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Seasonal assembly of skin microbiota driven by neutral and selective processes in the greater horseshoe bat

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Abstract

Skin microbiota play an important role in protecting bat hosts from the fungal pathogen *Pseudogymnoascus destructans*, which has caused dramatic bat population declines and extinctions. Recent studies have provided insights into the bacterial communities of bat skin, but variation in skin bacterial community structure in the context of the seasonal dynamics of fungal invasion, as well as the processes that drive such variation, remain largely unexplored. In this study, we characterized bat skin microbiota over the course of the bat hibernation and active season stages and used a neutral model of community ecology to determine the relative roles of neutral and selective processes in driving microbial community variation. Our results showed significant seasonal shifts in skin community structure, as well as less diverse microbiota in hibernation than in the active season. Skin microbiota were influenced by the environmental bacterial reservoir. During both the hibernation and active season stages, more than 78% of ASVs in bat skin microbiota were consistent with neutral distribution, implying that neutral processes, that is, dispersal or ecological drift contributing the most to shifts in skin microbiota. In addition, the neutral model showed that some ASVs were actively selected by the bats from the environmental bacterial reservoir, accounting for approximately 20% and 31% of the total community during hibernation and active season stages, respectively. Overall, this research provides insights into the assemblage of bat-associated bacterial communities and will aid in the development of conservation strategies against fungal disease.

KEYWORDS
active season, environmental bacterial reservoir, hibernation, neutral model, *Pseudogymnoascus destructans*, skin microbiota

1 | INTRODUCTION

Host-associated bacterial communities play an essential role in protecting hosts from pathogens by modulating and contributing to innate immune defences (Gensollen et al., 2016; Thaiss et al., 2016). In some adverse or stressful environmental conditions, host immune function may be downregulated and the microbiota may serve as the main defence against invading pathogens (Kaltenpoth & Engl, 2014; Koehler et al., 2013). Conversely, the disruption of resident bacterial taxa, a process known as dysbiosis, can cause the host to be more susceptible to pathogen infection and will eventually negatively impact host health (Khosravi & Mazmanian, 2013).

White-nose syndrome (WNS) is an emerging infectious disease in hibernating bats caused by the fungal pathogen *Pseudogymnoascus destructans* (*Pd*), which infects bats' dermal tissues and has caused dramatic population collapses in North America (Hoyt et al., 2021;

Lorch et al., 2011; Meteyer et al., 2009; Minnis & Lindner, 2013). Interestingly, not all bat species have experienced declines, and specific bacteria are likely responsible for disease resistance within some populations, especially considering that bacterial isolates from bat skin can inhibit the growth of *Pd* (Grisnik et al., 2020; Hoyt et al., 2015; Micalizzi et al., 2017). Moreover, bacterial additions to bat skin can ameliorate the symptoms of WNS (Hoyt et al., 2019). Such bacterial protection has been associated with the production of particular bacterial metabolites such as phenazine-1-carboxylic acid (Li, Li, Hoyt, et al., 2022). In addition, with respect to *Pd*, infection prevalence and fungal loads exhibit seasonal transmission dynamics that may be driven by changes in host physiology, specifically hibernation (Langwig et al., 2015). Given the defensive roles of bacterial communities, seasonal variation of these skin microbiota may be responsible for such disease dynamics, as in previous studies on the cutaneous fungal pathogen *Batrachochytrium dendrobatidis*, which caused the decline and extinction of amphibian populations (Longo et al., 2015; Rebollar et al., 2020). Therefore, understanding the response of skin microbiota to natural epidemics of known infectious pathogens is critical for providing information concerning the host's ability to maintain its health in the face of disease. Despite recent advances in our understanding of the potential role of skin microbiota in patterns of resistance and susceptibility to *Pd* (Ange-Stark et al., 2019; Lemieux-Labonté et al., 2017, 2020), we still have a limited understanding of seasonal dynamics of the community structure and function of bat skin microbiota in the context of *Pd* infection.

Disentangling the processes regulating the variation in bacterial community structure is a significant step towards achieving an integrated understanding of community assembly and is a key goal of microbial ecology research, especially considering the relevance of microbial communities to animal health. The variation in bacterial community structure could be driven through deterministic and stochastic processes (Kohl, 2020; Nemergut et al., 2013; Vellend, 2010). Deterministic processes arise as a result of the filtering of bacteria via ecological selection, which affects the fitness of the bacteria and thus determines the composition of the bacterial community. For example, particular sets of biotic and abiotic conditions, such as elevation and temperature, have the potential to constrain the distribution of taxa (Bletz et al., 2017; Li et al., 2019). However, the identification of the factors that affect deterministic process remains a fundamental challenge. In contrast, stochastic processes involve ecological drift and random dispersal between the environmental bacterial reservoir and hosts, which are not the result of environmental factors determined adaptations. Actually, the importance of stochastic processes in influencing bacterial community structure is not emphasized, mainly because it is technically challenging, in part due to the difficulty in the methods used to define stochasticity. The neutral model of community ecology provides an ideal approach to understanding the stochastic and deterministic processes that determine bacterial community assembly. Neutral (stochastic) processes, including passive dispersal and ecological drift, assume an equal opportunity for all bacteria to spread from a source pool to the host and to be lost or removed from the host. Selective (deterministic) processes, including positive or negative selection, are forces that drive host-associated bacterial colonization and survival due to preference for species with different fitness. Thus far, the neutral model has successfully been applied to the understanding of bacterial community assembly, including fish, horses, amphibians and humans, and has been increasingly recognized for its neutral processes in shaping bacterial community assembly (Fountain-Jones et al., 2020; Heys et al., 2020; Stothart et al., 2021; Venkataraman et al., 2015; Wilber et al., 2020). However, the ecological processes that regulate bacterial community assembly on chiropteran skin are poorly understood. Especially for bats under the pressure of *Pd* invasion, whether the host undergoes selection for specific taxa from the environment to provide resistance to pathogens needs to be further explored.

