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Gut microbiota of insectivorous bats in Japan harbor Chlamydophila psittaci and Shiga toxin-producing Escherichia coli: host species and location shape community structure

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Abstract

Background

The gut microbiota of bats is increasingly studied for its ecological and public health significance, yet factors shaping gut microbial communities, such as host species, environmental location, and ecological variation, remain poorly understood in insectivorous bats in Japan. We characterised the gut microbiota of three insectivorous bat species (*Miniopterus fuliginosus*, *Myotis macrodactylus*, and *Rhinolophus ferrumequinum*) collected from three sites in Yamaguchi Prefecture, using 16S rRNA gene (V3–V4) High-throughput amplicon sequencing. We further screened for pathogenic bacteria using species-specific nested PCR.

Results

The gut microbiota was dominated by *Pseudomonadota* (63.6%) and *Bacillota* (22.3%). A global core of ten genera accounted for 74.5% of total relative abundance across all three bat species. Nested PCR detected DNA of *Chlamydophila psittaci*, *Bacillus cereus*, and Shiga toxin 1-producing *Escherichia coli*. PERMANOVA revealed that both host species ($R^2 = 0.166$, $p = 0.001$) and sampling location ($R^2 = 0.132$, $p = 0.001$) significantly influenced community composition based on Bray-Curtis dissimilarity, while alpha diversity did not differ significantly among species or locations. Functional profiles predicted by PICRUSt2 showed no significant differentiation among species ($p = 0.114$) or locations ($p = 0.412$), suggesting potential functional redundancy despite taxonomic variation. Co-occurrence network analysis revealed species- and location-specific patterns in the connectivity of core and potentially pathogenic taxa.

Conclusions

This study provides the first detection of *Chlamydophila psittaci* DNA in bats, alongside other pathogenic species, *Bacillus cereus* and Shiga toxin-producing *Escherichia coli*. In addition, we provide a comprehensive characterisation of the gut microbiome of insectivorous bats in Japan and show that both host species and sampling location significantly influence gut community composition. Meanwhile, predicted metabolic functions remained conserved, consistent with potential functional redundancy. The detection of pathogenic bacteria underscores the value of integrating wildlife microbiome surveillance into One Health frameworks. Future studies incorporating culture-based methods and whole-genome sequencing would strengthen the assessment of zoonotic potential.

1 Background

The mammalian gut harbours diverse and complex microbial communities that play fundamental roles in host physiology and health [1]. Gut microbes contribute to nutrient metabolism [2], behavioural modulation [3], and defence against pathogens [4], and an alteration in the gut microbiome (dysbiosis) can compromise host health [5]. The composition of the gut microbiota is shaped by a combination of host-related and ecological factors [1]. Diet is a major driver of the gut microbiota structure, as different food resources provide distinct nutrients that support the growth and survival of specific microorganisms [6]. Long-term adaptation to local dietary resources leads to selection of microbial communities specialised in nutrient utilisation that benefit both the host and the microbes [6]. In addition, host-related factors such as body weight, age, sex, physiology, and seasonal demands play a significant role in shaping gut microbial composition [1, 7]. Geographical location further influences the gut microbiota through variation in environmental conditions and available food resources [8]. While most studies have focused on defining these factors in humans and captive wild animals, there is a need to consider how these factors shape the gut microbiota of free- ranging wildlife in their natural environment.

Bats (order Chiroptera) represent the second largest mammalian order after Rodentia. They comprise approximately 20% of global mammalian diversity and play critical ecological roles [9]. Owing to their taxonomic, ecological and dietary diversity, bats serve as an excellent study system for investigating how host identity and ecological factors shape the gut microbiome [10]. Approximately 70% of bat species are insectivorous, with a significant interspecific variation in diet composition [11]. Due to their differences in body size, feeding strategies, foraging habitats, and echolocation, insectivorous bats exploit distinct prey niches, leading to a dietary diversity that likely shapes their gut microbiota [12]. Hence, insectivorous bats are an excellent model for defining host and ecological effects on gut microbiota.

Bats are the only mammals capable of true flight, and their ecological diversity and long-distance migratory behaviour increase their exposure to and potential dissemination of diverse pathogenic microorganisms [13]. Consequently, they are recognised as natural reservoirs of several emerging and re-emerging zoonotic pathogens. Bats have recently gained global attention due to their zoonotic association with Severe Acute Respiratory Syndrome Corona Virus (SARS-CoV) and Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV 2) [14]. To date, bat borne viruses have been extensively studied. In contrast, the prevalence and abundance of bat borne bacteria have been underexplored despite their significance in disease transmission and public health [14]. Furthermore, elucidating how the energetic demands of flight influence the gut microbiota of bats compared with non-flying mammals remains important. Several pathogenic bacteria have been isolated from bats worldwide, including *Campylobacter* [15], *Salmonella*, *Shigella*, *Yersinia* [16] and *Enterobacter* [17]. Climate change and increasing human encroachment into wildlife habitats have intensified bat-human interactions, thereby elevating the risk of zoonotic spillover [18]. Hence, understanding the microbiota of wildlife species is crucial for elucidating the mechanisms driving cross-species transmission and developing effective mitigation strategies to prevent future zoonotic outbreaks [19].

Several reports have documented the detection of pathogenic bacteria in bats in Japan [20–22]. However, no studies have comprehensively characterised the gut microbial composition and the factors shaping the gut microbiota of bats in Japan. This study employed high-throughput amplicon sequencing to

characterize the gut microbiota of three insectivorous bat species (*Miniopterus fuliginosus*, *Myotis macrodactylus*, and *Rhinolophus ferrumequinum*) collected from three locations in Yamaguchi, Japan. The primary objectives of this study were to; (i) Characterise the microbial composition of the gut microbiota of bats in Japan, (ii) Assess whether the gut microbiota of bats in Yamaguchi harbour pathogenic bacteria, and (iii) Evaluate the effects of host identity and geographical location on the gut microbiota.

2 Methods

2.1 Study design

Sampling was conducted in 2021 and 2022 in two tunnels in Iwakuni city (Muikaichi tunnel located at 34°17'31.27"N, 131°58'15.58"E and Yamane tunnel located at 34°21'02.76"N, 132°00'06.08"E) and in one cave in Mine city (Beppu cave located at 34◦14'35.67"N, 131◦15'46.76"E) (Fig. 1). Seventy-five bats were captured using nets under permits No. 429-1 and No. 165-1 issued by the local government. The captured bats belonged to three species: the eastern bent-wing bat (*Miniopterus fuliginosus*, n = 37), the eastern long-fingered bat (*Myotis macrodactylus*, n = 15) and the greater horseshoe bat (*Rhinolophus ferrumequinum*, n = 23). Species identification was based on morphological characteristics. After capture, bats were euthanised and necropsied, and a section of the distal large intestine was collected. All samples were stored at -80°C until analysis. Sampled bats composed of 42 males and 33 females.

2.2 DNA extraction

In the laboratory, 50 µl of sterile phosphate buffered saline (PBS) were added into the intestine samples, vortexed briefly, and allowed to thaw to release the faecal content. We extracted total genomic DNA from the faecal content using the DNeasy® PowerLyzer® Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The glass beads provided in the kit were replaced with zirconia beads and homogenisation of the samples was done using a bead crusher at 2,800 rpm for 2 minutes. DNA concentration was determined using Thermo Scientific Nanodrop lite plus Spectrophotometer (Thermo Fisher Scientific, USA).

2.3 Amplification of the 16S rRNA gene

The hypervariable region V3-V4 of bacterial 16S rRNA gene was amplified using universal primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). The PCR mixtures (50 µl) consisted of 25 µl of 2x Mighty Amp buffer ver.3, 0.3 µm of each primer, 1 µl Mighty Amp DNA Polymerase ver.3 (Takara, Shiga, Japan), 25 ng of genomic DNA and PCR grade water to complete the reaction. *Francisella novicida* genomic DNA was used as the positive control, and PCR grade water (with no template) was used for negative control. The PCR cycling conditions consisted of an initial denaturation at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 15 s, extension at 68°C for 30 s, and a final extension at 68°C for 5 minutes. Purification of PCR product was done using Takara Nucleospin® Gel and PCR Cleanup kit (Macherey Nagel, Düren, Germany) and amplicons visualised on 1% agarose gel after electrophoresis. Initially, PCR was performed using 30 cycles of denaturation, annealing, and extension. However, amplification was unsuccessful despite optimization of other conditions. We therefore suspected that the samples contained relatively low biomass and increased the number of cycles to 40. Under these conditions, amplification was successful in only 31 out of 75 samples.

