

Article

Comparative Analysis of Gut Microbiota in Two Cucurbit Leaf Beetles Reveals Divergent Adaptation Strategies Linked to Host Plant Range

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Simple Summary

The relationship between insects' dietary habits and their gut microbiota remains poorly understood. We compared the gut microbiomes of two closely related beetles: the polyphagous *Aulacophora indica* and the oligophagous *Aulacophora lewisii*. Using cultivation, we found that *A. indica* had a higher abundance and diversity of culturable bacteria. In contrast, high-throughput sequencing revealed that *A. lewisii* possessed a richer and more diverse overall gut microbiota. Their community compositions differed notably at the genus level. Functional prediction further showed that *A. indica* had higher gene abundances in core metabolic pathways, such as carbohydrate and amino acid metabolism. These results highlight distinct, diet-adapted gut microbial strategies between a polyphagous and an oligophagous beetle.

Abstract

Insects' gut microbiota and their hosts share a mutually dependent symbiotic relationship. However, how insect dietary breadth relates to microbial diversity remains unclear. This study compared the gut bacterial communities of the polyphagous *Aulacophora indica* and the oligophagous *Aulacophora lewisii*. Using an integrated approach of cultivation, 16S rRNA high-throughput sequencing, and bioinformatic analyses, we assessed their composition, diversity, and functional potential. Using cultivation-based methods revealed that *A. indica* showed a greater abundance and diversity of culturable bacteria, dominated by Proteobacteria and Actinobacteria, compared to *A. lewisii* (Proteobacteria and Firmicutes). In contrast, high-throughput sequencing revealed the opposite pattern: *A. lewisii* exhibited significantly higher overall species richness and diversity. This apparent paradox highlights the methodological complementarity between cultivation and sequencing. Furthermore, the community composition differed notably at the genus level. Functional prediction via PICRUSt2 v2.2.0 indicated that core metabolic pathways, including carbohydrate metabolism, amino acid metabolism, and energy metabolism, were more enriched in *A. indica*. In summary, this study reveals systematic multi-dimensional differences in the gut microbiomes of these beetles, providing a theoretical foundation and microbial resources for understanding ecological adaptation and developing targeted control strategies based on gut microbiota.

Keywords: cucurbit leaf beetles; gut microbiota; 16S rRNA; microbial diversity; functional prediction; feeding difference



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1. Introduction

Herbivorous insects represent a vital component of terrestrial ecosystems and constitute an evolutionarily successful and highly diverse group within the insect class [1]. They inflict substantial economic losses on agriculture by feeding plant tissues and transmitting pathogens [2,3]. Representative orders such as Lepidoptera, Coleoptera, and Hemiptera frequently target food crops and cash crops, posing serious threats to agricultural and economic development. Through long-term co-evolution, complex microbial communities have established within the guts of these insects, forming a symbiotic system that co-evolves with their hosts and plays a central role in key physiological processes, including nutrient acquisition and immune regulation [4–7].

In particular, the gut microbiota of Coleoptera (beetles), the largest insect order, has garnered significant research attention. Studies across diverse coleopteran have revealed intricate associations between gut microbial composition and host diet, ecology, and phylogeny. For instance, in burying beetles (*Nicrophorus vespilloides*), gut microbes can be used to inhibit decay and preserve food resources [8]; in xylophagous longhorn beetles (*Anoplophora glabripennis*), specific bacterial taxa aid in lignocellulose degradation [9]; and in herbivorous pests like *Hylobius abietis*, the gut microbiome is implicated in host plant adaptation [10]. These investigations underscore that gut microbiota is a crucial interface through which beetles interact with their dietary resources. However, systematic comparisons of gut microbiome structure and function between closely related beetle species with divergent feeding breadths remain scarce, limiting our ability to discern microbial-driven adaptation mechanisms.

The focal subjects of this study are two important cucurbit pests: *Aulacophora indica* and *A. lewisii*. Although these two closely related species belong to the same family (Coleoptera: Chrysomelidae) [11–13], they exhibit strikingly different feeding habits: *A. indica* is polyphagous, damaging not only cucurbits such as cucumber and pumpkin but also various vegetables from families including Brassicaceae, Solanaceae, and Fabaceae [14]; in contrast, *A. lewisii* is oligophagous, feeding primarily on sponge gourd [15]. Despite differences in their dietary ranges, both species primarily infest plants of the Cucurbitaceae and exhibit a common preference for cucurbit vegetables. This distinct dietary divergence makes them an ideal natural system for investigating how host plant range shapes the gut microbial community. However, current research has primarily focused on the feeding behavior and control strategies of these two pests [15,16], while a systematic understanding of the composition, function, and dietary adaptation role of their gut microbiota is lacking. This knowledge gap constrains a microbiome-informed perspective on their ecological adaptation and hampers the exploration of potential microbial resources for pest control.

Insects provide intestinal microorganisms with a stable habitat and nutritional base, while the gut microbiota reciprocates with diverse functional contributions. This microbial community not only participates in host nutritional metabolism, assisting in the breakdown of complex carbohydrates and the synthesis of essential nutrients [17,18], but can also directly influence insect growth, development, and reproduction [19–21]. Furthermore, it is recognized as a key factor in regulating the host's adaptability to its host plants [22,23], serving as an important mediator of insect–plant interactions and a potential driver of the evolution of host dietary breadth [24]. In many insect systems, specific bacterial strains have been shown to produce antimicrobial substances that can help the host resist pathogens and natural enemies [25,26], thereby enhancing its ecological adaptability [27,28]. Beyond these beneficial effects, gut bacteria may also act as opportunistic pathogens under particular condition [29–31]. In recent years, the potential application of insect gut microbiota in the targeted control of agricultural pests has gained increasing attention. Precise modulation of the insect gut microbiome represents a promising strategy for managing pest

populations [32]. For instance, the injection of *Serratia marcescens* into the hemocoel of *Apolygus lucorum* causes high pathogenicity [33]. In *Drosophila melanogaster*, *Acetobacter* and *Lactiplantibacillus plantarum* can suppress host oviposition and development through endocrine signaling pathways [34], highlighting how gut microbes may serve as internal levers for population regulation. These principles offer a promising framework for devising novel control approaches for other pests.