In this study, we sampled *Rhinolophus ferrumequinum*, a widespread bat species in China that has shown high resistance to *Pd* infection (Hoyt et al., 2020), to explore the seasonal dynamics of the structure and function of skin microbiota, and we sought to determine which processes drive skin microbiota assembly over a longitudinal time course spanning approximately 1 year. Specifically, our goals were to (1) investigate whether the annual hibernation cycle affects bat skin microbiota, (2) examine the relationship of skin microbiota with environmental bacterial reservoir and (3) determine the importance of neutral and selective processes in shaping bat skin microbiota.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We collected epidermal fungal, bacterial and environmental samples from a wild population of *R. ferrumequinum* in Jilin Province between December 2017 and October 2018 with a longitudinal time course that spanned two physiological stages of bats, namely the hibernation and active season stages. In total, we collected 44 bat skin samples during the active season stage, of which 3 and 11 samples were collected in June and July (summer) and 15 and 15 samples in September and October (fall), respectively. The hibernation swab samples were from our previously published data for *R. ferrumequinum* spanning multiple periods, including early winter hibernation (9 individuals in December), middle winter hibernation (20 individuals in January and 14 individuals in February) and late winter hibernation (20 individuals in early April; Li, Li, Dai, et al., 2022). Additionally, we collected 40 environmental samples during the hibernation and active season stages. The detailed sampling information at each time point is summarized in Table 1. All sample collection methods were approved by the Laboratory Animal Welfare and Ethics Committee of Jilin Agricultural University.

During the summer and fall active seasons, adult bats with sealed epiphyseal gaps were captured using ultra-high-pressure sterilized (121°C/30 min) nets during each collection event as bats flew out of

TABLE 1 Sample size of skin bacterial community and environmental samples as well as sampling time point information in this study.

Sampling dates	Season	Stage	Bat samples	Environmental samples	Temperature (°C)
Dec 5, 2017	Early winter	Hibernation	9	5	8.3 ± 0.39
Jan 17, 2018	Middle winter	Hibernation	20	5	8.02 ± 0.26
Feb 27, 2018	Middle winter	Hibernation	14	5	8.31 ± 0.13
Apr 8, 2018	Late winter	Hibernation	20	5	8.67 ± 0.16
Jun 15, 2018	Summer	Active season	3	5	24.9 ± 1.51
Jul 19, 2018	Summer	Active season	11	5	29.4 ± 1.94
Sep 20, 2018	Fall	Active season	15	5	26.5 ± 1.33
Oct 19, 2018	Fall	Active season	15	5	18.9 ± 4.02

cave at night and removed from the nets with clean latex gloves. A fresh pair of sterile latex gloves was worn for each animal to avoid cross-contamination of *Pd* or bacteria. We sampled bats by dipping sterile polyester swabs in sterile water and then swabbing five times along the forearm and muzzle for *Pd* detection according to the methods of previous studies (Hoyt et al., 2016, 2020). Skin microbiota samples were collected by swabbing the propatagium (the patagium present from the neck to the first digit) and plagiopatagium (the portion found between the last digit and the hindlimbs) of the wing membrane, as these two body parts are directly exposed to environment, and swab tips were stored in individually labelled sterile tubes containing 500 μL of RNAlater (TIANGEN, Beijing, China). In addition, environmental samples were collected by swabbing the cave walls for 20 s in linear strokes (approx. 5 cm) using swabs without dipping them in sterile water according to the previous study and were preserved in the same way (Lemieux-Labonté et al., 2017). The temperature of bats was recorded by using a Fluke 62 MAX IR Thermometer (Fluke, Everette, WA, USA). After sampling, the bats were released together directly at the cave.

2.2 | Molecular methods and sequencing

We extracted DNA from fungal swab samples using Qiagen DNeasy blood and tissue kits (Qiagen, Hilden, Germany) and tested for the presence of *Pd* using qPCR with a cut-off of 50 cycles due to low-level infection intensity (Hoyt et al., 2016; Muller et al., 2013). All samples were run in duplicate, with eight negative controls (blanks) and one positive control (20 pg/uL) derived from the isolate *Pd* ATCC MYA-4855 as quantification standards on each plate. *Pd* loads were calculated according to the following formula: ng of *Pd* = −3.348*Ct + 22.049 (Langwig et al., 2015). All negative controls had no *Pd* detected during the entire experiment.

For bacterial swab samples, DNA was extracted from each bacterial swab using E.Z.N.A™ Mag-Bind Soil DNA kits (OMEGA Bio-Tek, Georgia, USA) following the manufacturer's instructions. Then, we amplified the V3-V4 region of the 16S rRNA gene with the universal primers 341F and 805R according to our previous study, and a unique combination of forward and reverse barcode sequences was added to 16S rRNA amplicons from each sample during this process (Li et al., 2022). The PCR products from each sample were quantified, pooled and subsequently sequenced by Illumina MiSeq (2 × 300 bp) at Sangon Biotech Co., Ltd. in Shanghai, China.

The total number of raw sequences generated was 10,508,899 (average 71,489 reads/sample [range from 41,299 to 140,357]) with an average length of 481 bp. Sequences were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2020.6; Bolyen et al., 2019). The demultiplexed paired-end reads were imported into QIIME2, and then the DADA2 plugin with default parameters (p-trunc-len-f 247 and p-trunc-len-r 240) was applied to quality filtering, denoising and removal of chimera and nonbacterial sequences, along with the generation of an amplicon sequence variant (ASV) table and representative sequences (Callahan et al., 2016). For diversity and compositional analysis, mitochondrial and chloroplastic sequences, as well as ASVs with a relative abundance less than 0.001%, were filtered out, resulting in a total of 11,723 ASVS remaining. The representative sequences were taxonomically assigned using QIIME2's feature-classifier classify-sklearn plugin, with a naïve Bayes classifier trained on the SILVA v.138 database (Quast et al., 2012). The align-to-tree-mafft-fasttree pipeline was used to build the phylogenetic tree (Price et al., 2010).

2.3 | Data analysis

We first used nonparametric Kruskal–Wallis tests to compare changes in *Pd* prevalence and loads over time, followed by Dunn's multiple comparisons test with Bonferroni correction in the *stat* and *FSA* packages of R.

