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RESEARCH

Microbial network assembly in bat flies with differing host specificity from North Africa

Alexandra Corduneanu[1,2] · Mohammed Lamine Bendjeddou[2] · Attila D. Sándor[2,3,4] · Andrei Daniel Mihalca[2] · Sándor Hornok[3,5] · Áron Péter[5] · Farouk Khelfaoui[6] · Justè Aželytè[7] · Dasiel Obregon[8] · Lourdes Mateos-Hernández[9] · Apolline Maitre[9] · Lianet Abuin-Denis[9,10] · Alejandra Wu-Chuang[9] · Myriam Kratou[11] · Mourad Ben Said[12] · Alejandro Cabezas-Cruz[9]

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Abstract

The study investigates the microbial composition of bat flies (Diptera: Nycteribiidae) collected from *Myotis punicus* in Algeria, focusing on the diversity and dynamics of their microbiota through network analysis. The analysis targets two genera, *Nycteribia* and *Penicillidia*, comparing oioxenous and stenoxenous species to understand host specificity's influence on microbial communities. Utilizing 16S rRNA sequencing, alpha and beta diversity metrics, and co-occurrence networks, the study assesses microbial diversity, community composition, and the impact of specific bacteria (endosymbionts, commensals, and pathogens) on network stability. Results reveal significant microbial community variations between genera and species, with *N. latreillii* exhibiting the most complex network. We showed that host specificity and feeding strategies significantly influence microbial diversity and interactions within bat flies. Robustness analysis through node removal simulations identifies the roles of key bacteria, such as *Wolbachia*, *Arsenophonus*, and *Bartonella*, in maintaining network stability. Findings highlight the complex interplay between these microorganisms and their hosts, offering insights into microbial ecology and vector-pathogen dynamics. The research underscores the importance of bat flies in shaping pathogen transmission networks, contributing valuable knowledge to wildlife ecology, disease control, and conservation strategies.

Keywords Microbiome · Bat flies · Network analysis · *Myotis punicus* · Endosymbionts · Commensals · Pathogens

✉ Alejandro Cabezas-Cruz
alejandro.cabezas@vet-alfort.fr

[1] Department of Animal Breeding and Animal Production, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Cluj-Napoca, Romania

[2] Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

[3] HUN-REN Climate Change: New Blood-Sucking Parasites and Vector-Borne Pathogens Research Group, Budapest, Hungary

[4] STAR-UBB Institute, Babes-Bolyai University, Cluj-Napoca, Romania

[5] Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest, Hungary

[6] Department of Biology, Faculty of Sciences, University Badji Mokhtar, B.P.12, 23000 Annaba, Algeria

[7] Nature Research Centre, Vilnius, Lithuania

[8] School of Environmental Sciences, University of Guelph, Guelph, Ontario N1G 2W1, Canada

[9] ANSES, INRAE, Ecole Nationale Vétérinaire d'Alfort, UMR BIPAR, Laboratoire de Santé Animale, Maisons-Alfort, France

[10] Animal Biotechnology Department, Center for Genetic Engineering and Biotechnology, Avenue 31 Between 158 and 190, P.O. Box 6162, 10600 Havana, Cuba

[11] Laboratory of Microbiology, National School of Veterinary Medicine of Sidi Thabet, University of Manouba, 2010 Manouba, Tunisia

[12] Department of Basic Sciences, Higher Institute of Biotechnology of Sidi Thabet, University of Manouba, 2010 Manouba, Tunisia

Introduction

Bats (Mammalia: Chiroptera) are the second most diverse and widely distributed group of Mammals, with nearly 1480 recognized species (Simmons and Cirranello 2020). As keystone species, they play essential ecological roles in pollination, seed dispersal, and insect population control (Ramírez-Fráncel et al. 2022). Their ability to fly enables them to travel long distances during seasonal migrations, while their social behavior—often forming large colonies—facilitates the transmission of pathogens (Dietz and von Helversen 2004; Drexler et al. 2011; Fleming et al. 2003). These traits, combined with their use of torpor and their role as host to a wide variety of ectoparasites (e.g., flies, fleas, ticks, mites) (Baker and Craven 2003; Dick and Patterson 2006; Hastriter 2016; Sándor et al. 2019), contribute to their complex and significant ecological functions.

Advances in molecular biology techniques have focused interest into the bat microbiomes, which are essential to bat health and disease ecology (Federici et al. 2022). Shaped by factors such as diet, habitat, and social behavior, these microbial communities influence immune function (Berman et al. 2023) and interactions with pathogens (Federici et al. 2022). While many studies have characterized bat microbiomes across various species and sample types—including feces (Dimkić et al. 2021), skin (Lemieux-Labonté et al. 2016), urine (Dietrich et al. 2018), and internal organs (Corduneanu et al. 2021; Ramos-Nino et al. 2021)—the microbiomes associated with permanent ectoparasites remain relatively understudied (André et al. 2023; Wilkinson et al. 2016). Exploring these associations is key to understanding both bat biology and zoonotic disease transmission.

Bat flies, belonging to the dipteran families Nycteribiidae and Streblidae (superfamily Hippoboscoidea), are among the most common bat ectoparasites (Szentiványi et al. 2019). Together with their bat hosts, these parasitic flies contribute to host–pathogen dynamics by harboring and potentially transmitting a wide range of pathogens, including bacteria (McKee et al. 2021; Sándor et al. 2018), viruses (Calisher et al. 2006), and other microorganisms (Corduneanu et al. 2017). However, our understanding of the composition and dynamics of microbial communities in ectoparasites, especially endosymbionts, commensals, and pathogens, remains limited, as do insights into the ecological and environmental factors shaping these interactions.

Hematophagous ectoparasites (e.g., ticks, mosquitoes, bat flies) harbor diverse bacterial communities that play key roles in parasite biology and influence host–pathogen relationships (André et al. 2023; Aželytė et al. 2022; Wu-Chuang et al. 2023). These bacteria can affect disease transmission, reproduction, and adaptation to blood-feeding (Hwang and Chang 2020; Maitre et al. 2022; White et al. 2013). For instance, certain bacteria in ticks and mosquitoes can alter vector competence (Bonnet and Pollet 2021). Endosymbionts, such as *Wolbachia*, play essential roles in ectoparasite development, reproduction (Kolo and Raghavan 2023), and pathogen blocking, as shown in mosquitoes for Dengue, Zika, and Chikungunya viruses (Caragata et al. 2021; Gul et al. 2024; Hoffmann et al. 2024). Similar symbionts have been detected in bat flies from Madagascar (Wilkinson et al. 2016) and Brazil (André et al. 2023), including *Wolbachia* and *Arsenophonus* spp., highlighting their potential roles in microbial transmission and host manipulation. Commensal bacteria, like *Acinetobacter* spp. and Enterobacteriaceae (e.g., *Pseudomonas*), also contribute to ectoparasite physiology and are common in bat flies (André et al. 2023). While often beneficial, these relationships may not be evolutionarily stable, as endosymbionts can lose functionality over time, potentially requiring replacement (Wernegreen 2017). Pathogenic microbes such as *Bartonella* spp., *Borrelia* spp., *Trypanosoma* spp., and *Plasmodium* spp. have also been found in bats and their ectoparasites (Schaer et al. 2013; Veikkolainen et al. 2014). *Bartonella* is widely studied due to transmission through bat flies (Fagre et al. 2023; McKee et al. 2021; Qiu et al. 2020). Human and animal infections with *Bartonella* have been reported in African regions where bat flies test positive for this pathogen (Bai et al. 2015; Billeter et al. 2012).

The relationship between bat flies and their chiropteran hosts offers a valuable model for exploring variability in microbial community structure and its underlying mechanism (Sebastián Tello et al. 2008). This study uses network analysis to examine the microbiomes of two bat fly genera with contrasting different feeding strategies: oioxenous bat flies, which feed exclusively on a single bat host (*Nycteribia latreillii* and *N. vexata*—on *Myotis punicus*), and stenoxenous bat flies (*Penicillidia dufourii*), which feed on multiple bat species (Gardner and Gardner 2024). We hypothesize that oioxenous bat flies exhibit lower microbial diversity and simpler microbial networks due to their specialized host range, whereas stenoxenous bat flies, which feed on multiple hosts, support more diverse and complex microbial communities. To evaluate this hypothesis, we applied network analysis and diversity metrics, exploring how host-parasite dynamics shape microbial networks across these different feeding strategies.

Materials and methods

Sampling and bat fly identification

The samples used in this study were collected from Maghrebian mouse-eared bats (*M. punicus*) captured near their roosts in Subterranean Lake Cave, at Bir Osman, Guelma province, Algeria (36°44′08″ N, 7°27′47″ E) in the spring season. Bat flies ($n = 22$) were visually inspected and carefully collected from the host's body in the field using sterile gloves and tweezers, then stored in labeled cryotubes containing 75% ethanol. Bat fly identification was performed under a binocular microscope using morphological keys described previously (Theodor and Moscona 1954). Each bat fly was identified to genus and species, and it was also sexed (Supplementary Table S7). Due to limited sample sizes for each sex within bat fly species, males and females were combined for analyses to improve statistical power. More detailed descriptions of protocols on sampling and bat fly identification are available in File S1.