2.4 Library preparations and sequencing

Illumina NextSeq primer sets were used for adding barcodes. The PCR mixtures (25 µl) consisted of 12.5 µl of KAPA HiFi HotStart Mix (Kapa, Cape Town, South Africa), 0.3 µm of each primer, 10 ng of PCR product DNA and PCR grade water to complete the reaction. The PCR cycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 10 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 minutes. Purification of the PCR product was done using magnetic beads AxyPrep™ Mag PCR Clean-Up (Axygen, Tewksbury, USA) according to the manufacturer's instructions. Illumina amplicon sequencing produced overlapping paired end reads (2x300bp), (Illumina, San Diego, CA).

2.5 Sequence data processing

Raw sequence data were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2, version 2024.10) software [23]. We performed sequence denoising using DADA2 [24]. Taxonomic assignment of amplicon sequence variants (ASVs) was conducted using a Naïve Bayes Classifier trained on Greengenes2 reference database (sklearn 1.4.2; 2024.09 backbone full-length release) [25, 26], taxa classified as *Chlamydophila* correspond to the currently accepted genus *Chlamydia* [27]. Sequences assigned to eukaryotes, chloroplast and mitochondria, or unclassified at the domain level were excluded prior to downstream analyses. We performed alpha and beta diversity analyses using the QIIME 2 diversity core-metrics plugin. Samples were rarefied to 140,000 sequences per sample to standardise sequencing depth. We assessed alpha diversity using Shannon diversity, Chao1 richness, Faith's phylogenetic diversity (PD), and Good's coverage metrics. Beta diversity was evaluated using Bray-Curtis dissimilarity, Jaccard distance, and weighted UniFrac distance metrics. Principal Coordinates Analysis (PCoA) was used to visualise beta diversity patterns.

2.6 Pathogenic bacteria

To assess the presence of pathogenic bacteria in the bat gut microbiome, we compared our 16S rRNA gene sequencing data with known pathogenic genera. Matching genera were identified as those potentially containing pathogenic species. To screen for the presence of pathogenic species in bat samples, selected samples with high relative abundances of target genera were screened for pathogenic bacteria using a two-step nested PCR approach. The PCR primers used are shown in Table 1. In the first PCR, a total reaction volume of 50 µl was used, which consisted of 2 µl of original sample, 25 µl of 2x Gflex PCR Buffer, 0.3 µm of each primer, 1 µl Tks Gflex DNA Polymerase (Takara, Shiga, Japan) and PCR grade water to complete the reaction. Samples with the desired band size were purified and used for a second reaction with nested PCR primers. The reaction setup was similar to the first PCR, with a template of 50 ng of purified PCR product. The amplification was carried out under the following conditions: an initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at different temperatures for different primer pairs (46°C – 60°C) for 15 s, extension at 68°C for 30 s, and a

final extension at 68°C for 5 minutes. Pathogenic bacteria tested included *Chlamydophila (*syn. *Chlamydia) psittaci*, *Bacillus cereus*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Chlamydophila pneumoniae*, *Streptococcus pneumoniae* and Shiga toxin-producing *Escherichia coli*.

Table 1
List of PCR primers used for detection of pathogenic bacteria in the bat samples

Pathogenic bacteria	Target gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)	Product size (bp)	Reference	Nested forward primer (5′-
Chlamydophila psittaci	ompA	ATGAAAAAACTCTTGAAATCGG	CAAGATTTTCTAGAYTTCATYTTGTT	1100	[28]	TTTCGCCCTACCTTCATGCT
Bacillus cereus	gyrB	TCATGAAGAGCCTGTGTACG	CGACGTGTCAATTCACGCGC	475	[29]	TGGTATTCAGGTTGAGGTT
Escherichia coli	stx1					TTGTACCTGGAAAGGTAAC

AT: Annealing temperature; ompA: Outer membrane protein A; gyrB DNA: gyrase subunit B; stx1: Shiga toxin 1

2.7 Statistical Analysis

Statistical computations were performed in R software v4.5.1 (R Core Team (2025), A Language and Environment for Statistical Computing, Vienna, Austria).

2.7.1 Microbial composition and diversity

The composition and relative abundance of the bat gut microbiome at the phylum, family, and genus levels were analysed using the "phyloseq" package [30]. We assessed the normality of alpha diversity data using the Shapiro-Wilk test, and tested group differences by Kruskal-Wallis or Wilcoxon rank-sum tests. *Post hoc* analysis was performed using Dunn's test from the "FSA" package [31], and *p* - values adjusted for false discovery rate (FDR) using the Benjamini-Hochberg correction. Adjusted $p < 0.05$ was considered statistically significant. We utilised Permutational analysis of variance (PERMANOVA) to test differences in beta diversity distances (permutations = 999) using the *adonis* function contained in the "vegan" package [32]. Since bat species were unevenly distributed across sites, we tested for species and location interactions and performed within-site analyses where multiple species co-occurred to isolate species effects independent of location. The homogeneity of multivariate dispersions was tested using the *betadisper* function, and multiple comparisons tested using the *pairwise.adonis* function. Differential abundance analysis of microbial taxa was performed using Linear Discriminant Analysis Effect Size (LEfSe)[33] in the ImageGP 2 webtool [34]. Taxa with a logarithmic LDA score of ≥ 2 and adjusted $p < 0.05$ were considered differentially abundant.

2.7.2 Prediction of microbial function

We predicted the functional potential of the microbial communities from ASVs and their abundances using PICRUSt2 (version 2.6.0) [35]. Functional pathway annotations were based on the MetaCyc database [36]. Statistical analysis and visualisation were performed using the "ggpicrust2" package [37]. Initially, Principal Component Analysis (PCA) was done to explore the overall variation of functional pathways among samples. Differential abundance analysis of functional pathways was carried out using the *pathway_daa()* function utilising the "ALDEx2" method [38]. We used the "ggplot2" package[39] for visualisation of relative abundances as stacked bar plots and heatmaps, alpha diversity analysis results as boxplots, and beta diversity analysis as PCoA ordination plots. The "ImageGP" package[34] was used to create Venn diagrams.

2.7.3 Microbial correlation analysis

We performed co-occurrence analysis of the bat gut microbiota using Sparse Correlations for Compositional data (SparCC), as implemented in FastSpar [40, 41]. Due to the limited sample sizes within groups, we treated resulting associations as exploratory. Before network inference, we filtered taxa at a prevalence threshold of 30% to avoid spurious results. Co-occurrence networks were generated using a correlation threshold $|r| \geq 0.6$, $p < 0.05$ and visualised in Cytoscape v3.10.3 [42]. In the networks, the nodes represent different bacterial genera, while the edges represent correlations between genera. Red edges represent positive correlations while blue edges represent negative correlations. The size of the nodes is proportional to degree (number of correlations), and correlations of the global core and pathogenic genera are highlighted.

3 Results

3.1 Samples and sequence output

A total of 75 bats were sampled from Mine and Iwakuni cities in Yamaguchi prefecture, Japan. Following DNA extraction and PCR amplification, 31 samples were successfully amplified and were retained for downstream analyses. Among sampling locations, 20 samples were from Yamane Tunnel, 6 from Muikaichi Tunnel, and 5 from Beppu Cave. Bat species representation included *Miniopterus fuliginosus* (n = 16), *Myotis macrodactylus* (n = 8), and *Rhinolophus ferrumequinum* (n = 7) (Table 2**)**. The final data set comprised 18 males and 13 females.

Table 2
Distribution of positive samples across bat species and sampling locations.

Location	Number of positive samples			
	M. fuliginosus	*M. macrodactylus*	*R. ferrumequinum*	Total
Beppu	0	1	4	5
Yamane	16	4	0	20
Muikaichi	0	3	3	6
Total	16	8	7	31

Sequencing 31 samples generated a total of 180,920,384 raw reads. After quality filtering and merging using DADA2, 23,338 ASVs were obtained. ASVs assigned to eukaryote, mitochondria and chloroplast were removed prior to downstream analyses, resulting in 22,965 ASVs. Taxonomic classification identified 21 phyla, 181 families and 286 genera across all samples. Species-specific analysis revealed that 62% of the obtained ASVs were unique to *M. fuliginosus*, 19% to *M. macrodactylus* and 14% to *R. ferrumequinum* (**Supplementary Fig. 1A**). Only 1% of the total ASVs were shared across all three species. Similarly, location-specific analysis showed that 68% of the total ASVs were unique to Yamane tunnel, 20% to Muikaichi and 7% to Beppu cave (**Supplementary Fig. 1B**), with only 1% of the total ASVs shared across all three locations. The good's coverage values exceeded 0.998 for all samples indicating sufficient sequencing depth to capture the majority of bacterial diversity present.

