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SHORT COMMUNICATION

Bacterial communities in the feces of insectivorous bats in South Korea

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Bats serve as vectors and natural reservoir hosts for various infectious viruses, bacteria, and fungi. These pathogens have also been detected in bat feces and can cause severe illnesses in hosts, other animals, and humans. Because pathogens can easily spread into the environment through bat feces, determining the bacterial communities in bat guano is crucial to mitigate potential disease transmission and outbreaks. This study primarily aimed to examine bacterial communities in the feces of insectivorous bats living in South Korea. Fecal samples were collected after capturing 84 individuals of four different bat species in two regions of South Korea, and the bacterial microbiota was assessed through next generation sequencing of the 16S rRNA gene. The results revealed that, with respect to the relative abundance at the phylum level, *Myotis bombinus* was dominated by Firmicutes (47.24%) and Proteobacteria (42.66%) whereas *Miniopterus fuliginosus* (82.78%), *Rhinolophus ferrumequinum* (63.46%), and *Myotis macrodactylus* (78.04%) were dominated by Proteobacteria. Alpha diversity analysis showed no difference in abundance between species and a significant difference ($p < 0.05$) between *M. bombinus* and *M. fuliginosus*. Beta-diversity analysis revealed that *Clostridium*, *Asaia*, and Enterobacteriaceae_g were clustered as major factors at the genus level using principal component analysis. Additionally, linear discriminant analysis effect size was conducted based on relative expression information to select bacterial markers for each bat species. *Clostridium* was relatively abundant in *M. bombinus*, whereas Mycoplasma_g10 was relatively abundant in *R. ferrumequinum*. Our results provide an overview of bat guano microbiota diversity and the significance of pathogenic taxa for humans and the environment, highlighting a better understanding of preventing emerging diseases. We anticipate that this research will yield bioinformatic data to advance our knowledge of overall microbial genetic diversity and clustering characteristics in insectivorous bat feces in South Korea.

Keywords: bacterial community, fecal analysis, insectivorous bats, microbiota, pathogens, zoonoses

Introduction

Bats are found in regions worldwide, with the exception of polar areas, and have adapted to diverse environments. They constitute the second-largest order of mammals, with approximately 1,400 known species (Schipper et al. 2008; Simmons and Cirranello 2023; Wilson and Mittermeier 2019). Bats play a critical role in regulating ecosystem services, such as pest control, pollination, and seed dispersion (Kasso and Balakrishnan 2013; Kunz et al. 2011; Ramírez-Fráncel et al. 2022). As flying mammals, bats act as potent vectors and natural reservoir hosts for numerous infectious viruses, bacteria, and fungi. These pathogens have also been detected in their excreta, such as guano, raising concerns regarding potential transmission to humans (Dietrich and Markotter 2019; Dietrich et al. 2018). Furthermore, studies have shown that bat feces and intestines may contain potentially pathogenic bacteria (*Bartonella*, *Campylobacter*, *Clostridium*, *Salmonella*, and *Shigella* species) capable of causing severe illnesses in hosts and other animals (Huang et al. 2022). Therefore, gaining new insights into the microbiome of bat guano from different locations is imperative. Even before the pandemic, studies had been conducted on zoonotic diseases resulting from the overlapping habitat use between bats and humans owing to habitat destruction caused by factors such as climate change, ur-

banization, and industrial and agricultural development (Brook and Dobson 2015; Brusse and Holmes 2022; O'Shea et al. 2014). Given the ease with which pathogens can spread into the environment through interactions with other animals, consumption of raw food, water contamination, and potential infection of humans, gaining an understanding of bat guano microbiota is of utmost importance (Wolkers-Rooijackers et al. 2019). As we prepare for a future marked by the constant threat of infectious diseases, understanding the mechanisms behind the emergence of pathogens from wild animals becomes a crucial issue for predicting their occurrence and preventing their spread (Dimkić et al. 2021).