DNA extraction and 16S rRNA sequencing for microbiota analysis

Genomic DNA was extracted from individual bat flies using the Nucleospin Tissue DNA extraction kit (Macherey-Nagel, Hœrdt, France), following the manufacturer's protocol for optimal DNA yield and quality. Each DNA sample was eluted in 70 µL of sterile water. The purified DNA samples were subsequently sent to Novogene Bioinformatics Technology Co. (London, UK) for amplicon sequencing of the bacterial 16S rRNA gene. Library preparation was performed using the NEBNext® Ultra™ II DNA Library Prep Kit (New England Biolabs, MA, USA), with the variable V4 region of the 16S rRNA gene being amplified using barcoded universal primers (515F/806R). Sequencing was carried out on the Illumina MiSeq platform, producing 251 base pair paired-end Reads. The raw 16S rRNA gene sequences obtained from the bat fly samples ($n = 22$) were processed and deposited in the Sequence Read Archive (SRA) under the Bioproject number PRJNA1123199 for future reference and accessibility. Extended methods on DNA extraction are provided in File S1.

Analysis of 16S rRNA sequencing dataset

The raw sequences (demultiplexed in fastq files) were denoised, quality trimmed, and merged using the DADA2 software (Callahan et al. 2016) implemented in QIIME2 (Bolyen et al. 2019), resulting in the identification of unique amplicon sequence variants (ASVs). The ASVs were aligned with q2-alignment of MAFFT (Katoh et al. 2002) and used to generate a phylogeny with q2-phylogeny of FastTree 2 (Price et al. 2010). Extended methods on the bioinformatic analysis are provided in File S1.

Controls, 16S rRNA sequence processing, and removal of contaminants

During the DNA extraction of the bat flies, two negative controls were included. These controls underwent the same DNA extraction protocol as the samples, but water was used as template instead. DNA amplification was then carried out under the same conditions as those for the bat fly samples. The package *decontam* (Davis et al. 2018) implemented in RStudio (Team Rs 2015) was used to detect potential contaminants in the samples using the statistical method *prevalence* (Table S1). Extended methods on removal of contaminants are provided in File S1.

Microbial diversity

To evaluate the diversity and community composition of the microbiota in bat fly samples, we analyzed both alpha diversity and beta diversity. We compared microbial richness between groups using pairwise Kruskal-Wallis tests ($p < 0.05$). Alpha diversity was assessed using Observed features (DeSantis et al. 2006), Faith's phylogenetic diversity index (Faith 1992), Shannon diversity index, and evenness as determined by Pielou's evenness index (Pielou 1966). In addition, beta diversity comparisons among groups were conducted using the Bray-Curtis dissimilarity index (Bray and Curtis 1957), with a PERMANOVA test ($p < 0.05$). Additionally, we calculated beta dispersion (refers to the variability in the composition of microbial communities across different samples or groups) using the *betadisper* function from the *Vegan* package in RStudio (version 4.1.3) (RStudio Team 2020; Team Rs 2015). Group differences were compared using an ANOVA ($p < 0.05$).

Bacterial co-occurrence networks, sub-networks, and Venn diagrams

Bacterial co-occurrence networks are invaluable for studying microbial interactions, community dynamics, and the processes that shape microbial community assembly (Cano-Argüelles et al. 2024). Co-occurrence networks were generated, based on the taxonomic profiles, for each bat fly's genus and each bat fly species. The correlation matrices were calculated using raw, unrarefied taxonomic data with the sparse correlations for compositional data (SparCC) method (Friedman and Alm 2012) implemented in RStudio (Team Rs 2015). Correlation coefficients with magnitude > 0.75 or < - 0.75 (for the weight of the interactions)

were used. In addition, co-occurrence subnetworks were also inferred using the SparCC method (Friedman and Alm 2012), but with a correlation coefficient of a magnitude > 0.60 or < - 0.60 for better resolution (it focuses on stronger correlations between taxa) of the microbial diversity. For these sub-networks, different bacteria were selected for analysis and assigned to groups: endosymbionts (*Arsenophonus*, *Wolbachia*) (Husník et al. 2017; Landmann 2019), commensals (*Acinetobacter*, Enterobacteriaceae) (Adegoke and Okoh 2012), and pathogens (*Bartonella*) (Veikkolainen et al. 2014).

We used the online Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to visualize the number of nodes and shared taxa between two bat fly genera and three bat fly species.

Extended methods on the microbiome analysis are provided in File S1.

Network robustness analysis using node removal

Network robustness was evaluated to assess the stability and resilience of microbial co-occurrence networks in bat flies, focusing on how removing specific bacterial taxa (endosymbionts, commensals, or pathogens) affects network structure and connectivity. This "in silico" approach simulated disturbances to determine whether networks are more vulnerable or resistant to invasion, with robust networks better able to maintain colonization resistance. We analyzed microbial networks from two bat fly genera—*Nycteribia* (*N. latreillii*, *N. vexata*) and *Penicillidia* (*P. dufourii*)—by removing nodes until 80% connectivity loss was reached, applying random or targeted (directed) attacks. Node removal was tested individually for *Arsenophonus* and *Wolbachia* (endosymbionts), *Acinetobacter* and Enterobacteriaceae (commensals), and *Bartonella* (pathogen). The modified networks were then compared with the original network using three directed attack strategies: (i) removing nodes based on decreasing betweenness centrality (BNC) values (Berry and Widder 2014), (ii) a cascading attack recalculating BNC values after each node removal, and (iii) removal based on degree centrality. Robustness analysis was performed in RStudio (v4.3.1) (Team Rs 2015) using the NetSwan package (Lhomme 2015), with networks generated in Gephi (v0.10.1) (Bastian et al. 2009). By using NetSwan, we could quantitatively compare the resilience of different networks and determine whether some bat fly microbiomes are more fragile or robust in the face of disruption, offering insights into ecological stability and potential vulnerability to pathogen invasion.

For inferential statistics, we restricted hypothesis testing to degree- and betweenness-targeted attacks, because they capture the two principal, and largely orthogonal, failure modes of biological networks: loss of hubs (degree) and loss of bridges/bottlenecks between modules (betweenness). In microbial association networks, degree heterogeneity and modularity dominate topology; consequently, these attacks typically produce the upper bound of damage under adaptive removal. Random and cascading attacks were retained as descriptive baselines/sensitivity analyses. Statistical comparisons were performed with paired *t*-tests, pairing each network with its corresponding taxon-removed version within the same bat fly lineage and attack mode (degree or betweenness). This pairing controls for network-specific baseline topology, isolating the effect of the focal taxon on robustness.

Results

Bat fly identification

Individuals of two bat fly genera and three bat fly species were collected from a total of 22 adults of *M. punicus* (mean parasite prevalence 40.1%, mean infestation 2.4, confidence interval (CI) 1.1–2.8). Two of these species, *N. latreillii* and *N. vexata*, are host-specific parasites of the Maghrebian mouse-eared bat, while the third, *P. dufourii*, is a stenoxenous taxon (nine bat hosts recorded in the region). A total of 22 individuals were included in the microbiome analysis (*N. latreillii*: $n = 4$ males; *N. vexata*: $n = 3$ females, 2 males; and *P. dufourii*: $n = 9$ females, 4 males).

Bat flies microbiota diversity and composition

The study aimed to elucidate and emphasize the influence of endosymbionts, commensals, and pathogens on their occurrence and diversity in the microbiota. To achieve this, both alpha and beta diversity analyses were conducted, comparing two bat fly genera and three distinct bat fly species. Alpha diversity was evaluated at the genus (Fig. 1A–D) and species (Fig. 1E–H) levels. At the genera level, no significant differences were detected among groups (all $p \geq 0.05$). The pairwise comparisons showed no significant differences between *Nycteribia* and *Penicillidia* for Pielou's evenness ($p = 0.15$), Faith's PD ($p = 0.10$), Observed features ($p = 0.89$), or Shannon diversity ($p = 0.30$). At the species level, no differences were observed between *N. latreillii* and *N. vexata* for Pielou's evenness ($p = 0.32$), Faith's PD ($p = 0.32$), Observed features ($p = 0.14$), or Shannon diversity ($p = 0.14$). Similarly, *N. latreillii* and *Penicillidia dufourii* did not differ for Pielou's evenness ($p = 0.90$), Faith's PD ($p = 0.30$), Observed features ($p = 0.33$), or Shannon diversity ($p = 0.49$). By contrast, *N. vexata* and *P. dufourii* differed significantly for Pielou's evenness ($p = 0.02$) and Shannon diversity ($p = 0.03$), whereas Faith's PD ($p = 0.10$) and Observed features ($p = 0.32$) were not significant. The bimodal distribution of

