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ORIGINAL ARTICLE

Microbial Communities in the Lungs of Bats in China

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ABSTRACT

Objective: Bats are the hosts of multiple pathogens, but the microbial composition of their lung tissues remains unknown. Our study investigated the species compositions and genera of important respiratory tract pathogenic bacteria in bat lung tissue.

Methods: A microbiota study was conducted in Hebei, Henan and Guizhou provinces in China. Lung tissues were collected from 104 healthy bats. The lung tissue was subjected to 16S ribosomal ribonucleic acid gene sequencing.

Results: We obtained 7,708,734 high-quality bacterial sequences from 104 healthy bats. Overall, the annotations indicated 55 phyla, 73 classes, 164 orders, 322 families and 953 genera. The lung microbiota was highly polymorphic and variable among bats from Hebei, Henan and Guizhou. The genetic characteristics of the main recognized respiratory pathogens in the samples were analyzed.

Conclusions: The findings indicate that the lungs of bats carry numerous bacteria with pathogenic importance. Pathogens disseminate through the respiratory tract in bats and are widely distributed among bats. Because bats prefer to inhabit areas placing them in close contact with humans, such as eaves and old buildings, further investigations are warranted to identify bat microbiota and their potential effects on humans.

Key words: microbial, 16S rRNA V3–V4, bats, lung tissue

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INTRODUCTION

Bats are among the most diverse and widely distributed mammals [1]. Because of their wide distribution, bats live in a variety of habitats, including caves, old buildings, trees and rock crevices, thus exposing them to various environments and microorganisms [2,3]. Owing to close interactions among bat species, bats are efficient carriers and natural hosts of many pathogens. In addition, bats have a long lifespan and reproduction rate [4], and can share habitats with humans [5]. Accelerated urbanization and destruction of the natural environment indirectly increases close human-animal contact, thereby increasing zoonotic disease risk. With the emergence of new infectious diseases, the relationship between bats and human disease has drawn substantial research attention [6-11]. Understanding the microbiome of bats is a very important and unexplored area of medical ecology. Studies on bat symbiotic bacteria have been conducted since the 1960s [12]. Research on bat microbiota at that stage was mainly based on culturing [13]. Those studies have been limited by the presence of large numbers of non-cultivable microorganisms. However, the development of sequencing technology has addressed this problem well.

Healthy lungs were previously believed to be sterile; consequently, lung tissue and airways were excluded from the list of organs at the beginning of the Human Microbiome Project [14]. However, later studies confirmed that the lower respiratory tract carries a similar density of bacteria to the upper small intestine [15,16], and healthy lungs are now generally accepted to have a microbiome. With deepening research on the pulmonary microbiota, studies have found that the microorganisms affect the occurrence of lung diseases [17]. In a study of the microbiota of bronchoalveolar lavage fluid, sputum and lung tissue, Marsh et al. have found the presence of a dynamic balance of microbial migration and elimination among various tissues [18]. Currently, studies on the animals' lower respiratory tract microbiota focus primarily on economically important animals, such as birds, cattle and horses [19-21], whose microbiota is much more diverse than previously expected, and notably includes bacteria that greatly influence animal and human health [22].

Despite many reports of infectious pathogenic microorganisms in bats worldwide [23], most studies have focused on the gut microbiota of bats [24,25], whereas little information is available on the microbial community in bat lungs. Consequently, we aimed to understand the microbiome of bat lung tissue.

MATERIALS AND METHODS

Sample collection

In the study, a total of 104 bat lung tissue samples were collected in October 2019 from caves in three regions of China: Hebei (HB, n = 64), Henan (HN, n = 20) and Guizhou (GZ, n = 20). The captured bats were identified by local experts on the basis of morphological characteristics and genetic identification [26], and all bats were confirmed to be Miniopterus fuliginosus. Through physical examination, all bats were considered healthy. To comply with laboratory animal management regulations, the bats were euthanized by cervical dislocation.

Extraction of genomic DNA and 16S rRNA gene amplification

The bats were dissected aseptically, and the lung tissues were homogenized: 1 ml of PBS was added to the lung tissue and vortexed, and DNA was extracted from an 200 μL aliquot; a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used for deoxyribonucleic acid (DNA) extraction. DNA concentration and purity were assessed on 1% agarose gels. Subsequently, the V3–V4 region of the 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using universal primers (F: 5′-CCTAYGGGRBGCASCAG-3′, R: 5′-GGACTACNNGGGTATCTAAT-3′) with a 6-bp barcode unique to each sample. The PCR products were detected with 2% agarose gel electrophoresis. After purification, the PCR products were sequenced on the Illumina HiSeq 2500 PE-250 platform.