Conventional methods for analyzing microbes involve bacterial culture; however, these methods do not capture the entire microbial diversity, especially uncultured microbes (Abdelfattah et al. 2018; Hahn et al. 2019). Therefore, using next-generation sequencing to explore intricate bacterial communities in guano has facilitated comprehensive research into these distinctive microhabitats, enhancing our understanding of their role in health and disease (Elie et al. 2023; Knight et al. 2018). This study aimed to establish a foundation for disease research within ecosystems by comparing the bacterial community compositions in the feces of four insectivorous bat species in South Korea during July and August. We also aimed to construct a bacterial genome database based on the bacterial data obtained from these samples.

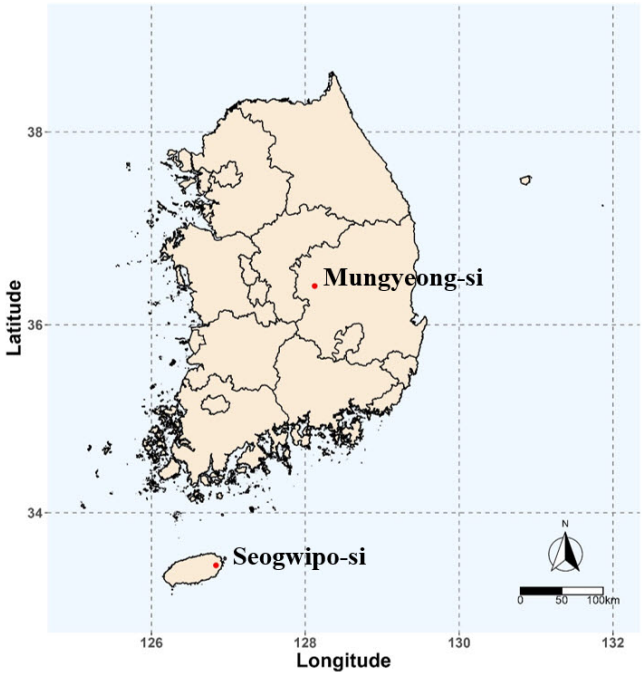

Fig. 1 Location of the study sites in South Korea.

Materials and Methods

Sample collection

Between July and August 2022, 84 samples of four bat species, *Myotis bombinus*, *Myotis macrodactylus*, *Miniopterus fuliginous*, and *Rhinolophus ferrumequinum*. The area where the four species of bats inhabit is located in Mungyeong-si (36°40'59.36"N-128°12'28.03"E) and Seogwipo-si (33°26'8.03"N-126°50'15.75"E), in South Korea (Fig. 1, Table 1). Bats were captured using mist nets and placed individually in clean cotton bags. Prior to release, the species, age, and sex of all captured bats were determined by, and they were tagged with a metal ring to mark them individually. Fecal samples were collected only if naturally excreted by the bats before they were placed in the cotton bag. Each fecal sample was immediately sealed in a 2 mL Eppendorf tube containing 100% ethanol and stored at –20°C until transported to the laboratory. The methods for capturing bats and collecting samples used in this study complied with the European Bat Monitoring Guidelines (Mitchell-Jones and McLeish 2004). This study received approval from the Research Planning Review Committee of the National Institute of Ecology (NIEIACUC-2021-001). We also ensured compliance with the Wildlife Protection and Management Act of Korea, as well as the Institutional Research Ethics Regulations and Guidelines. Permissions for all handling and sampling was obtained from the seven local governments each year. Academic survey approval was granted by Mungyeong-si (No. 2022-00004, 28 January 2022) and Seogwipo-si (No. 2022-2, 18 January 2022).

