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Uncovering gut microbial diversity in *Ligidium* woodlice and its correlation with *Rickettsiella*

Jin Wang¹, Qidi Zhu¹, Chao Jiang^{2*} and Weichun Li^{1,2*}

Abstract

Background Woodlice are a key group of macroarthropods in soil ecosystems, performing crucial ecological functions mediated by their microbiota. The gut microbial diversity has been investigated in several woodlice species, but the gut bacterial flora of the genus *Ligidium* remains unexplored. This study aims to characterize gut microbial diversity in *Ligidium* and its related influencing factors, thereby supporting their potential application as bioindicators for monitoring soil ecosystem health and enhancing bioremediation strategies.

Methods The gut microbiota of 102 terrestrial isopod samples representing 12 *Ligidium* species was characterized by high-throughput sequencing of the 16 S rRNA V3–V4 region using the Illumina NovaSeq platform. Microbial community analyses were performed using QIIME2 (v.2022.11) based on SILVA Release 138 database. Statistical analyses (PERMANOVA, PERMDISP and Spearman correlations) were conducted to examine ecological patterns.

Results Our analysis of intestinal contents from 102 *Ligidium* samples identified 2,781 amplicon sequence variants dominated by Actinomycetota (55.6%), Pseudomonadota (40.6%), and Bacillota (1.2%). The gut bacterial composition showed significant differences among *Ligidium* species. Host species was the primary driver shaping the gut microbial community structure in *Ligidium*, explaining 43.28% of variation. Geographic factors act as significant substantial variables, accounting for approximately 36.3% of the explained variation. The high abundance of *Rickettsiella* explained about 12.58% of community variation and emerged as the main source of intraspecific heterogeneity, as indicated by a 51.3% reduction in the PERMDISP F-value after it was removed. Furthermore, a significant negative correlation between the *Rickettsiella* relative abundance and the Shannon diversity index (Dataset 3: $\rho = -0.2108$, $p < 0.05$; dataset 4: $\rho = -0.2648$, $p < 0.01$), supporting the association of high *Rickettsiella* abundance with the reduced gut microbial diversity.

Conclusion As a key detritivorous isopod in undisturbed leaf litter ecosystems, *Ligidium* is anticipated to harbor exceptionally high gut microbial richness, reflecting its specialized saprophagous niche. This study presents the first comprehensive, multi-sample and multi-species analysis of gut microbial diversity in *Ligidium*, revealing that host species is the primary determinant of microbial community structure, while geographical habitat and *Rickettsiella* significantly contribute to inter-group variation. Spearman correlation analysis demonstrated a significant negative

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correlation between the *Rickettsiella* relative abundance and the Shannon diversity index. The high *Rickettsiella* abundance was associated with the reduced gut microbial diversity and altered the community composition.

Keywords Gut microbiota, Isopoda, Ligiidae, Oniscidea, 16S rRNA, *Rickettsiella*

Background

Woodlice (scientific name: Oniscidea), a suborder within crustacean Isopoda, comprises more than 4,100 described species across 568 genera and 38 or 39 families [1, 2]. The suborder inhabits nearly all terrestrial ecosystems except Polar Regions and elevations above 4,800 m [2, 3]. As major soil macrofauna, they play essential roles in leaf litter decomposition, nutrient cycling, and the structuring of soil microbial communities. In particular, their feeding and burrowing activities, microbial ingestion, and feces contribute to microbial dispersal and habitat modification [3–11].

The suborder relies on gut microbiota associations to fulfil critical ecological roles, particularly lignocellulose decomposition. The taxonomic profiling of microbiota-associated CAZymes in *Armadillidium vulgare* revealed that Proteobacteria (56%) and Bacteroidetes (36%) were the dominant bacterial phyla driving lignocellulose degradation [7]. Key degradative functions were attributed to Flavobacteriales, Micrococcales, and Burkholderiales, which significantly contributed to glycoside hydrolases (GH), carbohydrate esterases (CE), and auxiliary activities (AA) enzyme families. Notably, Vibrionales and Cytophagales also played major roles in lignocellulose degradation, while Bacteroidetes-dominated PULs (e.g., GH3/GH43-containing clusters) and cellulosome complexes (e.g., dockerin-linked GH5/CE1) indicated specialized microbial strategies for lignocellulose breakdown [7, 12–16]. These microbial consortia provide essential nutritional support to the host via cellulose-hydrolyzing enzyme production. Functioning as transient symbionts, nutrient reservoirs, or mutualistic partners, gut microbes further contribute to host physiological adaptation and fitness [17]. In addition, members of Oniscidea maintain specialized symbiotic relationships with intracellular bacteria such as *Wolbachia*, which can modulate host immunity, interfere with sexual determination pathways, and enhance pathogen resistance and longevity [12, 14]. Current research on the gut microbiota of woodlice indicates that microbial diversity is predominantly localized in the hindgut. The dominant phyla such as Actinomycetota, Pseudomonadota, and Bacteroidota collectively constitute the majority of the community, with Bacillota and Mycoplasmatota present as minor components [8, 10, 13, 14, 16, 18–21]. Although the gut microbiota has been characterized in eighteen oniscidean species, the relative importance of factors such as host phylogeny, diet, and habitat in shaping microbial community structure remains poorly understood.

The genus *Ligidium* Brandt, 1833 (Oniscidea: Ligiidae) comprises 65 species and exhibits a fragmented distribution spanning continental Eurasia and North America [1, 22, 23]. In the field, *Ligidium* members inhabit microhabitats characterized by high humidity, including the moist leaf litter in the subtropical evergreen broad-leaved forest, the edge of some wetlands and under the vegetation near the water [24–26]. As an important group within Oniscidea, *Ligidium* species play a vital ecological role as decomposers in forest ecosystems, in which gut microbiota are likely to critical functional supports for organic matter breakdown. Nevertheless, the gut bacterial flora of this genus remains uncharacterized, representing a notable knowledge gap in soil invertebrate symbiosis research.

In this study, we employed 16 S rRNA amplicon sequencing to characterize the gut microbiota across twelve *Ligidium* species. Our objective was to systematically explore their gut microbiota and establish a foundation for understanding cross-kingdom microbe-microbe interactions within detritivorous ecosystems.

Materials and methods

Sample collection and identification

A total of 102 specimens were collected from nineteen sites across China. All samples were stored in absolute ethanol. Species were identified by an integrative taxonomic approach as described in Wang et al. [22]. Detailed collection information and species identification are listed in Table S1. All studied specimens are deposited in the Insect Museum, Jiangxi Agricultural University, Nanchang, China (JXAUM).

