Captivity causes taxonomic and functional convergence of gut microbial communities in bats

Yanhong Xiao, Guohong Xiao, Heng Liu, Xin Zhao, Congnan Sun, Xiao Tan, Keping Sun, Sen Liu, Jiang Feng

1 Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal University, Changchun, Jilin, China
2 Institute of Resources & Environment, Henan Polytechnic University, Jiaozuo, Henan, China
3 College of Life Science, Jilin Agricultural University, Changchun, Jilin, China

Corresponding Authors: Yanhong Xiao, Jiang Feng
Email address: xiaoyh767@nenu.edu.cn, fengj@nenu.edu.cn

Background: Diet plays a crucial role in sculpting microbial communities. Similar diets appear to drive convergence of gut microbial communities between host species. Captivity usually provides an identical diet and environment to different animal species that normally have similar diets. Whether different species’ microbial gut communities can be homogenized by a uniform diet in captivity remains unclear. Methods: In this study, we compared gut microbial communities of three insectivorous bat species (Rhinolophus ferrumequinum, Vespertilio sinensis, and Hipposideros armiger) in captivity and in the wild using 16S rDNA sequencing. In captivity, R. ferrumequinum and V. sinensis were fed yellow mealworms, while H. armiger was fed giant mealworms to rule out the impact of an identical environment on the species’ gut microbial communities. Results: We found that the microbial communities of the bat species we studied clustered by species in the wild, while the microbial communities of R. ferrumequinum and V. sinensis in captivity clustered together. All microbial functions found in captive V. sinensis were shared by R. ferrumequinum. Moreover, the relative abundances of all metabolism related KEGG pathways did not significantly differ between captive R. ferrumequinum and V. sinensis; however, the relative abundance of “Glycan Biosynthesis and Metabolism” differed significantly between wild R. ferrumequinum and V. sinensis. Conclusion: Our results suggest that consuming identical diets while in captivity tends to homogenize the gut microbial communities among bat species. This study further highlights the importance of diet in shaping animal gut microbiotas.
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†Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal University, Changchun, Jilin, China
‡College of Life Science, Jilin Agricultural University, Changchun, Jilin, China
§Institute of Resources & Environment, Henan Polytechnic University, Jiaozuo, Henan, China

†These authors contributed equally to this paper.
*Corresponding Authors:
Yanhong Xiao
Jingyue Street, Changchun, Jilin, 130117, China
Email address: xiaoyh767@nenu.edu.cn

Jiang Feng
Jingyue Street, Changchun, Jilin, 130117, China
Email address: fengj@nenu.edu.cn
Abstract

**Background:** Diet plays a crucial role in sculpting microbial communities. Similar diets appear to drive convergence of gut microbial communities between host species. Captivity usually provides an identical diet and environment to different animal species that normally have similar diets. Whether different species’ microbial gut communities can be homogenized by a uniform diet in captivity remains unclear.

**Methods:** In this study, we compared gut microbial communities of three insectivorous bat species (*Rhinolophus ferrumequinum*, *Vespertilio sinensis*, and *Hipposideros armiger*) in captivity and in the wild using 16S rDNA sequencing. In captivity, *R. ferrumequinum* and *V. sinensis* were fed yellow mealworms, while *H. armiger* was fed giant mealworms to rule out the impact of an identical environment on the species’ gut microbial communities.

**Results:** We found that the microbial communities of the bat species we studied clustered by species in the wild, while the microbial communities of *R. ferrumequinum* and *V. sinensis* in captivity clustered together. All microbial functions found in captive *V. sinensis* were shared by *R. ferrumequinum*. Moreover, the relative abundances of all metabolism related KEGG pathways did not significantly differ between captive *R. ferrumequinum* and *V. sinensis*; however, the relative abundance of “Glycan Biosynthesis and Metabolism” differed significantly between wild *R. ferrumequinum* and *V. sinensis*.

**Conclusion:** Our results suggest that consuming identical diets while in captivity tends to homogenize the gut microbial communities among bat species. This study further highlights the importance of diet in shaping animal gut microbiotas.