Data analysis

The reads were compared with the Silva database (https://www.arb-silva.de/) [27] by using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) [28] to detect and remove chimeras [24]. Sequence analysis was performed in Uparse software (v7.0.1001, http://drive5.com/uparse/) [29]. Sequences with ≥97% homology were clustered into operational taxonomic units (OTUs). The OTU sequences were annotated with taxonomic information from the Silva database (v132; https://www.arbsilva.de/) [30], on the basis of the Mothur algorithm [31]. Alpha diversity was used to analyze the complexity of bat lung tissue species diversity, including Observed-species, Shannon, Chao1, ACE Simpson and phylogenetic diversity whole_tree (PD_whole_tree). Beta diversity was applied to analyze the microbial community composition through non-metric multidimensional scaling (NMDS) and principal co-ordinate analysis (PCoA). Alpha and beta diversity analyses were calculated with QIIME (v1.7.0) and displayed with R software (v2.15.3).

Data availability statement

The data generated in this study have been deposited in the NCBI BioProject repository (accession number PRJNA855058) and the BioSample database (accession numbers SAMN29480867 to SAMN29480970).

Ethical approval

The collection of bats for microbiological studies was approved by the ethical committee of Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention (No. ICDC-2019012).

RESULTS

Microbiota composition determination by OTU analysis

A total of 104 lung tissue samples were collected from bats from three locations (HN, HB and GZ) in China; 7,708,734 sequences were obtained (S1 Table), ranging in length from 403 to 425 bp (average, 410.9 ± 6.1 bp). Each sample yielded 50,354 to 100,735 reads (average, 74,122 reads). The 7,708,734 sequences were clustered into 10,372 OTUs, with an average of 1,190.1 ± 417.9 OTUs per sample. Overall, these 10,372 OTUs were annotated into 55 phyla, 73 classes, 164 orders, 322 families and 953 genera.

Bacterial profiles

A total of 68.33% of the total reads were assigned to 164 orders. The top ten orders with the highest RA accounted for most of the total (S1A Fig), including Clostridiales (23%), Bacteroidales (21%), Lactobacillales (14%), Enterobacteriales (4%), undentified_Gammaprotebacteria (3%), Ktedonobacterales (2%), Pseudomonadales (2%), Sphingomonadales (2%), Selenomonadales (2%) and Bacillales (1%).

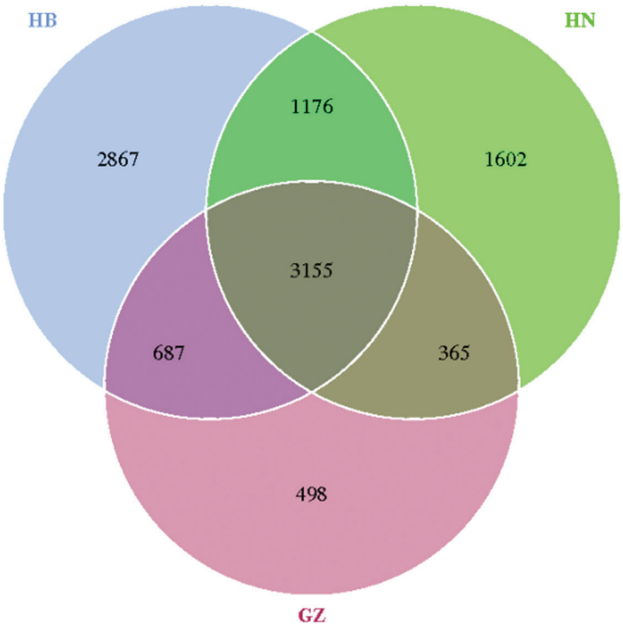

FIGURE 1 | A Venn diagram for the number of OTUs from 104 bats. Circles represent region groups, and numbers indicate the numbers of OTUs. Overlapping areas are shared between groups, and areas without overlap are unique to each group.