DNA extraction and 16S rRNA gene polymerase chain reaction

DNA extraction was performed on less than 200 mg of feces using a QIAamp DNA Fast DNA Stool Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. The extracted gDNA was stored in a –20°C freezer. Metagenomic DNA was extracted and amplification of the V3–V4 region of the bacterial 16S ribosomal RNA (16S rRNA) gene

Table 1 Number of fecal samples collected from individuals of each bat species in the two study sites

Site	Number of sampled individuals				
	Myotis bombinus	*Myotis macrodactylus*	*Miniopterus fuliginosus*	*Rhinolophus ferrumequinum*	Total
Mungyeong-si	8	-	27	4	39
Seogwipo-si	3	13	22	7	45
Total	11	13	49	11	84

was conducted using barcoded universal primers (Fadrosh et al. 2014). This genetic region provides abundant information for classifying microbial communities (Gevers et al. 2012). These amplicons were sequenced on an Illumina MiSeq platform using 2 × 250 base pairs (Illumina, San Diego, CA, USA), which provides fully overlapping paired-end reads.

Statistical analysis

Microbiome profiling was performed using the 16S-based microbiome taxonomic profiling platform with the PKSSU4.0 database of EzBioCloud Apps (CJ Bioscience, Inc. Seoul, Korea) (Yoon et al. 2017). Chimeric, low-quality, and non-target amplicons were automatically excluded from the analysis. The operational taxonomic unit was defined as a group of sequences exhibiting greater than 97% similarity to each other. All results were compiled using Excel 2016 of MS office (Microsoft, Redmond, WA, USA) for Windows. Alpha diversity indices, including Shannon, Chao index, were calculated using the Kruskal–Wallis test. Principal component analysis (PCA) was conducted using the singular value decomposition method. Data visualization and statistical analysis was performed using the R software R v4.3.1 and the following packages: ggplot2, pheatmap, ggfortify, autoplot, and ggpubr. Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al. 2011) was employed to determine taxonomic differences among the four bat species using the EzBioCloud Apps. Permutational multivariate analysis of variance (PERMANOVA) analysis, a non-parametric multivariate statistical method, was used to assess the significance of differences in microbial communities (Xia and Sun 2017), with p-values < 0.05 considered significant.

Results

Taxonomy

A total of 3,634,385 sequences successfully passed all quality control filters, with a range of 8,531 to 92,883 sequences per sample (median 43,370, mean 43,266). The median good's coverage was 97.9%. The fecal microbiota community of 84 bats was examined, and a total of eight phylum levels were identified. Among these, Proteobacteria dominated in the four species overall. *Myotis bombinus* was primarily dominated by Firmicutes (47.24%) and Proteobacteria (42.66%), and *M. fuliginosus* (82.78%), *M. macrodactylus* (78.04%), and *R. ferrumequinum* (63.46%) were primarily dominated by Proteobacteria (Table 2). At the genus level, a total of 96 genera were found, and *M. bombinus*, *M. macrodactylus*, *M. fuliginosus*, and *R. ferrumequinum* exhibited differences in their microbial communities (Fig. S1). The heatmap analysis results showed that *Clostridium* (Firmicutes) and *Asaia* (Proteobacteria) distinguished the microbial communities of these four bat species at the genus level (Fig. 2).

Alpha diversity

Richness and evenness were examined using the Chao and Shannon indices, which are alpha diversity indices, to compare the community diversity of fecal microbiota among bat species (Fig. 3A). There was no significant difference in Chao, an indicator of species richness, among the four bat species ($p > 0.05$), but a significant difference between *M. bombinus* and *M. fuliginosus* ($p < 0.05$) was observed in the Shannon index.

Beta diversity

PCA, based on the abundance of sequences at the genus level, revealed a clustering within the fecal microbiota community. It showed differences among bat species (Fig. 3B), but no regional differences were observed (Fig. S2). The first primary components, PC1 and PC2, accounted for 29.51% and 14.83% of the variance, respectively. This showed differences in bacterial community composition, with *M. bombinus* being distinct from other bat species and closely associated with *Clostridium* as the primary factor. In contrast, *M. fuliginosus*, *M. macrodactylus*, and *R. ferrumequinum* were clustered based on *Asaia* and Enterobacteriaceae_g.