Introduction

Trillions of microorganisms reside in animal guts, and these microorganisms constitute the animal’s gut microbiota, which is important for animal health (Flint et al. 2012). Ley et al. (2008a) found that animals who were closely related taxonomically had more similar gut microbial compositions. Phylogenetic congruence of microflora communities and their hosts was also observed among bat families (Ingal et al. 2018; Phillips et al. 2012). These studies indicated that host evolutionary history strongly impacts gut microbiome compositions. Although gut microbial communities are host-specific, they can be influenced by the host’s diet, developing immune system, chemical exposures and initial colonizers (Donaldson et al. 2015). Diet has been suggested to have the greatest impact on microbiota assembly (Donaldson et al. 2015). Diet shapes the gut microbial community by providing substrates that differentially support or enhance the growth of specific microbe (De Filippo et al. 2010; Scott et al. 2013; Wang et al. 2013). Taxonomic compositions of the gut microbial communities of different host species with similar diets appeared to converge in some studies (Carrillo-Araujo et al. 2015; Delsuc et al. 2014; Muegge et al. 2011). Ley et al. (2008a) also found that animals with similar diets (i.e., herbivores, carnivores, omnivores) had more similar gut microbiome compositions.

Wild animals in captivity are usually housed under uniform conditions that include identical diets and environments (Hale et al. 2018). This represents a rapid and dramatic dietary and
environmental change to the animals. The gut microbiome has been reported to rapidly respond
to an altered diet (David et al. 2013). However, whether different species’ gut microbiomes will
respond similarly to the uniform conditions of captivity remains uncertain. A study comparing
the gut microbial diversity in two woodrat species in the wild and in captivity found that the
microbial communities in these species did not converge (Kohl et al. 2014). Principal coordinate
analysis results showed that the microbial signatures of the captive woodrats still clustered by
species (Kohl et al. 2014). Woodrats are herbivores, which represents only one mammalian
dietary type. Carnivores represent another important dietary type, of which, insectivores are
thought to represent the ancestral condition for placental mammals (O’Leary et al. 2013).
However, whether the taxonomic compositions of different insectivorous species’ microbial gut
communities tend to converge under identical dietary and environmental conditions remains
unclear. In addition, the two wood rat species that Kohl et al. (2014) studied were closely related.
Given the phylogenetic distance among the hosts in the present study, it is unclear whether a
homogenous diet/environment or the host’s evolutionary history more strongly impacts the
microbiome community composition.

Bats (order Chiroptera) are the second largest mammalian group (Wilson & Reeder 2005).
Most bats are insectivores, which is also thought to be the ancestral condition for bats (Dawson
& Krishtalka 1984). To determine whether diet/environment or evolutionary more strongly
impacts the microbiome, we sampled feces (guano) from three bat species from three families
(Rhinolophidae, Vespertilionidae and Hipposideridae): the greater horseshoe bat (Rhinolophus
ferrumequinum), the Asian parti-colored bat (Vespertilio sinensis) and the great Himalayan leaf-
nosed bat (Hipposideros armiger) in the wild and in captivity. We then compared the bacterial
communities in both the wild and captive samples between these three species. We captured bats
in the wild, brought them back to the laboratory and housed them in identical environments but
provided different food. Rhinolophus ferrumequinum and V. sinensis were fed the same food
(yellow mealworms), while H. armiger were provided giant mealworms, thus forming a
comparison to eliminate the impact of environment on the gut microbiome. Given that diet
strongly influences microbiome composition and similar diets appear to drive convergence of gut
microbial communities between host species (Delsuc et al. 2014; Muegge et al. 2011), we
predicted that the gut microbiome compositions of captive R. ferrumequinum and V. sinensis
under identical environmental and dietary conditions would converge with each other but would
differ from captive H. armiger. In addition, taxonomy and function are decoupled in microbial
ecosystems (Graham et al. 2016; Inkpen et al. 2017; Louca et al. 2016). Microbial functions may
converge despite the microbial community’s taxonomic compositions varying among host
species (Phillips et al. 2017). Thus, microbial functions were also predicted and compared among
different bats species both in the wild and in captivity to investigate whether the gut microbiome
function converges in the captive bats.

**Material and Methods**

**Field sampling of bats**

All three bat species are insectivores. Rhinolophus ferrumequinum feeds preferentially on
lepidopterans, particularly the noctuid species, which constitute approximately 41% of the bat’s diet (Jones 1990). The bats also eat coleopterans, which constitute approximately 33% of their diet, of which, dung beetles and cockchafers are often consumed (Jones 1990). The dietary composition of *V. sinensis* mainly comprises Lepidoptera (mean relative percentage: 32.8%), Diptera (27.5%) and Coleoptera (22.6%), but the proportion of each order varies seasonally (Fukui & Agetsuma 2010). *H. armiger*’s diet mainly comprises 31.59-37.21% Coleoptera and 15.38-22.87% Lepidoptera (Han & He 2012).

