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Ecological and biological determinants of fecal bacterial diversity in bats across four continents and phylogenetic screening for pathogen−affiliated bacterial lineages

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ABSTRACT

Objective: To characterize the diversity and composition of bacterial communities in bat fecal samples under distinct ecological and biological conditions and to investigate the phylogenetic relationships of selected bacterial lineages with clinically relevant taxa.

Methods: Publicly available 16S rRNA gene sequencing data from 194 fecal samples covering 20 bat species from South America, Europe, Asia, and Africa were reanalyzed using DADA2 and the SILVA database. Alpha and beta diversity, taxonomic composition, and community differences according to host diet, habitat, sex, and geographic region were evaluated. Phylogenetic analyses were performed on amplicon sequence variants assigned to *Pseudomonas*, *Staphylococcus*, *Clostridium*, and *Streptococcus*.

Results: A total of 3 778 110 reads passed quality filtering, representing 123 bacterial families and 203 genera. Proteobacteria (57%) and Firmicutes (40%) predominated, with *Pseudomonas*, *Staphylococcus*, and *Carnobacterium* among the most abundant genera. Microbial composition and diversity varied significantly according to diet, habitat, sex, and geographic region. Frugivorous bats showed the highest alpha diversity, whereas piscivorous bats showed the lowest, and *Rousettus aegyptiacus* exhibited the greatest bacterial richness. Bats inhabiting forest and cave habitats possessed higher bacterial richness, while communities from freshwater and coastal aquatic habitats were less diverse. Males showed higher relative abundance of *Staphylococcus*, whereas females were enriched in *Pseudomonas*. Distinct taxonomic signatures were linked to dietary guilds. Insectivorous bats were enriched in Gammaproteobacteria, Pseudomonadales, and Moraxellaceae; frugivorous bats were enriched in Lactobacillales and *Streptococcus*; and piscivorous bats were enriched in Firmicutes, including Staphylococcaceae and *Clostridium*. Sixty-two genera containing species with recognized pathogenic potential were identified. Phylogenetic analyses revealed amplicon sequence variants affiliated with *Pseudomonas*, *Clostridium*, and *Streptococcus* clustering with or related to clinically relevant lineages, such as *Clostridioides difficile* and *Streptococcus pneumoniae*.

Conclusions: Bat fecal bacterial communities are highly diverse and vary significantly under different ecological and biological conditions. Detection of bacterial genera containing clinically relevant species and the phylogenetic proximity of selected amplicon sequence variants to known pathogens support further genomic, functional, and epidemiological studies to clarify their pathogenic and zoonotic relevance.

KEYWORDS: Bat gut microbiome; Bacterial diversity; Ecological drivers; Zoonotic potential; Chiroptera; Gut microbiota; 16S rRNA sequencing; Bacterial pathogens; One Health

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Summary

Question: How do ecological and biological factors influence the diversity of the bat fecal bacteriome and what is its association with bacterial lineages of zoonotic potential?

Findings: The reanalysis of 194 fecal samples from 20 bat species using 16S rRNA gene sequencing showed that diet, habitat, sex, and geographic region significantly influenced composition and diversity of bacterial community. Sixty-two potentially pathogenic genera were identified, and phylogenetic analyses revealed amplicon sequence variants related to clinically relevant species, including lineages associated with *Clostridioides* and *Streptococcus*.

Meaning: These findings indicate that bats harbor ecologically structured bacteriomes with potential zoonotic and epidemiological relevance.

1. Introduction

Bats (order Chiroptera) are widely recognized as natural reservoir hosts for a broad range of emerging pathogens that pose significant threats to both veterinary and human health. Several high-impact viral diseases, including Ebola virus, Marburg virus, Nipah virus, Hendra virus, severe acute respiratory syndrome coronavirus (SARS-CoV), and SARS-CoV-2, have been linked to bats as their primary reservoirs and probable evolutionary origins[1]. Despite harboring an exceptional diversity of viruses, bats generally remain asymptomatic upon infection with many pathogens that cause severe disease in other mammals. In contrast, their role in bacterial pathogen transmission remains comparatively underexplored[1,2]. The lack of overt inflammatory responses in bats is hypothesized to reflect their unique evolutionary trajectory, which has facilitated long-term co-evolution with a wide array of pathogens[3].

Although research has predominantly focused on bat-borne viruses, bats also host rich and diverse bacterial communities with potential relevance to public health[4]. Metagenomic studies of bat feces and urine samples have identified several zoonotic and potentially pathogenic bacterial genera, such as *Bartonella*, *Clostridium*, *Corynebacterium*, and *Rickettsia*[5,6]. These findings indicate that the impacts of bacterial microbiota on bat health and disease dynamics remain poorly understood. Previous studies have reported complex and diverse bacterial communities across different bat species, reflecting variation in host dietary habits and habitat preferences[6]. Moreover, unique biological and ecological traits of bats, such as powered flight, migratory behavior, long lifespan, high sociality, and adaptation to diverse ecological niches, may drive bacterial genetic variation and shape microbial community structure[6,7]. Collectively, these features may facilitate cross-species microbial transmission and enhance pathogen dispersal[8].

Given that host ecology and diet are major determinants of microbiota variation, the present study evaluated 16S rRNA sequencing data derived from fecal samples of bat populations covering different geographic regions, sexes, and ecological habits. We assessed bacterial richness and diversity using 16S rRNA gene sequences and examined the phylogenetic proximity of detected strains to clinically relevant bacteria. Our analyses expand upon previous studies conducted using the same sample set, providing additional insights into bat-associated microbiomes[6,9,10]. Furthermore, our findings reveal a complex bacterial assemblage with intrinsic pathogenic potential, which merits epidemiological concerns. Aerial mobility, a defining characteristic of bats, may play a critical role in the spread of zoonotic diseases and the emergence of infectious-disease outbreaks.

2. Methods

2.1. Samples, species and public data collection

We conducted a comprehensive survey of publicly available datasets of bat fecal microbiome, focusing on 16S rRNA sequencing data. Following data quality assessment and strict screening based on our research objectives, a total of 194 bat fecal samples were selected, including 87 samples from the Dataverse Academic Portal[6], 96 samples from the NCBI SRA database (project accession number PRJEB47836)[9], and 11 samples from the Dryad Digital Repository[10]. The final dataset comprised specimens from 20 bat species: *Barbastella barbastellus*, *Miniopterus schreibersii*, *Myotis bechsteinii*, *Myotis emarginatus*, *Myotis myotis*, *Myotis nattererii*, *Myotis capaccinii*, *Myotis daubentonii*, *Myotis vivesi*, *Myotis blythii*, *Myotis ricketti* (= *Myotis pilosus*), *Rhinolophus hipposideros*, *Rhinolophus blasii*, *Rhinolophus ferrumequinum*, *Pipistrellus pipistrellus*, *Eptesicus bottae*, *Hypsugo ariel*, *Noctilio leporinus*, *Pipistrellus kuhlii*, and *Rousettus aegyptiacus*. Samples were obtained from bats captured across diverse geographic regions, including South America (two species), Europe (14 species), Asia (three species), and Africa (one species), and were stratified according to sex- and diet-based classification. Foraging behavior was inferred from capture habitats (Supplementary Table 1)[6,9,10]. Four piscivorous bat species originating from well-characterized fishing colonies distributed across four geographically distinct regions were included; notably, fishing behavior is thought to have evolved independently within these lineages[9]. In addition, facultative fishing species exhibiting both insectivorous and piscivorous feeding strategies were classified as piscivores to ensure consistency across analytical groupings.