3.2 Pathogenic bacteria

We analysed the bat gut microbiota for pathogenic bacteria using both amplicon sequencing data and targeted conventional PCR for specific pathogenic species (Fig. 2). Several genera containing potential pathogenic species were detected at varying relative abundances (Fig. 2A). Some of the genera included *Chlamydophila*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Yersinia*, *Bartonella*, *Bacillus*, *Escherichia*, and *Vibrio*. Among them, *Chlamydophila* exceeded 1% mean relative abundance across samples. Genus *Staphylococcus* was identified as part of the global core, while bat species *M. fuliginosus* and *R. ferrumequinum* harboured *Chlamydophila* as part of their core bacteria. This suggests that bats in Yamaguchi could be potential reservoirs of these particular pathogenic genera, with possible implications for environmental dissemination and cross-species transmission.

To screen the presence of specific pathogenic species in the bat gut microbiota, nested conventional PCR targeting species-specific genes was performed. These assays for pathogenic bacteria detected DNA of *Chlamydophila psittaci*, *Bacillus cereus* and Shiga toxin 1 -producing *Escherichia coli* in the bat gut samples (Fig. 2B).

3.3 Alpha and Beta diversity

Alpha diversity analyses indicated limited differences among groups (Fig. 3, **Supplementary table 1**). The taxonomic richness and evenness reflected by Shannon index was not statistically significant in host species and sampling locations ($p > 0.05$). However, pairwise comparison was statistically significant between Yamane tunnel and Beppu cave (adjusted $p = 0.037$) (Fig. 3D). No significant differences were observed between male and female bats (Wilcoxon rank-sum test, $p = 0.26$) (**Supplementary Fig. 3A**). Chao1 richness showed no significant variations on bat species, sampling locations (Fig. 3B, E), and between male and female bats (**Supplementary Fig. 3B**). Phylogenetic diversity measured by Faith's PD varied significantly across locations (Kruskal-Wallis, $p = 0.048$) (Fig. 3F) but not among bat species ($p = 0.058$) (Fig. 3C). Dunn's *post hoc* analysis indicated that *M. fuliginosus* had a higher diversity compared to *R. ferrumequinum* ($p = 0.030$), and that samples from Yamane tunnel were more diverse than those from Beppu cave ($p = 0.021$).

Beta diversity analyses based on Bray-Curtis, Jaccard and weighted UniFrac distances revealed significant differences in bacterial community composition among groups (Fig. 4, Table 5). Bray-Curtis PCoA plots demonstrated that the bacterial communities from Beppu cave clustered within the samples from Yamane and Muikaichi tunnels. Microbial community composition differed significantly by host species (PERMANOVA, $p = 0.001$, $R^2 = 0.166$) and sampling locations ($p = 0.001$, $R^2 = 0.132$) (Fig. 4A). Inclusion of an interaction term revealed a significant species - location interaction ($R^2 = 0.052$, $p = 0.014$), indicating that the effect of species on community composition varied among locations. To address this confounding, we conducted within-site species comparisons. At Yamane tunnel, community composition differed significantly among species ($R^2 = 0.191$, $p = 0.002$), demonstrating host-associated differences in microbial composition under shared environmental conditions (**Supplementary table 2**). No significant differences in dispersion were observed in both variables, host species, $p = 0.843$ and location $p = 0.778$. Pairwise comparisons confirmed that bacterial community composition differed significantly between *M. macrodactylus* and *M. fuliginosus* ($p = 0.015$), and between *M. macrodactylus* and *R. ferrumequinum* ($p = 0.030$) (**Supplementary Fig. 4**).

Analyses using Jaccard and weighted UniFrac distances similarly indicated a significant effect of the bat species and sampling location on the overall bacterial community structure (all, $p = 0.001$). However, tests of homogeneity of dispersions were statistically significant ($p < 0.05$), indicating that some observed differences may partly reflect unequal within-group variation (Fig. 4, Table 5).

Table 5
Effects of host species and sampling location on bacterial community structure assessed by PERMANOVA.

Distance metric	Variable	*p*-value	R^2	Dispersion test
Bray-Curtis	Species	0.001	0.166	0.843
	Location	0.001	0.132	0.778
Jaccard	Species	0.001	0.09	0.043
	Location	0.001	0.09	0.003
Weighted Unifrac	Species	0.001	0.34	0.0009
	Location	0.001	0.21	0.138

3.4 Overall bacterial composition

The dominant phyla in the overall bat gut microbiome (mean relative abundance > 1%) were *Pseudomonadota* (63.6%), *Bacillota* (22.3%), *Desulfobacterota* (3.0%), *Fusobacteriota* (2.5%) and *Chlamydiota* (1.4%) (Fig. 5A). Phylum *Pseudomonadota* predominated across most bat species and sampling locations except in Beppu cave, which was dominated by phylum *Bacillota* (**Supplementary Fig. 2A**). At the family level, *Enterobacteriaceae* was the most abundant group, followed by *Clostridiaceae*, *Enterococcaceae*, *Streptococcaceae*, *Desulfovibrionaceae*, *Fusobacteriaceae*, *Pasteurellaceae*, *Moraxellaceae and Chlamydiaceae* (Fig. 5B). Similarly, the genus level composition was largely driven by *Hafnia* (45.6%) (Fig. 5C), which dominated most bat species and sampling locations. An exception was *M. macrodactylus*, where *Dwaynesavagella* was predominant, and Beppu Cave, where genus *Lactococcus* prevailed (**Supplementary Fig. 2C**).

Using a prevalence threshold of 80%, we identified the core microbiota at the genus level for each bat species. *R. ferrumequinum* harboured 18 core genera, while *M. fuliginosus* and *M. macrodactylus* harboured 12 core genera each. Ten genera were shared among all three bat species and across all sampling locations, constituting the global core. These shared genera included *Hafnia, Dwaynesavagella, Serratia, Enterococcus*, *Lactococcus*, *Acinetobacter*, *Staphylococcus*, YIM-78166, *Vespertiliibacter* and *Corynebacterium*, and together accounted for 74.5% of the total microbial community across samples.

Of the 286 genera identified, only 57 were shared among the three bat species (Fig. 5D). *M. fuliginosus* harboured the highest number of unique genera (n = 100), followed by *M. macrodactylus* (n = 42), while *R. ferrumequinum* contained the fewest (n = 26). Across the sampling locations, 42 genera were shared among all three locations, with Yamane tunnel showing the highest number of unique genera (n = 122), followed by Muikaichi tunnel (n = 30) and Beppu cave (n = 25) (Fig. 5E).

LEfSe analysis identified a total of 5 genera and 2 orders with significant differential abundance among bat species (Table 3). Genus *Staphylococcus* and order *Mycobacteriales* were differentially abundant in *M. fuliginosus*, while *Aeromonas*, *Alphaproteobacteria*_un and order *Mycoplasmatales* were differentially abundant in *M. macrodactylus*. Genus *Paraclostridium* and *Bacilli*_un were differentially abundant in *R. ferrumequinum*. Among sampling locations, only *Paraclostridium* was differentially abundant, specifically in Beppu cave (Table 4).

Table 3
Differentially abundant taxa of the bat species identified by LEfSe analysis.

Taxon name	LDA Score	Adj. *p* value	Relative abundance (%)		
			Miniopterus fuliginosus	***Myotis macrodactylus***	***Rhinolophus ferrumequinum***
Staphylococcus	3.817	0.04469	1.2228	0.0124	0.0701
Paraclostridium	3.461	0.04469	0.0010	0.0221	0.3933
Aeromonas	3.371	0.04469	0.0028	0.4132	0.0044
*Bacilli*_un	4.432	0.01101	10.8896	8.0144	20.9992
*Alphaproteobacteria*_un	3.419	0.00817	0.0396	0.4414	0.0245
Order level					
Mycobacteriales	3.623	0.04469	0.7752	0.0903	0.0307
Mycoplasmatales	2.699	0.04469	0.0155	0.1240	0.0154
LDA score > 2, $p < 0.05$					

Table 4
Differentially abundant taxa among sampling locations identified by LEfSe analysis.

Taxon name	LDA Score	Adj. *p* value	Relative abundance (%)		
			Yamane tunnel	Muikaichi tunnel	Beppu cave
Paraclostridium	3.497	0.02850	0.0008	0.0551	0.5199
LDA score > 2, $p < 0.05$					

3.5 Predicted microbial function

To assess whether host identity and environmental factors influence the microbial function of the bat gut microbiota, functional profiles were inferred from 16S rRNA gene sequences using PICRUSt2. PCA of predicted pathway abundances revealed minimal separation in the pathway distances among the bat species (PERMANOVA, $p = 0.114$; Fig. 6A), as well as among the sampling locations ($p = 0.412$; **Supplementary Fig. 5**). The most abundant predicted metabolic pathways across all samples included peptidoglycan maturation (meso-diaminopimelate containing), adenine and adenosine salvage III, inosine 5'-phosphate degradation, glycolysis IV and purine ribonucleosides degradation (Fig. 6B). Differential abundance analysis revealed 4 pathways that were significantly enriched among bat species: 2,3-dihydroxybenzoate degradation, C4 photosynthetic carbon assimilation cycle NADP-ME type, photorespiration and TCA cycle VII (acetate producers) (Fig. 6C). In contrast, no pathways were significantly differentially abundant among sampling locations.