Eight greater horseshoe bats, 7 Asian parti-colored bats and 8 great leaf-nosed bats were collected from Jilin, Heilongjiang and Guizhou, China, respectively, during the summer of 2018. Bats were collected from one group of each species. Fecal samples were collected from these bats in the field. During the summer of 2017, we collected 10 greater horseshoe bats from three groups, of which, 3 bats were from Jilin, 1 from Liaoning, and 6 from Shannxi, China. Ten Asian parti-colored bats in one group and 10 great leaf-nosed bats in one group were collected from Heilongjiang and Shannxi, China, respectively, during the summer of 2017. These bats were returned to the laboratory, and different bat species were housed in separate cages for 4-6 months before collecting their fecal samples. Details on the bats collected are shown in Table 1.

### Collection of fecal samples

Fecal samples were used because dietary signals in the microbiome are more easily detected in fecal samples than in intestinal samples (Ingala et al. 2018). Bats were captured in the field using mist nets placed at cave entrances, immediately recovered from the nets, and placed in separate clean holding bags to await processing. We recorded each bat’s sex, age, weight, forearm length, and reproductive condition (Table 1 and DataS1). Feces were collected directly from the bottom of the holding bags and placed in sterile tubes using sterile forceps, then stored in dry ice before transport to the laboratory. The bags were checked frequently to ensure the samples’ freshness. In the laboratory, the greater horseshoe bats and the Asian parti-colored bats were fed yellow mealworms (*Tenebrio molitor*), while the great leaf-nosed bats were fed giant mealworms (*Zophobas morio*) for comparison. We kept the bats for 4-6 months, and collected their fecal pellets less than 15 minutes after defecation in the laboratory. Each bat’s sex, age, weight, forearm length, and reproductive condition was recorded (Table 1 and DataS1), then the bats were placed in separate clean cages, which were placed on sterile brown paper. Feces were collected from the brown paper and placed in sterile tubes, then temporarily stored in liquid nitrogen. The brown paper was checked frequently to ensure the feces’ freshness. All samples were stored in -80°C until DNA extraction.

Sampling was conducted with permission from the local forestry department. The National Animal Research Authority of Northeast Normal University, China (approval number: NENU-20080416) and the Forestry Bureau of Jilin Province, China (approval number: [2006]178) approved all study protocols.

### DNA extraction

Fifty-three fecal samples were used, including 23 from the wild bats and 30 from the captive bats.
DNA was extracted from all fecal samples using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc.) per the manufacturer’s instructions and stored at -20°C for further analysis. Extracted DNA was measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis to estimate DNA quantity and quality, respectively.

**16S rDNA amplicon pyrosequencing**

The V3-V4 region of the bacterial 16S rRNA genes were amplified via PCR using the forward primer, 338F (5’-ACTCCTACGGGAGGCAGCA-3’), and the reverse primer, 806R (5’-GGACTACHVGGGTWTCTAAT-3’) (Dennis et al. 2013). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5µl of Q5 reaction buffer (5×), 5µl of Q5 High-Fidelity GC buffer (5×), 2 µl of dNTPs (2.5 mM), 1 µl of each forward and reverse primer (10 µM), 0.25 µl of Q5 High-Fidelity DNA polymerase (5U/µl), 2 µl of DNA template, and 8.75 µl of ddH$_2$O. The PCR conditions consisted of initial denaturation at 98°C for 2 min, followed by 25 denaturation cycles at 98°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The individual PCR products were then pooled in equal amount, and sequenced using the paired-end 2×300 bp method on the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). All raw sequences were deposited into the NCBI Sequence Read Archive under accession number SRR8238420 - SRR8238472.