2.2. 16S raw data processing

The sequences were organized into R1 (forward) and R2 (reverse) files and subjected to quality validation. Low-quality bases,

particularly those located at the read terminals, were trimmed, and sequence error rates were assessed through data filtering and sorting. All sequences were processed independently according to dietary grouping. R (v4.1.2) and the DADA2 package (v1.16.1)[11] were used to process and analyze the 16S rRNA sequences *via* a custom analytical pipeline. Forward and reverse reads were merged to obtain unique amplicon sequence variants (ASVs). Chimera detection and removal were performed during ASV table construction. After completion of all quality-control procedures, a total of 3 778 110 sequences were retained, with an average of 19 677 reads per sample.

Taxonomic annotation was performed against the SILVA database V138.1[12], with classification assigned down to the genus level. The ASV count table with 194 bat feces samples, sample metadata, and the taxonomic assignment table were imported into the Microbiome Analyst[13] for data processing and tabular output, including identification of microorganisms and analysis of diversity profiles. Additionally, diversity analyzes were performed based on the relative abundance of each ASV (calculated as the read count of a given ASV divided by the total read depth per sample library). For statistical analyses, heat tree analysis was used to visualize the hierarchical taxonomic structure using median abundance, and the non-parametric Wilcoxon rank-sum test (α=0.05)[14] was used to detect taxonomic differences between microbial communities. Separate workflows[15] were applied to compute and visualize alpha- and beta-diversity indices, respectively. One-way analysis of variance (ANOVA) (α=0.05) and permutational multivariate analysis of variance (PERMANOVA) (α=0.05) were also used for single-factor statistical comparisons and covariate-adjusted linear regression analyses.

2.3. Phylogenetic analysis of bacterial genera

To investigate the pathogen-related association of the bacterial taxa detected, phylogenetic analyses were conducted. Four genera containing well-documented pathogenic species and exhibiting high relative abundance in bat fecal samples were selected: *Pseudomonas*, *Staphylococcus*, *Clostridium*, and *Streptococcus*. For each genus, all ASVs targeting the V4 hypervariable region of the 16S rRNA gene were retrieved. A reference database was constructed for each genus, comprising confirmed and potential pathogenic bacterial species capable of infecting humans, animals, or plants. Full-length 16S rRNA sequences from reference taxa were aligned against the ASV dataset using MAFFT version 7[16], and resulting multiple sequence alignments were manually curated using BioEdit version 7.2.5[17]. Maximum likelihood phylogenetic trees were subsequently reconstructed in MEGA7 version 7.0.26[18] based on combined datasets containing ASVs (V4 region) and reference sequences, with branch robustness assessed by bootstrap analysis based on 1 000 replicates. Final phylogenetic trees were visualized and annotated using iTOL[19].

2.4. Statistical analysis

Statistical analyses were performed to assess differences in microbial composition and diversity among the evaluated groups. Relative abundance was calculated for each ASV as the number of reads assigned to a given ASV divided by the total number of reads in the corresponding sample. Differences in the relative abundance of bacterial taxa between groups were assessed using the non-parametric Wilcoxon rank-sum test. Alpha-diversity metrics were compared using ANOVA, whereas differences in overall microbial community composition based on beta-diversity were evaluated using PERMANOVA. Multivariable linear regression analyses were additionally performed to assess associations while accounting for potential confounding factors. All available covariates included in the sample metadata, including sex, diet, geographic region, and foraging behavior, were considered in the multivariable analyses. Heat tree analyses were used to visualize differences in the hierarchical taxonomic structure of microbial communities based on median taxon abundance. Statistical analyses and diversity assessments were conducted using MicrobiomeAnalyst and the corresponding R-based workflows described above. Statistical significance was defined as $P<0.05$.

3. Results

3.1. Bacterial profile of the fecal microbiota of bats

Analysis of the fecal microbiome of 20 bat species based on 16S rRNA sequencing data revealed a bacterial community dominated by two major phyla, Proteobacteria (57%) and Firmicutes (40%), which together accounted for 97% of the total identified taxa (Figure 1A). Across all samples, 123 bacterial families and 203 genera were detected (Supplementary Tables 1 and 2). In addition to these dominant phyla, a minor fraction of microbial diversity was observed at low relative abundances (<1%), including Actinobacteriota, Bacteroidota, and several low-abundance phyla, such as Deinococcota, Spirochaetota, Campylobacterota, Patescibacteria, Verrucomicrobiota, Fusobacteriota, Desulfobacterota, Halobacterota, Chloroflexi, Cyanobacteria, Thermoplasmatota, and Synergistota. A subset of sequencing reads remained taxonomically unassigned, indicating the presence of poorly characterized or potentially novel microbial taxa. At the family level, Peptostreptococcaceae predominated in samples from the piscivorous species *Myotis vivesi* and *Myotis ricketti*. The highest family-level bacterial diversity was

observed in the frugivorous bat *Rousettus aegyptiacus* sampled in South Africa. In contrast, species such as *Rhinolophus hipposideros* (arthropod-feeding; forested habitats in Europe), *Rhinolophus blasii* (insectivorous; freshwater environments), *Hypsugo ariel* (insectivorous; desert habitats), and *Noctilio leporinus* (facultative piscivore–insectivore; predominantly whitewater habitats) exhibited high bacterial abundance but relatively low diversity at the family level. Rhodanobacteraceae and Thermoactinomycetaceae were the rarest families detected, each accounting for less than 1% of the processed dataset. At the genus level, the most prevalent taxa were *Pseudomonas*, *Staphylococcus*, *Carnobacterium*, and *Lonsdalea*. Consistent with the family-level patterns, *Rousettus aegyptiacus* showed the greatest genus-level diversity, while high relative abundances were also observed in the insectivorous species *Myotis myotis*, *Myotis bechsteinii*, and *Barbastella barbastellus*. Analysis of the core microbiome revealed that no taxon was consistently present across all samples at a relative abundance threshold of ≥1%.

Relative abundance patterns were further examined by stratifying samples according to biological and ecological factors, including diet, habitat, and sex. At the genus level, insectivorous bats exhibited the highest microbial diversity (165 genera), with *Pseudomonas* representing the most abundant taxon (781 085 sequences) (Figure 1B). Frugivorous bats displayed a distinct microbial composition, also comprising 165 genera, with *Streptococcus* as the dominant genus (112 139 sequences) (Figure 1C). In contrast, piscivorous bats harbored the lowest bacterial diversity (16 genera), with *Clostridium* predominating (20 859 sequences) (Figure 1D). Habitat exerted a strong influence on microbial community structure. Bats foraging in forests and open fields exhibited the highest relative diversity (204 genera each), with *Pseudomonas* (502 480 sequences) and *Acinetobacter* (14 141 sequences) as the most abundant genera, respectively (Figure 2A and 2B). Conversely, bats captured in caves and desert habitats showed distinct microbial profiles, characterized by a predominance of *Staphylococcus* (298 808 sequences) in cave-dwelling bats (Figure 2C) and *Romboutsia* (1 517 sequences) in desert-dwelling bats (Figure 2D). Among piscivorous bats, individuals foraging in freshwater and coastal water environments exhibited greater *Bartonella* enrichment (19 220 sequences and 6 434 sequences, respectively) (Figure 2E and 2F). Sex-stratified comparisons further revealed distinct microbial signatures, with male bats showing enrichment of *Staphylococcus* (338 648 sequences) (Figure 3A) and female bats dominated by *Pseudomonas* (168 689 sequences) (Figure 3B).