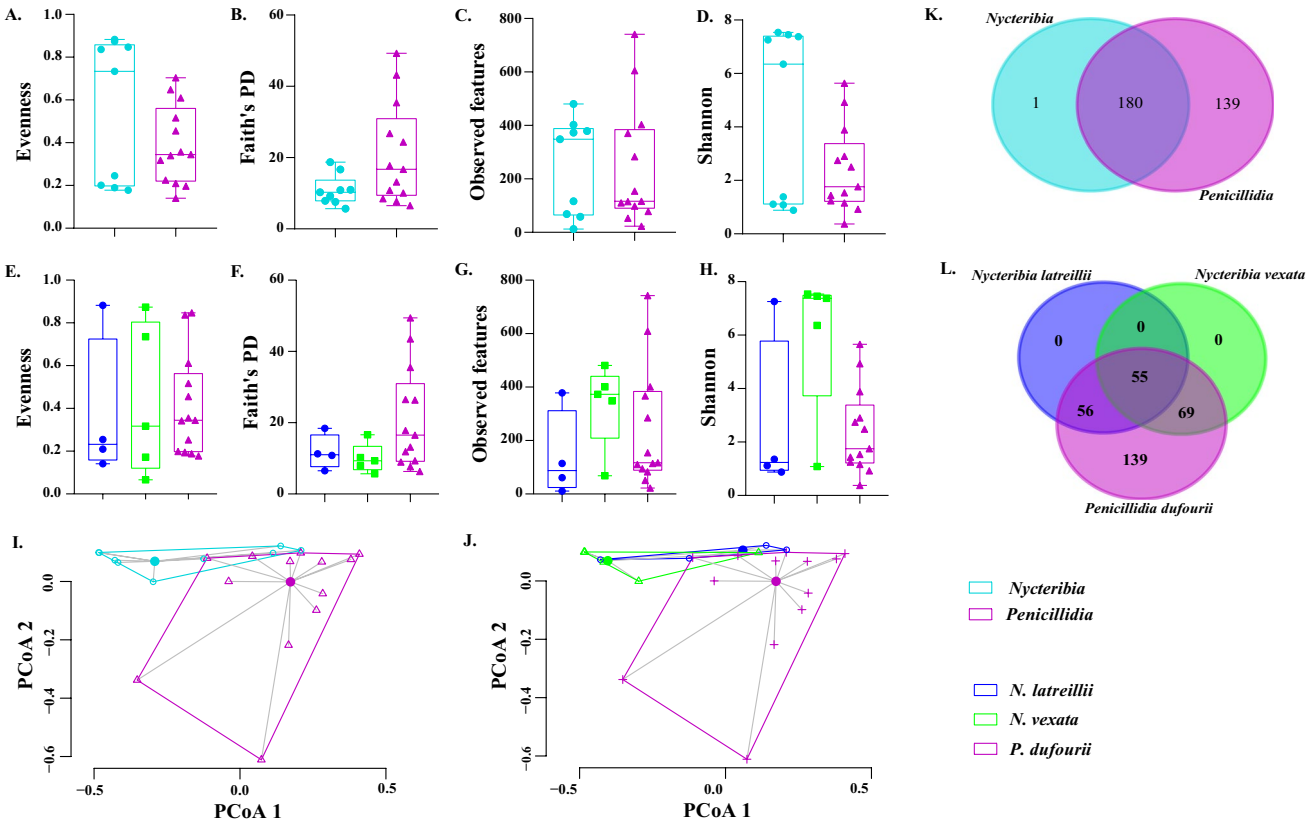

Fig. 1 Comparison of microbial diversity. Comparison of different metrics between two bat fly genera (**A**–**D** blue—*Nycteribia* and pink—*Penicillidia*) and three bat fly species (**E**–**H** blue—*N. latreillii*, green—*N. vexata*, and pink—*P. dufourii*): Pielou's evenness index (**A** genera, **E** species), **B** Faith's phylogenetic diversity (PD) (**B** genera, **F** species), **C** Observed features (**C** genera, **G** species), and **D** Shannon entropy (**D** genera, **H** species). Comparison of beta–diversity using beta dispersion, calculated from Bray-Curtis distance matrix for bat genera **I** *Nycteribia* (blue) *Penicillidia* (pink) and three bat fly species **J** *N. latreillii* (blue), *N. vexata* (green), and *P. dufourii* (pink) represented in PCoA plot. Venn diagrams showing the number of shared and unique taxa within **K** two bat fly genera and **L** three bat fly species

alpha diversity metrics observed in *Nycteribia* likely reflects variation within the genus, possibly driven by differences between species or individual host factors. Such heterogeneity suggests that microbial richness and evenness are not uniform across all samples, highlighting the complexity of bat fly microbiota. However, given the limited sample size, further studies with larger datasets are needed to confirm and explore the drivers of this pattern.

Beta diversity, which examines differences in microbial community composition, was analyzed using the Bray-Curtis dissimilarity index. This analysis revealed significant variations in microbial community structure between the two bat fly genera (PERMANOVA, $p = 0.001$; $F = 11.34$) and among the three bat fly species (PERMANOVA, $p = 0.001$; $F = 7.67$). These results highlight that while the overall richness and evenness of microbial communities were consistent across genera and species, the composition of these communities varied significantly, suggesting genus- and species-specific microbial assemblages (Table 1). However, beta dispersion analysis, which assesses the variability of microbial communities within groups, found no significant differences in the dispersion of communities between the two genera (ANOVA test, $p = 0.53$) or among species (ANOVA test, $p = 0.77$) (Fig. 1I, J).

Regarding the number of shared taxa between the two bat fly genera (*Nycteribia* and *Penicillidia*), there was a substantial overlap, with 180 shared taxa (Fig. 1K) compared to *Nycteribia* ($n = 1$, Fig. 1K). Comparison of the number of unique and shared bacterial taxa between three bat fly species revealed that only *P. dufourii* had unique taxa ($n = 139$) among the three bat fly species; the number of shared taxa among all species was 55 (Fig. 1L). These results underscore the substantial taxonomic diversity within the genus *Penicillidia*.

Comparative analysis and local connectivity in the microbial community

Bacterial co-occurrence networks were employed to characterize the bacterial community structure within two

Table 1 Fraction of nodes removed to achieve 80% connectivity loss across commensals Enterobacteriaceae and *Arsenophonus* networks

Conditions	Node removal	Loss in connectivity*			
		Random	Directed		
			Betweenness	Degree	Cascading
Nycteribia	Enterobacteriaceae	0.46	0.23	0.36	0.21
	wo(Enterobacteriaceae)	0.47	0.21	0.32	0.21
	Acinetobacter	0.48	0.23	0.36	0.21
	wo(*Acinetobacter*)	0.46	0.22	0.37	0.22
Nycteribia latreillii	Enterobacteriaceae	0.43	0.25	0.35	0.16
	wo(Enterobacteriaceae)	0.45	0.26	0.32	0.18
	Acinetobacter	0.47	0.25	0.33	0.16
	wo(*Acinetobacter*)	0.47	0.26	0.33	0.18
Nycteribia vexata	Enterobacteriaceae	0.39	0.21	0.17	0.12
	wo(Enterobacteriaceae)	0.4	0.22	0.17	0.12
Penicillidia dufourii	Enterobacteriaceae	0.51	0.37	0.46	0.26
	wo(Enterobacteriaceae)	0.5	0.36	0.43	0.25
	Acinetobacter	0.48	0.37	0.4	0.26
	wo(*Acinetobacter*)	0.51	0.39	0.39	0.25

*Threshold of connectivity loss 0.8

distinct bat fly genera and three species (Table 2, Fig. 2). Examination of the networks within each bat fly genus/species revealed different patterns of bacterial co-occurrence, highlighting variations in bacterial structure among bat fly genera/species (Fig. 2). Analysis of the topological features of the networks revealed a higher number of nodes ($n = 47$) in the genus *Nycteribia* and a higher presence of edges ($n = 148$) (Table 2) in the genus *Penicillidia* within microbial co-occurrence networks derived from the microbiota of these two bat fly genera. Across various bat fly species, *N. latreillii* exhibited the most complex co-occurrence network (Fig. 2C), contrasting with the least complex network observed in *N. vexata* (Fig. 2D). Furthermore, *N. latreillii* exhibited the highest number of nodes ($n = 89$) and edges ($n = 375$), while *P. dufourii* exhibited the lowest node number ($n = 45$) (Table 2). Conversely, *N. vexata* displayed the lowest edge count ($n = 52$) (Table 2). Moreover, analyzing other topological parameters, we observe that *N. latreillii* shows the highest modularity (1.252) (Table 2), which indicates a more compartmentalized or community-structured microbial network, with distinct modules that are less interconnected. In contrast, *P. dufourii* has a lower modularity (0.362), indicating a microbial network with less apparent modular separation (Table 2). The average clustering