3.6 Microbial correlation analysis

Microbial co-occurrence patterns were explored using correlation network analysis for each bat species (Fig. 7) and sampling locations (**Supplementary Fig. 7**). The resulting networks revealed species-specific and location-specific patterns of bacterial co-occurrence, with marked differences in network complexity and taxonomic composition. Among bat species networks, *R. ferrumequinum* exhibited the most complex network, whereas *M. fuliginosus* showed the simplest. Networks were constructed at a correlation threshold $|r| \geq 0.6$ and $p < 0.05$. Positive correlations predominated across all species, particularly in *M. fuliginosus*, while *R. ferrumequinum* showed the highest proportion of negative correlations. Species-specific differences were observed in the integration of global core taxa within the broader microbial network. Among the ten global core taxa identified, only five were involved in significant correlations in the microbial network of *M. fuliginosus*, six in *R. ferrumequinum*, and *M. macrodactylus* showed the highest representation, with integration of seven core taxa in its network. Only one global core, *Serratia*, was consistently observed in the microbial networks of the three bat species. Pathogenic bacteria also exhibited species-specific network integration. *M. fuliginosus* and *R. ferrumequinum* networks showed integration of *Chlamydophila*, while no pathogenic bacteria were present in the microbial network of *M. macrodactylus*. Similarly, the location-specific networks differed in network complexity and taxonomic composition (**Supplementary Fig. 7**). Beppu cave, for instance, had the simplest network structure with no integration of global core and pathogenic genera. The most complex network was observed in Muikaichi tunnel, while integration of pathogenic taxa was observed only in Yamane tunnel.

4 Discussion

4.1 Pathogenic risks of the bat gut microbiome

The gut microbiota of bats has increasingly been recognised as a potential reservoir of pathogenic bacteria. To our knowledge, we report the detection of *Chlamydophila psittaci* DNA in bats for the first time, suggesting that bats may represent a previously underrecognized host. Other pathogenic species detected were *Bacillus cereus* and Shiga toxin 1-producing *Escherichia coli* (STEC) (Fig. 2B). Potentially pathogenic genera detected by 16S rRNA sequencing included *Staphylococcus*, *Streptococcus*, *Clostridium*, *Yersinia*, *Bartonella* and *Vibrio* (Fig. 2A), similar to previous studies [43–45]. Our findings together with previous reports emphasize the importance of incorporating bacterial surveillance into One Health frameworks, which have traditionally focused on viral pathogens.

Chlamydophila psittaci causes psittacosis in humans and avian Chlamydiosis in birds. In humans, psittacosis typically presents with influenza-like symptoms, and in the absence of proper treatment, can progress to severe and occasionally fatal respiratory disease [46]. To date, *C. psittaci* has not been previously reported in bats. The detection of *C. psittaci* DNA in this study therefore expands the known host range of this pathogen and warrants further investigation into its epidemiology in bat populations. *Bacillus cereus* is a Gram-positive, rod-shaped, spore-forming bacterium, with certain strains causing foodborne illnesses. This bacterium has been isolated from the gut microbiota of fruit bats and lesser horseshoe bats using culture-dependent methods [47]. We detected *B. cereus* in the bat gut microbiota by targeting the *gyrB* gene. Our findings together with previous reports suggest its persistence within bat gastrointestinal communities and underscores the importance of microbiota-based surveillance for pathogenic bacteria in bats. Shiga toxin-producing *Escherichia coli* (STEC) are essential gastrointestinal pathogens causing bloody diarrhoea with a haemolytic uremic syndrome, mediated by the production of Shiga toxins 1 and 2. Ruminants are the natural reservoirs of STEC, and human infection typically occurs through consumption of food or water contaminated with cattle faeces. Thus far, STEC have not been isolated from bats, but have been reported in other wildlife species in Portugal [48]. Nevertheless, multiple studies have documented the presence of multidrug-resistant *E. coli* in bat populations worldwide, indicating that bats may serve as reservoirs and disseminators of antimicrobial-resistant pathogens [49–51]. In the present study, we detected Shiga toxin 1- producing *E. coli* in the gut microbiota of bats, highlighting a previously unreported potential public health risk at the bat-human interface.

Although genera containing clinically relevant species were detected by 16S rRNA gene sequencing, species-specific PCR assays did not confirm the presence of *Staphylococcus aureus*, *Yersinia enterocolitica*, *Chlamydophila pneumoniae*, or *Streptococcus pneumoniae*. This discrepancy likely reflects the limited taxonomic resolution of short-amplicon 16S rRNA gene sequencing, which generally identifies bacteria at the genus level rather than reliably distinguishing pathogenic species. The presence of a genus does not necessarily imply the presence of its pathogenic members. Additionally, low

abundance of specific species or absence of targeted genes may have contributed to the lack of PCR amplification. These findings highlight the importance of combining amplicon sequencing with species-specific molecular assays when assessing zoonotic risk.

Together, our findings reinforce the need for integrated wildlife-livestock-human surveillance systems. Understanding the ecological dynamics and potential transmission routes of these bacterial pathogens in wildlife is essential for early detection and prevention of zoonotic outbreaks. These findings contribute valuable insights to the growing body of evidence highlighting the role of wildlife in zoonotic spillover events [52] particularly in Japan.

4.2 Diversity

Alpha diversity visualization revealed strong inter-individual variation within each species and within each location, with a broad overlap among groups (Fig. 3). Statistical analysis showed no significant differences in the microbial richness and evenness among bat species and sampling locations, similar to a previous study on insectivorous bats [19]. The taxon richness and evenness variation between Yamane tunnel and Beppu cave may reflect differences in sample size between sites or distinct ecological characteristics associated with each habitat. Consistent with findings from a previous study [44], no significant differences in the alpha diversity (Shannon, Chao1 and Faith's PD) were detected between male and female bats (all $p > 0.05$) (**Supplementary Fig. 3**). Phylogenetic diversity varied significantly across locations ($p < 0.05$) but not among bat species. Although microbial communities showed similar levels of richness and evenness, differences in phylogenetic diversity indicate that these communities are composed of evolutionarily distinct taxa. In contrast to alpha diversity, beta diversity differed significantly by PERMANOVA analyses based on Bray-Curtis, Jaccard and weighted UniFrac distances (all $p < 0.05$) among samples, indicating that both species abundance and phylogenetic relatedness varied across groups (Fig. 4). Host identity has been shown to significantly shape the gut microbiota of insectivorous bats [1]. In this study, both host species and sampling locations significantly shaped the structure of the gut microbiota. However, these factors were interrelated in our sampling design, and their effects were partially confounded. The significant interaction suggests that host-associated differences are context-dependent. Despite this, within-site analyses demonstrated clear species-specific differences under shared environmental conditions. The clustering of Beppu cave samples within the broader distribution of the other two sampling locations emphasized the low intra-variability and low diversity of gut microbiome in the cave habitat, as observed by Shannon index. Moreover, the overlapping clusters could possibly indicate a core microbiome conserved across these populations, with site-specific taxa contributing to the observed beta diversity differences [1].

Variations in microbial abundance across sampling locations were observed at the genus level, with *Paraclostridium* showing significant enrichment in samples from Beppu Cave (Table 4). Species within this genus are typically linked to anaerobic environments and have been detected in both soil [53] and faecal microbiomes, indicating that the unique environmental conditions or dietary variations associated with the cave habitat may play a role in their enhanced abundance. Pairwise comparisons across sampling locations were not statistically significant, while among the bat species, *M. macrodactylus* differed significantly from the other two species. The incomplete overlap of bat species across sampling locations constrains the ability to fully isolate host and environmental variations in a global model. Although the inclusion of interaction terms and site-specific analyses help address this shortcoming, future studies with balanced sampling would give stronger conclusions.

