked to high lipid production [83], which may contribute to energy homeostasis and thermoregulation [84] in *L. timidus*. On the other hand, Ustilaginomycetes, found here to be more

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abundant in *L. europaeus*, are well-known to infect vascular plants, especially grass families like Poaceae [85]. Indeed, *L. europaeus* are primarily grazers and have a diet that heavily relies on grasses [69], possibly explaining the higher abundance of this fungal class, as well as Pezizomycetes (phylum Ascomycota) in their gut compared to *L. timidus*. Again, molecular diet analysis of the faecal pellets would allow us to confirm that the patterns in fungal diversity and composition are mainly due to differences in feeding preferences.

In this study, host species identification was conducted using mitochondrial DNA (mtDNA) barcoding, which is currently regarded as the most reliable approach for species assignment from faecal samples, owing to the high copy number of mitochondrial genomes per cell and their relatively high mutation rate which allow for good taxonomic discrimination, even between closely related species [86, 87]. However, because mtDNA barcoding does not allow discrimination between hybrids and their maternal parental species, we cannot exclude the possibility that a small number of the analysed samples may correspond to hybrid individuals. However, the occurrence in northern European and Alpine regions has been estimated to occur at a frequency of only 2-5% [34, 88, 89]. Therefore, while hybridization is frequently associated with substantial shifts in the composition and diversity of gut microbial communities [90, 91, 92], we do not know yet how the observed differentiation in composition and predicted functional potential between *L. europaeus* and *L. timidus* could be translated into the gut microbiota of their hybrids. *L. europaeus* and *L. timidus* hybrids might exhibit transgressive microbiome configurations, not directly resembling the composition and the predicted functional potential between parental species as seen for hybrids between subspecies *Mus musculus musculus* × *M. m. domesticus* [90], intermediate microbiome configurations (e.g. *Centropyge* spp.; [91]), or asymmetric, parent-biased microbiomes as seen for *Cervus elaphus* [92]. Therefore, at present we are unable to predict the most likely microbiota configuration of *Lepus* hybrids, or its influence on host nutritional behavior, disease susceptibility, or adaptive potential to changing environments. Genotyping of faecal samples could resolve the issue by identifying hybrid individuals; however, many more samples would be needed since the success rate of genotyping from hare faecal samples is only about 50%, and the occurrence of hybrids is so low and unpredictable.

Within-species variation in microbial diversity across an elevational gradient

Elevation did not appear to affect the alpha diversity of prokaryote and fungal communities hosted by the two *Lepus* species, and only marginally impacted (Tab. S3) beta diversity estimates of *L. europaeus* prokaryote gut communities. The resilience of the hare gut prokaryote microbiota to variation in elevation and associated environmental variables was unexpected based on studies performed on other mammal species like wild house mice [93], pikas [94], ungulates [95]; macaques,

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humans and domestic dogs [96]. However, the beta diversity of the prokaryote community structure was in striking contrast to that observed in the gut fungal communities of both species; in fact, marked differences in fungal microbiota composition were detected using both Bray-Curtis (e.g. accounting for ASV abundances) and Jaccard (e.g. based on ASV presence/absence) indices, indicating that variation in fungal composition across elevations reflected changes in both the shared abundant and rare components of the environmental fungal community. Furthermore, we found elevation to be a significant driver of fungal diversity even in pairwise comparisons between neighbouring elevations (e.g. 1000m vs 1500m a.s.l. for *L. europaeus* and 2000 m vs 2500 m for *L. timidus*). Consistently, Mantel tests highlighted low but significant correlations between the composition of overall fungal communities and pre-sampling precipitation, temperature and plant richness. Moreover, several fungal ASVs in *L. europaeus*, including seven matching the coprophilous genus *Sporormiella* (phylum Ascomycota), that uses herbivore dung as a primary substrate [97], was significantly associated with these environmental variables. Interestingly, variation in the relative abundance of *Sporomiella* in the gut of the lagomorph *Ochotona curzoniae* (i.e. plateau pikas) captured at different elevations was also reported by Tang et al. [98]. However, only a few fungal taxa are considered gut residents [99, 100] and environmental fungi ingested with diet are almost certainly more exposed to abiotic variation. Consistently, differences in soil fungal communities driven by elevation have been reported frequently [79, 101, 102]. Additionally, the survival of many fungal taxa relies on interactions with plants and soil [103, 104, 105]. Therefore, the high number of taxa common to both species (80% of generated sequence reads, Fig. S1), together with the marked association with elevation and related environmental variables (Fig. 5, Tab. S5), indicate a strong contribution of diet (and possibly geophagy) to the gut fungal assembly in these two *Lepus* species, as suggested by Grieneisen et al. [106] for a primate hybrid zone (see also [107]).