Table 2 Topological features of the microbial co-occurrence networks

Topological parameters	Bat flies species			
	Nycteribia	*Nycteribia latreillii*	*Nycteribia vexata*	*Penicillidia/ Penicillidia dufourii*
Nodes	47	89	51	45
Edges	131	375	52	148
Positive edges	131	253	43	148
Negative edges	0	122	9	0
Network diameter	5	6	9	5
Average degree	5.574	8.427	2.537	6.578
Weighted degree	4.47	2.483	1.34	5.247
Average path length	2.094	2.977	3.574	2.083
Modularity	0.391	1.252	0.807	0.362
Number of modules	6	16	13	4
Average clustering coefficient	0.572	0.452	0.302	0.61

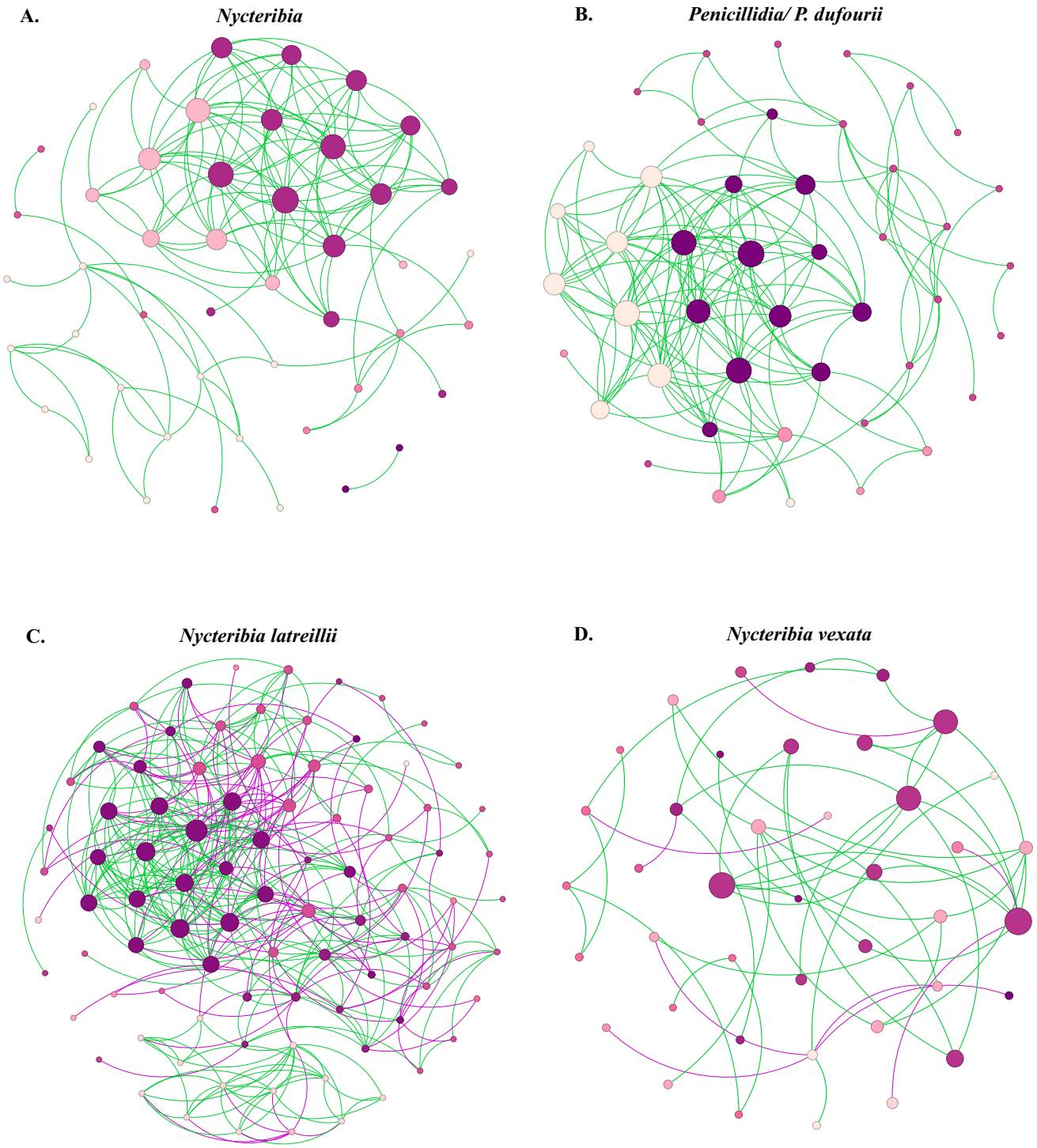

Fig. 2 Microbial co-occurrence networks of different bat flies. The networks were constructed using the *SparCC* method. Both significant negative (pink edges) and significant positive (green edges, SparCC > 0.75 or < - 0.75) are represented. The node size indicates the Eigenvector centrality, and the color is based on the modularity class. Nodes with the same colors belong to the same cluster, and only the nodes with at least one connecting edge are displayed. The panels are representing the visualization of **A** *Nycteribia*, **B** *Penicillidia/P. dufourii*, **C** *N. latreillii*, and **D** *N. vexata*

coefficient measures the degree to which nodes tend to cluster together. *P. dufourii* has the highest clustering coefficient (0.61) (Table 2), which implies a greater tendency for the microbes to form closely interconnected groups. On the other hand, *N. vexata* has a much lower coefficient (0.302) (Table 2), suggesting a less cohesive network with fewer

connected microbial communities. The network diameter and average path length reflect the "spread" of the network. *N. vexata* has the longest diameter (9) and average path length (3.574) (Table 2), indicating that its microbial network is more dispersed and connections between microbes are more distant, compared to the more compact networks in other species, like *P. dufourii* with a diameter of 5 and an average path length of 2.083 (Table 2). These networks demonstrate structural differences in the bacterial community among bat fly species, particularly between different genera, with *Nycteribia* exhibiting a microbial network with higher connectivity and complexity based on network parameters.

Variation in the local connectedness of several endosymbionts, commensal, and pathogenic bacteria was observed in the co-W genera and species (Figs. 3, 4, and 5). The selected bacterial taxa were chosen based on previously published data and were visualized alongside other interacting taxa. We first examined two common endosymbionts—*Arsenophonus* spp. (Fig. 3A, Table S2) and *Wolbachia* spp. (Fig. 3B, Table S3). The number of directly connected nodes co-occurring with *Arsenophonus* spp. was higher in *Penicillidia* ($n = 27$) than in *Nycteribia* ($n = 3$), while *Wolbachia* spp. showed more connections in *N. latreillii* ($n = 22$) than in other species. Visualization of co-occurrence for the commensal *Acinetobacter* spp. was not possible in *N. vexata*, as this bacterium was absent from its microbiota. In *N. latreillii*, *Acinetobacter* spp. had the highest number of co-occurring neighbors (Fig. 4A, Table S4). A similar pattern was observed for Enterobacteriaceae, where the number of co-occurring taxa was also highest in *N. latreillii* (Fig. 4B, Table S5). For the pathogen *Bartonella* spp., only one interaction was observed in each of the bat fly genera (*Nycteribia* and *Penicillidia*), but *N. latreillii* again showed a greater number of associated taxa (Fig. 5, Table S6). Overall, *N. latreillii* consistently showed a higher number of co-occurrence connections across all bacterial groups examined, suggesting more densely connected local sub-networks in this species' microbiota.

Influence of endosymbionts, commensals, and pathogens on the assembly and robustness of bat fly microbiota

1.1. Influence of *Wolbachia* and *Arsenophonus* endosymbionts on bat fly network robustness

To assess the potential structural role of the endosymbionts *Wolbachia* and *Arsenophonus* in microbial network connectivity, we analyzed and compared the robustness of bat fly genera and species networks before and after in silico node removal. Two scenarios were considered: networks including all taxa and networks with *Wolbachia* ("woWol")