Table 2 The mean relative abundance of fecal microbiota in bat species at the phylum level

Phylum	Mean relative abundance (%)			
	Myotis bombinus (n = 11)	*Myotis macrodactylus* (n = 13)	*Miniopterus fuliginosus* (n = 49)	*Rhinolophus ferrumequinum* (n = 11)
Actinobacteria	-	1.09	2.87	2.05
Bacteroidetes	-	1.98	3.24	3.21
Chlamydiae	2.84	6.52	2.91	-
Firmicutes	47.24	7.99	6.02	2.55
Fusobacteria	-	-	-	1.93
Proteobacteria	42.66	78.04	82.78	63.46
Synergistetes	1.16	-	-	1.35
Tenericutes	4.43	3.76	1.95	25.31
Etc. (under 1% in average)	1.67	0.62	0.24	0.14

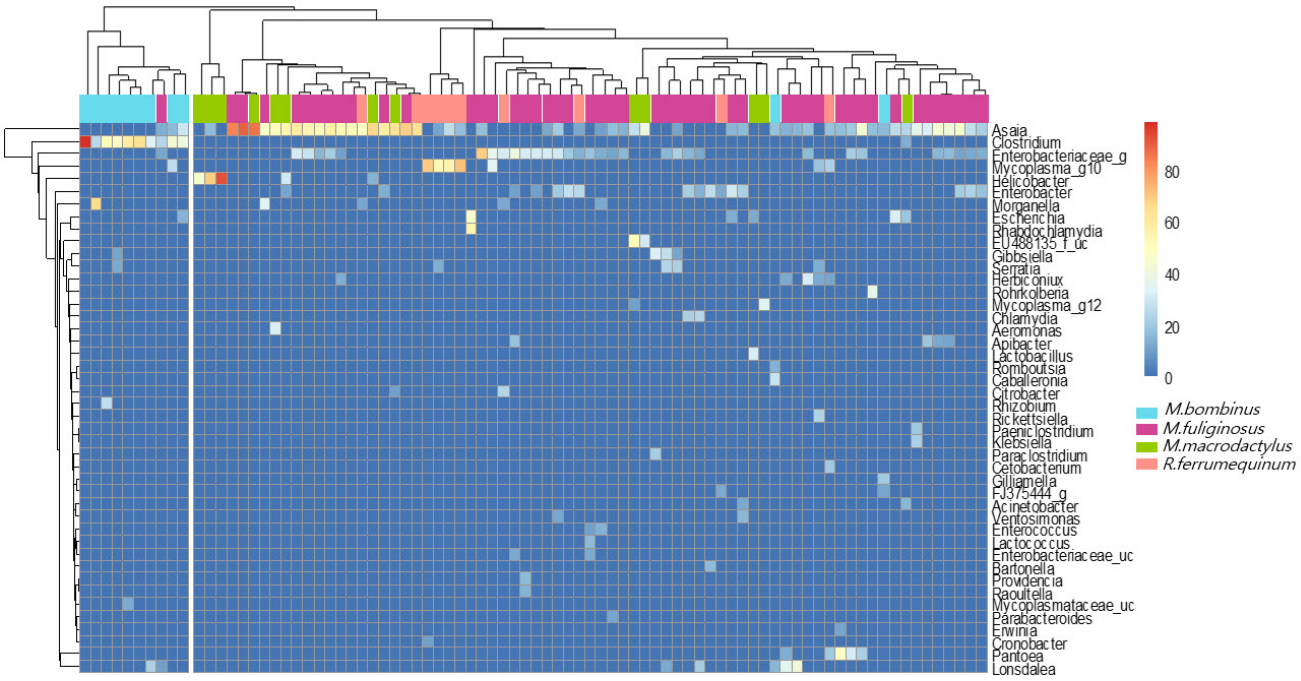

Fig. 2 Relative abundance heatmap at the genus level. Relative abundance (over 10%) is represented as a percentage value, with the color scale indicating relative abundance (%).