A total of 93.82% of the total reads were assigned to 322 families. The top ten families with the highest RA accounted for 60% of the total, and mainly included Streptococcaceae (12%), Peptostreptococcaceae (9%), Prevotellaceae (8%), Ruminococcaceae (7%), Lactobacillaceae (7%), Enterobacteriaceae (5%), Lachnospiraceae (4%), unidentified Clostridiales (3%), Mycoplasmataceae (3%) and Bacteroidaceae (2%). The distribution of the top 30 families is shown in S1B Fig.

A total of 96.38% of the total reads were assigned to 791 genera. The top ten genera with the highest RA accounted for 43% of the total, and mainly included *Lactococcus* (12%), *Paeniclostridium* (7%), *Lactobacillus* (7%), *Faecalibacterium* (3%), unidentified *Clostridiales* (3%), *Plesiomonas* (3%), *Bacteroides* (3%), *Mycoplasma* (2%), *Acinetobacter* (2%) and *Agathobacter* (1%). The distribution of the top 30 genera is shown in S1C Fig, and the RA of the 30 genera in each bat is shown in S2 Fig. The top 100 genus level species phylogenetic relationships are shown in S3 Fig.

Microbiota variation by location

A total of 10,372 OTUs were clustered by using a 97% identity threshold. The microbiota from bat lungs from