DNA extraction

The specimens were thoroughly rinsed with sterilized water, after which digestive tracts were aseptically dissected using sterilized tweezers with particular attention to complete hepatopancreas removal. The gut exterior was repeatedly rinsed with sterile water and intestinal walls were removed to maximize the removal of host tissues, thereby minimizing DNA contamination. For each species, intestinal contents from 4 to 8 randomly selected individuals (matched by body size and sex) were homogenized to form a composite sample. This pooling procedure was independently repeated to generate 4–6 biological replicates per species. The samples of intestinal contents stored in liquid nitrogen for preservation. Total genomic DNA was extracted by OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA)

following the bacterial protocol. The quality and purity of extracted DNA were assessed by 0.8% agarose gel electrophoresis and quantified using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Amplicon library preparation

PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338 F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') [27]. Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contain 5 µL of buffer (5×), 0.25 µL of Fast pfu DNA Polymerase (5U/µL), 2 µL (2.5 mM) of dNTPs, 1 µL (10 µM) of each forward and reverse primer, 1 µL of DNA template, and 14.75 µL of ddH₂O. Thermal cycling consisted of initial denaturation at 98 °C for 5 min, followed by 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 5 min at 72 °C. Negative controls were included in each PCR run to monitor potential contamination. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illumina NovaSeq platform with NovaSeq 6000 N Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Sequence data and statistical analysis

16S rRNA gene sequence processing and amplicon sequence variant generation

Microbiota bioinformatics analysis was conducted using QIIME2 v.2022.11 [28] and R packages (v.3.6.0). The raw sequencing data were first demultiplexed with the demux plugin, followed by the removal of primers using the cutadapt plugin [29] (parameters: 20% error tolerance, minimum 90% primer overlap, discard untrimmed reads). The sequences were subsequently processed with the DADA2 plugin [30] for quality filtering, denoising, merging, and chimera removal (parameters: trim-left-f = 0, trim-left-r = 0, trunc-len-f and trunc-len-r were dynamically optimized using 98% sequence length coverage with R1/R2 reduction ratios of 20%/80% to prioritize overlap integrity, resulting in truncation positions approximately between 220 and 230 bp; min-overlap = 8, max-ee-r = 4, n-reads-learn = 1,000,000). Non-singleton amplicon sequence variants (ASVs) were generated by excluding singleton ASVs from the resulting dataset. Taxonomy was assigned to ASVs using the classify-sklearn naïve

Bayes taxonomy classifier in the feature-classifier plugin [31] against the SILVA Release 138 [32] under default parameters. Following taxonomic assignment, all ASVs classified as Chloroplast or Mitochondria were removed. The number of taxa contained in each of the seven levels of domain, phylum, class, order, family, genus, and species in samples were counted and visualized in R.

Dataset construction and filtering strategy

Four datasets were constructed to support downstream analyses:

Dataset 1. Retained non-singleton ASVs from genera with >0.01% mean relative abundance and >5% sample prevalence.

Dataset 2. Rarefied from dataset 1 to an even depth of 15,389 sequences per sample, corresponding to the lowest sequencing depth among all samples.

Dataset 3. Derived from Dataset 1 by removing all *Rickettsiella*-assigned ASVs and excluding samples with sequence counts below 5,000 or insufficient rarefaction curve saturation (e.g., the samples LQS1, LQS2, LQS4, LQS5, ZXS6, LP3, DFD1, DFD2, DFD4, LCQ2, and LCQ5). The final feature table was rarefied to 7,966 sequences per sample.

Dataset 4. Derived from Dataset 1 by removing all *Rickettsiella*-assigned ASVs and rarefied to 5,395 sequences per sample to balance the retention of sample size with the requirement for curves nearing an asymptote. The samples with sequence count below 5,395 (LQS4, LQS5, LP3, DFD1, and DFD4) were excluded.

The adequacy of dataset 1 was devalidated using the rare curve function from the R package vegan to evaluate sequencing depth (version 2.7-2) [33], and defined by a slope < 0.01 in the terminal 10% of sequences.

The adequacy of datasets 2–4 was validated by rarefaction curves for Observed species and Good's coverage (parameters: --p-steps 10, --p-min-depth 10, --p-iterations 10). Dataset 1 was used for species composition analysis; dataset 2 was employed to calculate alpha diversity indices and conduct PCoA analysis; datasets 2–4 were collectively used for PERMANOVA and PERMDISP analyses to examine the effects of host species, geographic factors, and *Rickettsiella* on the microbial community structure; datasets 3 and 4 were separately used to calculate the Shannon index and perform Spearman correlation analysis with the relative abundance of *Rickettsiella*.

Microbial diversity and statistical analysis

Alpha diversity was assessed using multiple indices, including Good's coverage index, Chao1 richness

estimator, Shannon diversity index, Simpson diversity index, Observed species, and Pielou's evenness index. All indices were calculated using QIIME2. Significant differences in alpha diversity indices between groups were evaluated using the Kruskal-Wallis test [34], with Dunn's post-hoc test adjusted by the Benjamini-Hochberg method for multiple comparisons, only results meeting more stringent thresholds ($p < 0.01$) are presented in figures for clarity [35]. Results were visualized using box plots.

Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Jaccard metrics and Bray-Curtis metrics [36, 37], and visualized via principal coordinate analysis (PCoA). The significance of the differentiation of microbiota structure among groups was assessed by PERMANOVA (Permutational multivariate analysis of variance) and PERMDISP using QIIME2 [38, 39]. UpSet plot and Venn diagram were generated to visualize the shared and unique ASVs among samples or species using R package "VennDiagram" and "UpSetR" based on the occurrence of ASVs across samples/species regardless of their relative abundance [40, 41]. Spearman's rank correlation analysis was performed on the GenesCloud platform (Shanghai Personal Biotechnology Co., Ltd; <https://www.genescloud.cn>) to assess both the correlation and statistical significance between specific bacteria relative abundance and the Shannon diversity index.

Results

The intestinal contents of 102 samples were successfully sequenced. The dataset 1 was rarefied to the minimum sequencing depth, and all the samples reached saturation (Fig. S1; Table S2). The rarefaction curves of datasets 2–4 approached saturation (Figs. S2–S7). All datasets are suitable for diversity analysis.

Taxonomic composition

Our analysis of intestinal contents from 102 *Ligidium* samples identified 2,781 amplicon sequence variants (ASVs) after applying prevalence and abundance filters (non-singleton ASVs, mean relative abundance $> 0.01\%$, prevalence $> 5\%$ of samples), which were taxonomically classified into 432 microbial species. The mean species richness per host species ranged from 85 to 187 based on dataset 1.

At the phylum level, 6–14 phyla were recognized within individual samples, and bacterial community composition in all the *Ligidium* samples consists of 14 phyla. The microbial community is dominated by Actinomycetota ($55.6 \pm 26.6\%$; range: 12.0–82.3%), Pseudomonadota ($40.6 \pm 28.2\%$; range: 9.9–86.4%), and Bacillota ($1.2 \pm 1.8\%$; range: 0.33–7.03%), collectively constituting $97.4 \pm 1.7\%$ (range: 93.8–99.6%) of the total classified microbiota.