To determine how the annual hibernation cycle affects the skin microbiota, the alpha diversity, including Shannon diversity, Faith's phylogenetic diversity and observed richness of each sample were computed by QIIME2's core-metrics-phylogenetic pipeline (–p-sampling-depth 20,451). The negative binomial generalized linear mixed model (since normal distributions of the data were rejected) of the *glmer.nb()* function or the linear mixed model of the *lmer()* function in R package *lme4* was used to compare alpha diversity between hibernation and active season stages, and significance

was tested with the *Anova()* function of the *car* package in R (Bates et al., 2015; Fox & Weisberg, 2018). Sampling time point was included in the models as a random effect. Variation in alpha diversity among sampling time points was also tested using nonparametric Kruskal–Wallis tests. Furthermore, Spearman's correlation analysis was used to test for an association between alpha diversity and *Pd* loads, and only infected individuals were included in this analysis. Then, we calculated Bray–Curtis dissimilarity matrices rarefied at 20,451 sequences/sample to measure the changes in beta diversity and conducted nonmetric multidimensional scaling (NMDS) in $k=2$ dimensions to visually represent the dissimilarity between samples using R package *phyloseq* (McMurdie & Holmes, 2013). The Bray–Curtis dissimilarity matrices are a standardized metric of compositional dissimilarity among ecological samples and are recommended as a robust measure of ecological distance for complex communities and so are widely used in skin microbiota studies (Avena et al., 2016; Council et al., 2016; Faith et al., 1987). Permutational multivariate analysis of variance (PERMANOVA) based on 999 permutations was implemented to assess differences in beta diversity between individuals from different stages and sampling time points using the *adonis()* function of the *vegan* package in R (Oksanen et al., 2020). Pairwise differences tests between each sampling time point with Bonferroni correction were implemented using the *pairwise. adonis()* function in R package *pairwiseAdonis* (Arbizu, 2017). The *betadisper()* function was used to evaluate the homogeneity of dispersion among sample groups. PERMANOVA was chosen because it has been shown to be more effective than other tests in detecting differences in community structure, even when group dispersions are heterogeneous. Mantel test with 9999 permutations was performed to test for associations between skin bacterial community structure and *Pd* loads, and only infected individuals were included in this analysis. In addition, an indicator species analysis using the ASVs with relative abundance >0.1% was performed in the *indicspecies* package to identify ASVs that were significantly associated with variability in skin bacterial communities between hibernation and active seasons (Cáceres & Legendre, 2009), and significance was assessed based on 9999 permutations. The *p* values for multiple comparisons were Bonferroni corrected. Only significant ASVs (P_{adj} <0.05 and *IndVal* >0.4) were considered as indicators according to a previous study (Lemieux-Labonté et al., 2017).

To address the relationship between bat skin microbiota and environmental bacterial reservoir, we first calculated the Shannon diversity as a measure of alpha diversity (–p-sampling-depth 16,995) based on previous analyses of the differences between the bat skin microbiota and environmental bacterial reservoir (Li et al., 2022). Then, the beta diversity was calculated using the Bray–Curtis dissimilarity matrices rarefied at 16,995 sequences/sample from bat and environmental samples. Differences in beta diversity between bat skin and environmental samples were statistically analysed using PERMANOVA, listing time as 'strata' with 999 permutations, and were visualized with NMDS in $k=2$ dimensions. Procrustes analysis was performed using the *procrustes()* function of the *vegan* package in R to assess the influence of the environmental bacterial reservoir on skin microbiota. Procrustes analysis is a technique for comparing the relative positions of points in two multivariate datasets to assess the statistical significance in the correlation between them (Peres-Neto & Jackson, 2001; Qin et al., 2021). For this, NMDS was performed separately on Bray–Curtis dissimilarity matrices of the skin microbiota and the environmental bacterial reservoir, and 9999 permutations were used to test the significance. Finally, to assess the processes that determine the variation of skin microbiota, a neutral model was used to quantify the importance of neutral and selective processes in shaping the skin microbiota. The basic assumption of the neutral model is that the probability of detecting an ASV on bat skin due to random dispersal and ecological drift is proportional to the relative abundance of that ASV in the environmental reservoir. Briefly, for each ASV shared between the bat skin and the environmental reservoir, a beta probability distribution was used to calculate the probability of ASV detection in the bat skin if it was present via dispersal and ecological drift. The shape parameters for the probability distribution were determined with an overall fitting parameter (Ntm) and the relative abundance of the ASV in environmental reservoir. Nt is the total community size, and m represents the probability of ASVs from environmental reservoir to bat skin. The value of this parameter was optimized using a least-squares approach to minimize the sum of all ASV residuals. We used the *Hmisc* package in R to calculate the variability around this predicted detection frequency based on 95% binomial proportional confidence intervals. In this way, ASVs were divided into three categories, namely neutrally distributed (consistent with model expectations; present via dispersal and ecological drift), over-represented (positively deviating from model expectations; selected by bats) and under-represented (negatively deviating from the model expectations; selected against or dispersal limited; Loudon et al., 2016; Venkataraman et al., 2015).

3 | RESULTS

3.1 | Seasonal variation of *Pseudogymnoascus destructans* infection

We did not detect the presence of *Pd* on bats in either December in early winter or June in summer. The prevalence increased significantly during hibernation stage and reached its peak (50%) in late winter and then decreased in active season stage (Figure 1a; Kruskal–Wallis test: Chisq = 32, P_{adj} <0.001). Infection prevalence in the active season remained stable between 18% and 28%. However, *Pd* loads on bats showed different trends from the prevalence pattern. The *Pd* loads remained relatively low (mean loads across sampling time points: −5.43 ± 0.74) compared to those from North American bats and showed no significant changes over different sampling time points for infected individuals (Figure 1b; Kruskal–Wallis test: Chisq = 7.69, $p=.17$).

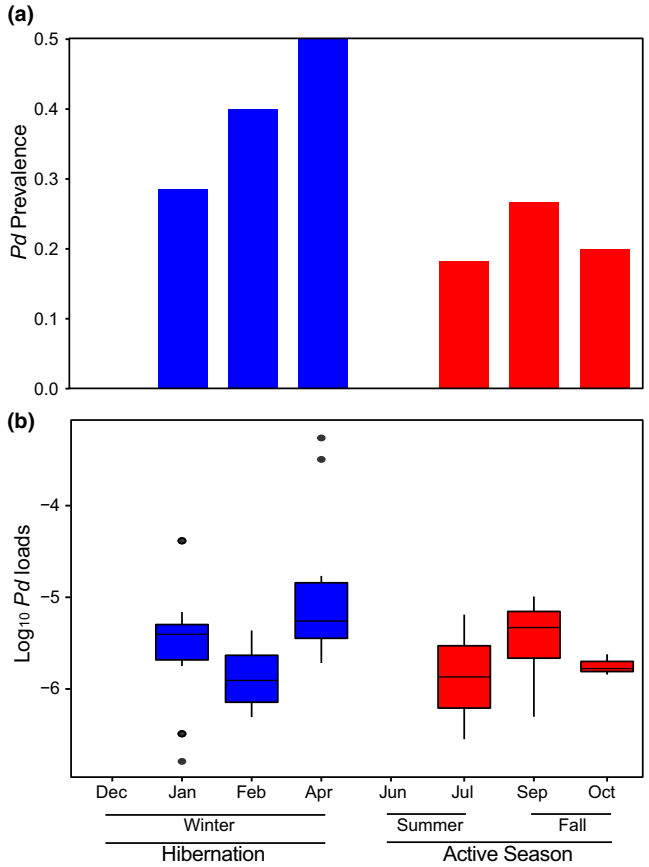

FIGURE 1 Seasonal prevalence and fungal loads of *Pd* on *R. ferrumequinum* across eight sampling time points. Prevalence of *Pd* (a) and fungal loads (b) on a log10 scale from winter in 2017 to fall in 2018.