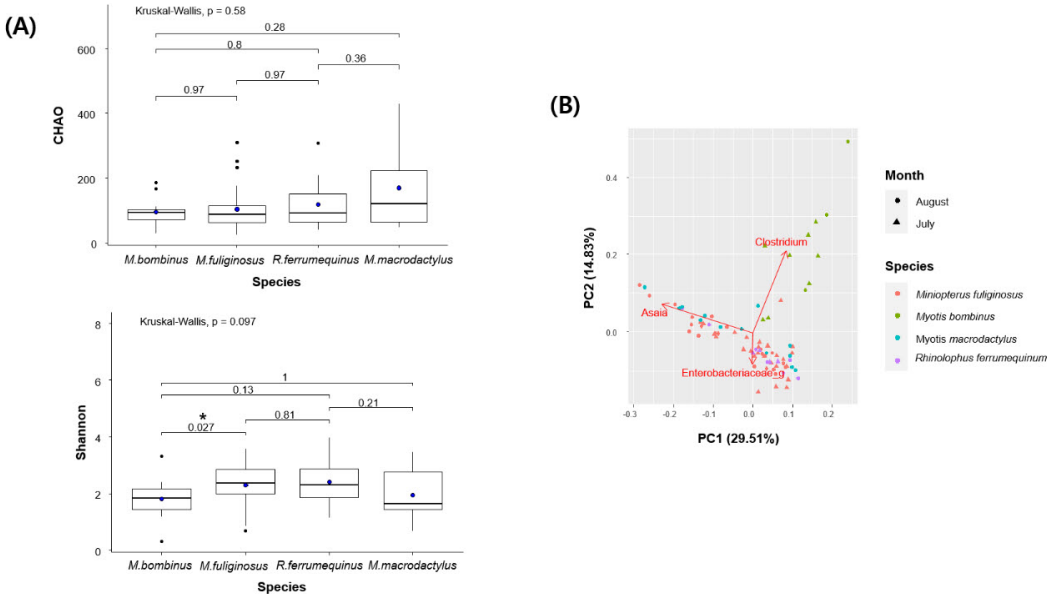

Fig. 3 Comparison of differences in fecal microbial community among the four bat species at the genus level. (A) Alpha diversity was calculated using the Chao and Shannon indices (*$p < 0.05$). (B) Principal component analysis based on the percentage contribution of the fecal bacterial microbiota. First two axes explained 29.51% and 14.83% of total variance, respectively.

Linear discriminant analysis effect size

The LEfSe was performed using relative expression information to select microbial markers. The LDA score threshold was set at 3.0 and filtered based on fales discovery rate corrected *p*-value ($p < 0.05$) (Table 3). Nineteen species were selected at the genus level in order of highest LDA score. Among common zoonotic pathogens the genera, *Clostridium* and *Mycoplasma*_g10 are *M. bombinus* (LDA score 5.314) and *R. ferrumequinum* (LDA score 5.095), respectively (Dimkić et al. 2021).

Discussion

Numerous studies have previously identified pathogens as common residents in guano (Gerbáčová et al. 2020; Veikkolainen et al. 2014; Wolkers-Rooijackers et al. 2019). As the characteristics of the host's intestinal microbial