The gut bacterial communities of each *Ligidium* sample included 64–274 identified genera. Among them, *Wolbachia* endosymbiotic bacteria were examined in approximately 71.56% of *Ligidium* samples. As shown in Fig. 1A, we provided the twenty most abundant genera to represent the taxonomic composition of gut microbiota in *Ligidium* species. The predominant genera were *Rickettsiella* ($21.91 \pm 34.54\%$; range: 0.02–81.22%), *Nocardioidea* ($5.38 \pm 3.28\%$; range: 0.768–9.815%), *Mycobacterium* ($3.85 \pm 2.16\%$; range: 0.622–7.571%) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* ($3.44 \pm 3.13\%$; range: 0.534–9.485%), *Microbacterium* ($3.23 \pm 2.24\%$; range: 0.408–8.193%), *Cellulomonas* ($2.78 \pm 1.86\%$; range: 0.305–6.842%), *Bradyrhizobium* ($2.54 \pm 1.92\%$; range: 0.105–6.998%), *Oerskovia* ($1.79 \pm 5.20\%$; range: 0.00–18.12%), *Pseudonocardia* ($1.77 \pm 1.89\%$; range: 0.030–6.353%), *Solirubrobacter* ($1.70 \pm 1.03\%$; range: 0.106–3.164%), 67–14 ($1.31 \pm 1.25\%$; range: 0.082–4.618%; an uncultured bacterial genus from the SILVA database), *Sanguibacter-Flavimobilis* ($1.26 \pm 3.50\%$; range: 0–12.34%), collectively representing $50.95 \pm 21.40\%$ (range: 29.32–89.06%) of the total sequences. The remaining genera, including *Nakamurella*, *Iamia*, *Aeromicrobium*, IMCC26256 (an uncultured bacterial genus from the SILVA database), *Conexibacter*, *Agromyces*, *Marmoricola* and *Actinoplanes*, each exhibited a mean relative abundance less than 1%.

Among the twelve analyzed *Ligidium* sample groups, the total number of observed ASVs significantly varied from 446 (group N2) to 2411 (group N3), and 40 ASVs were common to all groups (Fig. 1B). These indicate a significant heterogeneity in bacterial species composition across gut microbiota of different *Ligidium* species. Furthermore, ASV_5824, ASV_46668 (unclassified *Microbacteriaceae*), ASV_65619 (unclassified *Cellulomonas*), ASV_31759 (unclassified *Microbacterium*), ASV_38245 (unclassified *Microbacterium*), and ASV_34422 (unclassified *Bradyrhizobium*) consistently maintained relative abundances around 1% across most *Ligidium* species. This remarkable interspecies stability strongly suggests the presence of a conserved core gut microbiota in this genus. We subsequently conducted a comparative analysis of gut microbial diversity across geographic populations of *Ligidium acuminatum* Li, 2022 (group N3) and *L. rotundum* Li, 2022 (group N9). Through species-specific core microbiota analyses, we identified 231 and 347 conserved ASVs shared among the samples from five collection sites of the group N3 and four collection sites of the group N9 respectively (Fig. 1C, and D), while samples from different locations maintained 152–179 and 88–322 location-specific ASVs. These results revealed every *Ligidium* species owns conserved core microbiota while demonstrating the habitat is a significant influencing factor to the gut microbial consortia of the genus.

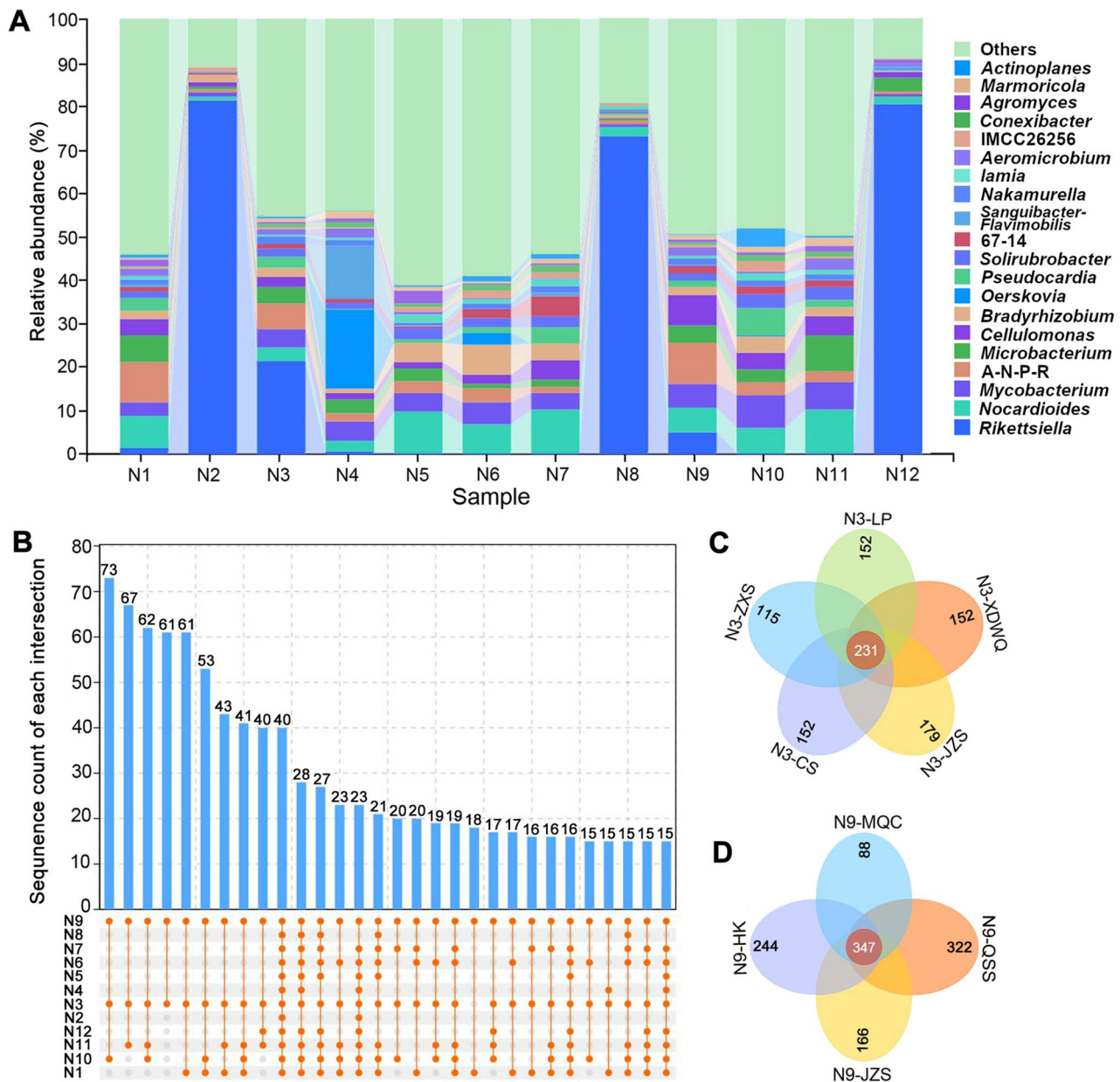


Fig. 1 Microbial community composition in *Ligidium* species. **A** Relative abundance of the top 20 bacterial genera across twelve *Ligidium* species (groups N1–N12), with *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* abbreviated as A-N-P-R for conciseness. The relative abundance refers to the relative abundance of 16 S rRNA gene copies. **B** Shared and unique microbial features among the twelve *Ligidium* species (groups N1–N12), visualized by an UpSet plot. **C** Microbial feature distribution across five geographical populations (CS, JZS, LP, XDWQ, and ZXS) of *L. acuminatum* Li, 2022 (group N3). **D** Microbial feature distribution across four geographical populations (HK, JZS, QSS, and MQC) of *L. rotundum* Li, 2022 (group N9). Venn diagrams overlap regions indicate numbers of shared microbial features between sample groups, while non-overlapping regions represent numbers of unique features. Complete sample abbreviations are provided in Table S1

Alpha diversity analyses

In the alpha diversity analysis, the groups N2 and N8 were excluded due to insufficient sample retention (less than four replicates per group). The results revealed significant variations of gut microbial diversity across *Ligidium* species by using the dataset 2 (Fig. 2). Amongst them, *Ligidium duospinatum* Li, 2022 (group N4) and *L. sichuanense* Nunomura, 2002 (group N10) with the

lowest and the highest microbial diversity respectively. In addition, the gut microbial diversity of *Ligidium* sp. 2 (group N5) and *Ligidium* sp. 5 (group N12) were lower than the other species (Fig. 2B; Table S3). The complete statistical results are provided in Table S2.