Given the substantial agricultural impact of cucurbit leaf beetles [13,15,35,36], and considering that chemical pesticides remain the primary control method [37] with associated environmental, health, and resistance risks [35,37,38], systematically analyzing and comparing the structural and functional differences in the gut microbiomes between *A. indica* and *A. lewisii* is of crucial importance for elucidating the ecological mechanisms underlying their adaptation to different diets, as well as for developing novel microbiome-based pest control strategies. In this study, we combine in vitro cultivation with high-throughput sequencing technologies to compare the structure, diversity, and functional potential of the gut bacterial communities in *A. indica* and *A. lewisii*. We hypothesize that, due to their distinct dietary breadths, the gut microbiota of the two beetles exhibit systematic differences in community structure, diversity, and core metabolic functions. This study aims to provide a theoretical foundation and microbial resources for developing novel microbial pesticides and screening bacterial strains with synergistic effects.

2. Materials and Methods

2.1. Insects and Sample Collection

All specimens of cucurbit leaf beetles used in this study were collected in September 2024 from Hedong Subdistrict, Qingyuan District, Ji'an City, Jiangxi Province, China (115.0395° E, 27.1041° N; elevation 57 m). Gut bacteria from *A. indica* and *A. lewisii* were isolated and identified via cultivation-based methods. Each specimen was surface-sterilized by immersion in 75% ethanol for 5 min, followed by three rinses with sterile water. The entire intestinal tract was aseptically dissected and transferred into a sterile, DNase/RNase-free microcentrifuge tube (Sangon Biotech, Shanghai, China) containing 100 µL of PBS for subsequent bacterial isolation. Following the same sterilization and dissection procedure described above, 16 gut samples were collected from *A. indica* and *A. lewisii* respectively (for each species: 8 male and 8 female individuals), all serving as independent biological replicates. Each sample was processed individually. After collection, the samples were immediately flash-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. To control potential exogenous contamination introduced during the experimental procedures, this study implemented negative controls. During sample processing, a “negative control” containing only sterile PBS was processed simultaneously to monitor potential contamination from the dissection and handling environment. Additionally, a “extraction blank” was included in the DNA extraction step to exclude background DNA introduced by reagents and the extraction process.

2.2. Isolation and Identification of Gut Bacteria

The tubes containing PBS and intestinal tissues were subjected to complete homogenization using a high-throughput tissue homogenizer (Shanghai Wanbai Biotechnology, Shanghai, China). The resulting suspension was gradient-diluted (with dilution factors ranging from 10^{-1} to 10^{-4}), and 50 µL aliquots from each dilution were spread onto Luria-Bertani (LB) solid medium. All plates were incubated at 37 °C for 12 h. It should be noted that this cultivation condition is selective and may favor fast-growing taxa; thus the observed diversity of culturable bacteria may not fully represent the entire gut microbial community. The colony-forming units (CFUs) on each plate and their corresponding

dilution factors were recorded, and morphologically distinct colonies were selected for purification. Each selected colony was inoculated into 5 mL of LB liquid medium and cultured with shaking (200 rpm) at 37 °C for 16 h. From each bacterial culture, 1 mL was mixed with 50% glycerol and stored at −80 °C, while the remainder was used for DNA extraction with the TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China). The 16S rRNA gene was amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3'). The 20 µL PCR mixture consisted of 10 µL of 2× San Taq PCR Mix (Sangon Biotech, China), 1 µL of each primer, 1 µL of DNA template, and 7 µL of sterile water. The PCR reaction program was as follows: initial denaturation at 95 °C for 3 min, followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were examined by 1% agarose gel electrophoresis, and qualified products were submitted to Sangon Biotech (Shanghai, China) for bidirectional sequencing. The resulting sequences were assembled and subjected to BLAST v2.13.0+ (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 10 July 2025) analysis using the National Center for Biotechnology Information (NCBI) for bacterial species identification. For taxonomic assignment, the top BLAST hits with a sequence identity $\geq 97\%$ and query coverage $\geq 95\%$ were used to assign isolates to the genus level, while hits with identity $\geq 99\%$ were used for species-level designation, following common practice in 16S rRNA-based bacterial classification.

2.3. 16S rRNA Full-Length Sequencing

The collected intestinal samples were disrupted using a tissue homogenizer at 55 Hz for 60 s to lyse the gut tissues. DNA was extracted using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). The concentration of DNA was measured with an ultra-micro-spectrophotometer (Shanghai Tripbiotech, Shanghai, China), and its quality was assessed by 1% agarose gel electrophoresis. The hyper-variable regions of the bacterial 16S rRNA gene were amplified using primers 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-RGYTACCTTGTACGACTT-3'). The 20 µL PCR mixture contained 10 ng of template DNA, 10 µL of 2× Pro Taq, 0.8 µL of each primer, and double-distilled water. The PCR reaction program consisted of initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 10 min. Each sample was tested individually, and the PCR products were checked by 2% agarose gel electrophoresis. The PCR products were purified with the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, USA) and quantified using a Quantus fluorometer (Promega Corporation, Madison, WI, USA). The purified PCR products were ligated with PacBio-compatible primers and SMRTbell adapters to construct circularized sequencing libraries. Following the standard workflow, qualified libraries were sequenced on the PacBio Revio system for HiFi circular consensus. The raw data was submitted to the NCBI Sequence Read Archive (SRA) (accession number: PRJNA1365039). Samples of *A. indica* and *A. lewisii* were labeled as Ye and Bl, respectively, with females and males denoted as Fe and Ma.

2.4. Bioinformatics Analysis

Following PacBio HiFi sequencing of the full-length 16S rRNA gene from the microbial communities, raw sequencing data were processed with the Circular Consensus Sequencing (CCS) module in SMRT Link v8.0 to extract and filter high-accuracy sequencing reads. The resulting high-quality HiFi reads were then processed using a denoising pipeline to resolve amplicon sequence variants (ASVs). Taxonomic analysis across multiple classification levels was performed based on the composition of these ASV feature sequences.

To assess species richness and diversity within the gut microbial communities of the samples, we calculated alpha diversity indices including ACE, Chao, Shannon, Simpson, Coverage, and Sobs. All indices were computed using Mothur v1.30 and visualized with the vegan package in RStudio v3.3.1 [39]. The Mann–Whitney U test in SPSS v26.0 was employed to test the significance of differences in the alpha diversity of gut bacteria between the two cucurbit leaf beetle species, with the significance level set at $p < 0.05$. Beta diversity analysis was performed using QIIME2. Within this framework, Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) were conducted via the vegan package in R to compare compositional differences in gut microbiota among samples. At the ASV level, the Bray–Curtis distance algorithm was used to calculate distances between samples, and Analysis of Similarities (ANOSIM) was applied to assess the significance of differences between sample groups.