Microbial abundance among bat species differed significantly in seven taxa (Table 3). *Staphylococcus* and the order *Mycobacteriales* were significantly enriched in *M. fuliginosus*, whereas *Aeromonas*, unclassified *Alphaproteobacteria*, and the order *Mycoplasmatales* were more abundant in *M. macrodactylus*. In contrast, *Paraclostridium* and unclassified *Bacilli* were predominant in *R. ferrumequinum*. These variations reflect that the gut microbiota composition is not only shaped by diet, but also host species differences such as physiology, immune status or diet preference shape the gut bacterial community structure [13, 17]. *Staphylococcus* forms a part of the skin and mucosal microbiota in both humans and animals. Most species within this genus exist as harmless commensals; however, *S. aureus* can become an opportunistic pathogen, causing skin and respiratory infections. Antimicrobial-resistant *S. aureus* strains have been isolated from the skin [54] and faeces [55] of bats, emphasizing the importance of studying microbial communities and their characteristics in wildlife reservoirs. The enrichment of *Staphylococcus* in *M. fuliginosus* observed in this study may reflect environmental exposure or food acquisition during foraging. *Aeromonas* is ubiquitous in both freshwater and marine environments and includes species capable of causing gastrointestinal infections in humans. Previous studies have detected *Aeromonas* in the faeces of hematophagous bats [56], and its abundance has been shown to be higher in bats that feed on both insects and fish compared to those that feed on insects only [57]. Consistent with our findings, a study in Korea involving the three bat species in this study reported a significant enrichment of *Aeromonas* in *M. macrodactylus* [19]. *Myotis macrodactylus* is strongly associated with riverine habitats and typically forages for food over rivers [58]. Therefore, the enrichment of *Aeromonas* in this species may reflect environmental exposure and/or ingestion of bacteria through their prey. Additionally, the enrichment of *Paraclostridium* in *R. ferrumequinum* in this study may reflect both habitat exposure and host-specific gut microbial selection process [59].

4.3 Bacterial composition

Multiple factors, including host species and environmental conditions, are known to influence gut microbiota composition [6, 60]. In this study, dietary composition was not directly assessed, and therefore its contribution to the observed microbial patterns could not be evaluated. Consistent with previous studies of insectivorous bats [17, 19], the gut microbiota in this study was dominated by *Pseudomonadota*, *Bacillota*, *Desulfobacterota*, *Fusobacteriota*, and *Chlamydiota* (Fig. 5A). The gut microbiota of bats differs significantly from that of other terrestrial mammals, in which the strictly anaerobic phylum *Bacteroidota* constitutes a larger proportion [16, 19]. Powered flight imposes high energy demands, and together with the short intestinal transit time, is thought to drive a shift of the gut microbiota from strict anaerobes (*Bacteroidota*) to facultative anaerobes (*Pseudomonadota*). Accordingly, bats harbour higher proportions of *Pseudomonadota* irrespective of their diet [13, 44]. This *Pseudomonadota*-dominated gut microbiota resembles that of birds [61], suggesting convergent microbial adaptations associated with flight. *Pseudomonadota* exhibit high functional variation, which enables hosts to maximize on microbial function while minimizing microbial diversity and biomass, an adaptation crucial for more sustained flight [62]. Phylum *Bacillota* the second dominant phylum, plays an important role in the metabolism of various nutrients resulting in products that are critical for energy homeostasis and intestinal health [63].

At the genus level, *Hafnia, Dwaynesavagella, Serratia, Enterococcus*, *Lactococcus*, *Vespertiliibacter*, *Acinetobacter*, *Staphylococcus*, YIM-78166 and *Corynebacterium* constituted the global core shared among the three bat species. The presence of a shared core microbiota suggests functional convergence driven by similar dietary niches rather than strict host phylogenetic constraints [64]. The high abundance of these genera in the insectivorous bats further supports the dominance of more metabolically flexible microbes over strict anaerobes [16]. Insectivorous bats are estimated to consume about 61% – 84% of their body mass, or at least 4.8g of arthropods per night [65]. Such high food intake necessitates a diverse and active microbiota to facilitate digestion and nutrient absorption. The genera such as *Hafnia* produce enzymes that allow the degradation of chitin, favouring digestion and the assimilation of nutrients in insectivorous bats [66], while *Enterococcus* and *Lactococcus* are lactic acid bacteria involved in carbohydrate fermentation to produce lactate, contributing to energy acquisition in the host [1, 67]. Bacterial genera such as *Serratia*, *Bacillus* and *Enterobacter* contain chitinolytic species, which facilitate the degradation of chitin from insect exoskeletons [66]. *Candidatus Dwaynesavagella* is a genus of uncultured segmented filamentous bacteria known to be involved in providing intestinal mucosal immunity by increasing the production of Th17 cells and their cytokines [68]. This suggests a potential role in immune regulation in bats by conferring increased protection against pathogens. Segmented filamentous bacteria have been described to increase pathogen resistance in mice and rats [69].

4.4 Predicted functional profiles of bat gut microbiome

Despite taxonomic differences, predicted microbial functions showed no significant variation among bat species and sampling locations (Fig. 6A, **Supplementary Fig. 5**). This functional stability suggests potential functional redundancy; whereby distinct microbial assemblages perform similar metabolic roles. In energetically demanding organisms such as bats, selective pressures associated with flight and insectivory may constrain functional variability [1]. Only four predicted pathways differed significantly among species (Fig. 6C), and none varied across sampling locations. This suggests that host identity has a modest influence on functional potential, whereas environmental effects are comparatively limited at the pathway level. The most abundant pathways overall included peptidoglycan maturation (meso-diaminopimelate containing), adenine and adenosine salvage III, inosine 5'-phosphate degradation, glycolysis IV and purine ribonucleosides degradation (Fig. 6B). The dominance of pathways associated with peptidoglycan maturation, nucleotide salvage, glycolysis, and purine degradation indicates a microbiome adapted for rapid growth and efficient energy extraction [17, 70]. High representation of glycolytic pathways is consistent with the elevated metabolic demands of flight, and pathways associated with carbohydrate and energy metabolism have been shown to be differentially abundant in insectivores compared to fructivores [17]. Similarly, enrichment of nucleotide salvage pathways suggests reliance on energy-efficient recycling mechanisms under shortage of resources [71]. Peptidoglycan maturation pathways may reflect bacterial adaptation to host immune pressures and dynamic gut conditions [70]. Collectively, these findings suggest that although taxonomic composition varies among hosts, core metabolic requirements associated with insect digestion and high-energy lifestyles constrain functional divergence. PICRUSt2 predictions are based on reference genome annotations and do not capture strain-level functional variation, horizontal gene transfer, or gene expression [35]. The apparent functional conservation should be interpreted as a hypothesis to be tested by shotgun metagenomics or meta transcriptomics.

4.5 Bacterial correlations

Correlation networks were used to interpret complex microbial interactions within the bat gut microbiota. The networks showed species-specific and location-specific differences of bacterial co-occurrence, with marked differences in network complexity, and the integration of the global core and pathogenic taxa within the broader network (Fig. 7, **Supplementary Fig. 7**). The marked variation in network complexity, ranging from the highly connected network observed in *R. ferrumequinum* to the sparse network in *M. fuliginosus*, suggests fundamental differences in microbial interaction dynamics and community stability among host species. More complex networks reflect greater ecological stability and resilience due to increased functional interconnectivity, whereas simpler networks may indicate more specialized or fragile microbial ecosystems that are potentially more susceptible to disturbance [72]. The predominance of positive correlations across all bat species suggests that cooperative interactions play a central role in structuring bat gut microbiomes. Such positive associations may reflect metabolic cross-feeding, niche complementarity, or shared ecological niches within the gut environment [73]. Moreover, the integration of core taxa in the microbial networks of the three bat species emphasizes their potential ecological roles and functional significance in the bat gut microbiome [74]. The integration patterns of pathogenic taxa varied among bat species and sampling sites. The varying associations of *Chlamydophila*, *Bacillus*, and *Escherichia* across different groups suggest that pathogen persistence and transmission potential are likely influenced by host identity and environmental factors. Limited connectivity of certain pathogens may indicate transient colonisation rather than stable ecological integration. Conversely, positive correlations within networks may facilitate their persistence in the ecosystem through cooperative interactions, biofilm formation or metabolic cross feeding. Overall, these results suggest that bat gut microbiomes are not only taxonomically distinct among species but are also organised into species-specific ecological networks shaped by host identity and habitat context. Although our findings are exploratory due to small sample sizes within groups, they suggest that future studies incorporating network analyses into microbiome research could offer valuable insights into microbial community stability and potential zoonotic risk dynamics [72].

The limitations of this study included the relatively small number of samples that were successfully amplified after V3-V4 region amplification due to relatively low biomass. Extraction blanks were not sequenced, precluding the use of in silico decontamination tools [75]. Given the low-biomass nature of these samples, contamination from extraction kits cannot be fully excluded [76]. Furthermore, functional profiles were inferred from 16S rRNA gene data rather than directly measured using metagenomic approaches and thus should be interpreted with caution. Future studies should integrate culture-based methods and shotgun metagenomic approaches to provide more robust insights into microbial function and pathogenic potential.

5 Conclusions

This study provides the first detection of *Chlamydophila psittaci* DNA in bats, alongside other pathogenic species, *Bacillus cereus* and Shiga toxin-producing *Escherichia coli*. In addition, we provide a comprehensive characterisation of the gut microbiome of insectivorous bats in Chugoku region, Japan, and show that host identity and environmental location are important factors shaping the gut microbiota composition. Although all studied species are insectivorous, dietary composition was not directly assessed in this study, and therefore its contribution to microbial variation remains unclear. Genomic evidence of

pathogenic bacteria in gut samples from bats highlights the importance of incorporating bacterial surveillance into wildlife studies focused on One Health. Together, these findings expand the current understanding of the gut microbiota of insectivorous bats and highlight the relevance of wildlife microbiomes in the context of zoonotic spillover risks. The detection of *C. psittaci* DNA in bats for the first time underscores the need for further investigation of the pathogenic potential of wildlife microbiomes at the human–wildlife interface and to evaluate the risk of cross-species pathogen transmission.