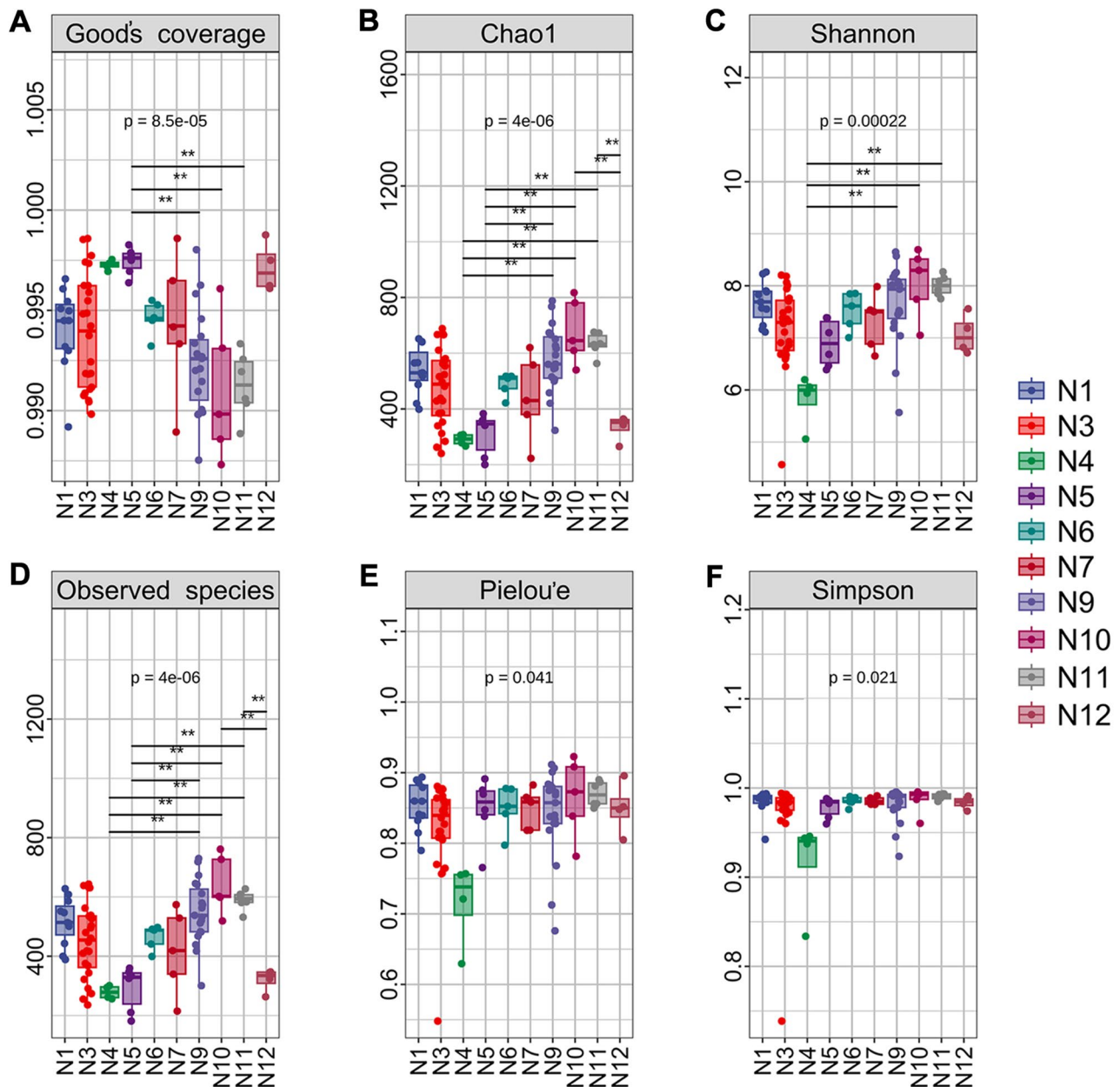


Fig. 2 Alpha diversity metrics of gut microbiota across ten *Ligidium* species (groups N1, N3–N7, N9–N12). (A) Good's coverage. (B) Chao1 index. (C) Shannon index. (D) Observed species. (E) Pielou's evenness index. (F) Simpson index. Boxplots show median values with interquartile ranges. Numbers above plots represent p-values from Kruskal-Wallis tests. Significant pairwise differences (Dunn's post hoc test with BH correction) are indicated: ** $p < 0.01$ ($p < 0.05$ are not shown). Complete sample abbreviations are provided in Table S1

Principal coordinate analysis (PCoA)

In the PCoA analysis, the groups N2 and N8 were excluded to maintain consistency with the alpha diversity analysis. The results revealed discernible separation trends among the remaining groups, while some overlaps were observed in the result of Bray-Curtis-based PCoA (Fig. 3A), and the Jaccard-based PCoA showed clearer segregations (Fig. 3B). The results reflected underlying differences in microbial community structure across *Ligidium* species. Notably, the group N12 characterized

by high *Rickettsiella* abundance was distinctly isolated in the Jaccard-based analysis. This pattern indicates that samples with high *Rickettsiella* composition harbor a structurally distinct microbial community.

PERMANOVA and PERMDISP analyses on interspecific microbial community

We conducted an analysis based on Bray-Curtis dissimilarity (Datasets 2 and 3) to assess the relative influence of host species, geographic factors, and *Rickettsiella*