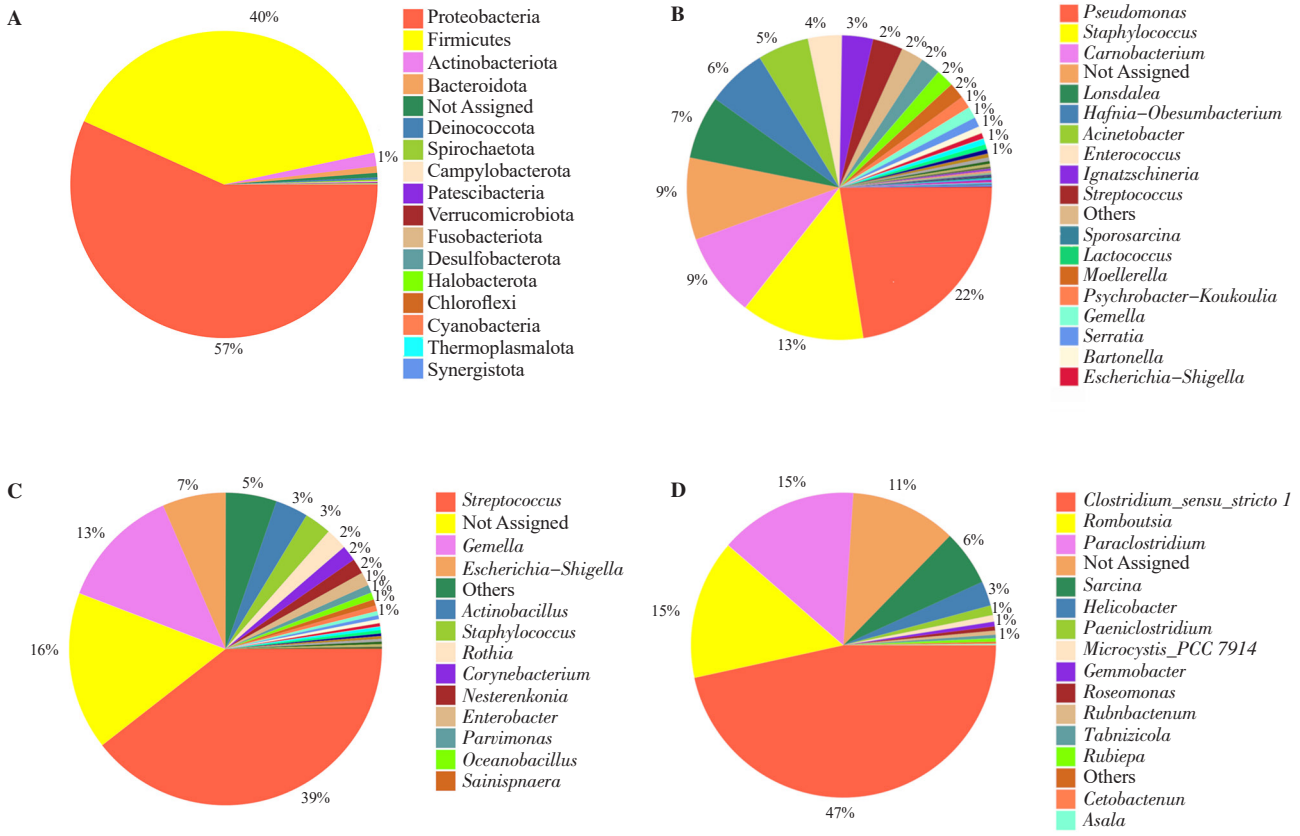


Figure 1. Relative abundance of the predominant bacterial genera from 20 bat species with distinct dietary habits. (A) All samples; (B) Insectivorous bats; (C) Frugivorous bats; (D) Piscivorous bats.

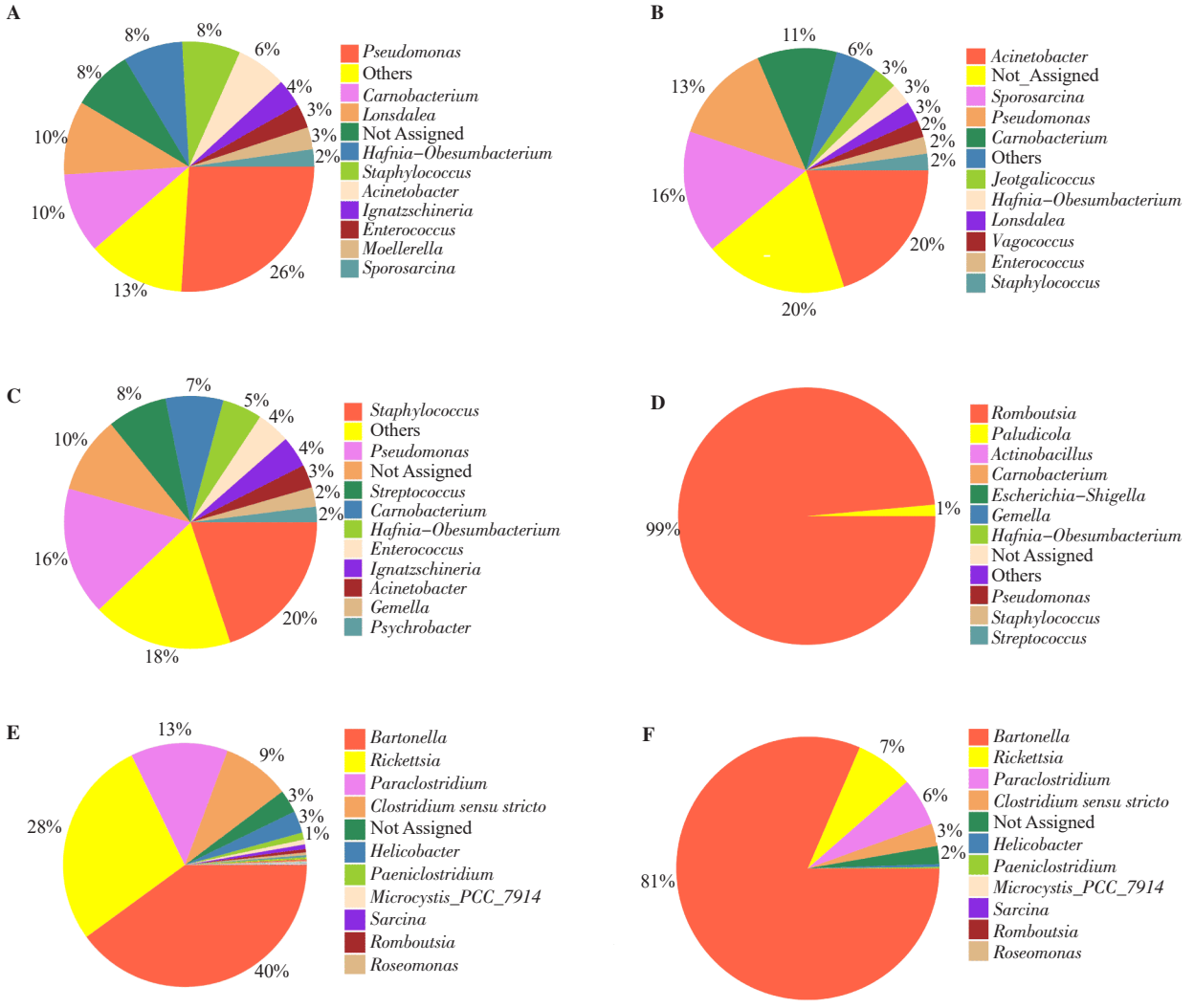


Figure 2. Relative abundance of the predominant bacterial genera from 20 bat species grouped according to habitat. (A) Forest-associated bats; (B) Open-area bats; (C) Cave-dwelling bats; (D) Desert-dwelling bats; (E) Freshwater piscivorous bats; (F) Coastal piscivorous bats.