6 List of abbreviations

DNA Deoxyribonucleic acid

PCR Polymerase chain reaction

rRNA Ribosomal ribonucleic acid

ASV Amplicon sequence variant

QIIME 2 Quantitative insights into microbial ecology 2

PICRUSt2 Phylogenetic investigation of communities by reconstruction of unobserved states

SparCC Sparse correlations for compositional data

Declarations

Ethics approval and consent to participate

Sampling was done under permits No. 429–1 and No. 165–1 issued by the local government.

Consent for publication

Not applicable

Availability of data and materials

Sequencing data that support the findings of this study have been deposited in the NCBI Sequence Read Archive under BioProject accession [PRJNA1450296].

Competing interests

The authors declare no competing financial interest.

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Authors' contributions

RMM: Formal analysis, Investigation, Visualization, Writing – original draft. BEC: Formal analysis, Investigation Writing – original draft. IUP: Data curation, Formal analysis, Validation, Visualization, Writing – review & editing. TS: Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing. MH: Data curation, Investigation, Writing – review & editing. HS: Resources, Writing – review & editing. MN: Resources. AT: Resources, Writing – review & editing. TY: Resources, Writing – review & editing. FM: Formal analysis, Writing – review & editing. JH: Formal analysis. KW: Validation, Writing – review & editing. MW: Funding acquisition, Supervision, Validation, Writing – review & editing.

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Figures

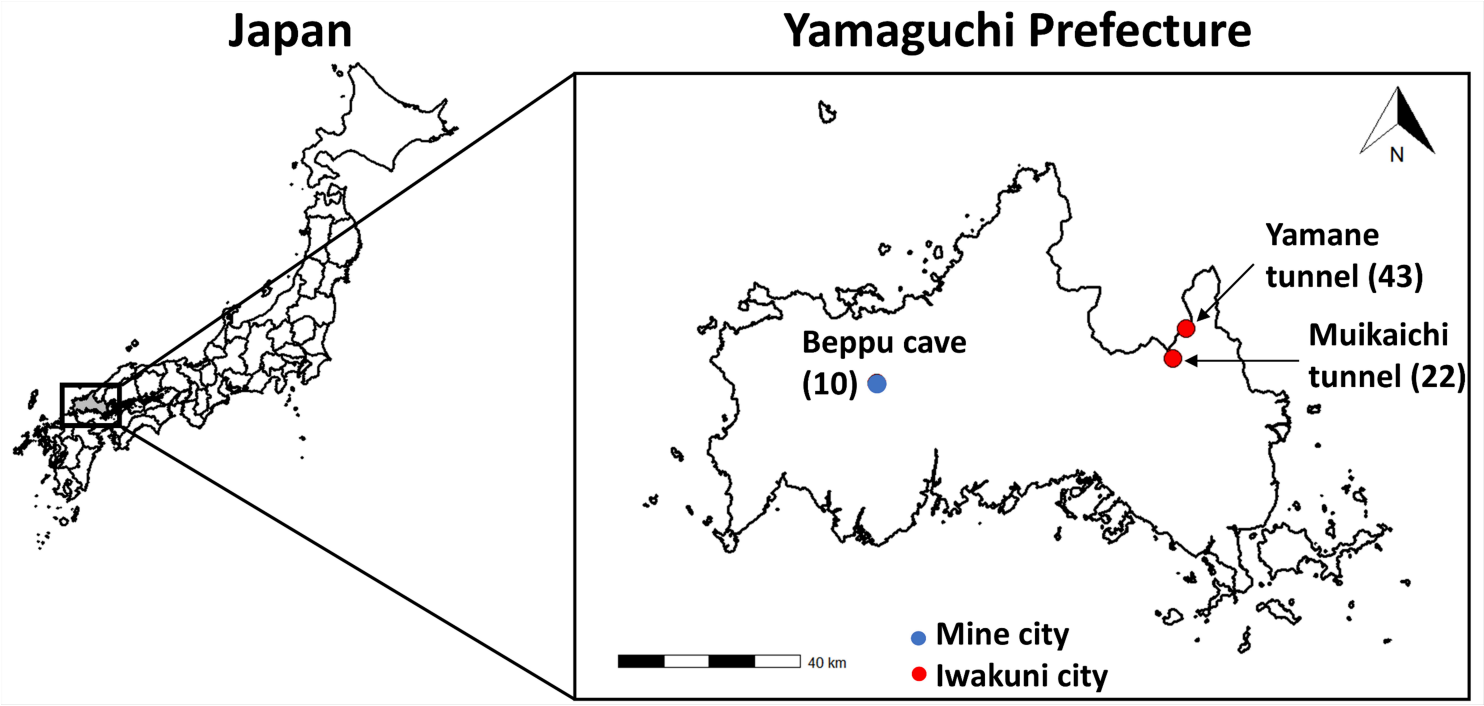


Figure 1

Sampling locations of bat samples in Yamaguchi Prefecture, Japan. The numbers in brackets indicate sample size per location. Three species of insectivorous bats were sampled, including *Miniopterus fuliginosus* (n = 37), *M. macrodactylus* (n = 15) and *R. ferrumequinum* (n = 23).

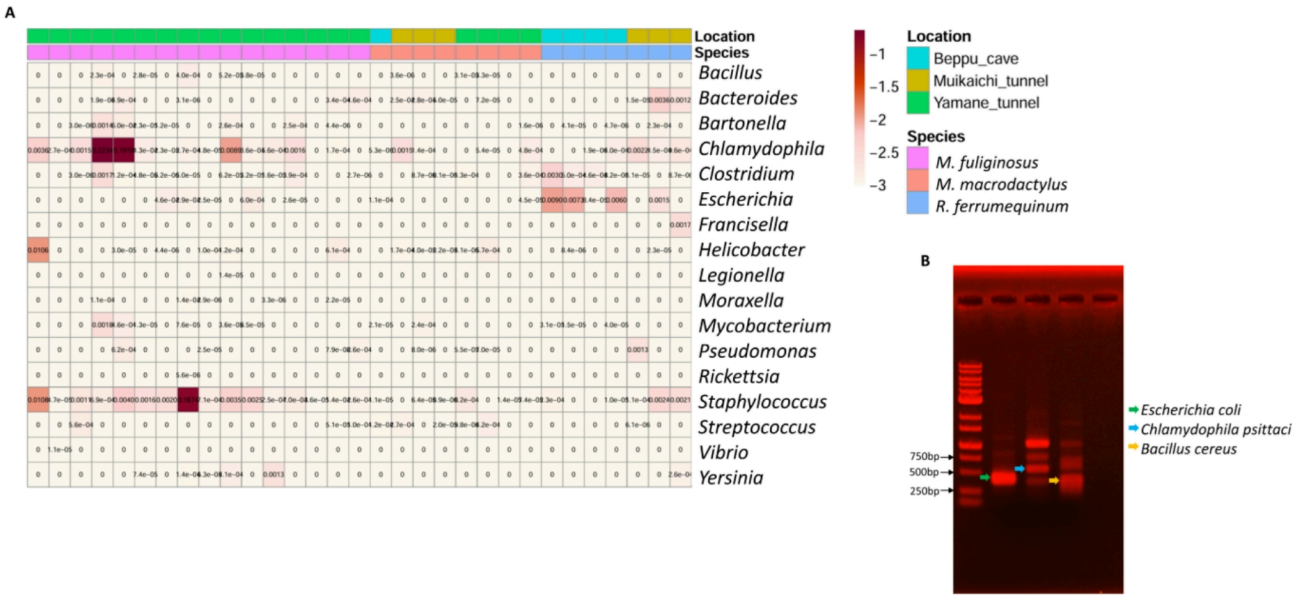


Figure 2

Pathogenic bacteria in the bat gut microbiota. (**A**) A heatmap showing per sample relative abundance of pathogenic genera in the gut microbiota using 16S rRNA sequencing data. (**B**) A cropped gel electrophoresis image of DNA of pathogenic species detected by nested PCR in gut samples of Yamaguchi bats. We detected Shiga-toxin producing *Escherichia coli*, *Bacillus cereus*and *Chlamydophila psittaci* using a two-step nested PCR approach. The image shown is from the second PCR reaction. The full gel image is presented in**Supplementary Fig. 6.**