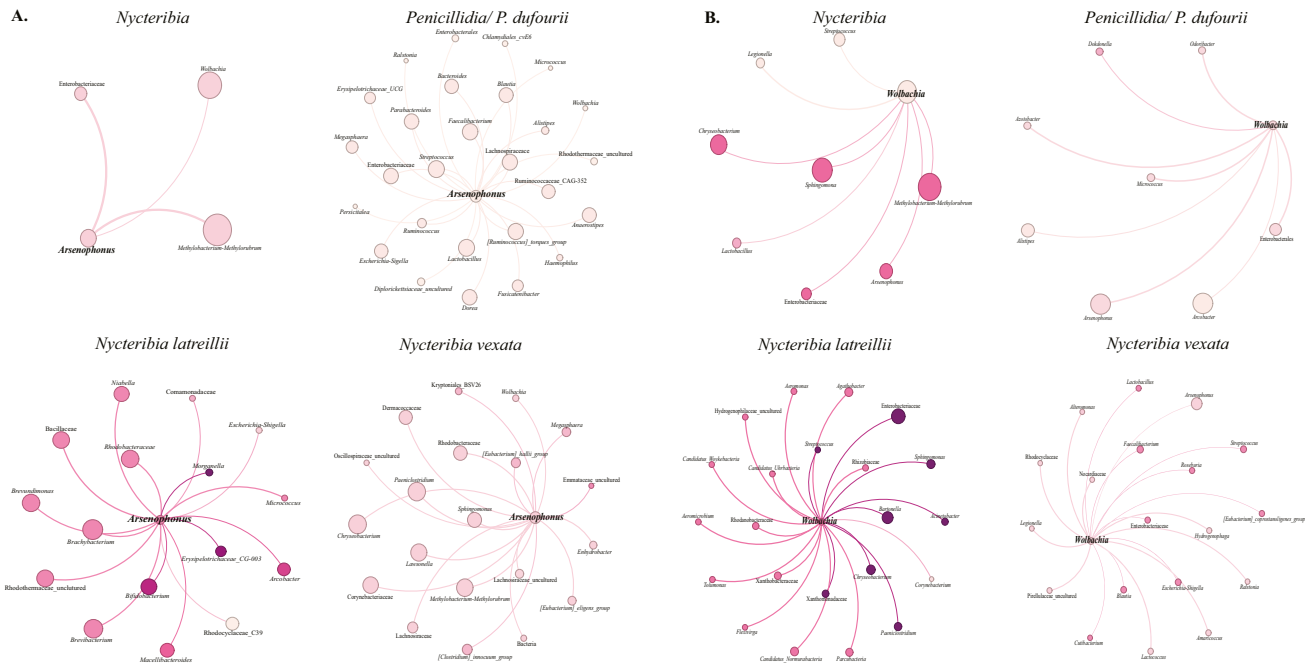

Fig. 3 Sub-networks of local connectivity of different endosymbionts were extracted from the co-occurrence networks. The sub-networks were constructed using the *SparCC* method (SparCC > 0.60 or < - 0.60). The node size indicates the Eigenvector centrality, and the color is based on the modularity class. Nodes with the same colors belong to the same cluster, and only the direct neighbors of the endosymbiont nodes are displayed. The panels show the local connectivity of the following endosymbionts found in different bat fly genera/species: **A** the visualization of *Arsenophonus* spp. and its connected taxa and **B** the visualization of *Wolbachia* spp. and its connected taxa

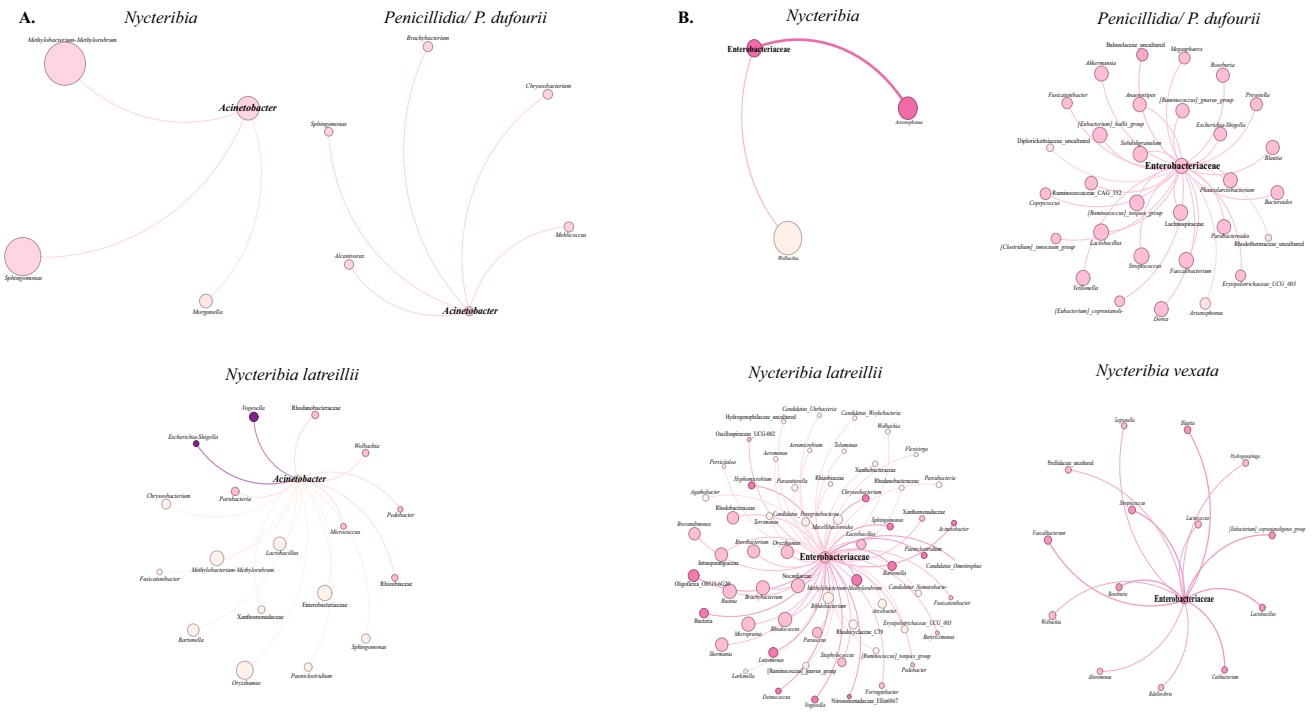

Fig. 4 Sub-networks of local connectivity of commensal bacteria *Acinetobacter* spp. were extracted from the co-occurrence networks. The sub-networks were constructed using the *SparCC* method (SparCC > 0.60 or < - 0.60). The node size indicates the Eigenvector centrality, and the color is based on the modularity class. Nodes with the same colors belong to the same cluster, and only the direct neighbors of the commensal bacteria are displayed. The panels show the local connectivity of the following commensals found in different bat fly genera/species: **A** the visualization of *Acinetobacter* spp. and its connected taxa and **B** the visualization of Enterobacteriaceae family and its connected taxa

or *Arsenophonus* ("woArs") removed (Figures S1, S2) (Table 3).

Networks for *Nycteribia* (genus), *N. vexata*, and *P. dufourii* retained connectivity under random and targeted node removal up to the 0.8 threshold, indicating high structural robustness even without *Wolbachia* (Figure S1A-P). In *N. latreillii*, robustness changed significantly only under betweenness-attacks ($p < 0.0001$, $R^2 = 0.7147$; Fig. 6). Removing *Arsenophonus* produced a similar pattern (Figure S2) with increased robustness in *N. latreillii* for betweenness ($p = 0.003$, $R^2 = 0.3865$; Fig. 6) and degree ($p = 0.0391$, $R^2 = 0.2158$; Fig. 7). In *N. latreillii*, *Wolbachia* and *Arsenophonus* act as load-bearing, high-centrality connectors whose presence concentrates flow through a few paths; removing them redistributes centrality and hardens the network to targeted loss. In other lineages (*Nycteribia* genus, *P. dufourii*), these endosymbionts are not universal scaffolds, implying greater redundancy in community wiring.

2.2. Influence of Enterobacteriaceae and *Acinetobacter* commensals on bat fly network robustness

When comparing bat fly microbial networks with and without selected commensals (e.g., Enterobacteriaceae and *Acinetobacter*), structural changes were observed primarily under targeted node removal (Figures S3 and 4). In *Nycteribia* genus network, the removal of Enterobacteriaceae significantly increased betweenness-attack robustness ($p = 0.0419$, $R^2 = 0.3843$; Fig. 6). In *N. latreillii*, taking out Enterobacteriaceae markedly increased degree-attack robustness ($p < 0.0001$, $R^2 = 0.8043$; Fig. 7). Removing *Acinetobacter* also increased robustness under betweenness ($p = 0.0256$, $R^2 = 0.2605$; Fig. 6) and degree attacks ($p = 0.001$, $R^2 = 0.4779$; Fig. 7) in N. *latreillii*. In *P. dufourii*, robustness to degree-based attacks increases after *Acinetobacter* removal ($p < 0.0001$, $R^2 = 0.9836$; Fig. 7). By contrast, *N. vexata* were comparatively stable. In *N. latreillii* (and for *Acinetobacter* in *P. dufourii*), these commensals function as vulnerability hubs: they help organize the network but at the cost of making it more attackable when high-centrality nodes are targeted. Their removal disperses connectivity across more alternatives, yielding greater resilience.