The clear differentiation between prokaryote and fungal communities in their resilience to environmental parameters could have interesting implications in relation to the observed elevation shifts in the distribution of *L. europaeus* and the associated potential reduction in habitat availability for *L. timidus*. The lack of differentiation in prokaryote communities across elevations suggests that gut functionality was being maintained along the entire gradient. Our data also suggested that *L. europaeus* and *L. timidus* had different feeding strategies (e.g. selecting different plant species) maintained along the same gradient, allowing them to co-exist in the same areas. However, we expect a selective feeding strategy to represent a potential vulnerability for *L. timidus*, particularly if climate change continues to alter the distribution of plant taxa in the *L. timidus* diet. Furthermore, the unique components of the *L. timidus* gut microbiota biodiversity also risk disappearing, with unknown impacts on the Alpine ecosystem. Future studies addressing the health impacts of diet, geophagy and

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other environmental factors across elevations are needed to disentangle the associations of these factors with the taxonomic and functional diversity of *L. europaeus* and *L. timidus* in the Alps, and consequently, of the implications for their survival during climate warming.

Conclusions

Our findings demonstrate that sympatric populations of *L. europaeus* and *L. timidus* maintained distinct, species-specific prokaryote and fungal gut microbiota in terms of taxonomy and structure, but not diversity, at least within our study area. Our data act as a baseline for future monitoring of the process of adaptation of these species to climate warming and associated environmental changes. Future studies should prioritize the characterization of gut prokaryote and fungal composition in hybrids of the two lagomorph species across multiple populations across the Alps. Moreover, future research should explore how climate-driven changes in soil microbiota and food plant composition may shape the future diversity and structure of host-associated gut microbiota in these Lagomorph species.

Data Availability

Sanger sequences have been deposited at NCBI GenBank with accession numbers PX122685 - PX122779. Sanger sequences of the mitochondrial D-loop generated in our study were made available to the editor and reviewers with the uploaded file: Submission2992130.txt.gz. The raw amplicon-sequencing data has been deposited at NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA1304890. Reviewers can access BioProject and associated SRA metadata at https://dataview.ncbi.nlm.nih.gov/object/PRJNA1304890?reviewer=rsuoanlfj6uo274kij1dik96rn.

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Author Contributions

BC, HCH, and GG conducted the sampling. LM, BC and GG completed the laboratory analyses. Data analyses were provided by LM and GG, with support from NP, TR, JS, PI and FNM; LM, HCH and GG drafted the manuscript. All authors edited and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

Consent for publication

Not applicable.

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Additional information

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Table 1. Results of PERMANOVA analysis comparing the faecal microbiota of *L. europaeus* and *L. timidus* at sympatric elevations. Microbial diversity was estimated using Bray-Curtis, Unweighted Unifrac (for 16S rRNA gene only) and Jaccard index (ITS2 locus only). For each microbial community and diversity index, statistical tests were performed by considering species, elevation and their interactions.

Microbial community (marker)	Diversity Index	Variable	R^2	F	p-value	Sig.
Prokaryotes (16S rRNA gene)	**Bray-Curtis**	Species	0.273	15.207	0.001	***
		Elevation	0.022	1.252	0.203	ns
		Species:Elevation	0.024	1.336	0.171	ns
		Residual	0.681			ns
	Unweighted Unifrac	Species	0.200	9.903	0.001	***
		Elevation	0.017	0.855	0.528	ns
		Species:Elevation	0.016	0.774	0.655	ns
		Residual	0.767			ns
Fungi (ITS2 locus)	**Bray-Curtis**	Species	0.240	13.659	0.001	***
		Elevation	0.052	2.967	0.007	**
		Species:Elevation	0.041	2.332	0.022	*
		Residual	0.667			ns
	Jaccard	Species	0.133	6.266	0.001	***
		Elevation	0.033	1.534	0.046	*
		Species:Elevation	0.029	1.364	0.085	ns
		Residual	0.806			ns

p-value ≤ 0.001: ***; p-value ≤ 0.01: **; p-value ≤ 0.05: *; ns: not significant.