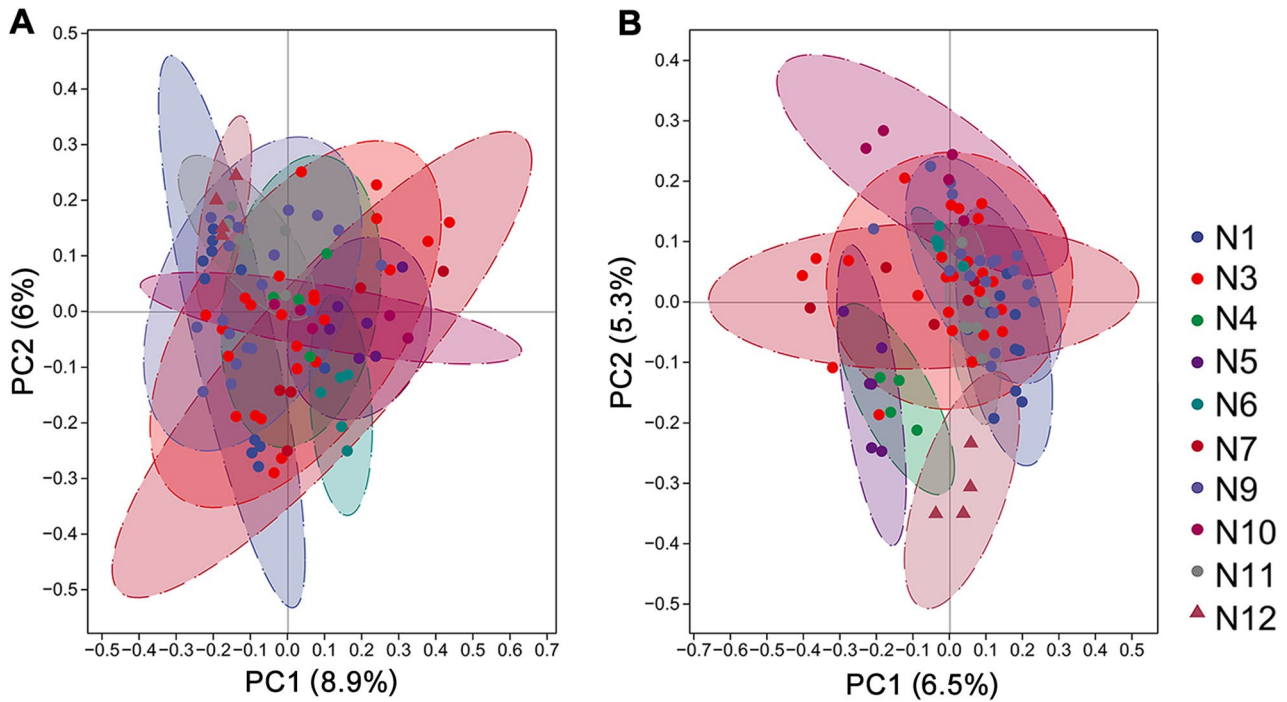


Fig. 3 Principal coordinate analysis (PCoA) of gut microbiota composition across ten *Ligidium* species (groups N1, N3–N7, N9–N12). **(A)** Bray-Curtis dissimilarity matrix. **(B)** Jaccard similarity matrix. Points represent individual samples, with shapes/colors corresponding to species groups. Axes indicate principal coordinates with percentage of explained variance in parentheses. Complete sample abbreviations are provided in Table S1

Table 1 Results of PERMANOVA and PERMDISP tests based on Bray-Curtis dissimilarities (999 permutations)

Groups	PERMANOVA			PERMDISP	
	Pseudo-F	R ²	Pr (> F)	F	P-value
All groups	5.3533	0.3955	0.001	19.5691	0.001
All groups exclude <i>Rickettsiella</i>	3.2419	0.2697	0.001	9.5300	0.001
All groups exclude N1, N3, and N9	7.9225	0.6314	0.001	7.5900	0.001
All groups exclude N1, N3, N9, and <i>Rickettsiella</i>	3.5604	0.4328	0.001	3.7565	0.007
N1	5.1869	0.3656	0.001	0.3941	0.553
N1 excludes <i>Rickettsiella</i>	5.128	0.363	0.001	0.3718	0.558
N3	2.7962	0.3475	0.001	0.2179	0.917
N3 excludes <i>Rickettsiella</i>	2.5592	0.3501	0.001	0.9965	0.441
N9	2.6263	0.3444	0.001	5.9734	0.011
N9 excludes <i>Rickettsiella</i>	2.667	0.3479	0.001	4.2167	0.021

Significant *p*-values (*p* < 0.05) are highlighted in bold. PERMANOVA tests for differences in group centroids, while PERMDISP tests for homogeneity of group dispersions. All analyses were conducted on rarefied datasets: dataset 2 (including *Rickettsiella*) and dataset 3 (excluding *Rickettsiella*). A comprehensive table of the detailed results is provided in Supplementary Tables S4, and S5

abundance, as well as their complex interactions, on the microbial community.

PERMANOVA revealed interspecific differences in the microbial community composition based on the host species (PERMANOVA: pseudo-F = 5.3533, *R*² = 0.3955, *p* = 0.001; Table 1; Table S4). Notably, the species-based groups such as N1, N3, and N9, which were collected from various geographic sites, showed a potential geographic influence on the microbial community. After removing these samples, the host species effect on the microbial community became more pronounced, even with significant differences in multivariate dispersion detected within each species (PERMANOVA: pseudo-F = 7.9225, *R*² = 0.6314, *p* = 0.001; PERMDISP: *F* = 7.59, *p* = 0.001; Table 1; Tables S4–S5). The result showed that the explained variation was 7.9 times as much as the residual variation (Tables S4), indicating the host species is a dominant factor on the divergence of microbial community when geographic interference is minimized. Moreover, the increase in explanatory power from 39.55% to 63.14% after excluding geographically widespread samples suggests that geographic factors obscure the host species effect.

Furthermore, the exceptionally high abundance of *Rickettsiella* in certain samples evidently contributed to interference, as confirmed by significant dispersion differences among species (PERMDISP: *F* = 19.5691, *p* = 0.001; Table 1; Table S5). After removing *Rickettsiella*

sequences, the explanatory power of species grouping on microbial community differences decreased significantly (PERMANOVA: pseudo- $F = 3.2419$, $R^2 = 0.2697$, $p = 0.001$; Table 1; Table S4), although community dispersion differences among species remained significant (PERMDISP: $F = 9.53$, $p = 0.001$; Table 1; Table S5). The decline in explanatory power from 39.55% to 26.97% indicates that *Rickettsiella* is an important driver contributing to microbial community differences among species. Moreover, The F-value decreased from 19.5691 to 9.53 shows that *Rickettsiella* is the main cause of intraspecific dispersion heterogeneity (PERMDISP: $F = 19.5691$ vs. $F = 9.53$, both $p = 0.001$; Table 1; Table S5).

In addition, we simultaneously removed the multi-geographic samples (groups N1, N3, and N9) and the *Rickettsiella* sequences to further validate the above conclusions. The results confirmed that host species remains a significant driver of microbial community composition (PERMANOVA: pseudo- $F = 3.5604$, $R^2 = 0.4328$, $p = 0.001$; PERMDISP: $F = 3.7565$, $p = 0.007$; Table 1; Tables S4, and S5), reinforcing the robustness of host species as an ecological determinant.

PERMANOVA and PERMDISP analyses on intraspecific microbial community

To elucidate the effects of geography and *Rickettsiella* abundance on intraspecific variation in the structure of microbial community, we analysed the microbiota structures of the multi-geographic samples (groups N1, N3, and N9) using PERMANOVA and PERMDISP tests.

In the groups N1 and N3, PERMANOVA revealed significant differences in microbial community composition across geographic sites, explaining 36.56% (group N1) and 34.75% (group N3) of the variance ($p < 0.001$) (Table 1; Tables S4–S5), while no significant heterogeneity in multivariate dispersion (PERMDISP, $p > 0.05$) (Table 1; Tables S4, and S5). This pattern suggests that the assembly of the groups N1 and N3 communities is primarily governed by deterministic processes, such as environmental filtering or dispersal limitation. In contrast, the geographic factors still explained a substantial portion of the variance in the group N9 (34.44%, PERMANOVA, $p < 0.001$), whereas significant heterogeneity in multivariate dispersion was detected (PERMDISP, $p < 0.01$) (Table 1; Tables S4, and S5).