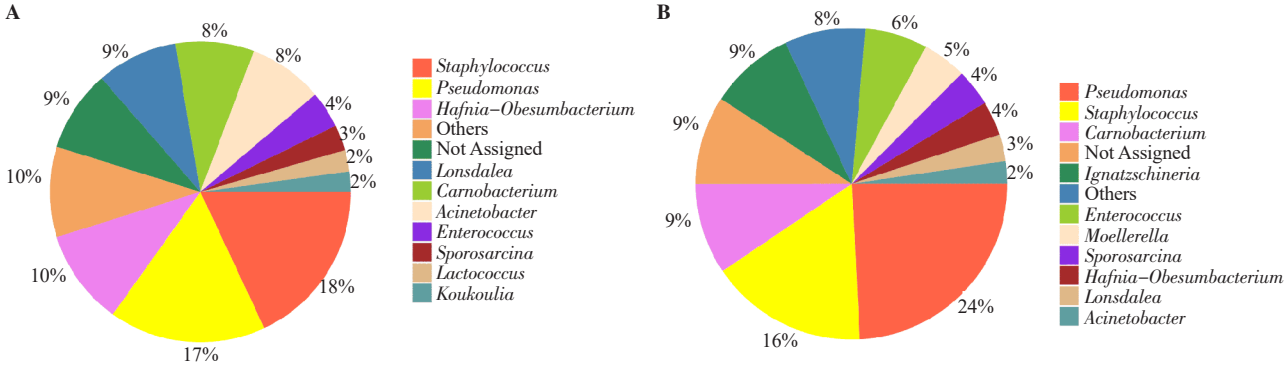


Figure 3. Taxonomic composition of the bacterial microbiota identified by 16S rRNA gene sequencing from 20 bat species grouped according to host sex. (A) Male bats; (B) Female bats.

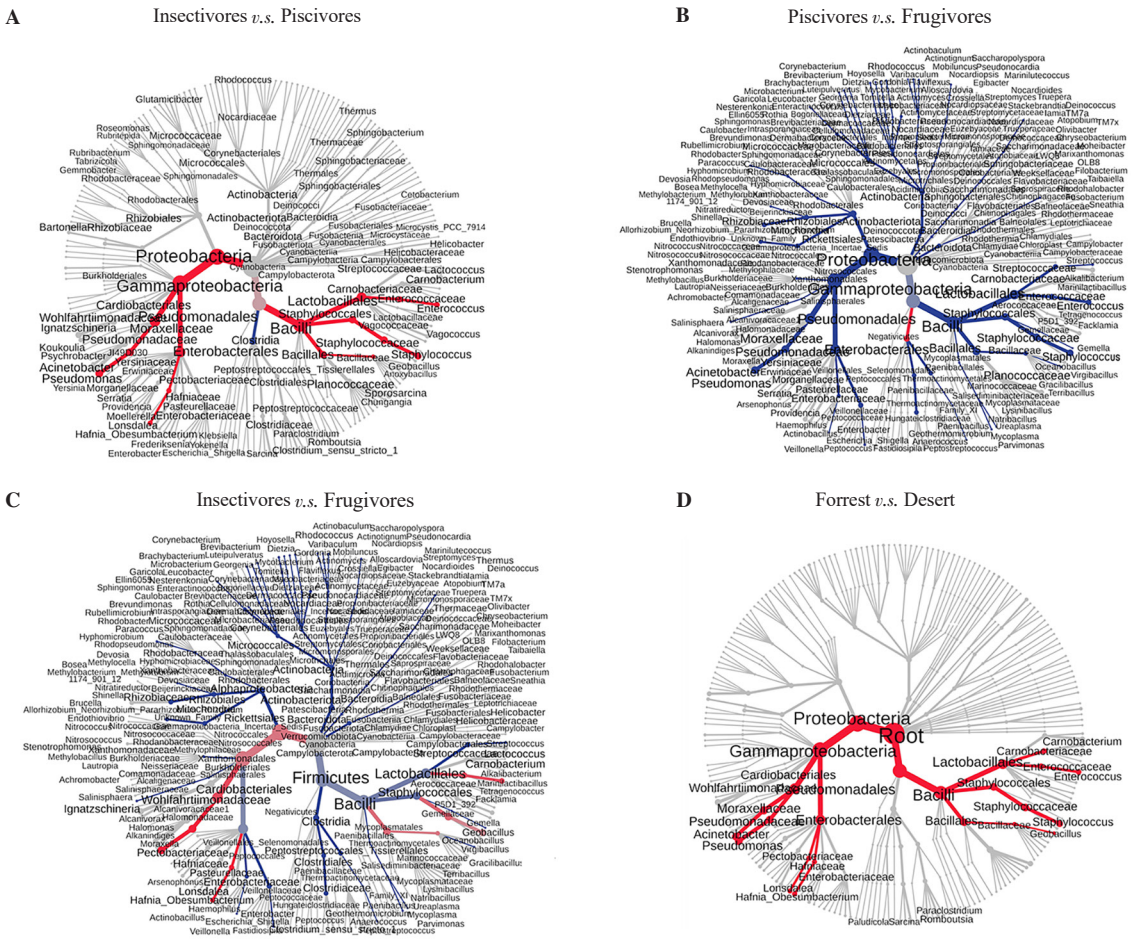


Figure 4. Heat tree analysis of differentially abundant bacterial taxa identified by 16S rRNA gene sequencing in the fecal microbiota of 20 bat species. (A) Differential bacterial taxa between insectivorous and piscivorous bats; (B) Differential bacterial taxa between piscivorous and frugivorous bats; (C) Differential bacterial taxa between insectivorous and frugivorous bats; (D) Habitat-associated differences between forest-dwelling and desert-dwelling bats; (E) Habitat-specific bacterial taxa in freshwater and coastal piscivorous bats.

3.2. Phylogenetic analysis of bat fecal microbiota communities

Heat-gradient phylogenetic tree analyses were conducted to compare divergent fecal microbiota abundance patterns among the 20 bat species (Supplementary Table 3). Comparison between insectivorous and piscivorous bats revealed significant microbial divergence (Figure 4; P=1.28×10^{-4}). Insectivorous bats were dominated by Proteobacteria, particularly Gammaproteobacteria, with marked enrichment of Pseudomonadales and Moraxellaceae. In contrast, piscivorous bats were enriched in Firmicutes, mainly Bacilli and Staphylococcaceae, showing reduced overall diversity but dominance of specific bacterial taxa.