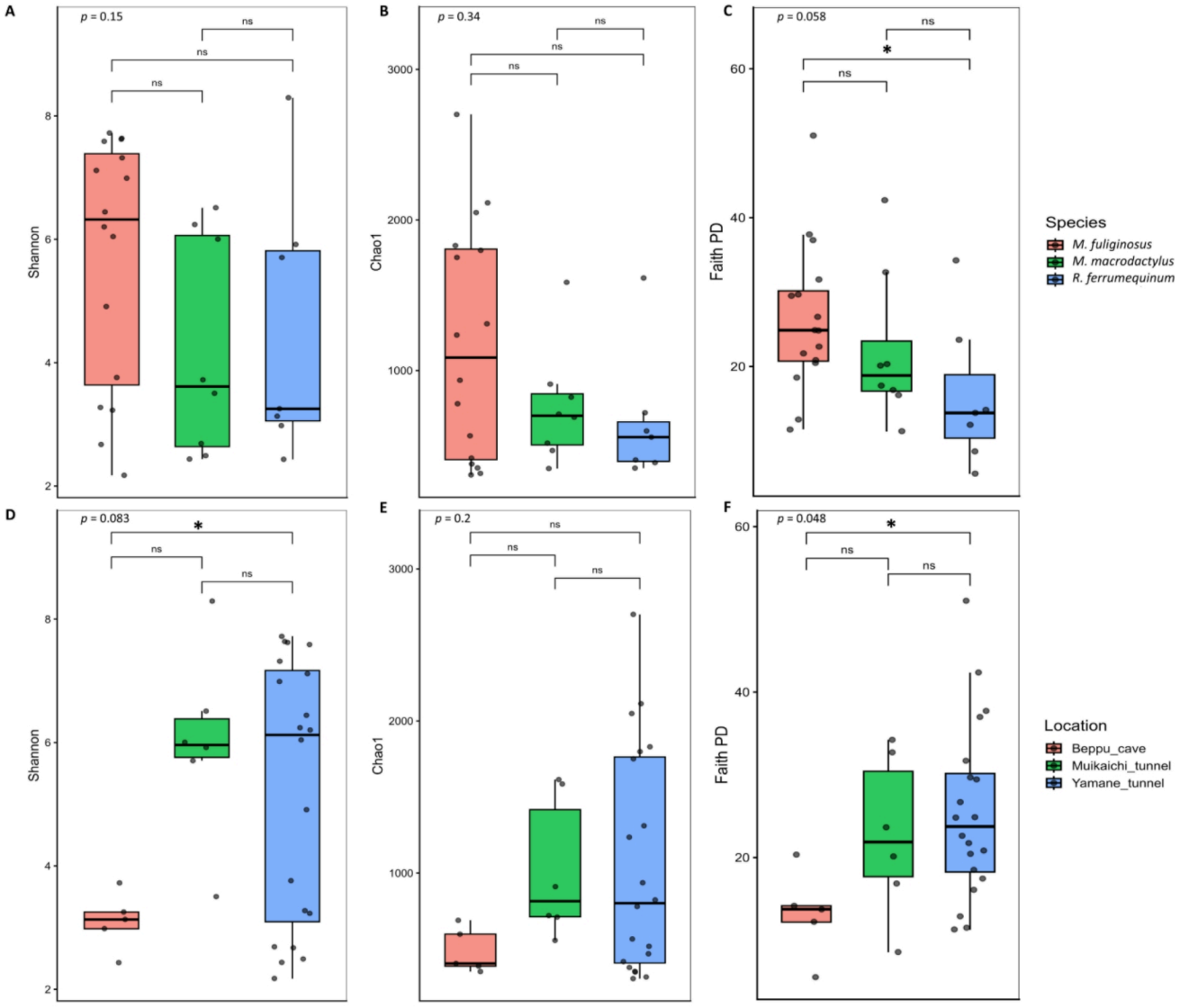


Figure 3

Alpha diversity of the bat gut microbiota assessed by Shannon, Chao1 and Faith's PD metrics. The upper panels show diversity on bat species and lower panels show diversity on sampling locations. The jitter points represent individual samples, and pairwise statistical significance between groups is shown using annotated brackets, (*$p < 0.05$, ns $p > 0.05$). Sample distribution among bat species included: *M. fuliginosus* (n = 16), *M. macrodactylus* (n = 8) and *R. ferrumequinum* (n = 7), while sampling sites: Beppu cave (n = 5), Muikaichi tunnel (n = 6) and Yamane tunnel (n = 20). Overall, the microbial diversity did not differ significantly across species and sampling sites, except Faith's phylogenetic diversity which differed across the sampling sites, $p = 0.048$. Groups' median and the 95% confidence interval are elaborated in **Supplementary table 1.**

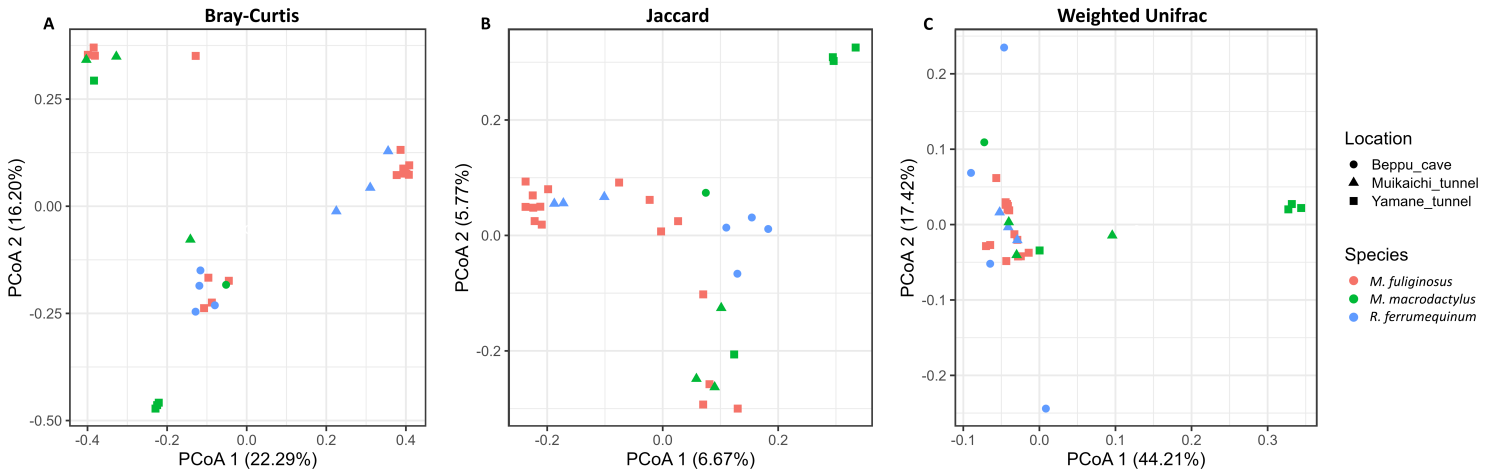


Figure 4

Principal Coordinate analysis (PCoA) based on Bray-Curtis, Jaccard and Weighted Unifrac distances depicting beta diversity clustering. Statistical analysis of Bray-Curtis distances revealed that microbial community structure varied significantly across bat species (PERMANOVA, p = 0.001, R^2 = 0.166) and sampling locations (p = 0.001, R^2 = 0.132), with the first two axes explaining 22.29% and 16.20% of the total variance. Similarly, Jaccard and weighted UniFrac distances indicated a significant effect of the bat species and sampling location on the overall bacterial community structure (both, p = 0.001).