**Sequence analysis**

Sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) (Caporaso et al. 2010). Briefly, raw sequences with unique barcodes were assigned to respective samples. Sequences shorter than 150 bp, having average Phred scores of <20, containing ambiguous bases, or sequences containing more than 8-bp mononucleotide repeats were regarded as low-quality sequences and removed (Chen & Jiang 2014; Gill et al. 2006). Paired-end reads were assembled using FLASH (Magoč & Salzberg 2011). Assembled sequences were trimmed of barcodes and sequencing primers. After chimera detection, the remaining trimmed and assembled sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using UCLUST (Edgar 2010). A representative sequence was selected from each OTU using default parameters. Representative sequences were aligned to the Greengenes Database (DeSantis et al. 2006) using the best hit (Altschul et al. 1997) to classify the taxonomy, which was conducted using BLAST. An OTU table was then generated to record each OTU’s abundance per sample and the OTU’s taxonomy. OTUs containing less than 0.001% of the total sequences across all samples were discarded. To minimize the differences in sequencing depth across samples, an averaged, rounded, rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth for further analysis.
Bioinformatics and statistical analysis

Sequence data were mainly analyzed using QIIME v1.8.0 and R v3.2.0. Beta diversity was analyzed to investigate the microbial communities’ structural variation across samples using UniFrac distance metrics (Lozupone & Knight 2005; Lozupone et al. 2007) and visualized via principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) (Ramette 2007). UniFrac is the only distance metric that considers the phylogenetic relationships between microorganisms, and UniFrac-based beta diversity has become a standard analytic method in microbiome studies. Therefore, we also chose the UniFrac distance to characterize the community structure in our study. Differences in the UniFrac distances for pairwise comparisons among groups were determined using Student’s t-test and the Monte Carlo permutation test with 1000 permutations, then visualized using box-and-whiskers plots. For UniFrac distance-based pairwise comparisons among groups, we used a very conservative Bonferroni post-hoc correction method to perform the multiple corrections and evaluate the significance of the comparison. Permutational multivariate analysis of variance (PERMANOVA) (McArdle & Anderson 2001) and analysis of similarities (ANOSIM) (Clarke 1993; Warton et al. 2012) were conducted using the R package “vegan” (v1.6.9) to assess the significance of the differentiation of the microbiota structures among groups. A Venn diagram was generated to visualize the shared and unique OTUs among groups using the R package “VennDiagram” (v2.4-6) (Chen & Boutros 2011) based on the occurrence of OTUs across groups regardless of their relative abundance (Zaura et al. 2009). Microbial functions were predicted using Phylogenetic Investigation of communities by Reconstruction of Unobserved States (PICRUSt, v1.0.0) (Langille et al. 2013) in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2004) based on high-quality sequences. The relative abundances of predicted functions in each sample were calculated based on the abundance matrix obtained via PICRUSt, and significant differences in each function’s relative abundances among different species were tested using analysis of variance (ANOVA) or the Kruskal-Wallis test (Wallis 1952). Results were considered significant at $p < 0.05$.

Results

Sequencing results

A total of 768,990 and 1,466,150 16S rDNA sequences were obtained from the microbiomes of the 23 wild and 30 captive bats, respectively, and the average sequence numbers per sample were 33,434 and 48,872, respectively. Rarefaction analysis demonstrated that the sequencing depth was sufficient for each sample (Fig. S1). A total of 3504 and 7057 OTUs were recovered at the similarity clustering threshold of 97%.

Shared microbial species were increased in captive bats

Venn diagrams were plotted to visualize the shared and unique OTUs (roughly equivalent to bacterial species) among three species of wild and captive bats. The captive bats we sampled shared more OTUs than did the wild bats (Fig. 1). A total of 2022 OTUs (approximately 29% of
the total OTUs) were shared by the three species in captivity, but only 228 OTUs (approximately 7% of the total OTUs) were shared by the wild bats. Approximately 71% of the OTUs from captive *V. sinensis* and *R. ferrumequinum* were shared, but only 18% were shared by these two species in the wild. The proportions of OTUs shared by *V. sinensis* and *H. armiger* were approximately 39% and 12% in captivity and the wild, respectively. Minimal difference was noted between the proportions of shared OTUs in the captive and wild *R. ferrumequinum* and *H. armiger*, of which, the proportions were nearly 36% and 29%, respectively.