Comparison between piscivorous and frugivorous bats showed marginal microbial community differentiation (Figure 4; P=0.05). Frugivorous bats displayed enrichment in Proteobacteria, including *Pseudomonas* and other members of Pseudomonadales, corresponding to higher microbial diversity and community complexity. Piscivorous bats again showed enrichment in Firmicutes, with predominance of *Staphylococcus* and other Bacillales, reflecting a more restricted gut microbiota.

Significant taxonomic differences were also detected between

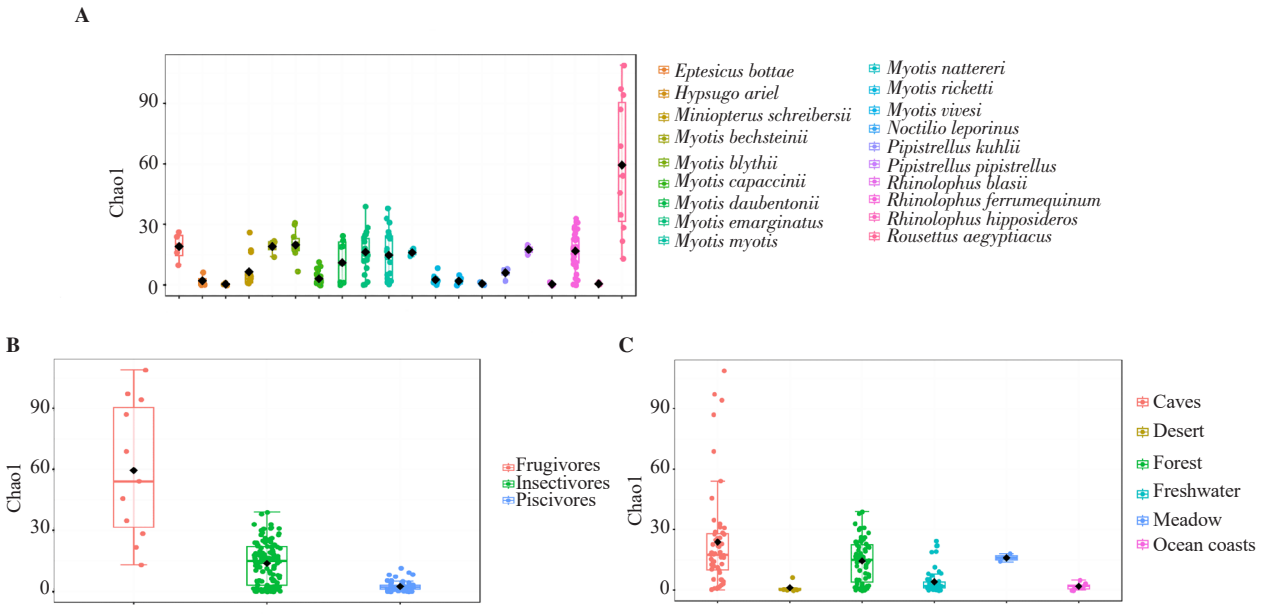


Figure 5. Alpha diversity of the fecal microbiota identified by 16S rRNA gene sequencing in 20 bat species, estimated using the Chao1 richness index. (A) Alpha diversity across individual bat species (ANOVA, $P < 0.001$); (B) Comparison of alpha diversity according to dietary guild; (C) Comparison of alpha diversity according to habitat type.

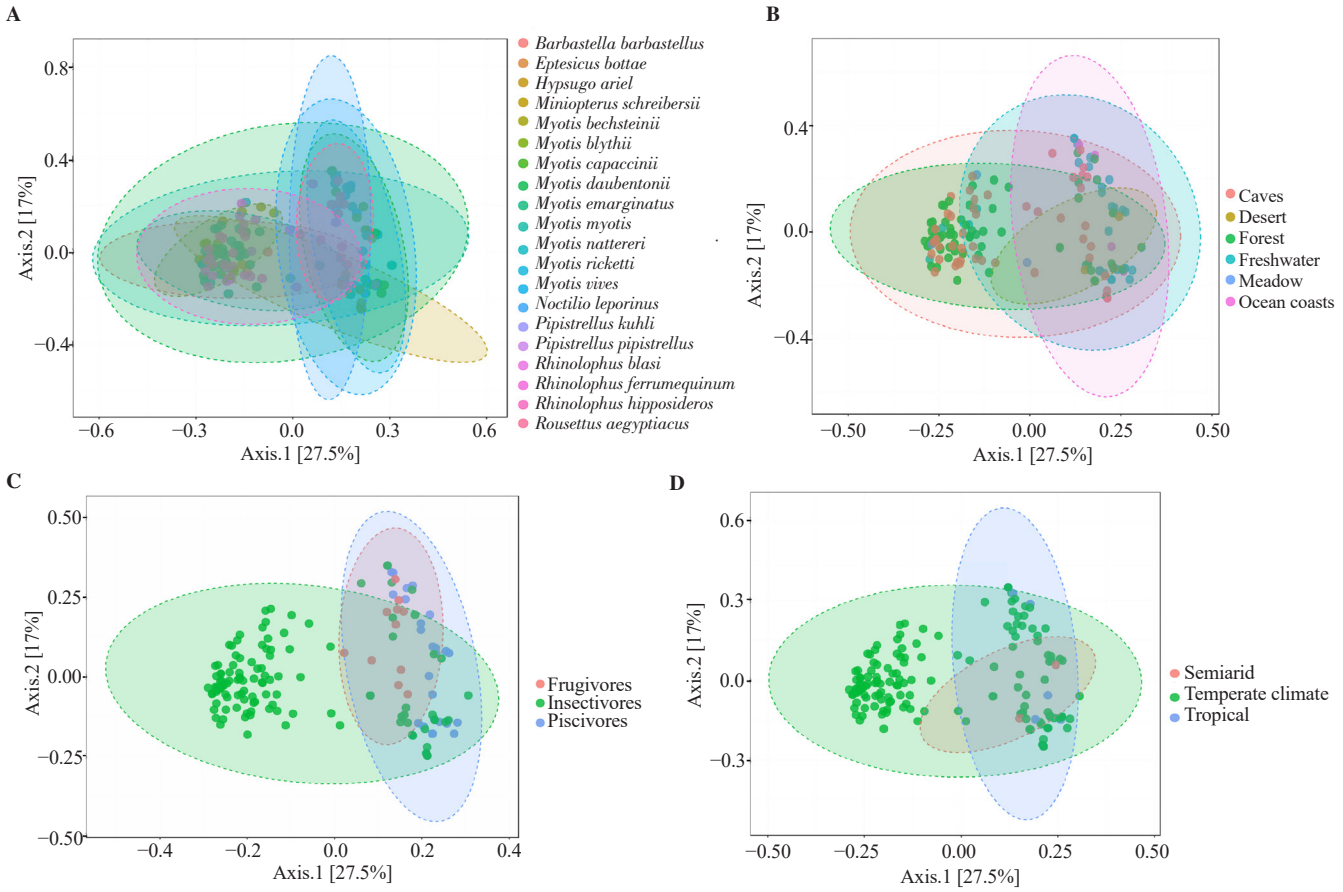


Figure 6. Beta diversity of the fecal microbiota identified by 16S rRNA gene sequencing from 20 bat species, visualized by principal coordinates analysis (PCoA). (A) Ordination of samples according to bat species; (B) Beta diversity grouped by habitat type (caves, desert, forests, freshwater, meadow, and ocean coasts); (C) Beta diversity grouped by dietary guild; (D) Beta diversity according to climatic region (semiarid, temperate, and tropical).

insectivorous and frugivorous bats (Figure 4; P=0.007). Both groups exhibited relatively high microbial diversity but distinct taxonomic signatures: insectivorous bats were enriched in Proteobacteria, particularly Gammaproteobacteria and Moraxellaceae, whereas frugivorous bats were dominated by Firmicutes, especially Lactobacillales, Staphylococcaceae, and *Streptococcus*. These patterns indicate that host gut microbial community composition is shaped by diet-driven selective pressures, with insectivorous bats adapted to protein- and chitin-rich diets and frugivorous bats harboring taxa specialized in the metabolism of fermentable carbohydrates.