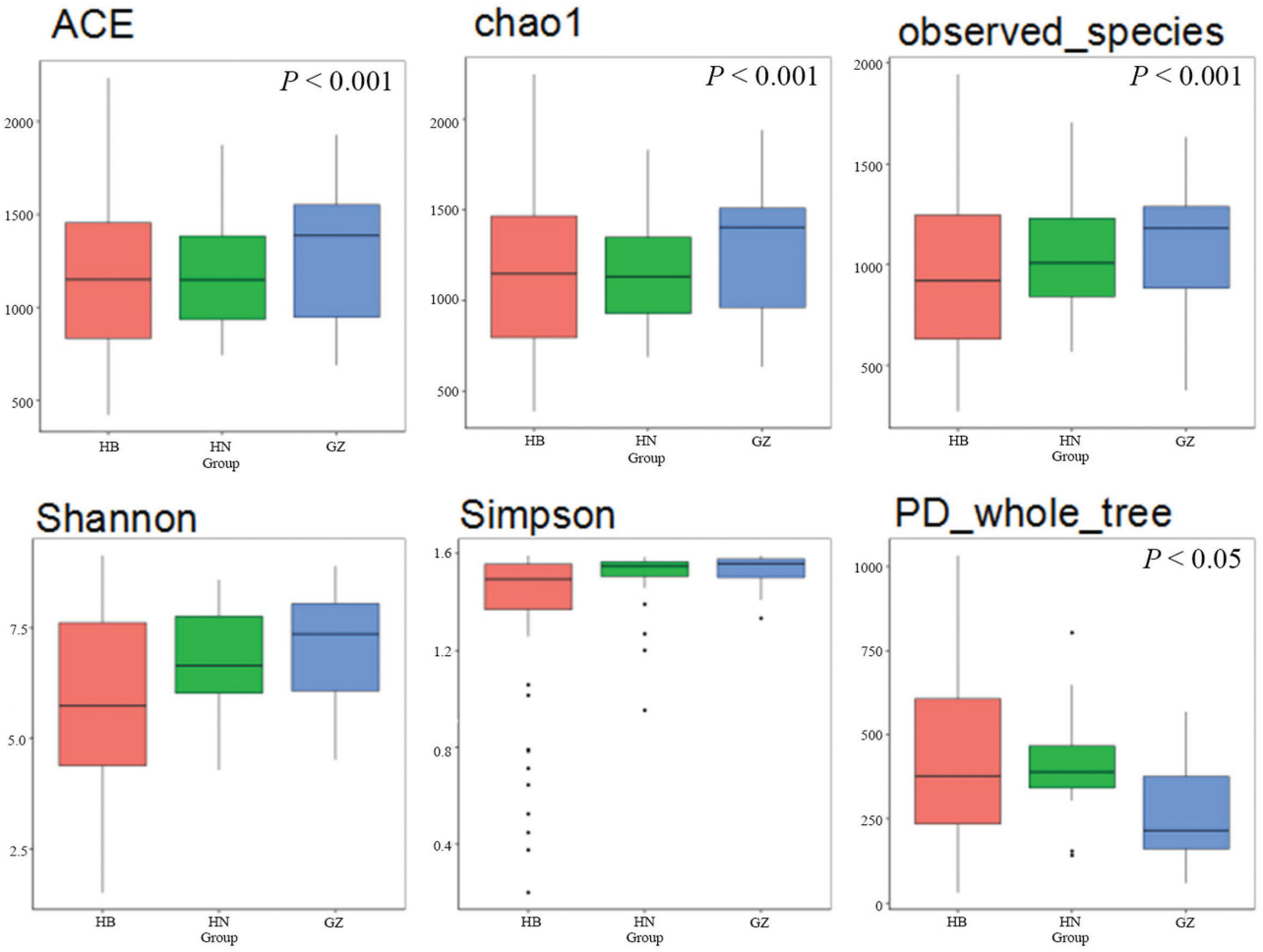

FIGURE 2 | Box plots of the six alpha diversity indices (ACE, Shannon, Chao1, Simpson, observed_species and PD_whole_tree) among bat samples from three regions.

the three locations included 3,155 common OTUs. HB samples contained 2,867 specific OTUs, HN samples contained 1,602 specific OTUs, and GZ samples contained 498 specific OTUs (Fig 1).

All six alpha diversity indices of lung tissue samples are shown in Fig 2. Significant differences in the complexity of species diversity were observed among the samples from HB, HN and GZ. Specifically, the observed-species ($P < 0.001$), ACE ($P < 0.001$) Chao1 ($P < 0.001$) and PD_whole-tree ($P < 0.05$) indexes showed significant differences. PCoA indicated that the composition of the microbiota significantly differed among locations (Fig 3A). No significant differences in the composition of the microbiota were observed for NDMS analysis in GZ and HN, but significant differences between HB and the other two locations were observed (Fig 3B).

The relationships between the regions and the microorganisms at the phylum level were determined through Spearman correlation analysis (Fig 4). In the figure, samples from each region had unique dominant bacteria. For example, HB samples showed a higher abundance of Fusobacteria, represented by Cetobacterium and Tenericutes, on the basis of Mycoplasma. Moreover, bat lung tissue samples that contained more Oxyphotobacteris, represented by unidentified Oxyphotobacteria, were more likely to come from HN. GZ samples were more likely to contain Acidobacteria, on the basis of Bryobacter and Bifidobacterium-based Actinobacteria. After further analysis, geographical location was found to have a significant effect on the diversity of the lung microbiota at the genus level in bats (Fig 5). Compared with HN and GZ samples, HB samples had higher levels of *Paeniclostridium*, *Mycoplasma*, *Plesiomonas*, *Lactococcus*, *Lactobacillus* and *Acinetobacter*. HN samples had higher levels of *Marinobacterium*, unidentified *Oxyphotobacteria* and unidentified *Enterobacteriaceae*. The compositions of the samples from both GZ and HN were highly similar, in agreement with the results of PCoA and NMDS analysis. Compared with the other two groups, GZ samples contained more *Bacteroides, Megamonas, Alistipes*.

Respiratory pathogen profile

In this study, the characteristics of the main recognized respiratory pathogens in bat samples were analyzed. In 104 bat lung tissue samples, nine candidate causative respiratory pathogens were detected: *Streptococcus*, *Neisseria*, *Orthomonas*, unidentified *Clostridium*, *Aeromoas*, *Vibrio*, *Acinetobacter*, *Morganella* and *Botox* (S4 Fig). Overall, the abundance of pathogenic bacteria in Hebei samples was higher than that in the other two groups. Hebei samples had higher levels of *Acinetobacter*, *Botox* and *Orthomonas* than samples from Henan and Guizhou. Henan samples had higher levels of *Vibrio* than the other two groups. The samples from Guizhou and Hebei generally contained unidentified *Clostridium*, and the highest levels were found in samples Hebei 30, 31 and 32. However, the genera *Streptococcus*, *Neisseria*, *Morganella* and *Aeromoas* were found in samples from various regions.