**Microbial compositions converged in captive bats fed the same food**

A NMDS based on unweighted beta diversity values indicated that the gut microbial communities in the wild bat were clustered strongly by bat species (**Fig. 2A**). However, the gut microbial community clustering was altered in the captive bats (**Fig. 2B**). In the captive bats, the gut microbial communities of the bats fed the same food (i.e., *V. sinensis* and *R. ferrumequinum* fed yellow mealworms) clustered together, while the gut microbial communities of *H. armiger*, fed giant mealworms, clustered alone. A PCoA based on unweighted UniFrac distances also demonstrated similar clustering results using NMDS based on unweighted UniFrac distances. In the wild bats, PC1, PC2 and PC3 accounted for nearly 42% of the variation, and samples were separated roughly by bat species (**Fig. S2A**). In the captive bats, PC1, PC2 and PC3 accounted for 48% of the variation in microbial composition (**Fig. S2B**).

Analyzing the differences in the UniFrac distances for pairwise comparisons among groups revealed that the differences between each pair group were significant in the wild samples (**Fig. S3A, Table 2**), while the differences between *V. sinensis* and *R. ferrumequinum* were not significant in the captive samples ($p = 0.381$ and $0.085$) (**Fig. S3B, Table 2**). However, the differences between *H. armiger* and the other two species were all significant in the captive samples (**Fig. S3B, Table 2**). Statistical analyses of the significance of the differentiation in the microbiota structure among the groups also yielded similar results. The differences among groups were significant both for the wild and the captive bats (all $p \leq 0.001$, **Table 3**). However, PERMANOVA and ANOSIM analyses cannot assess the significance of the differentiation between pairwise groups when more than two groups are analyzed. Thus, we did not find that the differences between *V. sinensis* and *R. ferrumequinum* were not significant in the captive samples based on the PERMANOVA and ANOSIM analysis results.

**Convergence of microbial function in captive bats fed the same food**

Finally, we predicted the microbial functions of wild and captive bats using PICRUSt, which yielded 5971 and 4771 KEGG pathways, respectively. Venn diagrams showed that in total 5495 KEGG pathways were shared among the wild bat samples, and 3964 were shared among the captive bat samples (**Fig. 3**). Unlike the wild bats, one hundred percent of the microbial functions in captive *H. armiger* were shared by the other two species, and all microbial functions in captive *V. sinensis* were shared by *R. ferrumequinum*. Thus, in terms of presence/absence, the microbial functions appeared converged in the captive bats.
Moreover, in terms of the relative abundance of functions, we found that the relative abundances of all metabolism-related KEGG pathways did not significantly differ between captive \textit{R. ferrumequinum} and \textit{V. sinensis}, while the relative abundance of “Glycan Biosynthesis and Metabolism” differed significantly between the wild \textit{R. ferrumequinum} and \textit{V. sinensis} (Fig. 4). In addition, except “Glycan Biosynthesis and Metabolism”, “Enzyme Families” and “Biosynthesis of Other Secondary Metabolites”, no significant differences were found in the relative abundances of any other metabolic pathways among the three bat species in the wild (Fig. 4A), while the relative abundances of all metabolism-related KEGG pathways except “Metabolism of Cofactors and Vitamins”, “Lipid Metabolism” and “Carbohydrate Metabolism”, differed significantly between captive \textit{H. armiger} and the other two bat species (Fig. 4B). This result indicated that the microbial functions converge in the captive bats fed the same food in terms of the relative abundance of functions.

\textbf{Discussion}

In this study we investigated the influence of identical diets under laboratory conditions on the gut microbial communities of three insectivorous bat species. Feces are sampled as a proxy for the gut microbiome in many studies of wild mammal microbiomes (Hale et al. 2018; Kohl et al. 2014; Phillips et al. 2017; Sommer et al. 2016). Moreover, more signals from the host’s diet are retained in fecal samples than in intestinal samples (Ingala et al. 2018). Thus, in our study we compared the microbial communities from fecal samples of three captive bat species, as well as the fecal microbial communities of their conspecific bats in the wild. The microbiome compositions of the bats in our study were mainly composed of Proteobacteria and Firmicutes, which occupied more than 80% of the microbiome (Fig. S4 and S5). This was consistent with previous work on bat microbiomes (Carrillo-Araujo et al. 2015; Ingala et al. 2018; Phillips et al. 2017; Phillips et al. 2012).