3.3. Influence of the pathogen *Bartonella* on bat fly network robustness

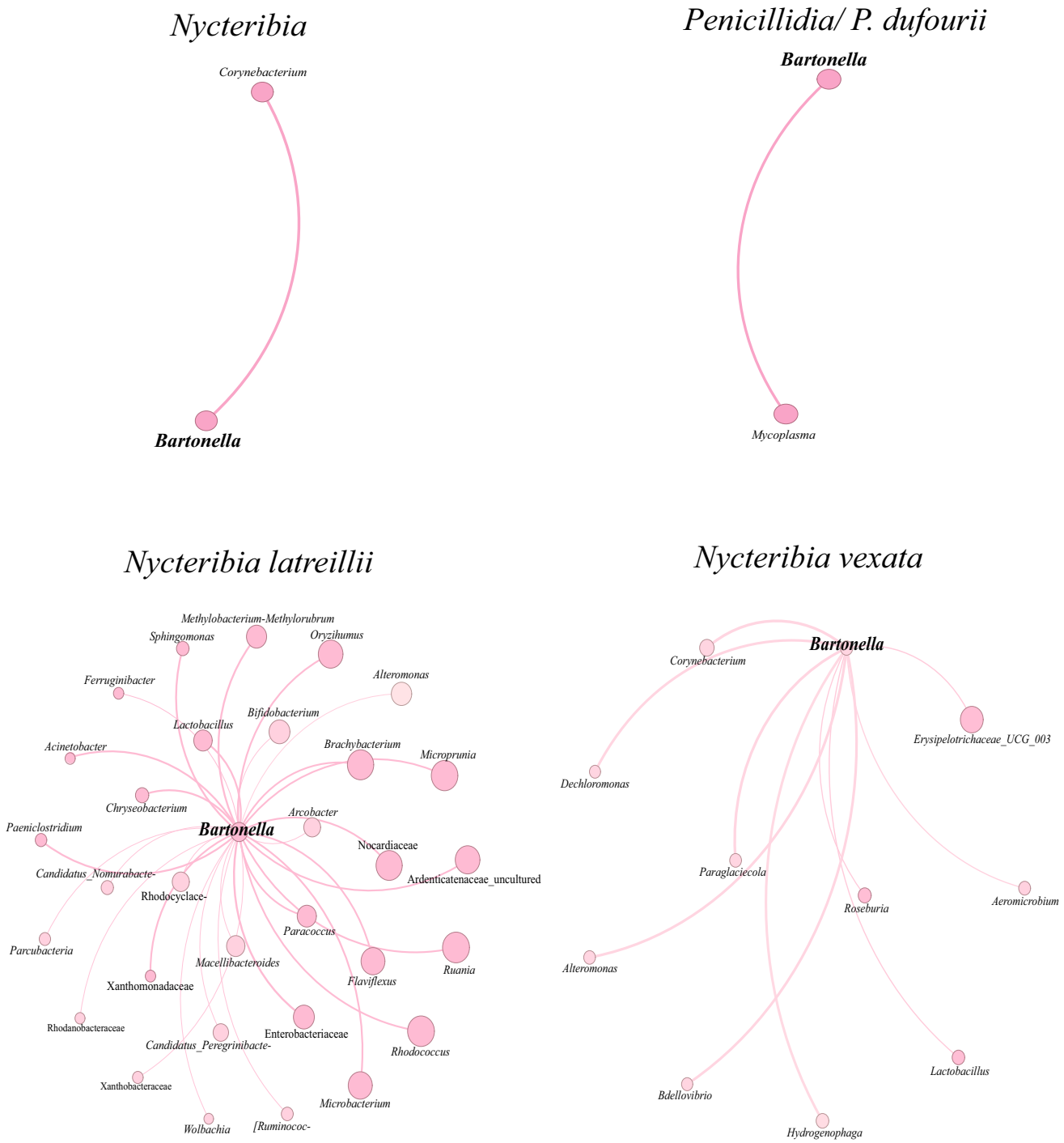

Fig. 5 Sub-networks of local connectivity of pathogen bacteria *Bartonella* spp. were extracted from the co-occurrence networks. The sub-networks were constructed using the *SparCC* method (SparCC > 0.60 or < - 0.60). The node size indicates the Eigenvector centrality, and the color is based on the modularity class. Nodes with the same colors belong to the same cluster, and only the direct neighbors of the pathogen bacteria are displayed. Nodes represent the visualization of *Bartonella* spp. and its connected taxa

To assess the structural influence of the pathogen *Bartonella* on bat fly microbial networks, we analyzed network robustness before and after its in silico removal (woBar) (Figure S5) (Table 4). In *N. latreillii*, removing *Bartonella* increased robustness under betweenness-based attacks ($p = 0.0160$, $R^2 = 0.2818$; Fig. 6). In *Nycteribia* (genus), removal decreased robustness under degree-based attacks ($p = 0.0312$, $R^2 = 0.4195$; Fig. 7). At the 0.8 connectivity-loss threshold, *N. vexata* and *P. dufourii* showed no significant change (Figure S5A–D). *Bartonella* integrates into bat

Table 3 Fraction of nodes removed to achieve 0.8 connectivity loss across endosymbionts *Wolbachia* and *Arsenophonus* networks

Conditions	Node removal	Loss in connectivity*			
		Random	Directed		
			Betweenness	Degree	Cascading
Nycteribia	*Wolbachia*	0.48	0.23	0.36	0.21
	wo(*Wolbachia*)	0.48	0.23	0.36	0.21
	Arsenophonus	0.48	0.23	0.36	0.23
	wo(*Arsenophonus*)	0.44	0.23	0.36	0.21
Nycteribia latreillii	*Wolbachia*	0.49	0.25	0.33	0.16
	wo(*Wolbachia*)	0.47	0.28	0.32	0.17
	Arsenophonus	0.49	0.25	0.33	0.16
	wo(*Arsenophonus*)	0.47	0.25	0.34	0.17
Nycteribia vexata	*Wolbachia*	0.36	0.21	0.17	0.12
	wo(*Wolbachia*)	0.33	0.19	0.16	0.11
	Arsenophonus	0.34	0.21	0.17	0.12
	wo(*Arsenophonus*)	0.30	0.23	0.15	0.10
Penicillidia dufourii	*Wolbachia*	0.48	0.37	0.46	0.26
	wo(*Wolbachia*)	0.51	0.37	0.46	0.26
	Arsenophonus	0.48	0.37	0.46	0.26
	wo(*Arsenophonus*)	0.51	0.37	0.46	0.26

*Threshold of connectivity loss 0.8

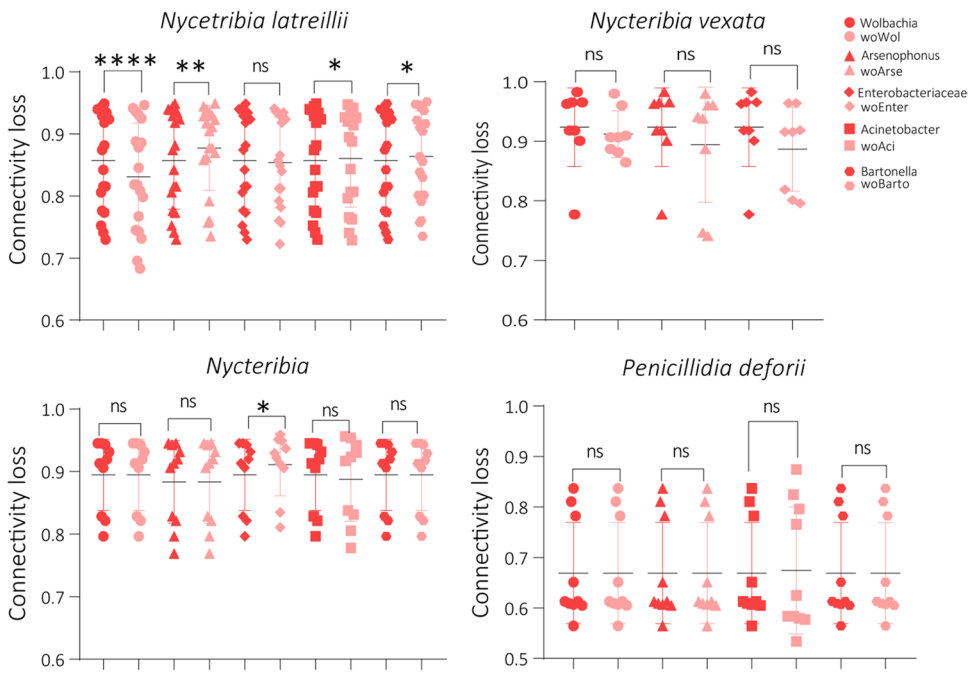

Fig. 6 Robustness of bat fly microbiome networks to betweenness-targeted attacks. For each lineage (*N. latreillii*, *N. vexata*, *Nycteribia* genus, *P. dufourii*), the original network (all taxa) is compared with *in silico* networks where a dominant taxon was removed (woWolbachia, woArsenophonus, woAcinetobacter, woEnterobacteriaceae, woBartonella). Robustness is summarized as connectivity loss under a betweenness-ordered node-removal attack (0 = fully connected, 1 = fully disconnected; higher values indicate lower robustness). Brackets denote paired *t*-tests between the original and its corresponding woTaxon network within each lineage; ns, not significant; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$

fly microbiomes in context-dependent ways: it can act as a bottleneck-forming connector that decreases network robustness (*N. latreillii*), or as a redundant, stabilizing component whose removal marginally decreases network stability (*Nycteribia* genus). The absence of effects in *N. vexata* and *P. dufourii* indicates robust, pathogen-tolerant architectures with distributed redundancy.