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Figure Captions

Figure 1. Map of the study area showing the 12 sampling plots and their respective elevations. Bottom right: large-scale overview of the sampling area. Top right: barplot showing the faecal pellet counts for the two study species across the four elevations (m a.s.l.). LEU: *Lepus europaeus*; LTI: *Lepus timidus*.

Figure 2. Relative abundances of faecal prokaryote (16S) and fungal (ITS2) taxa. (**a**) Phyla based on 16S rRNA gene data and (**b**) classes based on ITS2 data. Each bar represents a sample, with colors indicating the most abundant taxa as detailed in the legend. Samples are arranged by species (LEU: *Lepus europaeus*; LTI: *Lepus timidus*) and elevation.

Figure 3. Differential abundance analysis of prokaryote and fungal communities between the two hare species. (**a**) Prokaryote phyla and (**b**) fungal classes with significant differences in relative abundance identified using LEfSE (microeco). The x-axis represents the Linear Discriminant Analysis (LDA) scores, indicating the magnitude of difference between group means. LEU: *Lepus europaeus*; LTI: *Lepus timidus*

Figure 4. Non-Metric Multidimensional Scaling (NMDS) analyses comparing prokaryote and fungal communities of *L. europaeus* and *L. timidus* faecal pellets. Panels (**a, c**) show prokaryote communities, while (**b, d**) show fungal communities. Dissimilarities were calculated using Bray-Curtis (**a, b**), Unifrac (**c**), and Jaccard (**d**) indices. Each dot represents a single sample (N=95). LEU: *Lepus europaeus*; LTI: *Lepus timidus*

Figure 5. Non-Metric Multidimensional Scaling (NMDS) and Spearman correlation analyses comparing prokaryote and fungal community structures across different elevations. (**a, b**) NMDS clustering of prokaryote and fungal communities in *L. europaeus* faecal samples*;* (**c, d**) NMDS clustering prokaryote and fungal communities in *L. timidus* faecal samples. Each symbol represents a single faecal sample, with different shapes indicating elevation; (**e**) Spearman correlation analysis between environmental variables (*x*-axis) and *L. europaeus* faecal fungal ASVs (*y*-axis). Correlation coefficients indicate the strength and direction of the relationships. Significant correlations are indicated (* p-value < 0.05; ** p-value < 0.005; *** p-value < 0.0005). LEU: *Lepus europaeus*; LTI: *Lepus timidus*.

Fig. 6. Functional diversity in *Lepus* spp. predicted from 16S rRNA gene sequences. (**a, b**) Shannon