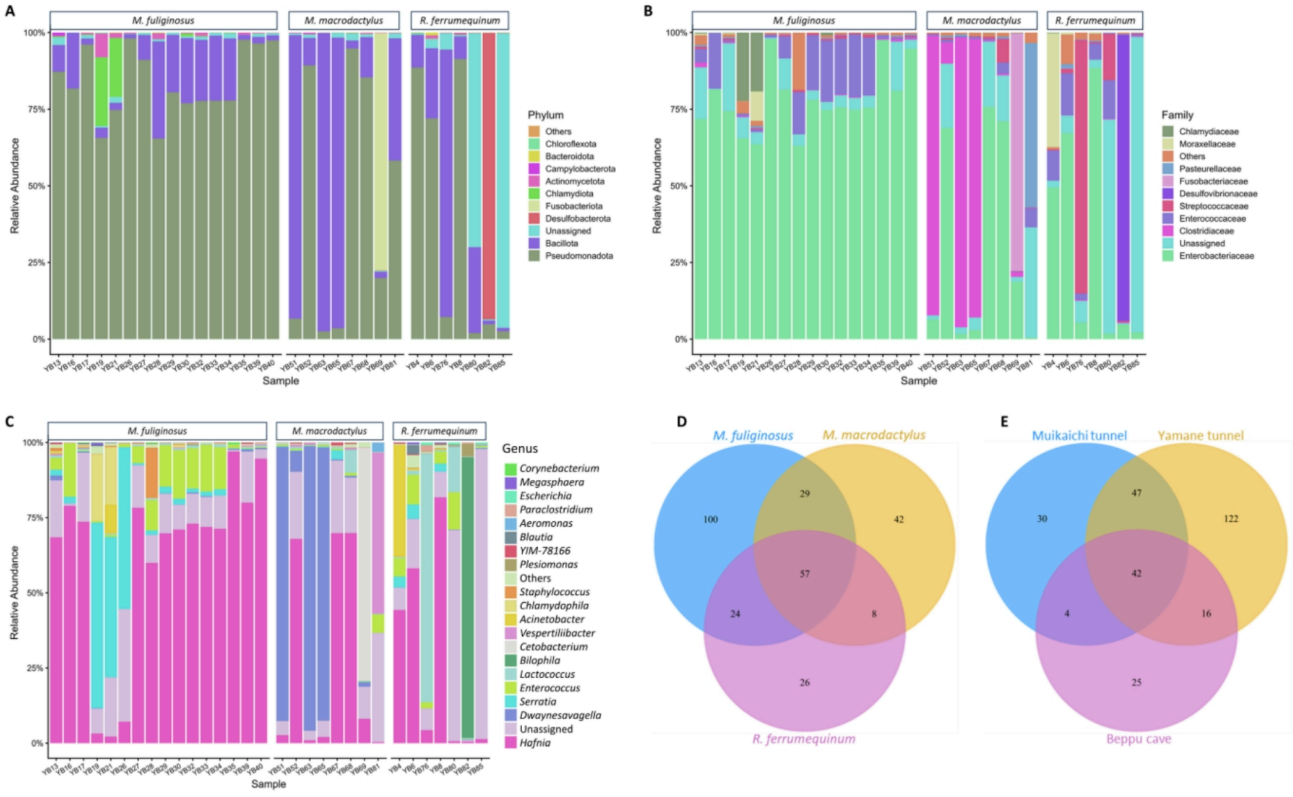


Figure 5

Microbial composition of the bat gut microbiota. Stacked bar plots of the relative abundance of bacteria, (**A**) Phylum level (**B**) Family level (**C**) Genus level, with the most abundant taxa positioned at the bottom. The Venn diagrams show the distribution of observed genera (n = 286) across (**D**) bat species and (**E**) sampling locations.

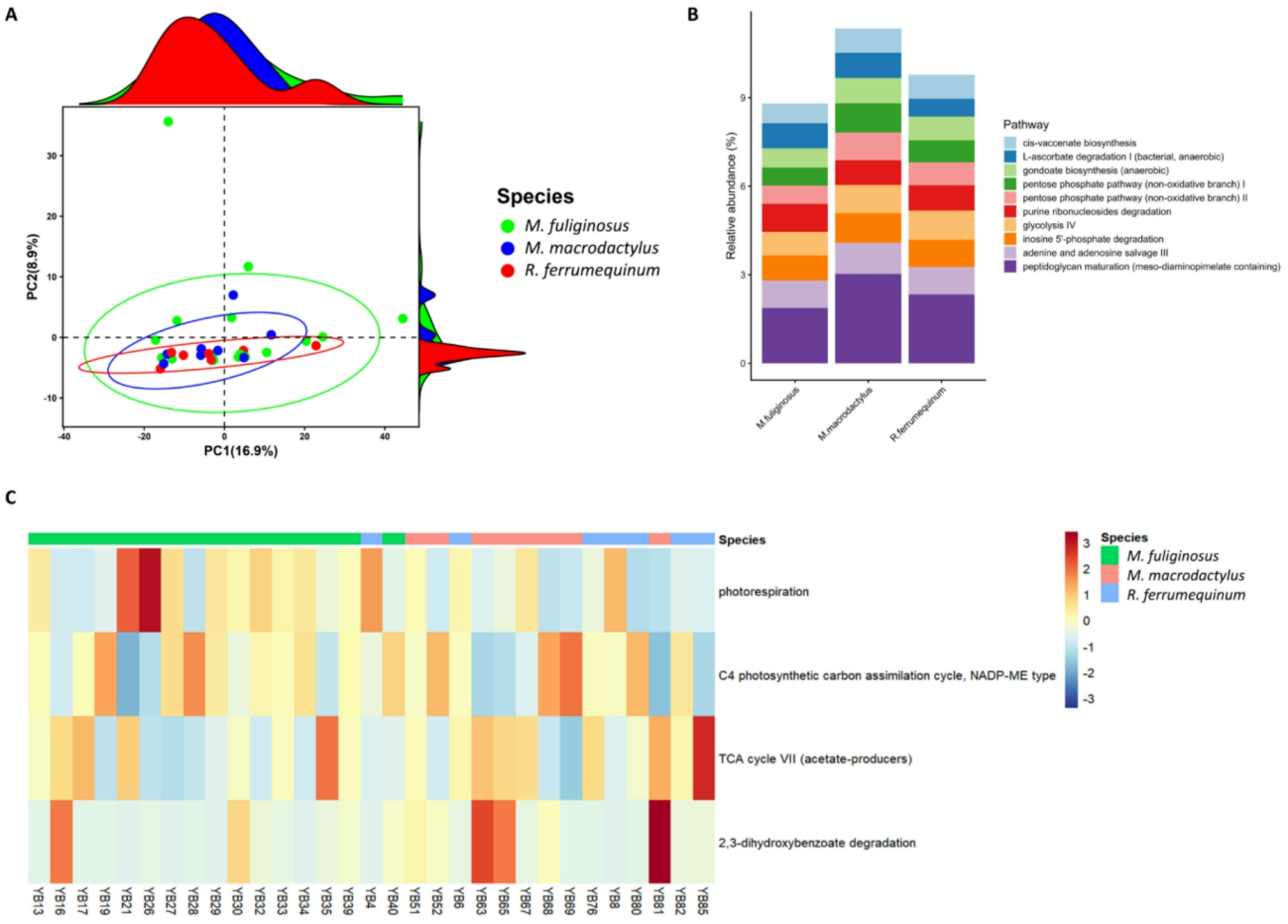


Figure 6

Predicted functional pathways of the gut microbiota inferred by PICRUSt2. (**A**) Principal component analysis of metabolic pathways showed no significant grouping among bat species (p = 0.114). (**B**) Stacked bar plot of the ten most abundant pathways. (**C**) A heatmap showing the relative abundances of the four pathways that were differentially abundant in different bat species, identified by ALDEx2, p.adjusted < 0.05.

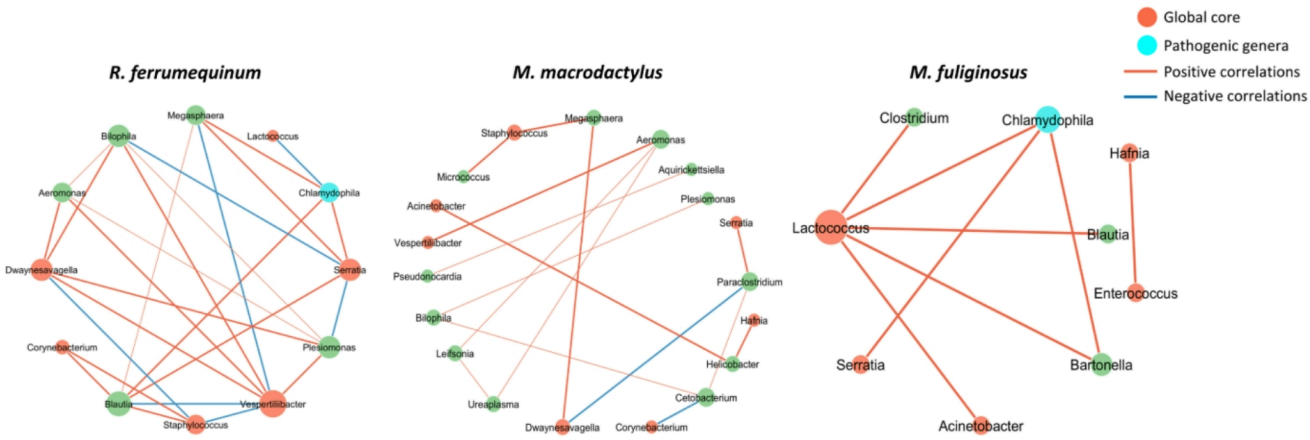


Figure 7

Microbial co-occurrence networks of the three bat species inferred using SparCC, $|r| \geq 0.6$, $p < 0.05$. The networks illustrate interactions between individual genera. Node size is proportional to degree (number of correlations). Red nodes show core bacteria, while blue nodes show detected pathogens. Red edges indicated positive correlations while blue edges indicate negative correlations, and correlation coefficients of core bacteria and detected pathogens are highlighted.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiguresIV.docx