3.2 | Taxonomic composition of the skin microbiota across seasons

Four main phyla were identified across all sampling time points, classified as Firmicutes, Proteobacteria, Actinobacteriota and Bacteroidetes (Figure 2a). The relative abundance of Firmicutes was higher in early winter (~78.8%), while that of Proteobacteria was higher in other periods (~64.3%). The relative abundance of phyla Cyanobacteria, Chloroflexi, Nitrospirota and Acidobacteriota was significantly greater in the active season compared with the hibernation stages (Mann–Whitney U tests, $p < .05$). At the genus level, the majority of ASVs assigned to Firmicutes in early winter were matched to *Enterococcus*, decreasing to undetectable levels in other periods (Figure 2b). In middle winter, ASVs were classified primarily into *Burkholderia* and *Pseudomonas* as well as an unclassified genus of the Alcaligenaceae family, whereas dominant groups in later winter were *Pseudomonas*, *Corynebacterium*, *Acinetobacter* and *Myroides*. Within the active season stage, the relative abundance of *Burkholderia*, *Ralstonia* and *Crossiella* was higher and significantly greater than in the hibernation stage (Mann–Whitney U tests, $p < .05$; Figure 2b).

3.3 | Variation of the skin microbiota across seasons

After controlling for sampling month using GLMM or LMM, we found significant differences in alpha diversity between hibernation and active seasons, that is, skin bacterial communities exhibited significantly lower average alpha diversity in hibernation than in the active season (Shannon diversity: Chisq = 4.30, $p = .038$; phylogenetic diversity: Chisq = 9.48, $p = .002$; observed richness: Chisq = 12.48, $p < .001$; Figure 2c and Figure S1a). We also found that the Shannon diversity of all four sampling months during hibernation was statistically indistinguishable, except for a significant difference between December and January (Wilcoxon, Dec. vs Jan.: $P_{adj} = 0.03$; Figure 2d). With respect to phylogenetic diversity and observed richness, pairwise comparisons among four sampling months during hibernation showed no significant differences, except for one comparison between January and April that was significantly different (Wilcoxon, Jan. vs Apr.: all $P_{adj} = 0.03$; Figure S1b). However, the alpha diversity of all four sampling months during active season was all statistically indistinguishable (Wilcoxon: all $P_{adj} > 0.05$; Figure 2d and Figure S1b). In terms of seasonal variation, early and late winter hibernators displayed significantly lower alpha diversity than summer or fall bats, while middle winter hibernators had lower phylogenetic diversity and observed richness values compared with fall bats (Figure S1b). It is worth noting that there was no correlation between *Pd* loads and alpha diversity ($p > .05$ for all sampling time points).

NMDS and cluster analysis derived from the Bray–Curtis dissimilarity matrices showed compositional differences among skin microbiota samples collected across sampling time points. NMDS revealed a clear separation between hibernation and active season stages, indicating that the total skin bacterial community structure of bats between hibernation and active season stages was significantly different (NMDS with stress = 0.17; PERMANOVA: pseudo-$F_{1,106} = 23.27$, $p = .001$; Figure 2e). With respect to seasonal variation, the bacterial community structure in hibernation stage was significantly different from those of the active season stage (PERMANOVA: pseudo-$F_{7,106} = 9.81$, $p = .001$), while the difference between the summer group and the autumn group was not significant (Table S1). Additionally, the dispersion values showed significant differences in the distance to the centroid over time, which could explain the differences between December with the least variation and the other sampling time point (*betadisper*: $p < .001$; Figure 2f). Contrary to our expectations, the Mantel test showed that there was no correlation between *Pd* loads and skin bacterial community structure ($p > .05$ for all sampling time points). In addition, we identified 73 ASVs spanning four phyla and six classes that were related to hibernation, and 32 ASVs spanning three phyla and four classes related to the active season (Table S2). Significant indicators at the genus level for the hibernation group were *Corynebacterium*, *Klebsiella*, *Myroides*, *Phreatobacter*, *Pseudomonas*, *Staphylococcus*, *Rickettsiella*, *Serratia*, *Rhodococcus*, *Arthrobacter* and *Acinetobacter*, while significant indicators at the genus level for the active season group were