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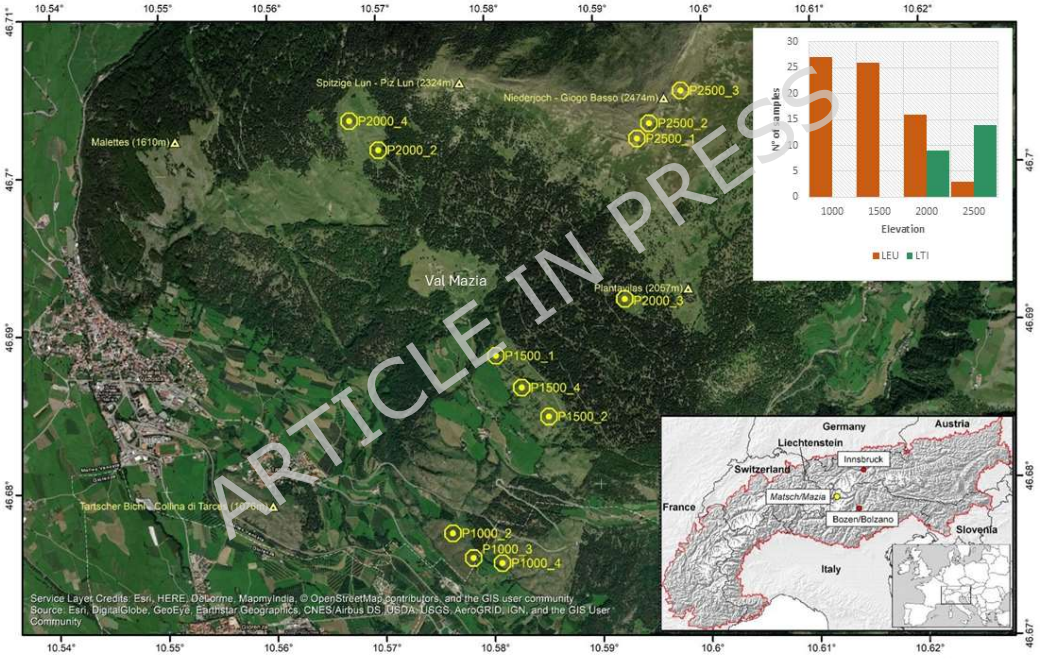
diversity of metaCyc pathways in faecal samples from *L. europaeus* and *L. timidus* collected at 2000 and 2500 m a.s.l., grouped by species (**a**) and elevation (**b**). Significant differences were assessed using the Wilcoxon rank-sum test. **: p-value ≤ 0.01; ns: p-value > 0.05. (**c**) Non-Metric Multidimensional Scaling (NMDS) based on Bray-Curtis dissimilarity estimates of metaCyc pathway abundances; (**d**) Heatmap of metaCyc pathways showing significant differences in abundance (e.g. Log2FC ≥ 1; FDR adj.p-value ≤ 0.01) between sympatric *L. europaeus* and *L. timidus* across elevations. Rows (i.e. metaCyc pathways; N=75) and columns (i.e. *Lepus* spp. faecal samples; N=42) are clustered using correlation distance and average linkage. Data are row-centered and scaled to unit variance. Panels (a-c) were created using ggplot2 [43] (**a, c**) and ClustVis [49] (**d**) and formatted using GIMP v2.10.18 (The GIMP Development Team, 2019). LEU: *Lepus europaeus*; LTI: *Lepus timidus*.



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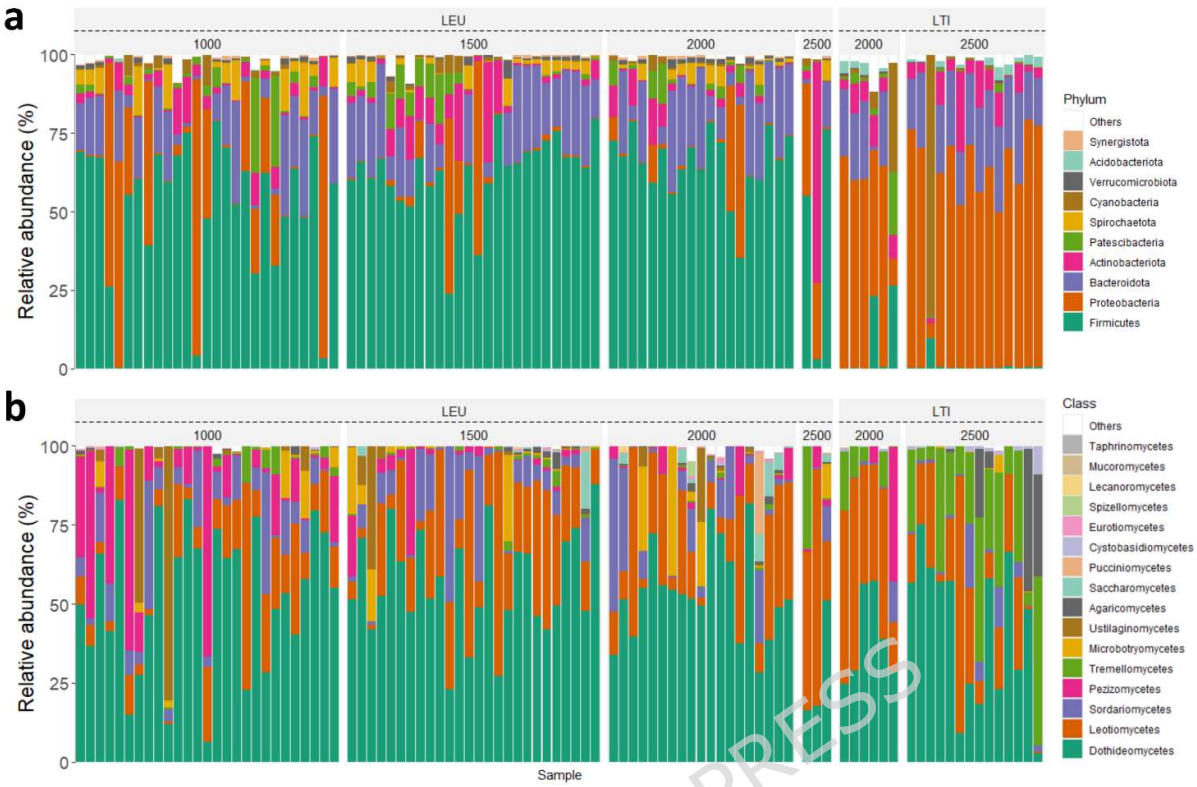