Furthermore, the species composition of the two sample groups was analyzed at the genus level using Python v2.7. To visually compare the dominant bacterial genera and their relative abundances, community bar plots were generated with R. Bar charts for specific gut bacterial genera were plotted and analyzed using the stats package in R. At the genus level, differential abundance analysis between groups was performed using the Wilcoxon rank-sum test. To control the False Discovery Rate (FDR) arising from multiple comparisons, p -values were adjusted using the Benjamini–Hochberg procedure. An adjusted $p < 0.05$ was considered statistically significant. Stacked bar plots were used to compare the proportions and relative abundances of three occurrence-based microbial categories—transient species (rarely detected), intermediate species (moderately frequent), and persistent species (consistently present)—thereby reflecting their differential stability or environmental sensitivity within the host gut. The Linear Discriminant Analysis Effect Size (LEfSe) method was performed to identify gut bacterial taxa with significantly different abundances between the two insects [40], using a logarithmic Linear Discriminant Analysis (LDA) score threshold of 2.0 and an α -value of 0.05 for the factorial Kruskal–Wallis test.

2.5. Functional Prediction of Gut Bacteria

The functional potential of the gut bacterial communities in cucurbit leaf beetles was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUST2) software [41]. First, the ASV feature table and corresponding abundance matrix for each sample were extracted from the QIIME2 analysis platform. Subsequently, these ASV feature sequences were aligned against a reference database and integrated into a phylogenetic tree to reconstruct phylogenetic relationships [42]. Based on this phylogenetic placement, the copy numbers of gene families and the 16S rRNA gene associated with each ASV were predicted [43]. This process ultimately produced a KEGG Orthology (KO) gene family abundance table. Following the hierarchical structure of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the KO identifiers were then systematically mapped and aggregated to the level 3 pathways (specific metabolic pathways), level 2 pathways (functional modules), and level 1 pathways (broad functional categories), thereby inferring the metagenomic functional composition of the gut bacteria in cucurbit leaf beetles and facilitating further investigation of abundance differences across these distinct functional categories. It is important to note that PICRUST2 predictions are derived from phylogenetic inference based on reference genomes and do not represent directly measured functional profiles.

3. Results

3.1. Isolation and Identification of Gut Bacteria from *A. indica* and *A. lewisii*

A total of 131 bacterial strains were isolated from the gut samples of the two *Aulacophora* species. We obtained 40 distinct 16S rRNA sequences via Sanger sequencing. Following BLAST analysis, these sequences were classified into 4 phyla, 10 families, and 19 genera (Table S1).

At the phylum level, Proteobacteria (23 species) and Firmicutes (8 species) dominated both beetles, whereas Actinobacteria (8 species) was prominent only in *A. indica*, and Bacteroidetes (1 species) was uniquely detected in *A. indica* (Figure 1A). Key compositional differences were observed at finer taxonomic levels. Within Proteobacteria, *A. indica* harbored a more diverse assemblage, including the families Enterobacteriaceae (16 species), Pseudomonadaceae, and Aurantimonadaceae, whereas only Enterobacteriaceae (9 species) was detected in *A. lewisii*. Among Firmicutes, Staphylococcaceae was the most predominant family in *A. lewisii*, whereas Bacillaceae showed the greatest species richness in *A. indica* (Table S1).

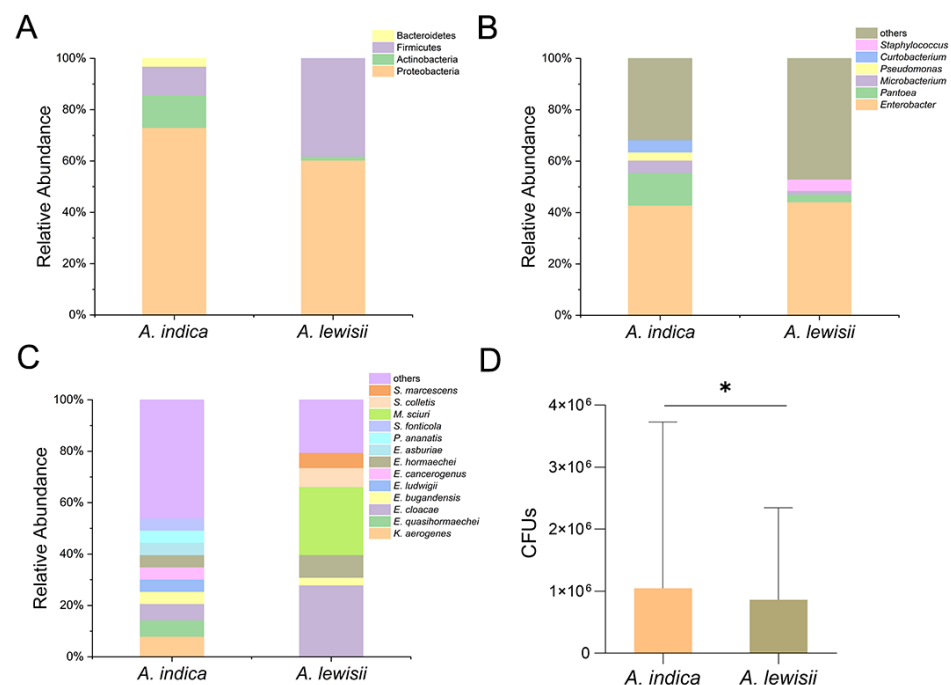


Figure 1. Composition and abundance of culturable gut microbiota. (A) Relative abundance of species composition at the phylum level. (B) Relative abundance of species composition at the genus level. (C) Relative abundance at the species level. (D) Colony-forming unit (CFU) count (* $p < 0.05$).

Further analysis was conducted of the genus-level composition of gut microbiota in the two cucurbit leaf beetles. Cultivation-based isolation revealed that *Enterobacter* was the most frequently isolated genus (57 strains, 43.51% of all culturable gut bacteria), followed by *Mammaliicoccus* (19 strains, 13.87%) and *Pantoea* (10 strains, 7.63%) (Table 1). The dominant bacterial genera observed within *A. indica* were *Enterobacter*, *Pantoea*, and *Microbacterium*, with the highest species richness observed within *Enterobacter*, including *Klebsiella aerogenes*, *Enterobacter quasihormaechei*, and *Enterobacter cloacae*. The dominant genera in *A. lewisii* were *Enterobacter*, *Pantoea*, and *Staphylococcus*, with the highest species richness represented by *E. cloacae*, *Enterobacter hormaechei*, and *Mammaliicoccus sciuri*. Although the two beetles shared similar profiles at the phylum level, their gut bacterial composition exhibited distinct differences at the species level (Figure 1B,C; Table S2).