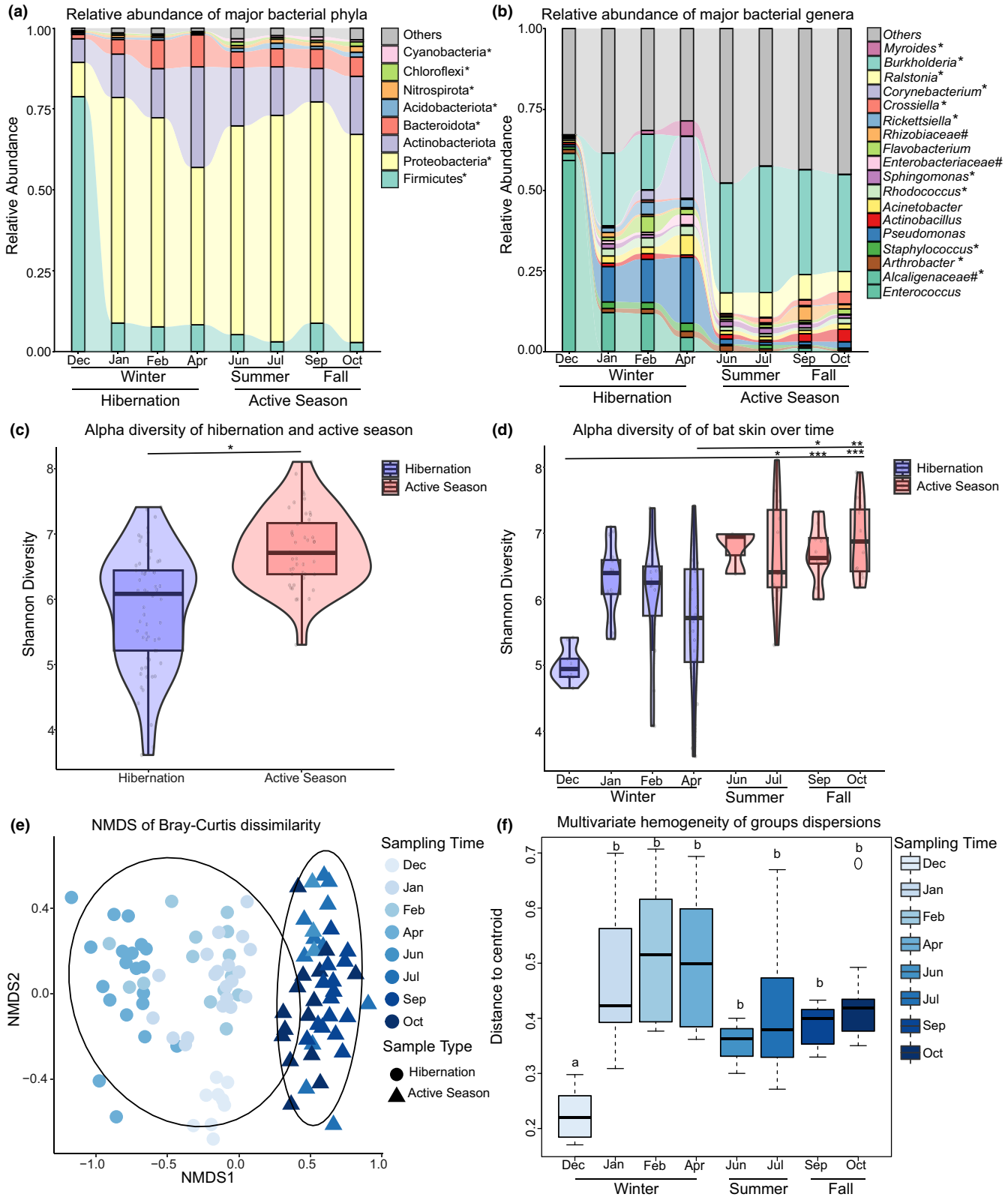

FIGURE 2 Skin bacterial communities of *R. ferrumequinum* across different seasons. Alluvial diagram of major taxa with relative abundance greater than 0.01 at the phylum level (a) and genus level (b). The symbol # represents ASVs that could only be classified to the family level. The asterisks (*) represent taxa with significant differences between hibernation and active seasons based on Mann–Whitney U tests. Comparison of alpha diversity between hibernation and active season (c) and across all sampling time points (d). The asterisks (*) represent significant differences in Shannon diversity between different groups based on GLMM or Kruskal–Wallis tests. (e) NMDS plot based on Bray–Curtis dissimilarity matrices coloured by sample period. Ellipses represent the 95% confidence intervals (CI). (f) Box plot of multivariate homogeneity of group dispersions (variances) across season groups. Different letters (a and b) represent statistically significant differences over sampling time points, as indicated by the Tukey post hoc tests.

Undibacterium, *Ralstonia*, *Smaragdicoccus*, *Chitinophaga*, *Crossiella*, *Actinobacillus*, *Burkholderia*, *Bradyrhizobium* and *Bosea* (Figure 3).

3.4 | Relationship between the environmental bacterial reservoir and skin microbiota

Across all environmental samples, the taxa were dominated by Proteobacteria (~34.88%) and Actinobacteriota (~27.86%), with lesser abundant phyla including Acidobacteriota (~7.76%), Nitrospirota (~6.06%), Bacteroidota (~5.39%), Chloroflexi (~5.17%), Planctomycetota (~3.75%) and Gemmatimonadota (~3.27%) (Figure 4a). The alpha diversity as measured by the Shannon diversity indicated that the environmental bacterial reservoir harboured significantly high alpha diversity in comparison with bat skin (Mann–Whitney U test, all $p < .05$, Figure 4b). The results of NMDS controlling for sampling months clearly distinguished between environmental and bat skin samples (NMDS with stress = 0.21; PERMANOVA: pseudo-$F_{1,146} = 10.73$, $R^2 = 0.07$, $p = .001$; Figure 4c), implying their difference in bacterial community structure. Procrustes analysis showed that the bat skin bacterial community was significantly correlated with the environmental bacterial community (PROTEST, $r = 0.76$, $p < .05$; Figure 4d), thus suggesting the significant impact of the environmental bacterial reservoir on the bacterial community of the bat skin.

3.5 | Fit to the neutral model of skin microbiota assembly

Using the neutral model of community ecology to analyse ASVs that were shared between bat skin and the environment, we found that up to 78.96% of the ASVs in bat skin microbiota during hibernation were consistent with a neutral distribution, while up to 87.53% of ASVs were neutrally distributed on bat skin in the active season (grey points in Figure 5). We also found that 14.48% or 6.56% of the ASVs deviated from the neutral prediction as being more or less frequent than predicted, respectively, in the hibernation groups, whereas 7.60% and 4.87% ASVs fell into the over-represented and under-represented categories in the active season groups (green points or red points in Figure 5). Furthermore, for the hibernation groups, we found that the 159 over-represented ASVs accounted for 5.88%, 30.66%, 23.02% and 19.46% of the total community during four sampling time points, while 72 under-represented ASVs only accounted for less than 5% of each community (Table 2). With respect to the active season groups, 92 over-represented ASVs accounted for 20.63%, 36.61%, 34.58% and 32.23% of the total community in the four sampling time points, but under-represented ASVs accounted for less than 5%. Additionally, 128 and 76 over-represented ASVs were identified at the genus level.