Comparing the microbial communities of fecal samples from three bat species in the wild revealed that the microbial signatures of \textit{R. ferrumequinum}, \textit{V. sinensis} and \textit{H. armiger} in the wild cluster by species when measured by principal coordinate analysis. Microflora communities of wildlife species are shaped by complex processes, including host phylogeny, dietary strategy and reproductive conditions (Phillips et al. 2012). Though \textit{R. ferrumequinum}, \textit{V. sinensis} and \textit{H. armiger} are all insectivores, wild bat diets are varied, species-specific and belong to different bat families. Thus, taxonomic compositions of gut microbial communities differ among bat species in the wild. Our result was consistent with the study of Phillips et al. (2017).

Comparing the microbial communities in fecal samples from three captive bats species showed that the microbial compositions of two bat species (\textit{R. ferrumequinum} and \textit{V. sinensis}) fed the same food converged markedly, while they differed from those of \textit{H. armiger} fed different food. This result highlighted the importance of diet on gut microbial communities. Diet shapes the gut microbiota by providing substrates that differentially support or enhance specific microbial growth (De Filippo et al. 2010; Scott et al. 2013; Wang et al. 2013). The gut microbiota can in turn enable their host to adapt to new dietary niches (Ley et al. 2008a). In this study, captive bats were fed mealworms, which is a novel and high-quality diet for the...
previously wild bats. Long-term dietary intake influences the structure and activity of gut microbiota (Duncan et al. 2007; Ley et al. 2006; Muegge et al. 2011; Walker et al. 2010; Wu et al. 2011). After 4-6 months’ feeding in the laboratory, the bats’ gut microbiotas should have adapted to the new diet. Feeding the bats the same food means that same substrates are provided to the gut microbiota; thus, the gut microbial signatures of captive *R. ferrumequinum* and *V. sinensis* should cluster together. Similar results were obtained in a study comparing the gut microbiotas of captive colobine monkeys. This study found that the gut microbial communities were more similar in the colobine species who consumed the same diet (Hale et al. 2018). In contrast, captive *H. armiger* were fed a different diet (giant mealworms) than were *R. ferrumequinum* and *V. sinensis*, and the gut microbial communities of captive *H. armiger* did not converge with the other two bat species. This result eliminated the impact of environment on the gut microbial communities because the three bat species were housed in identical environments in the laboratory. Further, this suggested that identical diets contribute to microbial community convergence in various bat species. However, fecal samples usually include bacteria that are ingested with the food (e.g., the commensal bacteria in the mealworms), and distinguishing these bacteria from the host-derived bacteria in the fecal microbiome is difficult. Thus, the gut microbiota in this study did not specifically refer to the host-derived bacteria. The microbial community compositions between species fed uniform diets may have converged due to changes in the compositions of the host-associated gut microbiome or a much larger shared component of the fecal microbiome based on a completely shared diet comprising mealworms and their commensal bacteria or both. Our results highlight the need for future studies to address this issue, for example, incorporating dietary classifications via metabarcoding and classifying the microbiomes of the invertebrate prey. In addition, our results differed from those obtained by Kohl et al. (2014), whose results suggested that microbial communities of various woodrat species clustered by species rather than converged together after being exposed to similar diets. The bat species in our study were insectivores, while the woodrat species in the work of Kohl et al. (2014) were herbivores. Herbivorous digestive systems contain multiple enzymes originating from different microbial species needed to process (hemi)celluloses, lignin-derivatives and insoluble starches, thus supporting a highly diverse ecosystem (Karasov et al. 2011). Moreover, bacterial diversity increases as the host diet diverges from carnivorous to omnivorous to herbivorous in mammalian guts (Ley et al. 2008b). Thus, we hypothesize that the higher bacterial diversity in herbivorous mammals allows them to retain more species-specific microbial communities in captivity than do mammals that eat animal-based diets. In addition, more unique bacteria make it difficult for microbial communities of different herbivorous species to cluster together although they have similar diets. Kohl et al.’s results (2014) indicating that 64% and 51% of OTUs were retained in the two captive woodrat species studied may support our hypothesis. Another possible explanation for the differences between the bats in this study and the woodrats in Kohl et al.’s study (2014) may be that the difference between the diets of captive bats and those in nature is larger than that of the woodrats. In xenarthrans (anteaters, sloths, and armadillos), especially myrmecophagous mammals (i.e., mammals that eat termites...
and ants), the effect of captivity on their gut microbiomes is especially noticeable in animals whose diets differ markedly in captivity and in nature (Superina 2011).