Habitat-driven differences were also evident. Comparison between forest-dwelling and desert bats yielded significant microbial differences (Figure 4; P=0.002). Forest-dwelling bats exhibited greater bacterial diversity, with enrichment of Proteobacteria (particularly Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, and *Acinetobacter*) and Bacilli (including Lactobacillales and Staphylococcaceae). In contrast, desert bats showed markedly reduced diversity and were dominated by Romboutsia, reflecting adaptive microbial shifts in response to nutritionally limited diets in extreme environments.

Finally, significant microbial divergence was observed between freshwater and coastal piscivorous bats (Figure 4; P=0.006). Freshwater bats were enriched in *Bartonella* (identified in the dataset although not highlighted in the figure), whereas coastal bats were dominated by Romboutsia, suggesting that aquatic foraging ecology directly influences gut microbiota composition by selecting for taxa adapted to distinct dietary resources.

3.3. Diversity profile of bacterial communities in the fecal microbiota of bats

To investigate differences in bacterial diversity of bat fecal microbiota, analyses were conducted focusing on patterns of alpha and beta diversity. Alpha diversity analysis revealed substantial variations in microbial richness across bat species, with distinct taxa exhibiting divergent diversity levels. Alpha diversity indices among bat species differed significantly (P<0.001), with *Rousettus aegyptiacus* exhibiting the highest microbial richness (Figure 5A). When samples were grouped by diet (frugivorous, insectivorous, and piscivorous), significant differences were also observed, with frugivorous bats showing the highest alpha diversity values (P<0.001) and piscivorous bats the lowest (Figure 5B). Habitat-based analyses further demonstrated that bats inhabiting caves and forests possessed significantly greater fecal microbial diversity (P<0.001), whereas bats from freshwater and coastal environments hosted less diverse and structurally simpler bacterial communities (Figure 5C).

Beta diversity analyses were performed to assess dissimilarity in microbial composition among samples. Microbial communities revealed partial clustering according to host species (Figure 6A) and habitat (Figure 6B). However, overlapping clusters indicated concurrent patterns of host or habitat specificity and microbiota sharing across conditions. PERMANOVA results confirmed significant overall compositional divergence among bat species (F=4.667, R^2=0.340, P=0.001). It demonstrated significant

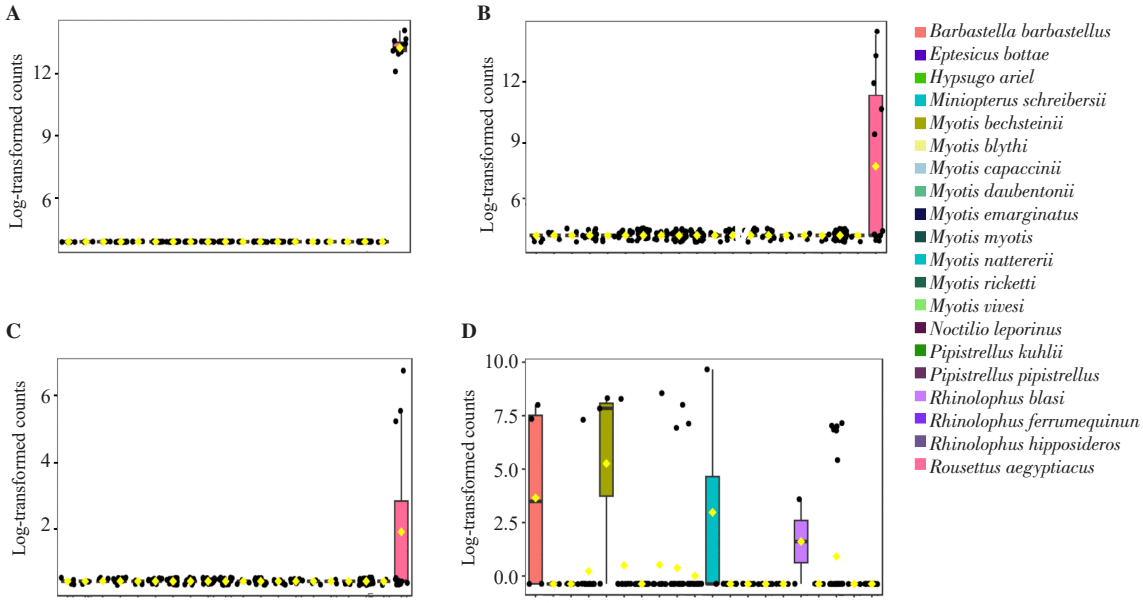


Figure 7. Distribution and relative abundance of potentially pathogenic bacterial genera identified by 16S rRNA gene sequencing in the fecal microbiota of 20 bat species. Boxplots show the log-transformed bacterial counts of (A) *Streptococcus*, (B) *Mycobacterium*, (C) *Escherichia-Shigella*, and (D) *Yersinia* across bat species.

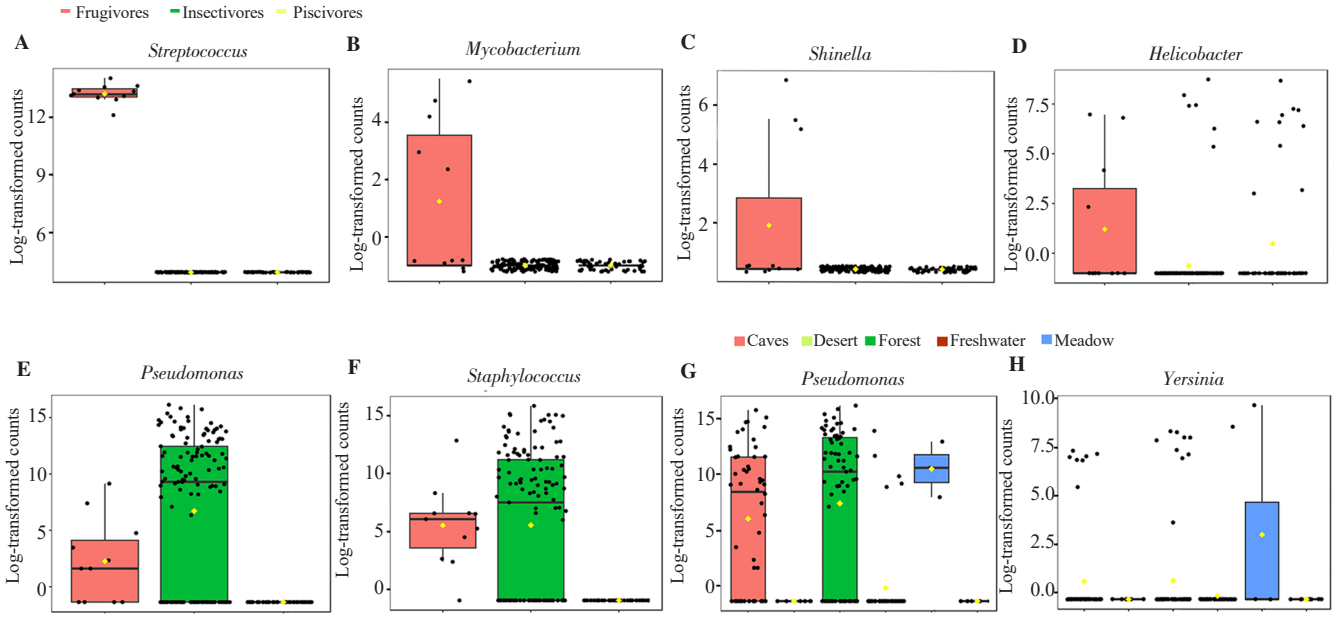


Figure 8. Single-factor analysis of bacterial genera across dietary guilds (A-F) and habitat types (G-H).