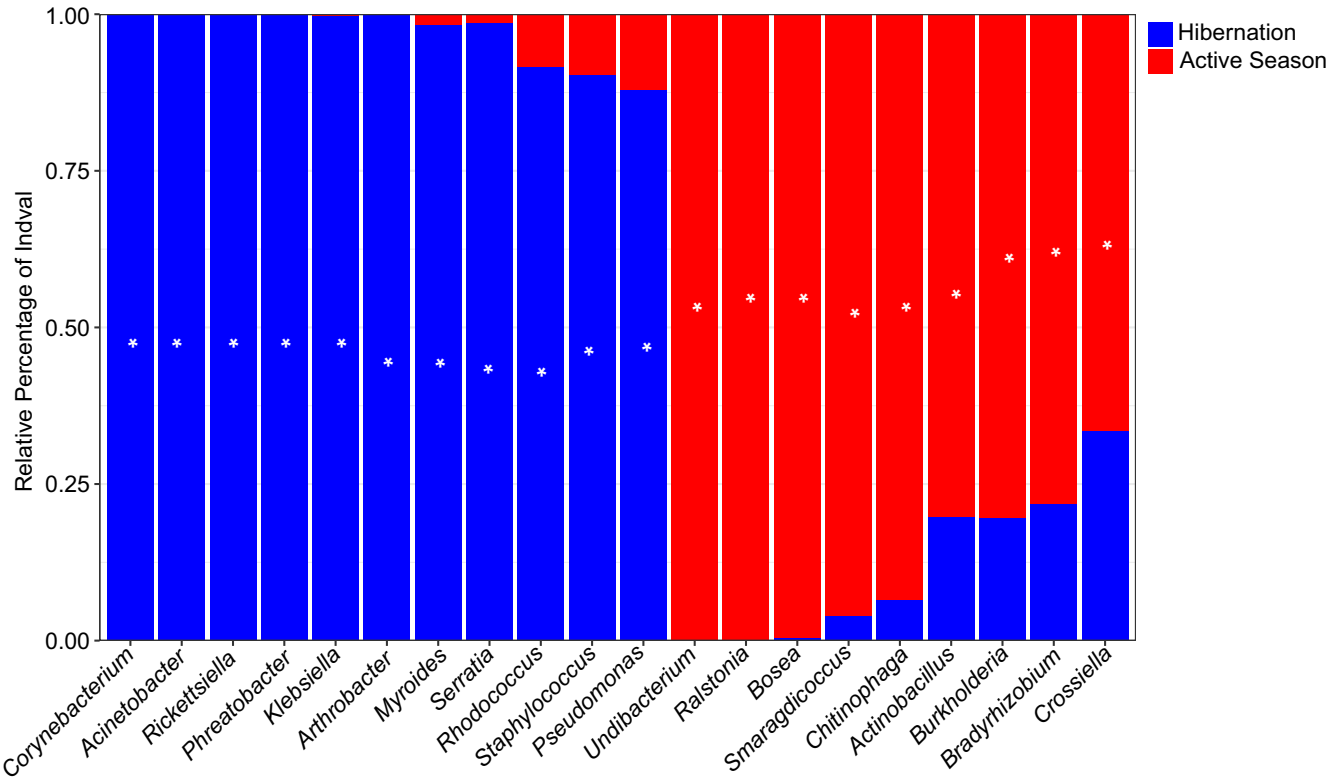

FIGURE 3 Indicator species of *R. ferrumequinum* skin microbiota in hibernation and active season groups. The bar chart displays the significant representative indicators with $P_{adj} < 0.05$ at the genus level.

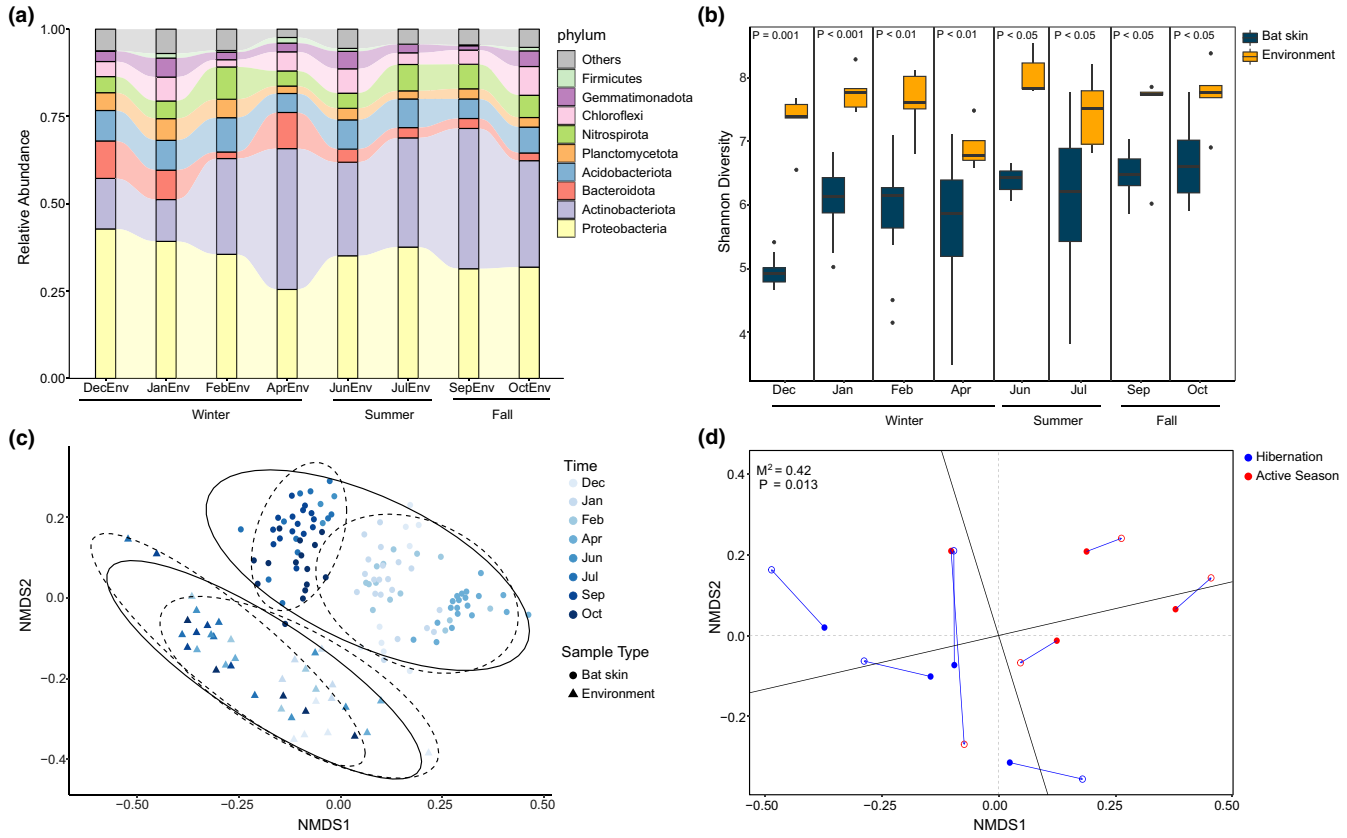

FIGURE 4 Diversity and structure of bacterial communities in bat skin and environmental samples. (a) Alluvial diagram of relative abundance of major bacterial phyla at each sampling time point. (b) Comparison of Shannon diversity between bat skin and the environmental bacterial reservoir across seasons. The *p* values are shown at the top of each paired comparison. (c) NMDS plot based on Bray–Curtis dissimilarity matrices showing beta diversity between bat skin and environmental samples. Ellipses represent the 95% confidence intervals (ci). (d) Procrustes analysis of the correlation between skin bacterial communities and the environmental bacterial reservoir based on Bray–Curtis dissimilarity matrices. Arrows point to the positions of skin bacterial communities in the transformed ordination based on the environmental bacterial reservoir. M2 and *p* values are provided at the top of the NMDS plot.