To evaluate the influence of *Rickettsiella* on the geographic effect, we repeated the PERMANOVA and PERMDISP analyses after removing all *Rickettsiella* sequences. The results shows that the geographic factors remain strongly significant in all groups with only minor changes in explanatory power (group N1: 36.56% vs. 36.30%; group N3: 34.75% vs. 35.51%; group N9: 34.44% vs. 34.79%; Table 1, Tables S4, and S5). These results indicate that *Rickettsiella* contributes to geographic

divergence, but it is not the sole driver. Notably, the heterogeneous dispersion in the group N9 persisted after excluding *Rickettsiella* (PERMANOVA: pseudo- $F = 2.667$, $R^2 = 0.3479$, $p = 0.001$; PERMDISP: $F = 4.2167$, $p = 0.021$; Table 1; Tables S4, and S5), indicating that the exceptional intraspecific variability in this group arises from inherent host factors or broader environmental interactions rather than from the influence of this single symbiont.

A new insight into the role of *Rickettsiella* in the gut microbiota of *Ligidium*

We conducted a systematic comparison of gut microbiota composition across all samples to evaluate the relationship between *Rickettsiella* abundance and microbial community structure, such as *Ligidium* sp. 1 (group N2), *Ligidium* sp. 3 (group N8), and *Ligidium* sp. 5 (group N12) were revealed to have exceptionally high relative abundances of *Rickettsiella* with mean values of 81.22%, 73.08%, and 80.48%, and peak values reaching 93.96%, 98.12%, and 90.11%, respectively. Amongst the *Ligidium* species, *L. acuminatum* Li, 2022 (group N3) was found to own the largest sample size and broadest representation of *Rickettsiella* abundance levels. Its samples JZS22, ZXS1, ZXS3, and ZXS5 showed *Rickettsiella* relative abundances exceeding 70%, and the sample LP3 reaching 95.16% (Fig. 4). In *L. acuminatum*, the samples with more than 70% *Rickettsiella* abundance exhibited reduced ASV counts, while those with low abundance (0.48%–8.48%) maintained normal diversity levels. We also observed the similar trends in *L. denticulatum* Shen, 1949 (group N1), and *L. rotundum* Li, 2022 (group N9).

Spearman correlation analysis between the relative *Rickettsiella* abundance (Dataset 1) and the Shannon diversity index across all samples (Dataset 3) revealed a significant negative correlation ($\rho = -0.2108$, $p < 0.05$; Table S6). To account for the exclusion of highly infected samples in dataset 3, we repeated the analysis using dataset 4, which confirmed the robustness of this negative correlation ($\rho = -0.2648$, $p < 0.01$; Table S7). These results suggest that *Rickettsiella* may competitively exclude or parasitically suppress other taxa. Notably, a *Rickettsiella* abundance of 20.63% in the sample DFD3 of *Ligidium* sp. 3 (group N8) coincided with reduced microbial diversity, indicating that relative abundances greater than or equal to 20.63% are associated with decreased gut microbial diversity in *Ligidium*. A threshold-stratified analysis further identified 20% as the critical threshold for *Rickettsiella*'s ecological impact, beyond which significant differences in alpha diversity first emerged. Based on this, we classified the samples into low-abundance group (group L) and high-abundance group (group H) using 20% as the cutoff.

We further examined the gut microbial diversity in *Ligidium* individuals classified into group L and group

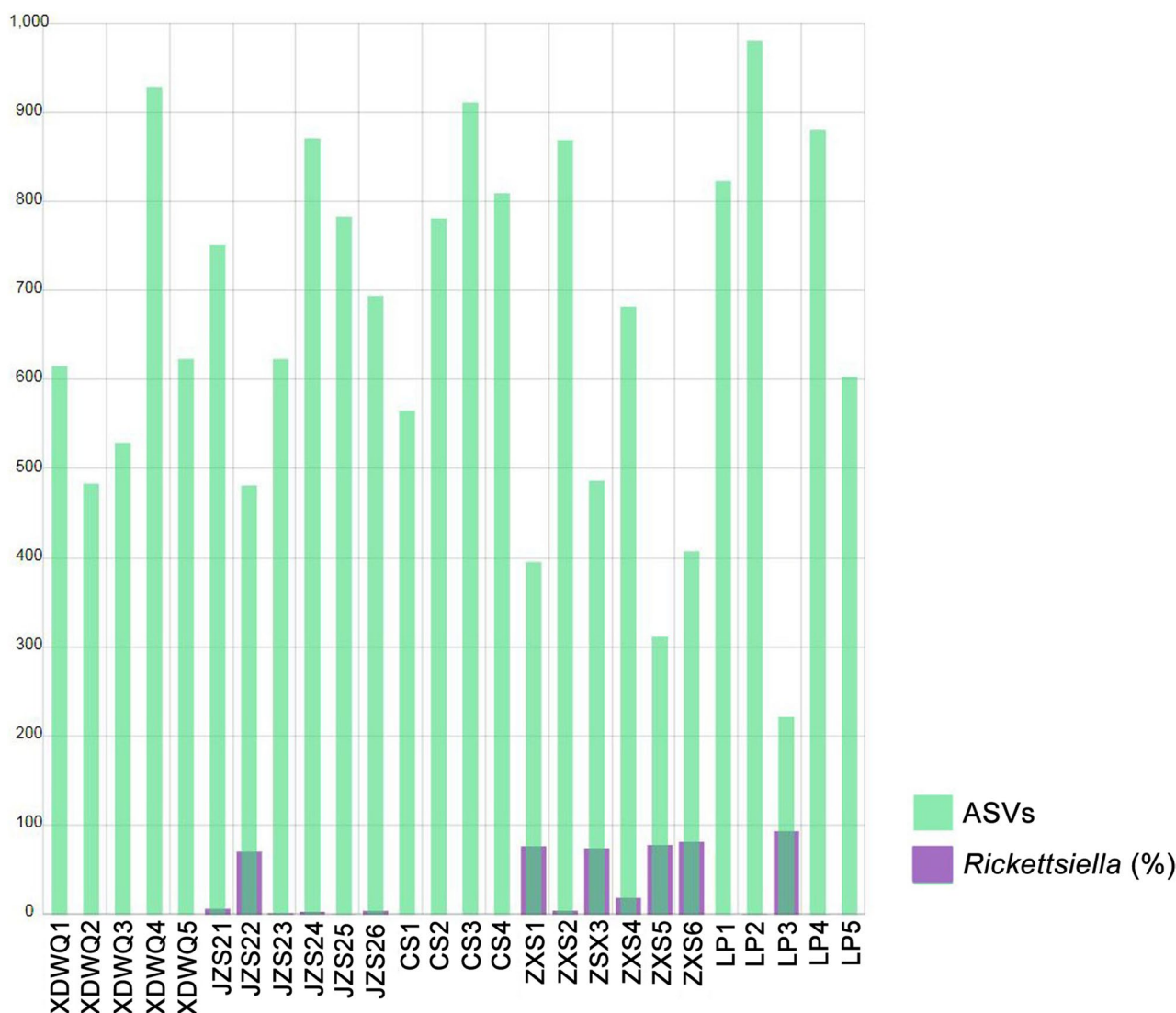


Fig. 4 Gut microbiota composition of *Ligidium acuminatum* Li, 2022 (group N3). Green bars: number of genus-level ASVs (species richness). Purple bars: relative abundance of *Rickettsiella* (%). Sample codes (e.g., XDWQ, JZS) denote geographical groups. Illustrates the relationship between overall microbial diversity and *Rickettsiella* abundance. Complete sample abbreviations are provided in Table S1