Table 3 Selected taxonomic markers at the genus level

Taxon name	LDA score	*p*-value	Relative abundance (%)			
			Myotis bombinus	*Myotis macrodactylus*	*Miniopterus fuliginosus*	*Rhinolophus ferrumequinum*
Clostridium	5.314	1.57×10^{-8}	43.209	1.231	0.614	0.000
Mycoplasma_g10	5.095	8.27×10^{-6}	2.345	0.008	1.451	24.727
Helicobacter	5.007	0.00011	0.718	20.146	0.135	0.182
Enterobacteriaceae_g	4.789	7.22×10^{-8}	1.355	2.608	14.831	7.755
Enterobacter	4.541	0.00036	0.864	2.231	7.729	6.555
EU488135_f_uc	4.497	0.00349	0.000	6.138	0.010	0.000
Cronobacter	4.161	0.01897	0.018	0.531	0.549	2.673
Herbiconiux	4.167	0.00874	0.336	0.008	2.704	1.800
Citrobacter	4.158	0.01117	0.000	0.992	0.649	2.327
Serratia	4.143	0.00115	2.864	0.146	2.965	2.273
Acinetobacter	4.116	0.00006	0.055	2.592	0.416	0.055
Enterobacteriaceae_uc	3.981	0.00005	0.145	1.323	2.092	1.873
Aeromonas	3.975	0.00035	0.000	2.500	0.006	0.009
Romboutsia	3.975	0.00704	1.500	0.008	0.092	0.336
Chlamydia	3.945	0.02031	0.000	0.000	1.510	0.000
Gilliamella	3.826	0.00004	1.873	0.008	0.133	0.000
Amoebophilaceae_uc	3.655	0.00058	0.000	0.031	0.002	0.982
Vespertiliibacter	3.287	3.13×10^{-6}	0.255	0.338	0.024	0.036
Rosenbergiella	3.105	0.00637	0.000	0.031	0.047	0.209

Linear discriminant analysis (LDA) effect size identified differentially relative abundance of the four bat species (LDA > 3, $p < 0.05$).

community are closely linked to their food resources, it can offer insights into changes in the host's ecological characteristics and habitat environment (Gong et al. 2021). Seasonal variations in diet can influence the diversity of gut microbes in animals, as observed in *R. ferrumequinum* (Xiao et al. 2019). Therefore, the composition of intestinal microbes in bats can help explain their ecological niche differences between species and changes in seasonal food resources. Bats serve as an excellent study system for exploring the role of microbes in shaping host physiology, evolution, and fitness due to their taxonomic, ecological, and dietary diversity (Ingala et al. 2018). Bat guano may harbor potential pathogenic bacteria, which is essential to investigate. Common pathogenic bacteria in bat guano include *Bartonella*, *Borrelia*, *Leptospira*, *Campylobacter*, *Clostridium*, and *Bacillus* (Dimkić et al. 2021).

Previous studies have consistently found that Proteobacteria and Firmicutes dominate the microbial community of bats, distinguishing them from other terrestrial mammals where strictly anaerobic bacteria from the phylum Bacteroidetes are relatively rare (Lutz et al. 2019; Rizzatti et al. 2017; Song et al. 2020; Sun et al. 2020). Representatives from the phyla Proteobacteria and Firmicutes were detected as dominant groups in the fresh fecal samples collected from bats (Gong et al. 2021). Our results revealed that *M. bombinus* had a dominant presence of Firmicutes (47.24%) and Proteobacteria (42.66%), whereas *M. fuliginosus* (82.78%), *M. macrodactylus* (78.04%), and *R. ferrumequinum* (63.46%) were dominated by Proteobacteria. In this study, the feces of four insectivorous bat species primarily inhabiting caves revealed the presence of Proteobacteria and Firmicutes, similar to previous research findings. This result indicates a distinct difference from that of mammals. Regarding the alpha diversity at the genus level, no significant difference in richness was observed, which indicates the abundance of bacterial communities in feces, among the four bat species. However, a significant difference in the Shannon index of evenness was observed between *M. bombinus* and *M. fuliginosus* ($p < 0.05$). PCA results from the beta diversity analysis revealed that *M. bombinus* differed from the other three bat species in terms of bacterial community composition. The presence of *Clostridium* (Firmicutes), *Asaia* (Proteobacteria), and Enterobacteriaceae_g (Proteobacteria) emerged as the main factors influencing this distinction. Although it is challenging to definitively attribute the differences in bacterial community composition to specific factors among bat species, it is possible that bats may be exposed to different bacteria through their habitat, food sources, and diet preferences (Vengust et al. 2018). *Clostridium* exists in all environments and can inhabit the intestines of both animals and humans, potentially posing a pathogenic risk even in the absence of overt disease symptoms (Rodriguez et al. 2016). In contrast, *Asaia* is associated with flowers and fruits, and is commonly found in Diptera, Hemiptera, Lepidoptera, and Hymenoptera that utilize plants. *Asaia bogorensis* has been identified as an opportunistic pathogen causing peritonitis and bacteremia in humans (Gonella et al. 2012; Rami et al. 2018). In particular, *Asaia* is present in high density in the intestines of female mosquitoes and the reproductive organs of males, leading to studies on the symbiotic microbial community in mosquitoes (Wilke and