4 | DISCUSSION

Host-associated bacterial communities are increasingly recognized for their resistance to pathogens in multiple hosts. Although an increased understanding of seasonal variations in infection prevalence and intensity exists (Langwig et al., 2015), the impact of temporal fluctuations on host microbiota and the processes driving such variations are not well understood. In this study, we explored seasonal variation in the skin microbiota of *R. ferrumequinum*, a seasonally hibernating bat species with high resistance to *Pd* infection, and quantified the importance of neutral and selective processes in bacterial community assembly.

4.1 | Skin microbiota differs between hibernation and active season

Our results showed that hibernation significantly altered skin microbiota, as manifested by lower average diversity and altered bacterial community structure and function. We found that the skin microbiota of bats in hibernation stage exhibited significantly lower alpha diversity than that in active season stage (Figure 2c and Figure S1). This might be due to the differences in host physiology and behaviour between hibernation and active seasons. During hibernation for nearly half a year, bats would have low body temperatures, which could restrict the growth of certain bacterial taxa on the skin (Sonoyama et al., 2009; Tong et al., 2020). Simultaneously, bats continuously consume stored fats during hibernation, leading to changes in physical conditions. In contrast, bats increase their food intake and move around during the active season, which in return may increase the exchange of bacteria between the skin and the gut or the environment (Antwis et al., 2014; Fitzpatrick & Allison, 2014; Wiggins et al., 2011). Additionally, NMDS based on Bray–Curtis dissimilarity showed clear clustering (Figure 2e), implying differences in skin microbiota structure between hibernation and active seasons. Furthermore, bats during early winter were found to branch away from all other groups. This trend indicates that early winter is a transitional season in which the skin microbiota contracts and specializes as it adapts to the alternative physical and behavioural conditions brought about by hibernation (Dill-McFarland et al., 2014).

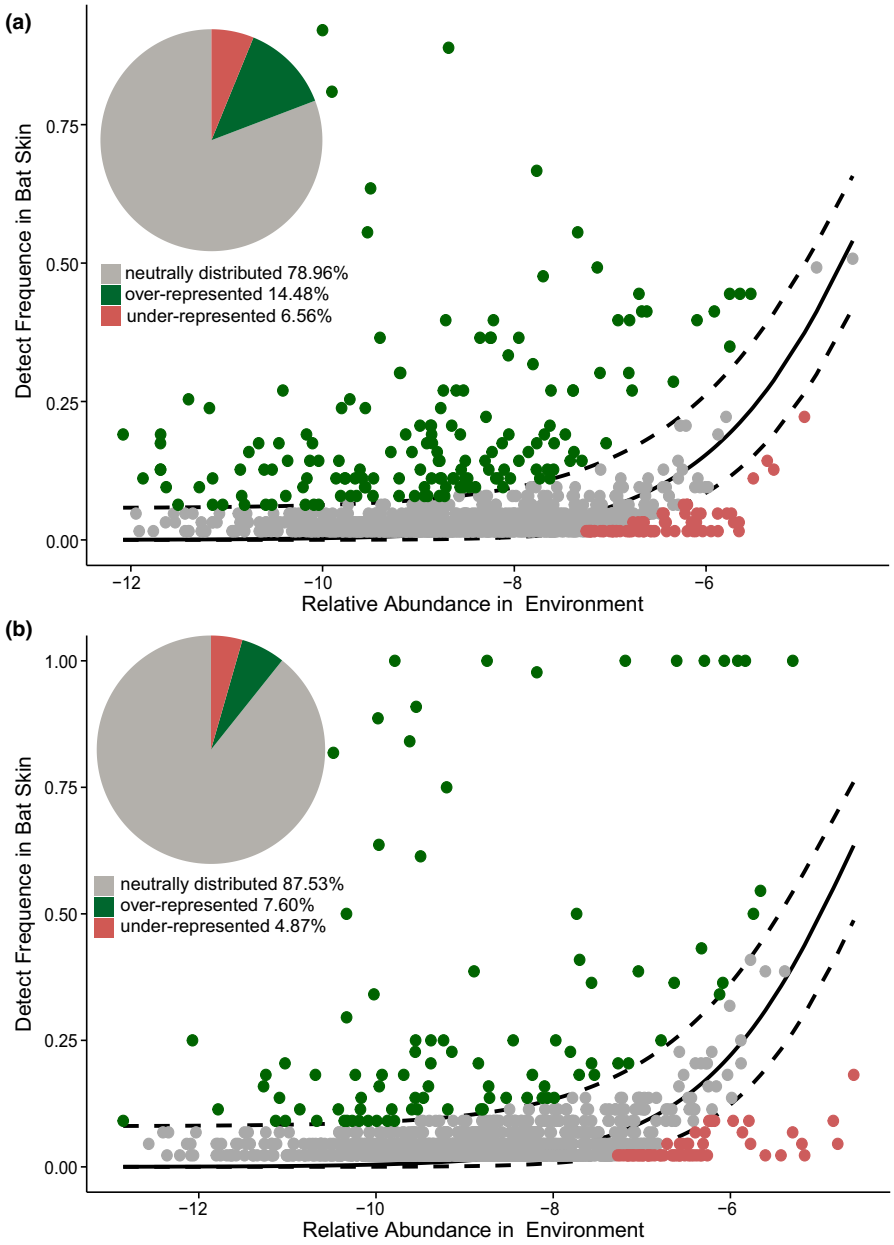

FIGURE 5 Implementation of the neutral model to *R. ferrumequinum* skin microbiota with the environmental bacterial reservoir as the source. ASVs that positively deviated from model expectations are shown in green points, while those that negatively deviated from the model expectations are shown in red points. Black solid lines represent 95% confidence intervals around the model predictions. The pie chart represents the relative importance of neutrally distributed, over-represented and under-represented ASVs on bat skin in hibernation (a) and active season (b) stages.