DISCUSSION

Bats are unique among mammals because of their biological and immunological characteristics. Bats provide unique niches for many pathogenic bacteria to co-evolve with them. With the increased density of humans and rapid development of modern transportation, the spread

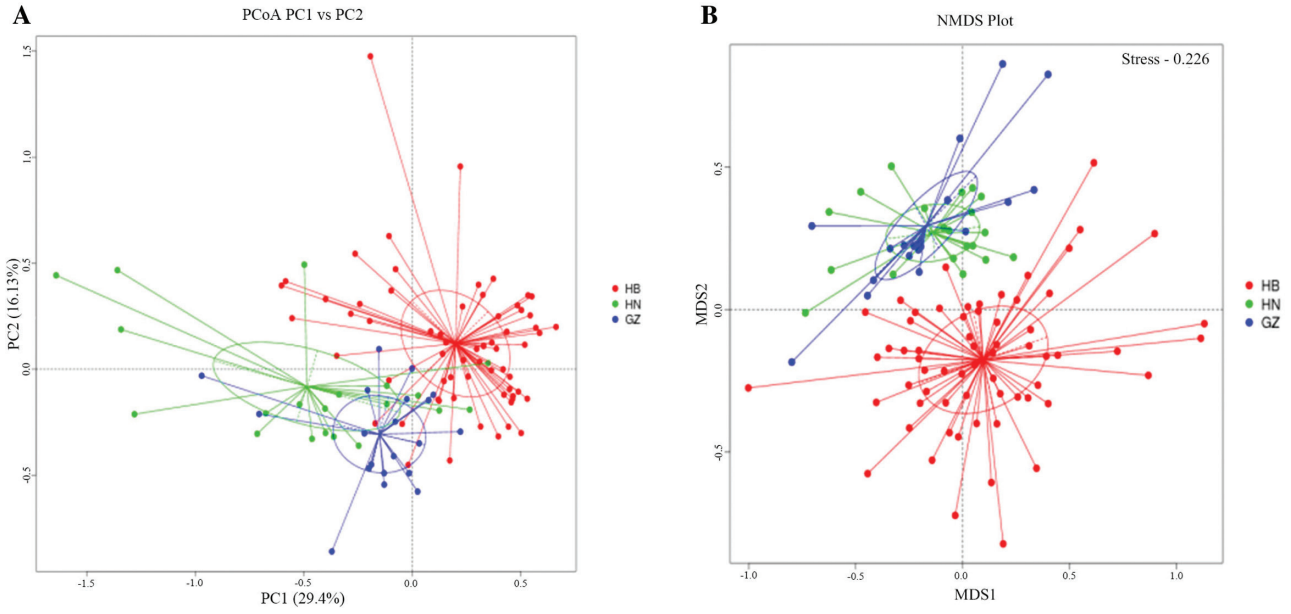

FIGURE 3 | Composition of the microbiota in samples from bat lung tissue. (A) Principal coordinate analysis. (B) Non-metric multidimensional scaling analysis.