The divergence or convergence of microbial community compositions differs from the divergence or convergence of their microbial functions. Different combinations of microbial lineages may achieve comparable community functions, meaning that microbial communities may differ in taxonomic composition but be similar in function (Phillips et al. 2017). In terms of their presence/absence, the unique microbial functions of the bats we studied were lost when the bats were taken from the wild into captivity, especially in *H. armiger* and *V. sinensis*. This may be due to the lack of a different environment or similar nutrient compositions in the mealworm larvae (Rumpold & Schlüter 2013) provided as food in the laboratory. However, in terms of the function frequency, we found that the relative abundances of most metabolic functions were similar, although the microbial community compositions differed among the three bat species in the wild. This finding was similar to that of Phillips et al. (2017) and supports the hypothesis of functional redundancy in the gut ecosystem, which is defined as functions conferred by multiple bacteria that can be shared across both related and unrelated bacterial species (Moya & Ferrer 2016). That is, although the microbial composition varies, different microbiotas may perform similar functions (Pérez-Cobas et al. 2013). Comparing the relative abundances of metabolic functions among the three bat species in captivity, the relative abundances of most metabolic functions were similar between *R. ferrumequinum* and *V. sinensis* but differed in *H. armiger*. Combining the result that the microbial compositions of *R. ferrumequinum* and *V. sinensis* converged together but diverged from *H. armiger*, we inferred that the different foods led to metabolic tuning of microbial functions and the identical diets which in captivity led to the convergence of both microbial compositions and microbial functions.

Unexpectedly, the number of OTUs found in the bats’ fecal samples was increased in the captive bats compared with those in the wild. In other words, the gut microbiota was more diverse in the captive bats than in the wild bats. This was surprising because the number of OTUs in the gut microbiome was expected to have been greatly decreased due to the single-food-source diet and captive conditions. This observation also contradicted the findings of previous studies on the influence of captivity on the animal microbial communities, which found that bringing animals into captivity resulted in a loss of microbial diversity (Redford et al. 2012; Kohl et al. 2014; Kueneman et al. 2016). Several points may explain this. First, the bat species may have been exposed to each other’s microbes due to their being in captivity in the same laboratory; thus, the overall community became more diverse because microbes were shared among species. Second, the bacteria ingested with the mealworms may have increased the diversity of the captive bats’ gut microbial communities. Although the microbial diversity was increased in the captive bats, the microbial function types were decreased in our study, indicating that selection by host diet primarily acts on metagenomic functions. Further research is required to investigate the possible reasons for this.

**Conclusions**
Comparing the results from PCoA and NMDS analyses between wild and captive bats suggests that the identical diets that were provided in captivity contributed to the taxonomy convergence of the gut microbial communities of *R. ferrumequinum* and *V. sinensis*. In addition, in terms of functional level, the identical diets while in captivity yielded more similar relative abundances of metabolic functions in the gut microbiomes of captive *R. ferrumequinum* and *V. sinensis* than in the wild bats, indicating that the identical diet while in captivity contributed to the convergence of the gut microbial community functions. Finally, the gut microbial diversity was surprisingly higher in the captive bats than in the wild bats. However, understanding why this phenomenon occurred requires further study. This study highlights the diet’s crucial role in shaping captive bat gut microbiotas.

**Acknowledgment**

We are grateful to Lixin Gong, Biye Shi, Zhongwei Yin and Chuantao Song for their great contributions to samples collection. Sequencing service was provided by Personal Biotechnology Co., Ltd. Shanghai, China. We thank Traci Raley, MS, ELS, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing a draft of this manuscript.

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**Table 1** *(on next page)*

Summary of samples included in this study.
| Sample type | Species            | Number | Sex     | Age            | Weight (g; mean ± SD) | Forearm length (mm; mean ± SD) | Site          |
|-------------|--------------------|--------|---------|----------------|-----------------------|--------------------------------|---------------|
| Wild        | *R. ferrumequinum* | 8      | 3M+5F   | Adults         | 18.44±1.41            | 60.55±0.92                     | Jilin         |
|             | *V. sinensis*     | 7      | F       | Adults         | 21.89±3.60            | 49.44±2.12                     | Heilongjiang  |
|             | *H. armiger*      | 8      | M       | 1Juveniles+7Adults | 67.03±9.01            | 95.82±2.58                     | Guizhou       |
| Captive     | *R. ferrumequinum*| 10     | 1M+9F   | 6Juveniles+4Adults | 25.84±5.39            | 60.83±1.35                     | Jilin/Liaoning/Shannxi |
|             | *V. sinensis*     | 10     | F       | Adults         | 21.33±3.83            | 50.87±1.21                     | Heilongjiang  |
|             | *H. armiger*      | 10     | 8M+2F   | Adults         | 69.12±8.08            | 95.64±3.38                     | Shannxi       |
Table 2 (on next page)