Table 1. Isolation frequency of culturable bacterial genera in the guts of *A. indica* and *A. lewisii*.

Genus	<i>A. indica</i>	<i>A. lewisii</i>
<i>Aureimonas</i>	1	0
<i>Brachybacterium</i>	1	0
<i>Curtobacterium</i>	3	0
<i>Enterobacter</i>	27	30
<i>Enterococcus</i>	2	2
<i>Klebsiella</i>	5	0
<i>Lactococcus</i>	2	2
<i>Leucobacter</i>	1	0
<i>Lysinibacillus</i>	0	1
<i>Mammaliicoccus</i>	1	18
<i>Microbacterium</i>	3	1
<i>Pantoea</i>	8	2
<i>Priestia</i>	1	0
<i>Pseudomonas</i>	2	0
<i>Rosellomorea</i>	1	0
<i>Serratia</i>	3	4
<i>Siccibacter</i>	0	5
<i>Sphingomonas</i>	2	0
<i>Staphylococcus</i>	0	3

Note: The numbers in the table indicate the count of bacterial isolates for each corresponding genus.

The number of culturable bacterial species was higher in *A. indica* (32 species) than in *A. lewisii* (16 species), with 8 bacterial species shared between them (Figure 2). The bacterial CFUs were significantly higher in *A. indica* ($1.045 \times 10^6 \pm 2.69 \times 10^6$ CFUs/individual) than in *A. lewisii* ($8.623 \times 10^5 \pm 1.48 \times 10^6$ CFUs/individual) (Mann–Whitney U test; $U = 309.5$; $p = 0.03$; $r = 0.47$; $n = 20$) (Figure 1D).

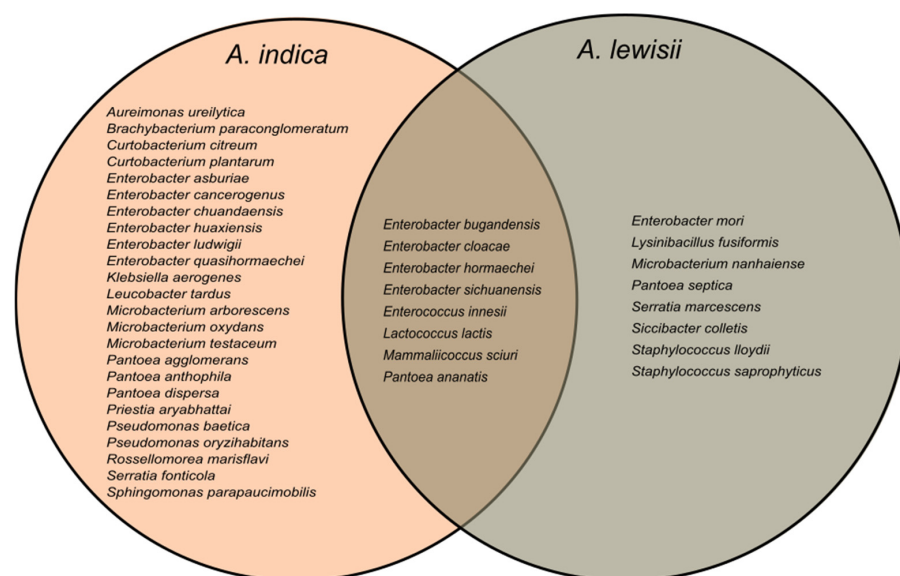


Figure 2. Venn diagram of culturable gut bacteria. The orange circle represents culturable gut bacteria unique to *A. indica*, the gray circle represents those unique to *A. lewisii*, and the overlapping region represents culturable gut bacteria shared by both species.

3.2. Diversity Analysis of Gut Microbiota

The gut microbiota composition and structure of *A. indica* and *A. lewisii* were characterized using PacBio HiFi 16S rRNA gene sequencing. Rarefaction curves plateaued for all

samples, indicating sufficient sequencing depth and adequate sample size for reliable data analysis (Figure S1).

Alpha diversity indices revealed significant differences in gut microbiota richness and diversity between the two species (Table 2 and Figure 3A–F). Specifically, the gut microbiota of *A. lewisii* exhibited significantly higher species richness than that of *A. indica*, as reflected by a greater observed number of species (Sobs index). In terms of diversity, *A. lewisii* also showed a markedly higher Shannon index and a lower Simpson index compared to *A. indica*, indicating not only a richer community but also a more even species distribution and a more complex microbial structure. Furthermore, the Coverage index exceeded 0.97 for all samples, with no significant difference between groups ($p > 0.05$), demonstrating sufficient sequencing depth to reliably capture the vast majority of microbial information present in the samples.