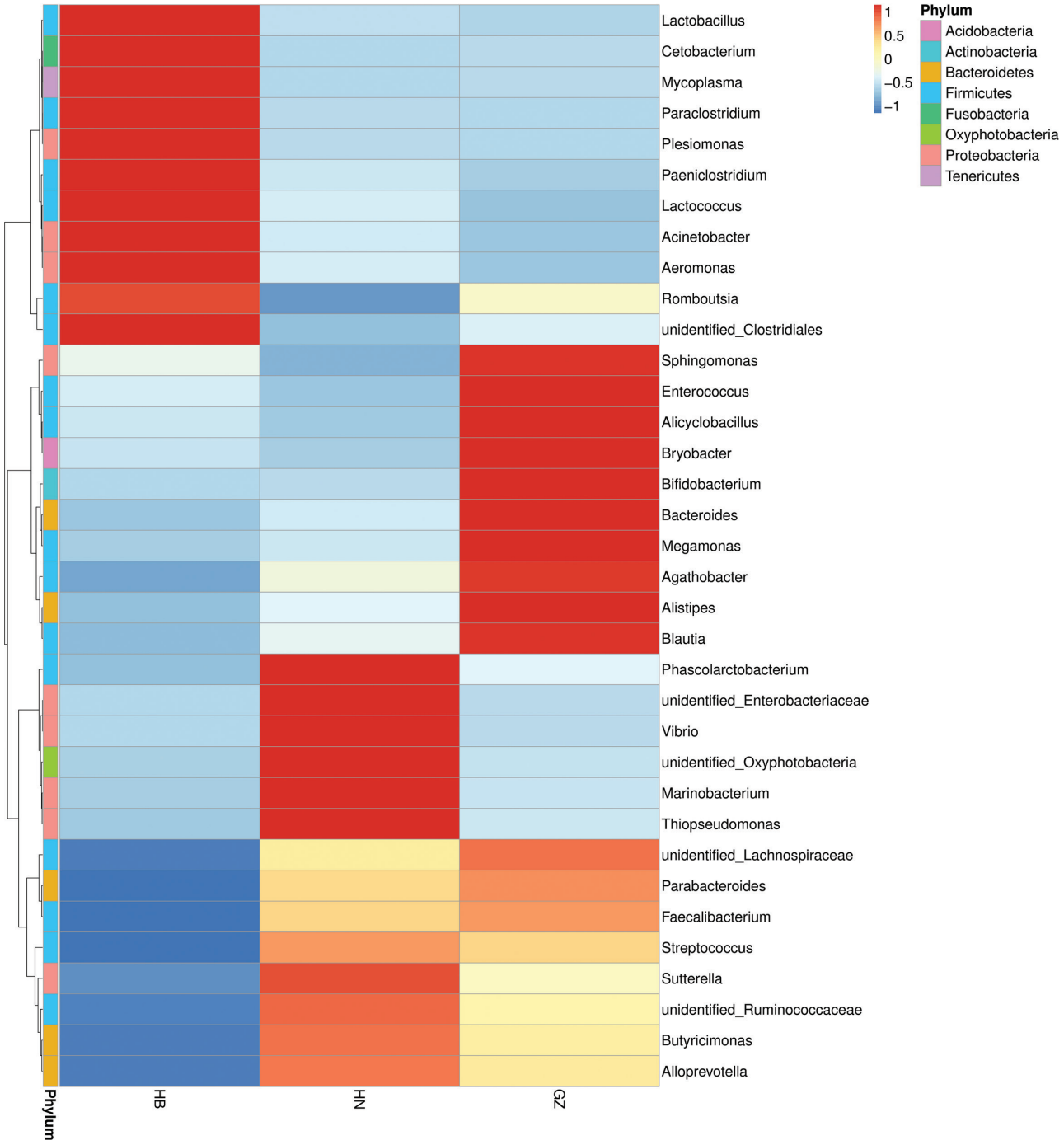

FIGURE 4 | Heat map showing the relationships among regions and the microbiota at the phylum level.

of bat-borne pathogenic bacteria to humans may cause diseases and social panic. In this study, we performed 16S rRNA amplicon sequencing to comprehensively detect of pathogenic microorganisms from 104 bat lung tissue samples. A substantial number of bacterial sequencing reads was detected in 27 of the 104 bats [32]. Of six alpha diversity indices, four showed differences among samples from Hebei, Henan and Guizhou. PCoA indicated that the composition of microbiota in Guizhou samples was highly diverse (Fig 3). Furthermore, nine genera containing candidate causative respiratory pathogens (*Streptococcus*, *Neisseria*, *Orthomonas*, unidentified *Clostridium*, *Aeromoas*, *Vibrio*, *Acinetobacter*, *Morganella* and *Botox*) were detected in the samples. The abundance of pathogenic bacteria, particularly *Acinetobacter*, *Botox*, unidentified *Clostridium* and *Orthomonas*, was higher in Hebei samples. Among the pathogenic bacteria, *Streptococcus* and *Neisseria* widely colonize the respiratory tract in humans and animals [33]. Additionally, new species were isolated from bats, but no evidence has indicated that these bacteria can be transmitted from bats to humans [34,35].