Marrelli 2015). Although the genus level analysis identified potential pathogenic bacteria, further studies employing polymerase chain reaction and bacterial culture at the species level are necessary to obtain accurate information.

The microbial diversity of bat feces is influenced by various factors, including host diet, age, phylogeny, gut type, and the reproductive stage of the host (Ley et al. 2008; Phillips et al. 2021; Zhang et al. 2010). The bat species included in this study, *M. bombinus*, *M. macrodactylus*, *M. fuliginosus*, and *R. ferrumequinum* are insectivorous bats that primarily consume insects as their main food source, reflecting differences in food preferences among bat species (Hayes and Loeb 2007). Insectivorous bats exhibit varying ecological niches within their habitats depending on environmental conditions and feeding behavior, as well as their preference for different food resources. Siemers and Schnitzler (2004) found that five species of *Myotis mystacinus*, despite their similar external appearances, occupied different ecological niches owing to their distinct feeding spaces, such as forest edges, the air, and grasslands. Additionally, the feeding guild of bats display variations in wing shape and echolocation based on their ecological niches, which, in turn, impact their food resources (Zou et al. 2022). Despite limited information, we established a database on the fecal bacterial communities of four insectivorous bat species in South Korea, revealing differences among these species. Our results also indicate differences in the composition of the fecal bacterial community among *M. bombinus*, *M. macrodactylus*, *M. fuliginosus*, and *R. ferrumequinum*. However, our study was limited to specific periods and only analyzed bacterial communities in feces, indicating the need for further research.

In conclusion, insectivorous bats inhabit in South Korea; however, research on the bacterial communities in bat feces in South Korea is not well-known. Therefore, this study analyzed the bacterial communities in the feces of four insectivorous bat species. We obtained foundational data on the bacterial communities in the feces of four bat species and confirmed that the composition of bacterial communities varies among bat species. These findings suggest that food sources and environmental conditions may influence differences in the bacterial communities in bat feces. Our study highlights the importance of investigating the bacterial communities in bat feces to gain insights into the overall microbial communities, assess the health status of bats, and identify the presence of potential pathogenic bacteria. This research lays the foundation for potential pathogenic bacterial studies related to bat health and contributes to expanding research in this field. However, future studies should consider food sources, age, and environmental influences to investigate bacterial communities. Subsequent studies will follow the same population annually to investigate the relationship between bat bacterial communities, food sources, and their health.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.23.060.

Figure S1. Relative abundance of fecal microbiota in bat species at the genus level. Others of bacterial genera, each with a relative abundance below 1%. **Figure S2.** Principal component analysis (PCA) based on the percentage contribution of the fecal bacterial microbiota between the study sites.

Abbreviations

PCA: Principal component analysis

LDA: Linear discriminant analysis

LEfSe: Linear discriminant analysis effect size

Acknowledgements

Not applicable.

Authors' contributions

IA and BK developed the concept of this study. IA and SJ analyzed and interpreted data regarding bacterial communities in the feces. IA, KK, and TWL participated in the investigation. IA and BK major contributor in writing, review and editing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Research Planning Review Committee of the National Institute of Ecology (NIEIACUC-2021-001). All academic survey permission was granted by Mungyeong-si (No. 2022-00004, 28 January, 2022) and Seogwipo-si (No. 2022-2, 18 January, 2022).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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