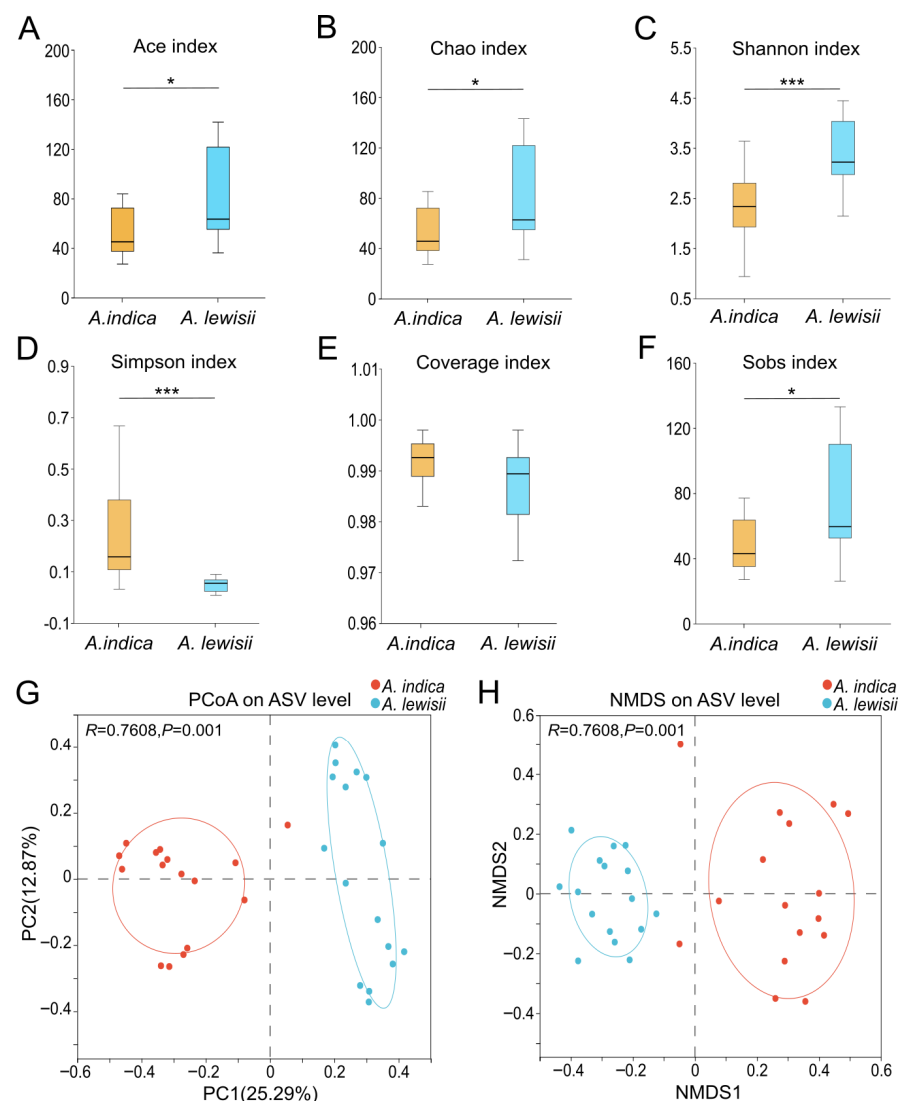


Figure 3. Alpha and beta diversity analysis of gut microbiota in *A. indica* and *A. lewisii*. (A–F) Boxplots of ACE, Chao, Shannon, Simpson, Coverage, and Sobs diversity indices. The x-axis represents the group names, and the y-axis indicates the value of each index. Significant differences between the two selected sample groups shown in the figure are denoted by asterisks (* for $0.01 < p \leq 0.05$, and *** for $p \leq 0.001$). (G) PCoA plot; (H) NMDS plot. Points of different colors represent samples from different groups. Red circles represent *A. indica*; blue circles represent *A. lewisii*. The closer any two sample points are, the more similar their species compositions are.

Table 2. Comparison of alpha diversity indices of gut microbiota between *A. indica* and *A. lewisii*.

Species	ACE	Chao	Shannon	Simpson	Coverage	Sobs
<i>A. indica</i>	58.76 ± 9.03 ^a	57.66 ± 7.99 ^a	2.35 ± 0.19 ^a	0.25 ± 0.05 ^a	0.99 ± 0.00 ^a	51.00 ± 5.87 ^a
<i>A. lewisii</i>	84.00 ± 9.44 ^b	82.89 ± 9.62 ^b	3.41 ± 0.17 ^b	0.07 ± 0.01 ^b	0.99 ± 0.00 ^a	76.44 ± 8.79 ^b

Note: In the table, lowercase letters “a” and “b” indicate the results of statistical comparisons between species indices. Groups sharing the same letter show no significant difference in indices, whereas those labeled with different letters indicate statistically significant differences.

Beta diversity analysis supported the above findings from the perspective of community structure differences. PCoA revealed clear separation along the first principal coordinate (PC1, 25.29%) and the second principal coordinate (PC2, 12.87%), with samples from *A. indica* and *A. lewisii* forming distinct clusters, indicating fundamental differences in their community composition (Figure 3G). NMDS similarly showed group-specific clustering, and the low stress value effectively validated the reliability of the grouping model (Figure 3H). ANOSIM analysis (based on Bray–Curtis distances) further confirmed that between-group differences were significantly greater than within-group differences ($R > 0$; $p < 0.05$).

3.3. Composition and Differential Analysis of Gut Microbiota

The community bar plot at the genus level visually compares the overall composition and relative abundance differences in gut microbiota between *A. indica* and *A. lewisii* (Figure 4A). *Enterobacter* and *Desulfotomaculum* were dominant genera in both beetles. The relative abundance of *Enterobacter* was similar between *A. indica* and *A. lewisii*. This result was consistent with the dominant genera identified using traditional culturing methods. However, *Desulfotomaculum* was notably less abundant in *A. indica* than in *A. lewisii*. Genera such as *Erythrobacter* and *Aromatoleum* were more abundant in *A. indica*, whereas *Pseudomonas* was more prominent in *A. lewisii*. Furthermore, clear visual contrasts were evident in the composition of other low-abundance bacteria between the two species. The genus-level differential analysis based on the Wilcoxon rank-sum test (with FDR correction) revealed that the gut microbiota of *A. indica* and *A. lewisii* differed significantly across multiple genera (FDR-adjusted $p < 0.05$) (Figure 4B). Notably, *Pseudomonas*, *Lactococcus*, *Serratia*, and *Stenotrophomonas* were significantly more abundant in *A. lewisii* (FDR-adjusted $p < 0.001$). The stacked bar chart (Figure 4C) reveals differences in the composition of three occurrence-based categories in the gut microbiota of *A. indica* and *A. lewisii*. In terms of relative abundance, both species were dominated by the intermittent type, followed by the transient type, with the persistent type being the least abundant. Specifically, the transient type was more abundant in *A. indica* (29.28%) than in *A. lewisii* (18.78%), whereas both the intermittent and persistent types were lower in *A. indica* (64.00% and 6.71%) compared to *A. lewisii* (72.88% and 8.34%). Regarding species number distribution, both beetles exhibited the highest number of species in the transient category (*A. indica*: 58.59%; *A. lewisii*: 51.76%), followed by the intermittent category (*A. indica*: 40.12%; *A. lewisii*: 47.02%). The number of persistent species remained very low in both insects (approximately 1.2–1.3%).

LEfSe identified distinct bacterial taxa that were statistically enriched in the gut microbiota of each species (Figure 5). According to a significance threshold of an LDA score > 2.0 , the taxa significantly associated with *A. indica* primarily belonged to the phylum Proteobacteria, including the orders Burkholderiales and Pseudomonadales, family Pseudomonadaceae, and genus *Pseudomonas*. In *A. lewisii*, significantly enriched taxa included the order Lactobacillales (Firmicutes) along with specific groups from Bacteroidetes and Actinobacteria.