H using datasets 3 and 4. As the results of the bacterial community composition (Fig. 5A) and the alpha diversity analyses (Fig. 5B), the gut microbial diversity of the group H was significantly lower than that of the group L. In the result of principal coordinate analysis (PCoA) based on Bray-Curtis distance and Jaccard dissimilarity matrix (Fig. 5C) showed clear separation between the two groups, suggesting that the relative abundance of *Rickettsiella* also affected the beta diversity of gut microbiota in *Ligidium*. Based on both dataset 3 (rarefied to 7,966 sequences) and dataset 4 (rarefied to 5,395 sequences), PERMANOVA also confirmed a significant divergence in microbial community composition between groups with high and low *Rickettsiella* relative abundance (Dataset 3: pseudo-F = 1.9309, $R^2 = 0.0212$, $p = 0.001$; dataset 4: pseudo-F = 2.669, $R^2 = 0.0273$, $p = 0.001$; Tables S4, and S5),

explaining 2.12% and 2.73% of the variance, respectively. At the same time, non-significant intraspecific dispersion was detected (Dataset 3: $F = 0.0045$, $p = 0.934$; dataset 4: $F = 0.0309$, $p = 0.859$; Tables S4, and S5). In summary, our comprehensive analyses provide compelling evidence that a high relative abundance of *Rickettsiella* (>20%) exerts a substantial negative impact on gut microbial diversity in *Ligidium*, likely through competitive exclusion mechanisms that lead to reduced alpha diversity and altered beta diversity patterns.

Discussion

Gut microbial diversity in woodlice

Prior to this study, gut microbial diversity was reported with three to twenty-five phyla in eighteen woodlice species [16, 19, 21]. In the present study, we revealed

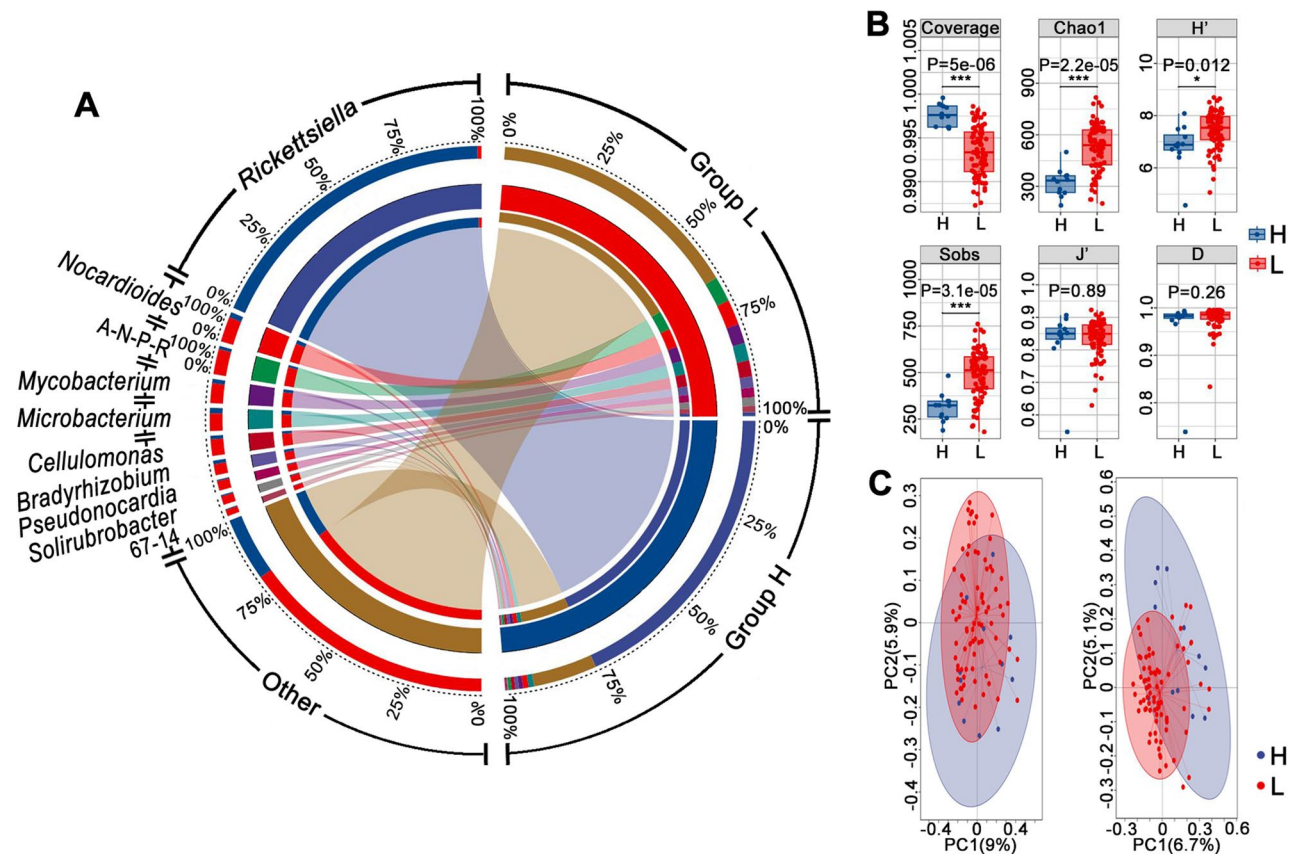


Fig. 5 Comparative analysis of gut microbiota in the high-abundance group (group H) and the low-abundance group (group L). **A** Taxonomic composition showing relative abundances of major bacterial taxa based on dataset 1 (blue: group H; red: group L), with *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* abbreviated as A-N-P-R for conciseness. **B** Boxplots of alpha diversity indices (left to right: Coverage: Good's coverage; Chao1: Chao1 index; H': Shannon index; Sobs: Observed species; J': Pielou's evenness; D: Simpson index) with $p < 0.05$, $***p < 0.001$ (Kruskal-Wallis test followed by Dunn's post hoc correction) based on dataset 2. **C** Beta diversity analysis visualized by principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity matrix (left) and Jaccard dissimilarity matrix (right) based on dataset 2.