Endosymbionts, commensals, and a pathogen do not play fixed "keystone" roles across bat fly lineages. Instead, network position and host lineage determine whether a dominant taxon is stabilizing, neutral, or destabilizing. Practically, these results argue for lineage-specific manipulation strategies: targeting high-centrality endosymbionts/commensals in *N. latreillii* could harden the microbiome against

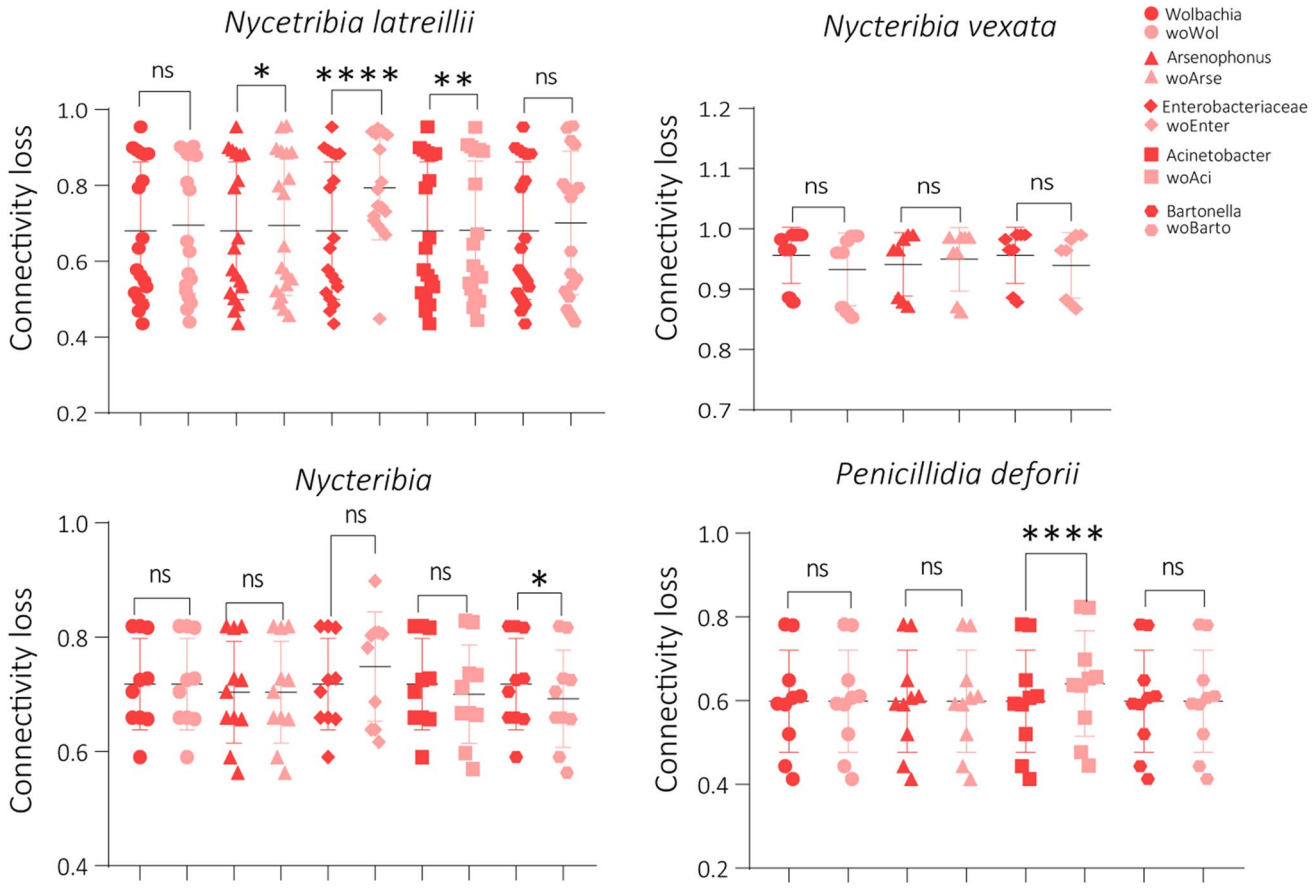

Fig. 7 Robustness of bat fly microbiome networks to degree-targeted (hub) attacks. For each lineage (*N. latreillii*, *N. vexata*, *Nycteribia* genus, *P. dufourii*), the original network (all taxa) is compared with *in silico* networks where a dominant taxon was removed (woWolbachia, woArsenophonus, woAcinetobacter, woEnterobacteriaceae, woBartonella). Robustness is summarized as connectivity loss under a degree attack (0 = fully connected, 1 = fully disconnected; higher values indicate lower robustness). Brackets show paired *t*-tests between each original network and its corresponding woTaxon network within a lineage; ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Table 4 Fraction of nodes removed to achieve 80% connectivity loss across pathogen *Bartonella* network

Conditions	Node removal	Loss in connectivity*			
		Random	Directed		
			Betweenness	Degree	Cascading
Nycteribia	*Bartonella*	0.48	0.23	0.36	0.21
	wo(*Bartonella*)	0.48	0.23	0.36	0.21
Nycteribia latreillii	*Bartonella*	0.48	0.25	0.33	0.16
	wo(*Bartonella*)	0.45	0.25	0.35	0.17
Nycteribia vexata	*Bartonella*	0.36	0.21	0.17	0.12
	Wo(*Bartonella*)	0.41	0.15	0.15	0.12
Penicillidia dufourii	*Bartonella*	0.46	0.37	0.46	0.26
	wo(*Bartonella*)	0.48	0.37	0.46	0.26

*Threshold of connectivity loss 0.8

invasion or cascade failure, whereas similar interventions may be unnecessary or counterproductive in other bat fly hosts.

We acknowledge that the relatively small sample size ($n = 22$), particularly for stenoxenous species, may limit the statistical power and resolution of the microbial

co-occurrence networks and robustness analyses. While our findings provide valuable preliminary insights into the microbial community structure of bat flies with varying host specificity, they should be interpreted with caution. Limited sample size can increase the likelihood of unstable correlations and reduce the precision of inferred microbial interactions. To mitigate these effects, we applied a high correlation threshold ($r > 0.75$), interpreted network metrics qualitatively, and approached robustness analyses as exploratory. Expanding sample sizes in future studies will be essential to deepen our understanding of bat fly microbiomes.

Discussion

Bats and their ectoparasites, particularly bat flies, are ideal models for microbiome studies due to their global distribution and role as pathogen reservoirs. This study investigated the microbial composition of three Nycteribiidae bat fly species (*N. latreillii*, *N. vexata*, and *P. dufourii*) collected from *M. punicus* in Algeria. These species differ in host specificity, ranging from oioxenous (host-restricted) to stenoxenous (generalist), allowing exploration of how host specificity and feeding ecology shape microbial community structure. Using diversity metrics and network analysis, we examined microbial diversity and interactions while acknowledging limitations including a narrow species scope, potential sampling bias, lack of functional validation, and possible spatial or temporal variability in microbial communities.

Previous studies have investigated the microbiomes of bat flies from both the Streblidae (André et al. 2023) and Nycteribiidae (Wilkinson et al. 2016) families, but to our knowledge, network analysis has not been applied in this context. Our co-occurrence network analysis indicated that *N. latreillii* exhibits the most complex microbial network, whereas *P. dufourii* showed the lowest number of edges (Fig. 2). This pattern may reflect a more evenly distributed microbial community in *N. latreillii*, allowing for a higher number of bacterial interactions and greater network complexity. In contrast, *P. dufourii* likely harbors more singleton taxa, bacteria present at low abundance or unique to some individuals, resulting in fewer co-occurrences and a less connected network. These differences in network structure suggest variation in microbial community composition across species, though functional implications remain to be tested. A significant limitation of this study is the variation in sample sizes among bat fly species, which likely influenced network topology. For example, the *N. latreillii* network ($n = 4$) contained 375 edges, while the combined *Nycteribia* dataset ($n = 9$) showed fewer edges, indicating potential overestimation of connectivity due to small sample size. Conversely, the larger sample size for *Penicillidia* ($n = 13$) may partly explain its higher number of inferred interactions. It is well established that co-occurrence network inference is sensitive to sample size, network density, and data structure (Berry and Widder 2014). Although *N. latreillii* is an oioxenous parasite, it exhibits higher microbial diversity (Fig. 2C). This may reflect either an ancestral microbial composition shaped by a long-term association with its host, *M. punicus* (Ruedi 2023; Stadelmann et al. 2004), or a functional adaptation aiding immune modulation in a specialized host environment. The phylogenetic distinctiveness of *M. punicus* might contribute to this unique microbial community. *N. latreillii* is also known from other European hosts (Szentivanyi et al. 2016), but these do not overlap geographically with North African populations, limiting gene flow (Ruedi 2023). In contrast, *Penicillidia* harbors the highest number of unique bacterial taxa (Fig. 1K, L), which may be linked to its broader host range and exposure to diverse microbes (Szentivanyi et al. 2016). Statistical analyses confirm differences in microbial diversity and composition across bat fly genera and species. Low sample size, especially in the case of stenoxenous bat fly species, can significantly affect the accuracy and reliability of microbial co-occurrence network analyses. As highlighted by Berry and Widder, small datasets are prone to statistical artifacts that can inflate network density and lead to misleading associations. In microbial networks, co-occurrence relationships are often inferred from correlation-based methods, which assume that observed patterns reflect true ecological interactions. However, with limited sample size, random variation or shared habitat preferences can produce apparent correlations that do not represent biologically meaningful relationships. Therefore, caution must be taken when interpreting networks derived from small datasets.