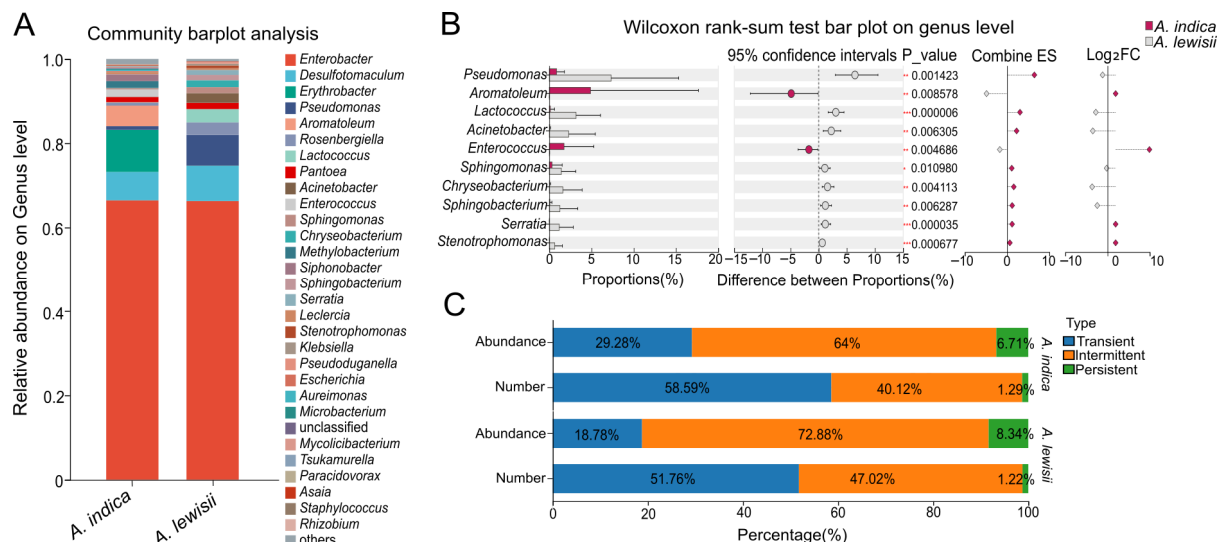


Figure 4. Analysis of gut microbiota composition and differences between *A. indica* and *A. lewisii*. (A) Genus-level community composition. Taxa with a relative abundance of less than 1% are grouped as “Others”. (B) Significantly differentially abundant genera between the two species (Wilcoxon rank-sum test, FDR-adjusted $p < 0.05$). Significance levels are denoted as follows: * for $0.01 < p \leq 0.05$, ** for $0.001 < p \leq 0.01$, and *** for $p \leq 0.001$. (C) Distribution of gut bacteria categorized by occurrence frequency (transient, intermediate, persistent).

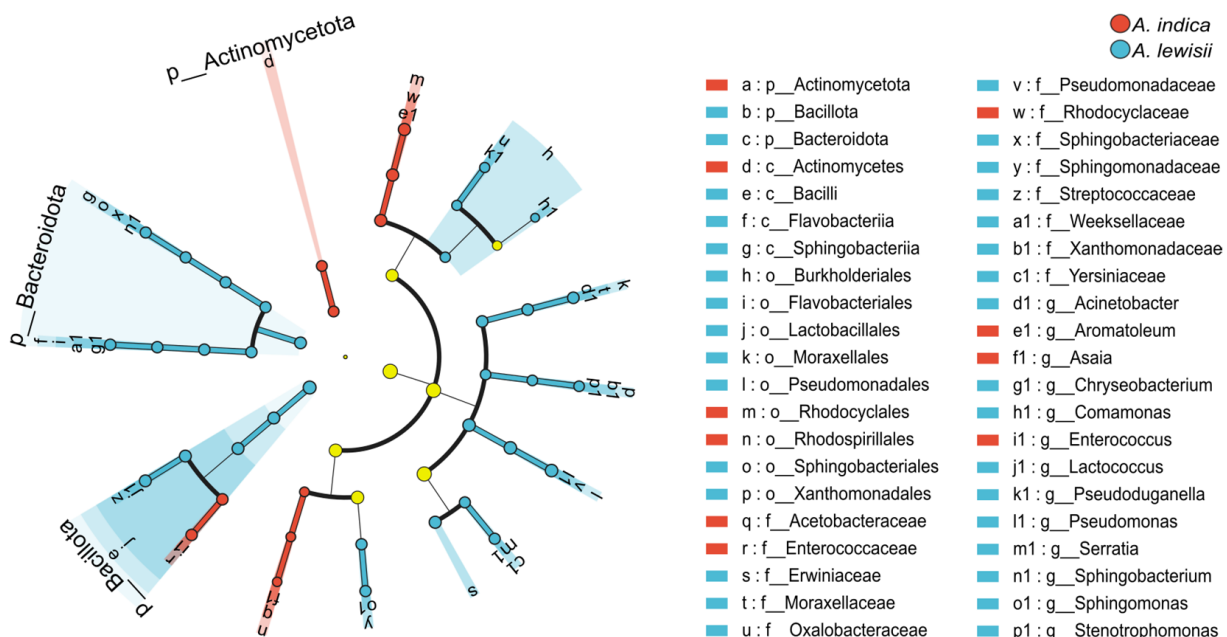


Figure 5. LEfSe multilevel species cladogram. Nodes of different colors represent microbial taxa that are significantly enriched in the corresponding group and significantly affect the differences between groups; light-yellow nodes represent microbial taxa that show no significant differences across groups or do not significantly contribute to intergroup differences.

3.4. KEGG Functional Prediction Analysis

The KEGG metabolic functions of the gut microbiota in the two leaf beetle species were predicted using PICRUSt2. The heatmap (Figure 6) visually presents the overall distribution and differential patterns of predicted gene abundances for core metabolic pathways at level 2 between the two sample groups. Overall, the gut microbiota of both *Aulacophora* beetles exhibited diverse metabolic potentials, including carbohydrate metabolism, amino acid

metabolism, energy metabolism, metabolism of cofactors and vitamins, lipid metabolism, nucleotide metabolism, and glycan metabolism. By visually comparing the color gradients in the heatmap, it can be observed that the predicted gene abundances for most of the above-mentioned core metabolic pathways show a trend of being higher in the *A. indica* group than in the *A. lewisii* group.