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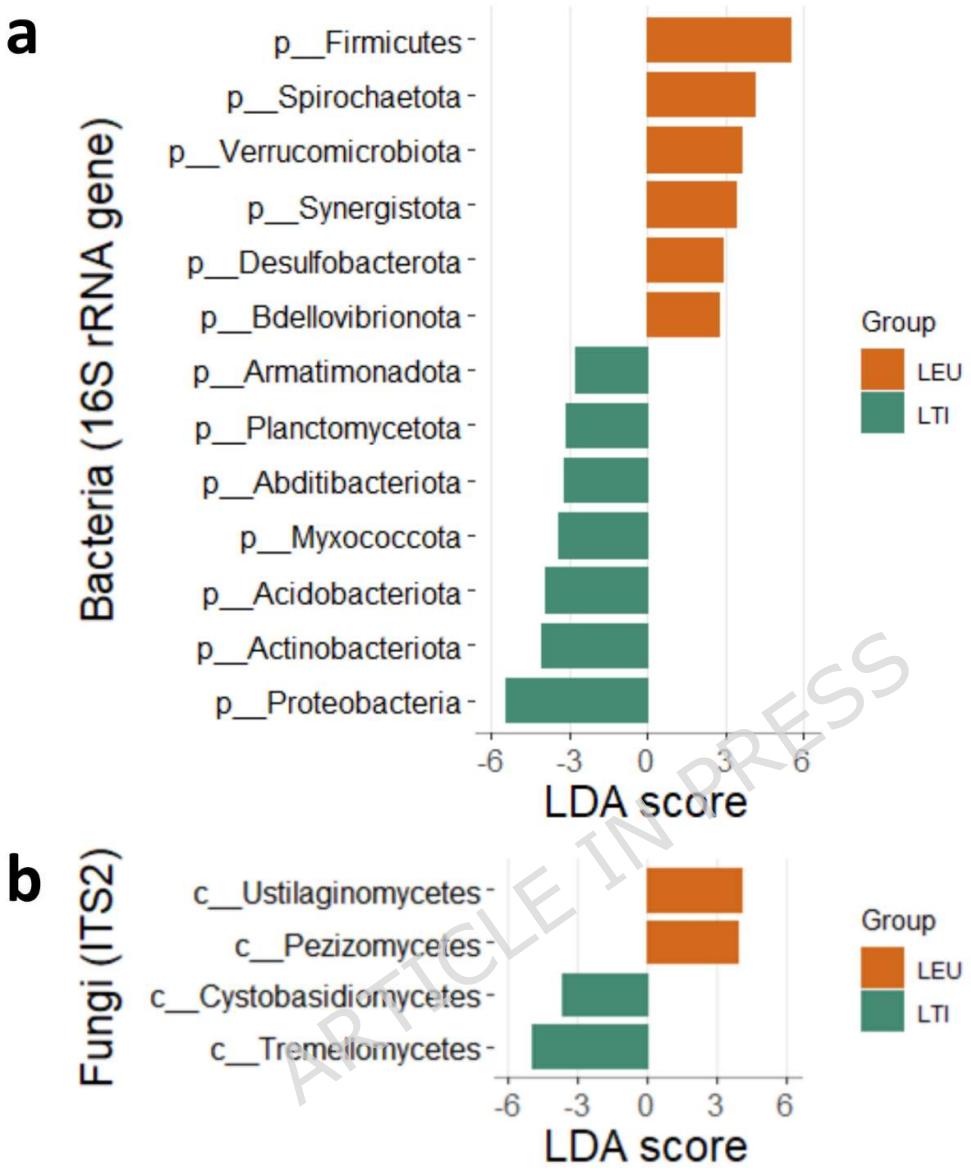