Facultative mutualistic microbes in bat flies have been linked to nutrition (Brownlie et al. 2009), immunity (Eleftherianos et al. 2013; Yadav et al. 2018), development (Liu and Guo 2019), and reproduction (Ma et al. 2014; Mondo et al. 2017), with *Wolbachia* among the most widespread endosymbionts (André et al. 2002; Bi and Wang 2020). Microbiome composition appears influenced by host family, species, and environment (Speer et al. 2022; Wilkinson et al. 2016). As obligate blood-feeders with vitamin B-deficient diets, bat flies likely rely on endosymbionts housed in bacteriocytes and female milk glands (Aschner 1946; Hosokawa et al. 2012). *Arsenophonus* spp. occurs in both Streblidae and Nycteribiidae (Duron et al. 2014; Hosokawa et al. 2012; Lack et al. 2011), acting as primary symbiont in Streblidae (Duron et al. 2014; Speer et al. 2022; Wilkinson et al. 2016), while *Wolbachia* is highly prevalent in Nycteribiidae from Madagascar and Brazil, though its role as a primary symbiont remains debated (Duron et al. 2014; Speer et al. 2022; Wilkinson et al. 2016). Other endosymbionts, including "Candidatus Aschnera chinzeii" and *Bartonella*, have also been

reported (Duron et al. 2014; Hosokawa et al. 2012; Morse et al. 2013). In our study, co-occurrence networks showed that *Arsenophonus* was more abundant in *P. dufourii*, *Wolbachia* in *N. latreillii* (Fig. 3), with *Bartonella*. Both interacted with commensals, with *Bartonella* co-occurring only with *Wolbachia*, consistent with findings from Madagascar and the Comoros (Qiu et al. 2020). Emergent properties like robustness and connectivity influence the behavior of complex microbial communities (Röttjers and Faust 2018). Robustness analyses showed that removing *Wolbachia* or *Arsenophonus* slightly increased network resilience in *N. latreillii*, suggesting these high-centrality taxa act as connectors whose loss redistributes connectivity. No such effect was seen in *Nycteribia* spp. or *P. dufourii*, implying greater redundancy. Thus, endosymbionts are not universal keystones but play lineage-specific structural roles. The distribution of *Arsenophonus* may reflect ancient co-diversification in oligoxenous species or horizontal transfer in stenoxenous ones (Ichinohe et al. 2011; Qiu et al. 2020; Ramírez-Fráncel et al. 2022). A single ancestral acquisition has been suggested for Nycteribiidae versus multiple for Streblidae (Qiu et al. 2020). Our data reveal variability even within Nycteribiidae, but their precise roles remain unclear (Ichinohe et al. 2011).

Commensal bacteria can prevent pathogen establishment through nutrient competition, inhibitory compounds such as bacteriocins (Rea et al. 2010, 2014), short-chain fatty acids (Gantois et al. 2006), bacteriophages (Duerkop et al. 2012; Minot et al. 2011), signalling molecules (Brandl et al. 2008; Gallo and Hooper 2012), and modulation of immunity (Ichinohe et al. 2011; Naik et al. 2012; Satoh-Takayama et al. 2008) and inflammation (Fanning et al. 2012; Furusawa et al. 2013). Beyond gut systems, commensals also influence ectoparasite ecology, as shown in tsetse flies (*Sodalis*) (Gashururu et al. 2023; Wamwiri et al. 2014), fruit flies (*Acetobacter*) (Onuma et al. 2023), house flies (*Providencia*, *Myroides*) (Voulgari-Kokota et al. 2022), and mosquitoes (*Serratia*) (Gao et al. 2023; Wu et al. 2019). Bat fly microbiomes remain unexplored, but Dipteran ectoparasites often harbor Acetobacteriaceae and Enterobacteriaceae (Wilkinson et al. 2016). We analyzed co-occurrence sub-networks in Nycteribiidae bat flies focusing on *Acinetobacter* (Acetobacteriaceae) and Enterobacteriaceae (Fig. 4). The oioxenous *N. latreillii* displayed the most complex network, where the commensals co-occurred with *Bartonella*, indicating species-specific microbial patterns. Robustness results showed that removing Enterobacteriaceae increased resilience in *Nycteribia* genus (betweenness) and *N. latreillii* (degree), while removing *Acinetobacter* increased robustness in *N. latreillii* (both) and in *P. dufourii* (degree). These results suggest that in certain hosts, these commensals act as vulnerability hubs: their centrality organizes the network but raises sensitivity to disruptions. By contrast, *N. vexata* showed stable robustness regardless of commensal removal, indicating a more distributed, resilient network structure. The ecological functions of these taxa remain unclear, but their roles in pathogen dynamics, digestion, and microbial community stability should be of further study.

Pathogenic bacteria can be part of ectoparasite gut microbiomes, causing host infections (Qiu et al. 2020), through dysbiosis, immune evasion, and toxic metabolites (Thiennimitr et al. 2011; Winter et al. 2013). Among these, *Bartonella* is highly relevant for One Health. It has been detected in bats and their flies by PCR and phylogenetics (André et al. 2023; Judson et al. 2015; Sándor et al. 2018), though rarely microbiome approaches. In our network analysis, *Bartonella* occurred mainly in *N. latreillii* (Fig. 3). While common in Nycteribiidae (Speer et al. 2022; Wilkinson et al. 2016) but variable in Streblidae (André et al. 2023; Morse et al. 2013; Speer et al. 2022), its links with endosymbionts may influence vector competence or metabolism (Morse et al. 2013; Zhu et al. 2014), and it may act as a primary or secondary symbiont (Speer et al. 2022). Broad detection across bats and flies likely reflects shared transmission or evolutionary history (Ikeda et al. 2017; McKee et al. 2021; Wilkinson et al. 2016). Robustness results show species-specific roles: removal decreased robustness in *N. latreillii* but increased robustness in *Nycteribia*, with no effect in *N. vexata* or *P. dufourii*. Thus, *Bartonella* may act either as a bottleneck or a stabilizer depending on network structure. Further metagenomic and longitudinal studies are needed to clarify its functional role in microbial dynamics and host health.

Conclusions

This study demonstrates that bat fly species within the Nycteribiidae family harbor distinct microbial communities, with *N. latreillii* exhibiting the most complex co-occurrence network. We identified key bacterial groups—including endosymbionts, commensals, and pathogens—showing species-specific interaction patterns within bat fly microbiomes. Through network analysis, we revealed how the removal of bacteria influences microbial community robustness under different simulated disturbances, highlighting species-dependent network resilience. These findings advance understanding of microbial community structure in bat fly ectoparasites, providing new insights into host-microbiome interactions and laying the groundwork for future research on microbiome function, ecology, and potential implications for disease dynamics and conservation.

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Author contribution Alexandra CORDUNEANU: data collection and analysis, visualization, writing-original draft. Mohammed Lamine

BENDJEDDOU: sample collection, bat fly identification, sample preparation, writing-review. Attila D. SÁNDOR: supervision writing-review. Andrei Daniel Mihalca: writing-review. Sándor HORNOK: writing-review. Áron PÉTER: data contribution. Farouk KHELFAOUI: writing-review. Justè AŽELYTÈ: data analysis. Dasiel OBREGON: software, writing-review. Lourdes MATEOS-HERNÁNDEZ: sample preparation. Apolline MAITRE: writing-review. Lianet ABUIN-DENIS: data analysis. Alejandra WU-CHUANG: writing-review. Myriam KRATOU: data analysis. Mourad BEN SAID: writing-review. Alejandro CABEZAS-CRUZ: conception of study, supervision, visualization, and writing-review.

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Data availability All data generated for this manuscript was deposited in the SRA repository under the Bioproject number PRJNA1123199.

Declarations

Ethics approval The field work was carried out under permission number "052687" issued on 13.05.2017 by the Environmental and Protection Directorate of Annaba and the National Park of El Kala. No live bat was harmed during the sample collection.

Competing interests The authors declare no competing interests.

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