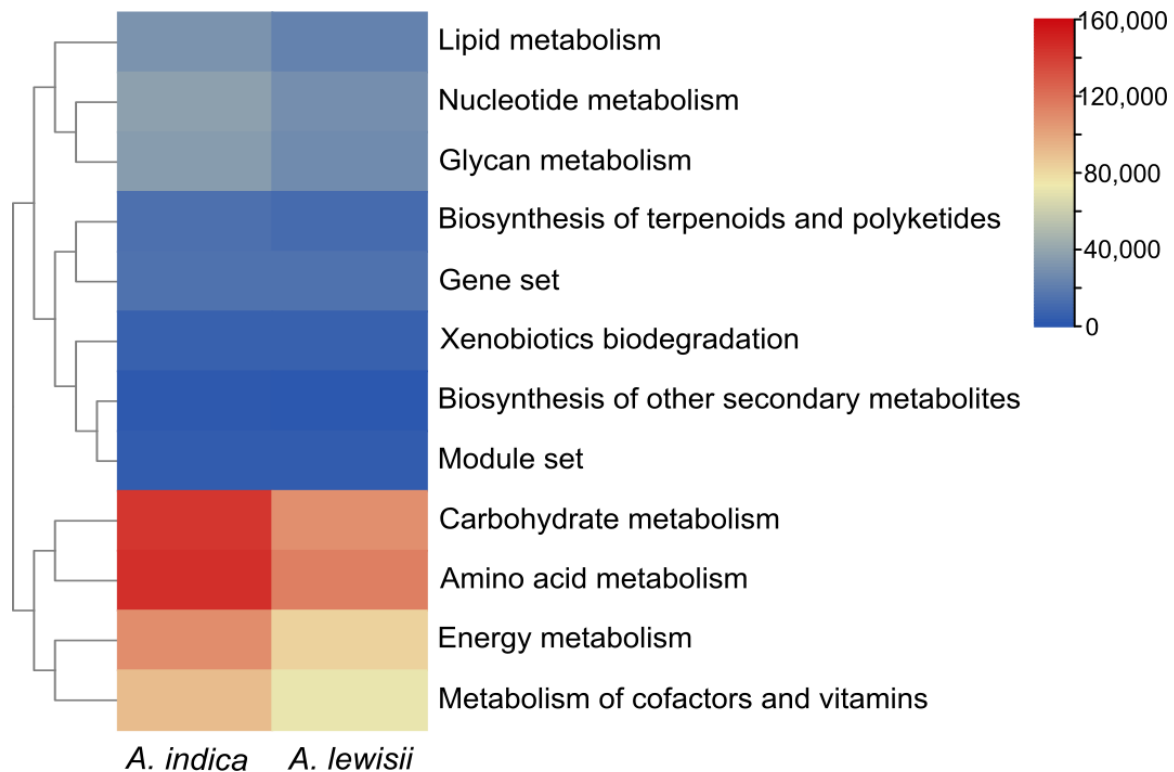


Figure 6. Prediction of KEGG metabolic functions.

4. Discussion

Numerous studies have demonstrated that interactions between insect gut microbiota and their hosts play crucial roles in host physiology, behavior, and ecology [18,29,30,44]. Cucurbit leaf beetles are common and destructive pests of cucurbit plants. In this study, we employed an integrated approach, combining traditional cultivation with high-throughput sequencing, to comprehensively analyze and compare the structure and diversity of gut bacterial communities in the polyphagous *A. indica* and the oligophagous *A. lewisii* [33,45,46]. Our findings reveal a phenomenon worthy of in-depth exploration: analysis based on high-throughput sequencing showed that the oligophagous *A. lewisii* exhibited significantly higher overall gut microbiota richness (ACE and Chao indices) and diversity (Shannon and Simpson indices) than the polyphagous *A. indica* (Figure 3A–D); however, traditional cultivation methods indicated higher culturable species richness and diversity in *A. indica*. This paradox precisely highlights the complementarity of the two methodological approaches and points to their underlying divergent ecological adaptation strategies.

Insect diet is a key determinant shaping the composition of gut microbial communities [47,48]. We propose that the higher overall diversity observed in the sequencing data for *A. lewisii* may be related to its stable feeding habit specialized to sponge gourd [35,49]. This stable dietary structure helps establish a relatively constant microecological milieu within its gut [50,51], thereby potentially accommodating a greater variety of microbial taxa, possibly including many unculturable or nutritionally fastidious obligate or symbiotic microorganisms. This may confer greater resilience and functional stability to its gut

ecosystem [6,7,19]. In contrast, the polyphagous *A. indica* feeds on plants from multiple families [14,52]. Its variable diet can lead to rapid changes in gut microbial community structure [53,54]. This variable environment may act as an “ecological filter,” tending to select for fast-growing “opportunistic” or generalist bacteria capable of rapidly adapting to different plant substrates. Such bacteria are often more readily culturable on conventional laboratory media (LB medium). This explains why *A. indica* showed higher abundance and diversity of culturable bacteria in the cultivation experiment, dominated by Proteobacteria and Actinobacteria. Results from both PCoA and NMDS (ANOSIM: $p < 0.05$) provided further evidence at the community structure level for the significant differences in gut bacterial composition between the two beetle species (Figure 3G,H). These findings strongly support the premise that diet is a key factor shaping gut bacterial community structure [47].

To contextualize our findings within the broader landscape of insect–microbe symbiosis, it is instructive to compare them with well-established models from other Coleoptera. The silkworm is a monophagous Lepidopteran model, harboring a simple yet stable gut microbiota dominated by Firmicutes and Proteobacteria, which is essential for nutrient digestion and host development [55]. This reliance on a conserved, functionally specialized microbiome parallels our observation of the oligophagous *A. lewisii*, which exhibited higher overall microbial diversity and stability, likely underpinning its adaptation to a consistent diet. Conversely, studies on polyphagous or ecologically specialized Coleoptera reveal diverse microbial strategies. For instance, in xylophagous beetles like *A. glabripennis*, the gut microbiota is enriched with genes for lignocellulose degradation [9], while *H. abietis* hosts bacteria capable of detoxifying conifer diterpenes [10]. *N. vespilloides* utilizes a vertically transmitted microbiota for carcass preservation, a function tied to its unique reproductive ecology [8]. Our study on *Aulacophora* beetles bridges these perspectives, demonstrating that even within the same genus, dietary breadth can drive fundamentally different microbial configurations—from a versatile, culturable, and metabolically enriched community in the polyphagous *A. indica* to a more diverse and stable community in the oligophagous *A. lewisii*.