differences between species with distinct dietary habits, such as insectivorous *Myotis daubentonii* and frugivorous *Rousettus aegyptiacus* (P=0.001; FDR=0.007). Although several bacterial communities overlapped across forest, grassland, desert, and cave habitats, a distinct microbial assemblage was identified as exclusive to piscivorous bats (P=0.001; FDR=0.008). Diet-driven microbial differentiation was further validated by PERMANOVA analysis (F=20.34, R^2=0.177, P=0.001). Core bacterial taxa were common to all three dietary groups, alongside a subset of genera exclusively associated with insectivorous bat hosts (Figure 6C). Finally, PERMANOVA analysis incorporating climatic variables detected weak but significant climatic effects on microbial community structure (F=1.119, R^2=0.012, P=0.034). A shared core microbiota was conserved across climatic regions; nevertheless, temperate environments harbored a distinct bacterial assemblage (P=0.004; FDR=0.012) (Figure 6D).

3.4. Potentially pathogenic genera in bat fecal microbiota

Genera containing pathogenic bacterial members with relatively high abundance were evaluated in relation to environmental factors, bat species, diet, and habitat. Interspecific comparison revealed a total of 62 bacterial genera. Samples obtained from the frugivorous bat *Rousettus aegyptiacus* harbored a higher diversity of potentially pathogenic taxa. This species exhibited significant enrichment of the genera *Streptococcus* (P<0.001), *Mycobacterium* (P<0.001), and *Shinella* (P<0.01) (Figure 7A-C). In contrast, other potentially pathogenic genera, such as *Yersinia*, were predominantly detected in insectivorous bats, including *Barbastella barbastellus*, *Myotis bechsteinii*, *Myotis nattereri*, and *Pipistrellus pipistrellus* (P<0.001) (Figure 7D), indicating occurrence of host-specific patterns of these pathogenic bacteria.

When examining differences across dietary guilds, univariable statistical analyses revealed 118 bacterial genera with statistically significant differences in relative abundance among feeding groups. Enrichment was primarily observed in frugivorous bats, particularly for *Streptococcus* (P<0.001), *Mycobacterium* (P<0.001), *Shinella* (P<0.001), and *Helicobacter* (P<0.05) (Figure 8A-D). Conversely, insectivorous bats exhibited marked enrichment of *Pseudomonas* (P<0.05) and *Staphylococcus* (P<0.001) (Figure 8E and F), indicating the influence of dietary specialization on the structure of potentially pathogenic bacterial communities.

Habitat-related patterns further supported the role of environmental context in shaping the distribution of these genera. The genus *Pseudomonas* (P<0.001) was significantly enriched in samples from cave and forest environments (Figure 8G), suggesting an association with more complex or sheltered habitats. In contrast, *Yersinia* (P<0.05) predominated in samples obtained from open grassland habitats (Figure 8H), highlighting the contribution of habitat-specific ecological pressures to the composition and abundance of potentially pathogenic bacteria in bat fecal microbiota.

3.5. Phylogenetic relationship of the main potentially pathogenic bacteria in the bat microbiota

Phylogenetic analyses based on 16S rRNA sequencing of bat fecal samples revealed a high number of ASVs, reflecting substantial bacterial diversity, particularly within the genera *Pseudomonas* (Supplementary Figure 1) and *Staphylococcus* (Supplementary Figure 2). Although *Staphylococcus* exhibited a high ASV richness, most

sequences did not cluster closely with the pathogenic reference strains included in the analysis, suggesting a predominance of non-pathogenic or distantly related lineages. In contrast, *Pseudomonas*-derived ASVs showed closer phylogenetic relationships to several disease-associated species. In the *Clostridium* phylogeny (Supplementary Figure 3 and Supplementary Table 4), three distinct ASVs clustered basally, appearing to represent ancestral lineages shared by known pathogenic *Clostridium* species. The majority of *Clostridium* ASVs formed a separate, well-supported clade, displaying a more distant relationship to established pathogens while sharing a common ancestor. Similarly, the phylogenetic reconstruction for *Streptococcus* (Supplementary Figure 4) revealed a closer association with multiple pathogenic species. Collectively, these findings for *Clostridium*, *Pseudomonas*, and *Streptococcus* indicate the presence of ASVs that may represent ancestral lineages of clinically relevant pathogenic bacteria within the bat fecal microbiota.

4. Discussion

Bats are widely recognized as natural reservoirs of viruses, and in recent years, increasingly comprehensive studies of bat-associated microbiomes have suggested that these mammals also host a wide diversity of bacteria, including potentially pathogenic strains[20]. Benefiting from flight capacity and migratory behavior, bats have successfully colonized a broad range of terrestrial and aquatic-associated habitats, ranging from forests, deserts, and urban environments to freshwater systems, coastal zones, and oceanic regions. Although bats harbor a remarkable diversity of pathogenic microorganisms, their long-distance mobility may facilitate the dispersal of infectious agents, including bacteria associated with human disease[6]. It has been proposed that the elevated metabolic rates and increased body temperatures required for flight have shaped bat immune systems, creating selective pressures that reduce microbial virulence while allowing bats to tolerate and carry a wide range of pathogens[21]. Moreover, adaptation to diverse dietary niches and variable ecosystem distributions has driven the evolution of complex and heterogeneous bacterial communities in bats[22]. In this study, we evaluated the complex interactions among sex, dietary preference, and geographic origin, assessing patterns of bacterial diversity in bats and exploring their reservoir potential for pathogenic bacteria.

Across all bat samples analyzed, members of the phyla Firmicutes (notably the families Staphylococcaceae and Carnobacteriaceae) and Proteobacteria (particularly Pseudomonadaceae and Pectobacteriaceae) dominated the fecal microbiota, consistent with previous studies of bat-associated bacterial communities[23]. Geographic variation was evident at the genus level, with *Pseudomonas* predominating at terrestrial sampling locations, whereas *Bartonella* and *Romboutsia* were more abundant in samples from freshwater and marine environments. Insectivorous bats exhibited a higher relative abundance of *Pseudomonas*, a pattern similar to that observed in bats foraging in wet habitats. This pattern differed from that of bats consuming a broader range of arthropods other than insects. Notably, *Pseudomonas* was more prevalent in female bats than in males, although it was abundant in both sexes. The genus *Paraclostridium*, significantly enriched in piscivorous bats and previously described as characteristic of piscivorous bats when compared with arthropodophagous bats[9], was also detected in insectivorous bats in our dataset. This finding indicates that no strictly diet-specific gut microbiota exists in these bat hosts.