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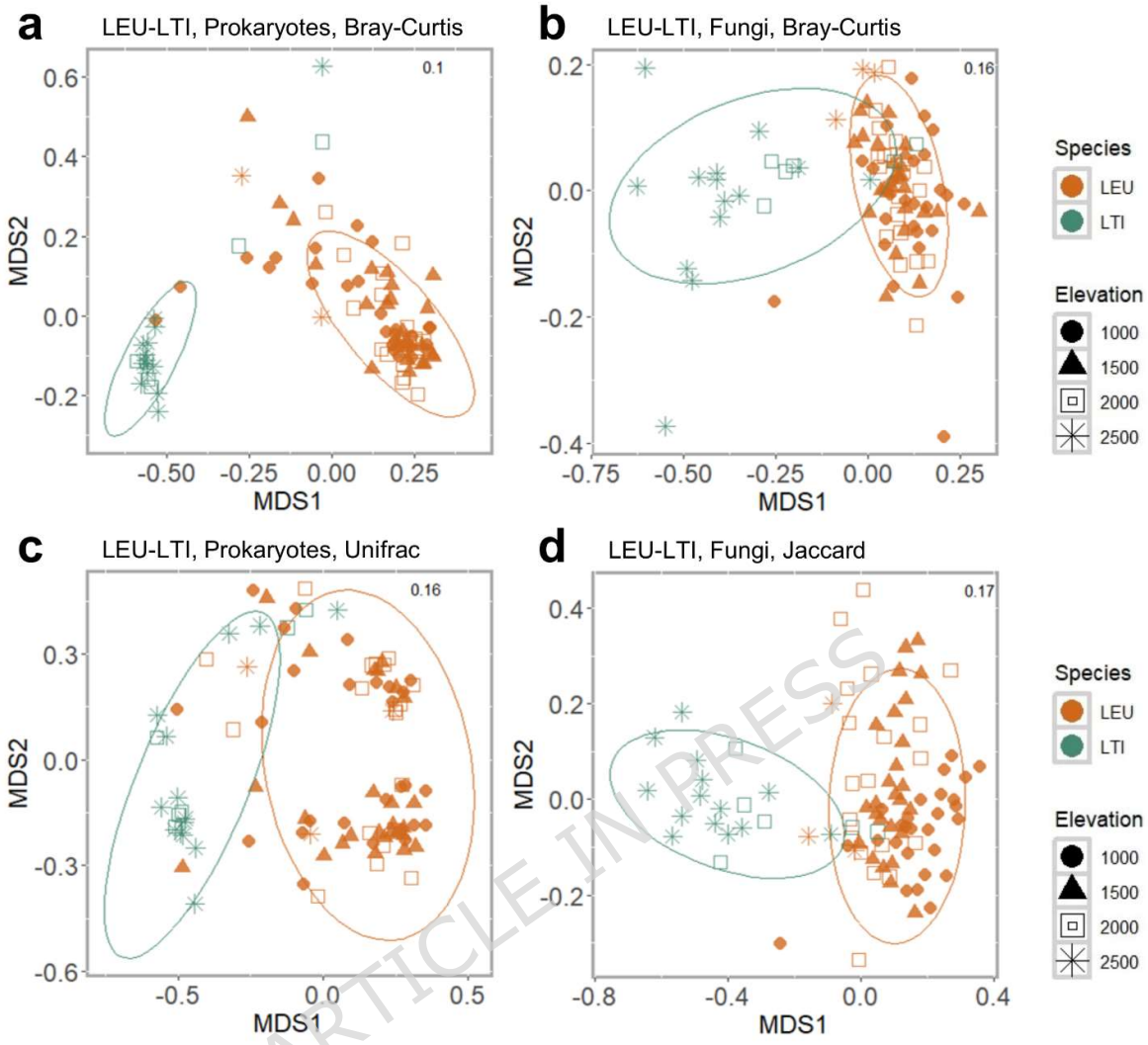

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