In this study, cultivation-based bacterial isolation revealed that Proteobacteria and Firmicutes were the dominant phyla shared in the guts of both *Aulacophora* species. This finding is consistent with reports from other beetles, such as *N. vespilloides*, *A. lucorum*, *Phalacrognathus muelleri*, and *H. abietis* [33,47,56,57]. These phyla are frequently listed among the most abundant bacterial communities associated with insect taxa [58–60]. We found that within Proteobacteria, Enterobacteriaceae was ubiquitous in the guts of both beetle species. This family is often reported as a dominant component of the gut microbiota in numerous insect species [61–63]. Notably, the relative abundance of Enterobacteriaceae was higher in *A. indica* than in *A. lewisii* (Figures 1B and 4A). This observation aligns with the functional prediction results, indicating that the gut bacterial communities of both species are involved in core metabolic pathways such as carbohydrate and amino acid metabolism and that the predicted functional gene abundances in these pathways were generally higher in *A. indica* than in *A. lewisii* (Figure 6). This pattern may correspond to the polyphagous feeding habit of *A. indica*, with Enterobacteriaceae potentially being better adapted to nutrient-rich and varied plant diets. Given that certain bacteria within Proteobacteria and Firmicutes have been shown to promote growth and nutrient acquisition in other insects [18,47,64], we hypothesize that the dominant bacterial genera identified in this study may similarly benefit cucurbit leaf beetles by facilitating their utilization of host plants. Furthermore, LEfSe revealed that taxa such as *Pseudomonas* were enriched in *A. indica* [25], which might contribute to the degradation of diverse plant secondary metabolites. In contrast, the enrichment of the order Lactobacillales and other taxa in *A. lewisii* could be associated with the insect’s high adaptation to its sole host plant (sponge gourd) [34]. These putative

functional links, while plausible based on comparative ecology, highlight specific microbial targets whose roles in host adaptation require future experimental confirmation.

The distinct gut microbial profiles of the two beetles may be shaped by their diets and offer a new perspective for developing targeted pest control strategies. For the polyphagous *A. indica*, its gut community is dominated by readily culturable, metabolically versatile bacteria, which presents an opportunity for disruption-based strategies. These key taxa can be targeted using specific antimicrobial compounds or engineered microbes to disrupt the host's gut microbiota for pest control [65]. In contrast, the oligophagous *A. lewisii* has a richer and more stable gut microbiota. Its weak point might be this very stability. We could attempt to disrupt this stability, for example by changing the gut conditions or introducing synthetic microbial mixtures to upset the natural balance [22]. Validation through microbial inoculation experiments is crucial to establish causal links between key microbial taxa and host fitness. Integrating such microbiome-mediated strategies with existing integrated pest management frameworks offers a promising and sustainable avenue to reduce reliance on chemical pesticides [66,67].

Taken together, validated by two complementary methodologies, our study revealed significant differences in the structure, diversity, and functional potential of the gut bacterial communities between *A. indica* and *A. lewisii*. It clearly established that Enterobacteriaceae occupy a dominant position in the guts of both beetle species, likely influencing their ecological performance. These findings provide valuable microbial resources and a crucial theoretical foundation for developing novel microbe-based pest control strategies. However, this study has some limitations. For example, our analysis focused solely on the adult stage and did not explore the composition and dynamic changes in the gut microbiota across other life stages, which may limit a comprehensive understanding of host–microbe interactions throughout the beetle's entire lifecycle. While 16S rRNA high-throughput sequencing can provide insights into community structure and allow for functional predictions, it does not directly validate microbial metabolic activities and function. Additionally, all insect samples were collected from the wild, and the specific host plant consumed by every beetle prior to collection was not recorded. This introduces uncertainty as to whether the observed microbial differences are solely attributable to the species' long-term dietary breadth adaptation or are partially influenced by short-term, recent dietary variations. Future studies will employ controlled laboratory rearing experiments to provide more direct causal evidence. Future research should also integrate multi-omics approaches, such as metagenomics and metabolomics, to empirically elucidate the function of key microbial communities and their interaction mechanisms with the host. Additionally, although intestinal dissection and DNA extraction were performed under sterile conditions, the potential for contamination from environmental or reagent sources cannot be entirely ruled out. Future studies should also conduct strain reintroduction assays to experimentally validate the roles of specific gut bacteria in host dietary adaptation, detoxification, and overall fitness, thereby establishing a more solid foundation for identifying precise targets for green pest control strategies.

5. Conclusions

This study employed both culture-dependent and culture-independent methods, revealing systematic multidimensional differences in the gut microbiota of two closely related leaf beetle species with distinct feeding habits: the polyphagous *A. indica* and the oligophagous *A. lewisii*. The combination of these approaches enabled the cultivation of both high-abundance and relatively low-abundance bacterial genera. *Enterobacter* was identified as the dominant bacterial genus in the gut microbial communities of both insect species. Notably, the composition of the gut microbiota differed significantly between the

two beetles. Furthermore, we predicted the gene abundances of core metabolic functions in their gut microbiomes, with *A. indica* exhibiting higher gene abundances than *A. lewisii*. These differences are likely associated with their respective host feeding ranges, providing a novel microbial perspective for understanding insect ecological adaptation. This study not only offers a concrete case for comparative insect microbial ecology but also lays an important theoretical and resource foundation for the future development of targeted pest management strategies based on gut microbiota.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biology15040314/s1>. Figure S1: Rarefaction analysis for intestinal samples of *Aulacophora indica* and *Aulacophora lewisii*; Table S1: Sequence homology comparison of the 16S rRNA of gut bacteria of *Aulacophora indica* and *Aulacophora lewisii*; Table S2: Isolation frequency of culturable gut bacterial strains from the *Aulacophora indica* and *Aulacophora lewisii*.

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Abbreviations

The following abbreviations are used in this manuscript:

LB	Luria–Bertani
CFU	Colony-forming unit
NCBI	National Center for Biotechnology Information
SRA	Sequence Read Archive
CCS	Circular Consensus Sequencing
ASVs	Amplicon sequence variants
PCoA	Principal Coordinate Analysis
NMDS	Non-metric Multidimensional Scaling
ANOSIM	Analysis of Similarities
FDR	False Discovery Rate
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear Discriminant Analysis
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2
KO	KEGG Orthology
KEGG	Kyoto Encyclopedia of Genes and Genomes

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