From an ecological perspective, fruit bats interact extensively with other organisms, including plants and animals, due to their feeding behavior and habitat use. This complex interaction network likely contributes to the higher and more distinct diversity of bacterial genera observed in the intestinal microbiomes of frugivorous bats compared with other dietary guilds. While zoonotic and potentially zoonotic pathogens of bats have been widely investigated, the broader ecological context of bat-associated pathogens remains poorly understood, particularly the diverse community of taxa that bats interact with[24,25]. Fruits themselves constitute complex microecosystems supporting diverse arthropod assemblages that often co-evolve with host plants[26]. In this context, fruit bats may ingest insects incidentally, which can constitute a meaningful nutritional resource[27–29]. These trophic interactions may facilitate the acquisition of viruses and bacteria associated with fruit-dwelling arthropods[30].

Alpha diversity analyses at both family and genus levels revealed that the frugivorous species *Rousettus aegyptiacus* from South Africa, a bat typically inhabiting terrestrial environments, harbored the most diverse bacterial communities. These findings were supported by both capture location and dietary preference. In contrast, bats with arthropod-based diets exhibited higher beta diversity indices and are recognized as major nocturnal consumers, playing key roles in regulating arthropod populations[31]. Their broad consumption of diverse arthropod taxa mirrors spatial variation in arthropod availability and abundance across habitats[32,33]. Such dietary breadth likely contributes to the elevated and differentiated abundance of bacterial genera observed in their microbiomes. For example, *Diplorickettsia*, a potential human pathogen previously isolated from Ixodes ticks, important vectors of human disease[34], was detected at high abundance in arthropod-feeding bats.

Arthropodophagous bats constituted the majority of dietary categories in our dataset. Terrestrial bat samples from Slovenia showed a distinct relative abundance of bacterial genera, including *Streptococcus*, *Mycobacterium*, *Pseudomonas*, *Mycoplasma*, and *Ureaplasma*, which were largely restricted to continental bat

populations. In addition, many bats frequently inhabit urban environments and often come into close contact with humans and domestic animals. The accumulation of bat guano in buildings potentially facilitates the maintenance and indirect transmission of these bacteria to humans[35,36]. Similar patterns were observed in bats inhabiting coastal and freshwater environments. Piscivorous bats foraging near oceans, lakes, and rivers exhibited high abundances of *Aeromonas*, a genus commonly found in freshwater and marine environments and associated with gastrointestinal disease, wound infections, and septicemia in humans[37].

The fecal bacteriome of bats revealed high diversity within the genera *Pseudomonas* and *Staphylococcus*, with numerous ASVs diverging from known pathogenic strains. In the case of *Pseudomonas*, a limited number of ASVs clustered near pathogenic species, whereas *Staphylococcus* ASVs were largely phylogenetically distant from established pathogens, despite their high overall diversity. Bats are known to harbor non-viral pathogens while remaining clinically asymptomatic, which supports their role as natural reservoirs of diverse bacterial taxa. Nevertheless, the magnitude of their contribution to disease spillover and zoonotic bacterial transmission remains insufficiently explored[5]. Most *Pseudomonas* ASVs occupied basal phylogenetic positions relative to reference pathogenic strains, with sequences such as *Pseudomonas*_467, *Pseudomonas*_485, and *Pseudomonas*_473 clustering closer to the outgroup than to pathogenic ingroups. These findings suggest that diversification of these clades may have co-occurred alongside their mammalian hosts. Several ASVs also clustered with pathogenic species infecting fungi (*e.g.*, *Pseudomonas tolassi*) and warm-blooded animals (*e.g.*, *Pseudomonas oryzihabitans*), as well as with putative ancestral lineages related to *Pseudomonas aeruginosa*[38].

Similarly, phylogenetic reconstructions of *Clostridium* and *Streptococcus* revealed basal lineages related to pathogenic taxa, including putative ancestral strains associated with *Clostridium difficile* (*Clostridium* 014) and *Streptococcus pneumoniae* (*Streptococcus* 012 and *Streptococcus* 034), both of which carry well-characterized virulence factors. These phylogenetic patterns support prediction from the "bat seeding hypothesis"; however, the present 16S rRNA-based analysis does not establish bats as ancestral sources of these pathogenic lineages. Further genomic analyses are required to disentangle their evolutionary histories and assess their potential epidemiological significance[39]. This long-term cohabitation and coevolution between bats and microbial symbionts may explain the capacity of bats to host infectious agents with little or no overt clinical signs[5,40]. Combined with the long lifespan of many bat species and their adaptability to diverse ecological niches, these traits underscore bats' potential contribution to the emergence and transmission of zoonotic pathogens[41].

The high immune tolerance of bats is thought to be a consequence of long-term coevolution with microbial pathogens, which has shaped distinctive immunological response profiles[42]. The bat immune system appears to limit immunopathology while maintaining effective pathogen control. Microbial community composition may therefore serve as an important predictor of immune response magnitude and infection severity[43]. Robust antiviral defenses in bats include restrained inflammatory cytokine expression, activation of interferon regulatory factors, and engagement of Toll-like receptor signaling pathways[41,44]. Additionally, bats exhibit reduced sensing of self and exogenous DNA, partly due to dampened expression of stimulator of interferon genes, a key adaptor protein in DNA-sensing pathways. This reduced functionality of stimulator of interferon genes may contribute to balanced immune responses, highlighting a fundamental distinction in immune tolerance between bats and other mammals[45].

In summary, our study shows that bats harbor complex fecal bacterial communities, whose composition and diversity vary significantly according to diet, habitat, sex, and geographic origin. Proteobacteria and Firmicutes predominated across all analyzed samples, with recurrent detection of genera such as *Pseudomonas*, *Staphylococcus*, *Clostridium*, and *Streptococcus*, some of which include species of recognized clinical relevance. Frugivorous and insectivorous bats exhibited richer bacterial communities than piscivorous bats, supporting a significant association between dietary habits and microbial diversity. Similarly, cave- and forest-associated bats showed higher bacterial richness than bats from freshwater, coastal, and desert environments, indicating an association between habitat characteristics and fecal microbiota composition.

The absence of a universally shared core microbiome further highlights the variability of bat-associated bacterial communities across ecological and biological contexts. Phylogenetic analyses identified ASVs within *Pseudomonas*, *Clostridium*, and *Streptococcus* that clustered with or were phylogenetically related to bacterial taxa of recognized pathogenic potential, including lineages related to *Clostridioides difficile* and *Streptococcus pneumoniae*. However, these phylogenetic relationships do not confirm pathogenicity or zoonotic spillover risk. Rather, they indicate the presence of bacterial lineages that warrant further genomic, functional, and epidemiological investigation. Collectively, these findings expand current knowledge of bat-associated bacterial diversity and reinforce the need for continued investigation of how ecological factors contribute to the distribution of bacterial taxa with potential clinical relevance.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

LPG, WAS Jr., and MPCS contributed to the conception and design of the study, definition of the intellectual content, literature review, data collection and analysis, statistical analysis, preparation and editing of the manuscript, as well as to the critical review of the final version of the paper. RAM, LSS, JPXB, and MPM contributed to the literature review, data analysis, statistical analysis, preparation and editing of the manuscript, as well as the critical review of the manuscript.

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