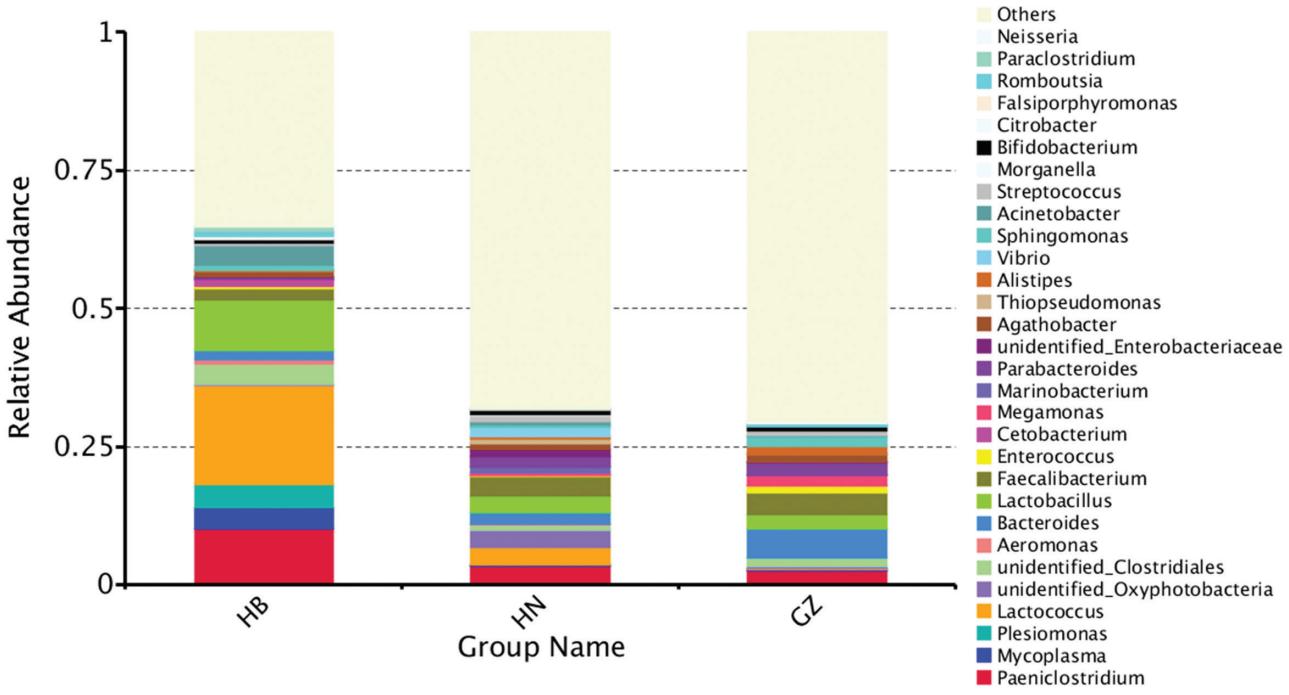

FIGURE 5 | Histogram of the stacking showing the RA of bacterial populations at the genus level in bat lung tissues from different regions.

Because this study is largely descriptive, further studies are needed to understand the differences in the structure of bats' lung microbiota and the surrounding environment. Future work would enhance the ability to culture representative pathogens and explore the differences in bacterial composition between bat lung tissue and the human body, focusing on the abundance of pathogenic bacteria, to find methods to prevent and manage clinical respiratory diseases.

In summary, we report the presence of many species of bacteria in samples of bat lung tissues, including nine genera of pathogenic bacteria. Because the habitat of bats overlaps with a range of human activities, more attention must be paid to the problem of respiratory infections. Measures should be taken to minimize contact of humans with bats and to promote respiratory safety during outings.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Avena CV, Parfrey LW, Leff JW, Archer HM, Frick WF, Langwig KE, et al. Deconstructing the bat skin microbiome: influences of the host and the environment. Front Microbiol. 2016;7:1753.
2. Wilder AP, Frick WF, Langwig KE, Kunz TH. Risk factors associated with mortality from white-nose syndrome among hibernating bat colonies. Biol Lett. 2011;7(6):950-953.
3. Gorbunova V, Seluanov A, Kennedy BK. The world goes bats: living longer and tolerating viruses. Cell Metab. 2020;32(1):31-43.
4. Marrotte RR, Gonzalez A, Millien V. Landscape resistance and habitat combine to provide an optimal model of genetic structure and connectivity at the range margin of a small mammal. Mol Ecol. 2014;23(16):3983-3998.
5. Dimkic I, Fira D, Janakiev T, Kabić J, Stupar M, Nenadić M, et al. The microbiome of bat guano: for what is this knowledge important? Appl Microbiol Biotechnol. 2021;105(4):1407-1419.
6. Ciminski K, Ran W, Gorka M, Lee J, Malmlov A, Schinköthe J, et al. Bat influenza viruses transmit among bats but are poorly adapted to non-bat species. Nat Microbiol. 2019;4(12):2298-2309.
7. Fan H, Walker AP, Carrique L, Keown JR, Martin IS, Karia D, et al. Structures of influenza A virus RNA polymerase offer insight into viral genome replication. Nature. 2019;573(7773):287-290.
8. Centers for Disease Control and Prevention. Update: outbreak of severe acute respiratory syndrome--worldwide, 2003. MMWR Morb Mortal Wkly Rep. 2003;52(12):241-246, 248.
9. Lu G, Liu D. SARS-like virus in the Middle East: a truly bat-related coronavirus causing human diseases. Protein Cell. 2012;3(11):803-805.
10. Luis AD, Hayman DTS, O'shea TJ, Cryan PM, Gilbert AT, Pulliam JRC, et al. A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? Proc Biol Sci. 2013;280(1756):20122753.
11. Pigott DM, Golding N, Mylne A, Huang Z, Henry AJ, Weiss DJ, et al. Mapping the zoonotic niche of Ebola virus disease in Africa. Elife. 2014;3:e04395.
12. Klite PD. Intestinal bacterial flora and transit time of three neotropical bat species. J Bacteriol. 1965;90:375-379.
13. Heard DJ, De Young JL, Goodyear B, Ellis GA. Comparative rectal bacterial flora of four species of flying fox (Pteropus sp.). J Zoo Wildl Med. 1997;28(4):471-475.
14. Dickson RP, Erb-Downward JR, Huffnagle GB. The role of the bacterial microbiome in lung disease. Expert Rev Respir Med. 2013;7(3):245-257.

15. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossely C, et al. Disordered microbial communities in asthmatic airways. PLoS One. 2010;5(1):e8578.
16. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Falkowski NR, Huffnagle GB, et al. Bacterial topography of the healthy human lower respiratory tract. mBio. 2017;8(1):e02287-16.
17. Mammen MJ, Scannapieco FA, Sethi S. Oral-lung microbiome interactions in lung diseases. Periodontol 2000. 2020;83(1):234-241.
18. Marsh RL, Kaestli M, Chang AB, Binks MJ, Pope CE, Hoffman LR, et al. The microbiota in bronchoalveolar lavage from young children with chronic lung disease includes taxa present in both the oropharynx and nasopharynx. Microbiome. 2016;4(1):37.
19. Bond SL, Timsit E, Workentine M, Alexander T, Leguillette R. Upper and lower respiratory tract microbiota in horses: bacterial communities associated with health and mild asthma (inflammatory airway disease) and effects of dexamethasone. BMC Microbiol. 2017;17(1):184.
20. Nicola I, Cerutti F, Grego E, Bertone I, Gianella P, D'Angelo A, et al. Characterization of the upper and lower respiratory tract microbiota in Piedmontese calves. Microbiome. 2017;5(1):152.
21. Shabbir MZ, Malys T, Ivanov YV, Park J, Shabbir MAB, Rabbani M, et al. Microbial communities present in the lower respiratory tract of clinically healthy birds in Pakistan. Poult Sci. 2015;94(4):612-620.
22. Man WH, de Steenhuijsen Piters WA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. Nat Rev Microbiol. 2017;15(5):259-270.
23. Muhldorfer K. Bats and bacterial pathogens: a review. Zoonoses Public Health. 2013;60(1):93-103.
24. Sun DL, Gao YZ, Ge XY, Shi ZL, Zhou NY. Special features of bat microbiota differ from those of terrestrial mammals. Front Microbiol. 2020;11:1040.
25. Yin Z, Sun K, Li A, Sun D, Li Z, Xiao C, et al. Changes in the gut microbiota during Asian particolored bat (Vespertilio sinensis) development. PeerJ. 2020;8:e9003.
26. Hebert PDN, Cywinska, A, Ball SL, deWaard JR. "Biological identifications through DNA barcodes." Proc Biol Sci. 2003;270(1512):313-321.
27. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-D596.
28. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27(16):2194-2200.
29. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 2011;21(3):494-504.
30. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996-998.
31. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792-1797.
32. Li B, Zhang X, Guo F, Wu W, Zhang T. Characterization of tetracycline resistant bacterial community in saline activated sludge using batch stress incubation with high-throughput sequencing analysis. Water Res. 2013;47(13):4207-4216.
33. Helmick KE, Heard DJ, Richey L, Finnegan M, Ellis GA, Nguyen A, et al. A Pasteurella-like bacterium associated with pneumonia in captive megachiropterans. J Zoo Wildl Med. 2004;35(1):88-93.
34. Takada K, Hirasawa M. Streptococcus dentirousetti sp. nov., isolated from the oral cavities of bats. Int J Syst Evol Microbiol. 2008;58(Pt 1):160-163.
35. Bandelj P, Knapic T, Rousseau J, Podgorelec M, Presetnik P, Vengust M, et al. Clostridioides difficile in bat guano. Comp Immunol Microbiol Infect Dis. 2019;65:144-147.