TABLE 2 Number of ASVs on bat skin that were consistently over-represented or under-represented and the corresponding proportions in the total community, as well as the numbers of each that match antifungal isolates at the genus level.

	ASVs status	Total ASVs	Proportion in total community	ASVs identified at genus
Hibernation	Over-represented	159	5.88% (Dec); 30.66% (Jan); 23.02% (Feb); 19.46% (Apr)	128
	Under-represented	72	4.78% (Dec); 2.66% (Jan); 3.76% (Feb); 3.71% (Apr)	52
Active Season	Over-represented	92	20.63% (Jun); 36.61% (Jul); 34.58% (Sep); 32.23% (Oct)	76
	Under-represented	59	3.41% (Jun); 4.15% (Jul); 4.77% (Sep); 4.38% (Oct)	54

4.2 | Skin microbiota assembly by dispersal and selection from the environmental bacterial reservoir

From an ecological perspective, the skin microbiota was assembled from the source pool through fundamental selection processes, namely drift and dispersal. Procrustes analysis revealed that there was a significant correlation between bat skin microbiota and environmental bacterial reservoirs, implying that environmental bacterial reservoirs provide a source for the assembly of bat skin microbiota. As the bat skin was in direct contact with the external environment, the skin microbiota was assumed to be susceptible to stochastic processes, such as randomly losing and acquiring

bacteria from environmental bacterial reservoirs. Supporting this, the neutral model of community ecology revealed that a majority of ASVs from bat skin, which were shared with the environmental bacterial reservoir fell into a neutral distribution (Figure 5), indicating that neutral processes, including random dispersal and ecological drift, are more important than selection in skin assembly. That is, it is plausible that during inactivity, such as hibernation, bat skin acquires a diverse bacterial community from the environment by contact. However, the bacterial communities of bat skin and the environment clustered separately in the NMDS based on Bray–Curtis dissimilarity, implying that the skin microbiota assembly did not fully mirror the bacterial community in the immediate environment, which is consistent with other bat or amphibian studies (Avena et al., 2016; Walke et al., 2014). Surprisingly, some ASVs that positively deviated from the model's expectations were indicative of bacteria that were selected by the host. Deviations from neutral patterns in bacterial communities have been attributed to community-level perturbations such as the specialization of bacterial species, changes in community composition across environmental gradients and measurable fitness differences between bacterial species, particularly for infectious diseases (Burns et al., 2016; Dumbrell et al., 2010; Langenheder & Székely, 2011; Venkataraman et al., 2015). Actually, *R. ferrumequinum* in Asia likely has had a long co-evolutionary relationship with *Pd* spanning thousands of years (Drees et al., 2017). Since exposure to *Pd* is likely a strong selective pressure on bats, it is conceivable that there has been ample time for selection for antifungal bacterial species on bats' skin. Accordingly, we found that a few ASVs were over-represented; these comprised a large proportion of the total community (Table 2) and were among the most abundant ASVs found on bat skin. Some of these ASVs are recognized for their antifungal activity, and specific abundant strains of *Corynebacterium*, *Pseudomonas*, *Rhodococcus* and *Arthrobacter* are known to inhibit *Pd* growth (Grisnik et al., 2020; Hoyt et al., 2015; Micalizzi et al., 2017). Enrichment of antifungal bacteria provides a mechanism against *Pd* infection, just as one theoretical study predicted that bacterial communities need a high enough population density of probiotics to resist pathogens (Scheuring & Yu, 2012).

The present study also showed that differences in skin microbiota did not correlate with *Pd* infection. This might be due to selection for bacteria with potential antifungal capabilities to resist fungal invasion and growth, contributing to stability and low fungal load. In contrast, North American bat species have just been exposed to this pathogen for the first time, so *Pd* invasion is currently restructuring the bat skin microbiota in this area (Ange-Stark et al., 2019; Lemieux-Labonté et al., 2017). Furthermore, it is worth noting that matching these ASVs to antifungal isolates does not necessarily mean that these bacteria exhibit antifungal activity, but rather that they are strong candidates for exhibiting antifungal activity. Follow-up research should cultivate those over-represented ASVs and test their ability to inhibit fungi. Additionally, some ASVs were under-represented on the bat skin, meaning that they were selected against by the host or were significantly dispersal limited. Some ASVs should be harmful to the host, and the skin may have evolved mechanisms to prevent the persistence of these ASVs despite exposure from the environment, or some resident skin bacteria could inhibit colonization of some environmental bacteria (Franzenburg et al., 2013; Fraune et al., 2010; Ward et al., 2009). Overall, our results revealed that both neutral and selective processes contributed to maintaining the bat skin microbiota, with neutral effects contributing most to the seasonal shifts in skin microbiota. However, considering the microbial variation among populations (Li et al., 2022), the results from one bat population are limited and may only explain a small proportion of variations in host microbiota (McGill et al., 2006). Furthermore, assessing and comparing multiple populations are necessary for enhancing the credibility of the relative roles of neutral and selective processes in shaping skin microbiota.

This study highlights that neutral processes dominate the assembly of skin microbiota of *R. ferrumequinum*, but under *Pd* infection pressure, the skin is colonized with bacteria from the environmental bacterial reservoir that potentially inhibit fungal growth. Our findings may not only provide insights into bat protection strategies but may also apply to amphibians and snakes, whose susceptibility to fungal diseases is linked to the skin microbiota (Rebollar et al., 2016; Walker et al., 2019). These findings represent a first step towards unravelling the skin microbiota–pathogen interaction and towards achieving a better understanding of the role of community assembly processes in the skin microbiota. Further research is needed to include more information about host traits in the analyses to integrate deterministic processes driven by the host.

AUTHOR CONTRIBUTIONS

AL performed laboratory work, conducted bioinformatics and data analysis and drafted the manuscript. AL, ZL and HL collected the samples. LJ and YX helped supervise laboratory procedure. KS and JF developed the study concept and supervised the project.

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DATA AVAILABILITY STATEMENT

All raw sequence data are deposited in the NCBI sequence read archive with accession no. SRP335652. The datasets generated and analysed during the current study are available in the Figshare repository (available at: https://figshare.com/s/d13c66f26c85bb82a9a9).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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