fourteen phyla through investigation of the gut microbial diversity in the genus *Ligidium*. At the phylum level, the dominant phyla in *Ligidium* guts are Actinomycetota, Pseudomonadota and Bacillota, collectively constituting 97.4% of the total classified microbiota, showing similar composition patterns to other woodlice species: *Porcellio scaber* (Pseudomonadota 84.1%, Bacteroidota 7.4%, Actinomycetota 3%) [13], *Porcellio dilatatus* (Actinomycetota/Pseudomonadota/Bacillota 87.4%) [16], and *Porcellionides pruinosus* (Pseudomonadota/Bacteroidota-dominant) [19]. Our analysis indicates that *Ligidium* exhibits a higher abundance of Actinomycetota, which may reflect an adaptation to its semi-aquatic detrital niche. The shared ASVs of 12 *Ligidium* species, including *Microbacteriaceae*, *Cellulomonas* and *Bradyrhizobium* representatives along with unclassified strains, may form a functional consortium that could potentially facilitate cellulose degradation and nitrogen fixation [42, 43]. It must be emphasized that these functional inferences are based on phylogenetic comparisons with related species and await experimental validation. The prevalence

of unclassified taxa (e.g., unclassified *Microbacteriaceae*) suggests possible unique microbial adaptations in *Ligidium* guts, offering new directions for understanding isopod-mediated organic decomposition processes. However, these preliminary observations require confirmation through targeted cultivation and omics-based studies. This aspect constitutes a recognized limitation in our present research framework. Notably, our results demonstrate substantially greater gut microbial richness than previously documented in other isopod studies. This pattern corresponds with *Ligidium*'s occupation of botanically heterogeneous semi-terrestrial habitats where increased environmental complexity theoretically supports greater microbial diversity. In contrast, previously examined synanthropic species (e.g., *P. pruinosus*, *A. vulgare*) inhabit stable environments with dietary monotony and potential antibiotic exposure, factors expected to constrain microbial diversity. This highlights the advantage of selecting wild *Ligidium* populations as study subjects and underscores their potential as biological indicators for monitoring soil microbial diversity. By

extensively sampling diverse wild *Ligidium* species rather than focusing on limited taxa, our study provides a more comprehensive characterization of natural isopod microbiome composition. With the change in global climate and the aggravation of soil pollution, *Ligidium* and other woodlice may be applied as biological indicators of soil health and soil bioremediation.

Taxonomic heterogeneity and environmental influences in the gut microbiota of *Ligidium*

In research of taxonomic composition in *Ligidium* guts, we discovered notable interspecific differences among twelve *Ligidium* species (Fig. 1). Based on comparative analyses of gut microbial diversity across geographic populations within the same species, we revealed a stable species-specific core microbiota of total sequence variants within individual population microbiomes (Fig. 1C, D). Moreover, shared ASVs with stable relative abundances were consistently observed across all 12 species. These results revealed that every *Ligidium* species owns conserved core microbiota, and the habitat was demonstrated as a significant influencing factor to the gut microbial consortia of the genus. These results align with reported geographic variations in the gut microbiome structure of *P. pruinosis* across Tunisian populations [21]. Similarly, distinct bacterial communities and population divergence in *A. vulgare* also reinforce this pattern [18]. Collectively, these findings support the existence of robust host-microbiota-environment interactions that are likely linked to terrestrial isopod nutritional ecology.

Symbiotic interactions and novel discoveries in *Ligidium* guts

Among the gut microbiota of woodlice, the symbiotic bacteria are commonly found and play crucial ecological mediators. Previous studies have revealed *Porcellio scaber* populations from contaminated areas with diverse symbiotic gut bacteria, suggesting their potential role in pollution tolerance [44]. Two hepatopancreas-colonizing symbionts, *Candidatus* Hepatoplasma crinochetorum and *Candidatus* Hepatincola porcellionum, have been demonstrated to bear distinct survival impacts on the woodlice. The former enhances host survival under nutrient-deficient conditions, while the latter reduces host longevity [4, 10, 45–47]. In this study, we discovered *Candidatus* Hepatoplasma crinochetorum in the hindguts in about one-fifth of *Ligidium* samples. This finding indicated the symbiont not only colonized in woodlice hepatopancreas, but also occurred in the hindguts. However, potential contamination from hepatopancreatic leakage during dissection cannot be ruled out and the relationship between *Candidatus* Hepatoplasma crinochetorum and *Ligidium* species has not been explored. *Candidatus* Hepatoplasma crinochetorum was detected exclusively,

with no occurrence of *Candidatus* Hepatincola porcellionum observed. This exclusive colonization pattern aligns with previous reports and strongly suggests competitive exclusion between these two taxa [12, 21]. In addition, *Wolbachia* endosymbiotic bacteria were found in most samples of *Ligidium*, which have been reported to affect the biological characteristics and functional traits of the hosts [12, 14]. Notably, our analyses revealed a significant negative correlation between the relative abundance of *Rickettsiella* and gut microbial diversity in *Ligidium* (Dataset 3: $\rho = -0.2108$, $p < 0.05$; dataset 4: $\rho = -0.2648$, $p < 0.01$; Tables S6, and S7). These findings strongly suggest a potential pathogenic role of this bacterium in woodlice, though the precise nature of host-microbe interactions warrants further investigation. Due to current technical limitations, we were unable to conduct rigorous validation of this hypothesis. Future studies employing axenic culture and experimental infection models will be essential to elucidate the mechanistic basis of this observed relationship.

Conclusions

The gut microbiota of *Ligidium* woodlice exhibits remarkable diversity. The results revealed gut bacterial composition has a significant heterogeneity in different *Ligidium* species. Notably, this study comprehensively analysed alpha and beta diversity to systematically elucidate the assembly mechanisms of gut microbiota in *Ligidium*. Our findings identify *Ligidium* species as the core ecological driver, accounting for 63.14% of variation after excluding geographic interference, with geographical distance and high-abundance *Rickettsiella* acting as significant confounding variables. Key findings include: (1) Significant microbial diversity differences were observed among 10 *Ligidium* species ($p < 0.01$). (2) Geographical isolation was revealed as a factor to drive intraspecific divergence (explaining 34.44%–36.56% of variation). (3) *Rickettsiella* relative abundance negatively correlated with Shannon index (Dataset 3: $\rho = -0.2108$, $p < 0.05$; dataset 4: $\rho = -0.2648$, $p < 0.01$; Tables S6, and S7). It significantly reduced alpha diversity ($p < 0.05$) and altered beta distribution patterns when *Rickettsiella* exceeding 20%, likely through competitive exclusion, independently contributing approximately 12.58% to interspecific variation. This research confirms the dominant role of host species genetic background in microbial community assembly, while also revealing the interactive effects of obligate endosymbionts and geographical factors in shaping microbial biogeographical patterns. Uncovering the gut microbial diversity in woodlice and its related influencing factors could support woodlice applied as biological indicators of soil health and soil bioremediation.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-025-04705-x>.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.
Supplementary Material 8.
Supplementary Material 9.
Supplementary Material 10.
Supplementary Material 11.
Supplementary Material 12.
Supplementary Material 13.
Supplementary Material 14.

Acknowledgements

We are grateful to all sample collectors for their collecting in the field. We are particularly indebted to two anonymous reviewers for their invaluable comments and suggestions on the manuscript.

Authors' contributions

J.W. and W.L. conceptualized the study. J.W. conducted laboratory work, sequence data and statistical analysis, data curation, and wrote the original draft. Q. Z. conducted the sample collection. C.J. and W.L. provided supervision, and acquired the funding and assumed administration of the study. All authors contributed to the review and editing, and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (grant nos. 32460124, 82474043), the Key Project at Central Government Level: The ability establishment of sustainable use for valuable Chinese medicine resources (grant no. 2060302), the Survey of Wildlife Resources in Key Areas of Xizang (Phase II) (grant no. ZL202303601) and the Postgraduate Innovation Special Fund Project of Jiangxi (grant no. YC2024-B116).

Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive [48] in National Genomics Data Center [49], China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA023569) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa/browse/CRA023569>.

Declarations

Ethics approval and consent to participate

Not applicable, since this article reports studies involving arthropods.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 17 April 2025 / Accepted: 31 December 2025

Published online: 11 January 2026

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