Results of Student’s t-test and the Monte Carlo permutation test of differences in the UniFrac distances for pairwise comparisons among groups.
| Group 1          | Group 2          | t statistic | p-value\(^a\) |
|------------------|------------------|-------------|---------------|
| Wild             | All within Group | -17.881     | 0.000***      |
| WFVs vs. WFVs    | WFVs vs. WFRf    | -16.090     | 0.000***      |
| WFVs vs. WFVs    | WFVs vs. WFHa    | -22.230     | 0.000***      |
| WFRf vs. WFRf    | WFVs vs. WFRf    | -8.363      | 0.000***      |
| WFRf vs. WFRf    | WFRf vs. WFHa    | -7.793      | 0.000***      |
| WFHa vs. WFHa    | WFVs vs. WFHa    | -12.663     | 0.000***      |
| Captive          | All within Group | -19.425     | 0.000***      |
| FVs vs. FVs      | FVs vs. FRf      | -2.499      | 0.381         |
| FVs vs. FVs      | FVs vs. FHa      | -15.337     | 0.000***      |
| FRf vs. FRf      | FVs vs. FRf      | -3.014      | 0.085         |
| FRf vs. FRf      | FHa vs. FRf      | -15.644     | 0.000***      |
| FHa vs. FHa      | FVs vs. FHa      | -48.674     | 0.000***      |
| FHa vs. FHa      | FHa vs. FRf      | -58.733     | 0.000***      |

\(^{a}\)p-value was corrected by Bonferroni method. ***(p-value≤0.001.**
**Table 3** (on next page)

Statistical analyses accessing significance of differentiation of microbiota structure among groups.
### Results of PERMANOVA analysis

|    | Df | Sums of Sq | F. Model | r²  | p-value |
|----|----|------------|----------|-----|---------|
| Wild | 2  | 2.139      | 4.565    | 0.313 | 0.001*** |
| Captive | 2  | 2.656      | 8.320    | 0.381 | 0.001*** |

### Results of ANOSIM analysis

|    | R statistic | p-value | Number of permutations |
|----|-------------|---------|-----------------------|
| Wild | 0.873       | 0.001*** | 999                   |
| Captive | 0.803       | 0.001*** | 999                   |

1. **p-value ≤ 0.001**.
Figure 1

Venn diagram of shared and unique OTUs in the fecal bacterial communities of three bat species.

(A) Wild bat’ fecal samples. (B) Captive bat’ fecal samples. WFVs, WFRf and WFHa represent fecal samples from V. sinensis, R. ferrumequinum and H. armiger collected from the wild respectively. FVs, FRf and FHa represent fecal samples from captive V. sinensis, R. ferrumequinum and H. armiger respectively.
Figure 2

Wild and captive bats’ fecal bacterial communities clustered using nonmetric multidimensional scaling analysis of the unweighted UniFrac distance matrix.

Wild (A) and captive (B) bats’ fecal bacterial communities clustered using nonmetric multidimensional scaling analysis. Each point corresponds to a fecal sample colored according to bat species with different symbols corresponding to host family (red circle = Hipposideridae, green square = Vespertilionidae, blue triangle = Rhinolophidae).
Figure 3

Venn diagram of shared and unique microbial functions in three bat species.

(A) Wild bats. (B) Captive bats. WFVs, WFRf, WFHa, FVs, FRf and FHA are defined in the legend for Fig. 1.
Figure 4

The relative abundance of microbial functions related to metabolism predicted by PICRUST.

(A) Wild bats. (B) Captive bats. WFVs, WFRf, WFHa, FVs, FRf and FHa are defined in the legend for Fig. 1. Different letters in each KEGG pathway indicate significant differences ($p < 0.05$) in the relative abundances of this function in different bats species.
