A peer-reviewed version of this preprint was published in PeerJ on 29 March 2019.

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Comparative analysis and characterization of the gut microbiota of four farmed snakes from southern China

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Background. The gut microbiota plays an important role in host immunity and metabolichomeostasis. Although analyses of gut microorganisms have been used to assess host health, as well as for disease prevention and treatment, no comparative study of gut microorganisms among several species of farmed snake is yet available. In this study we characterized and compared the gut microbiotas of four species of farmed snakes (Naja atra, Ptyas mucosus, Elaphe carinata, and Deinagkistrodon acutus) using high-throughput sequencing of the 16S rDNA gene in southern China and tested whether there was a relationship between gut microbiota composition and host species. Results. A total of 629 operational taxonomic units (OTUs) across 22 samples were detected. The dominant bacterial phyla were Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria; the dominant bacterial genera were Bacteroides and Cetobacterium. This was the first report of the dominance of Fusobacteria and Cetobacterium in the snake gut. Our phylogenetic analysis recovered a relatively close relationship between Fusobacteria and Bacteroidetes. Alpha diversity analysis indicated that species richness and diversity were highest in the gut microbiota of D. acutus and lowest in that of E. carinata. Significant differences in alpha diversity were detected among the four farmed snake species. The gut microbiotas of conspecifics were more similar to each other than to those of heterospecifics. Conclusion. This study provides the first comparative study of gut microbiotas among several species of farmed snakes, and provides valuable data for the management of farmed snakes. In farmed snakes, host species affected the species composition and diversity of the gut microbiota.
Comparative analysis and characterization of the gut microbiota of four farmed snakes from southern China

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Running head: Gut microbiota in farmed snakes

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Brief information of the corresponding author: Prof. Daode Yang, from Central South University of Forestry and Technology, Changsha, Hunan, China, with his research focusing on wildlife conservation and nature reserve management.
**Background.** The gut microbiota plays an important role in host immunity and metabolic homeostasis. Although analyses of gut microbiotas have been used to assess host health, as well as for disease prevention and treatment, no comparative study of gut microbiotas among several species of farmed snake is yet available. In this study we characterized and compared the gut microbiotas of four species of farmed snakes (*Naja atra*, *Ptyas mucosus*, *Elaphe carinata*, and *Deinagkistrodon acutus*) using high-throughput sequencing of the 16S rDNA gene in southern China and tested whether there was a relationship between gut microbiota composition and host species. **Results.** A total of 629 operational taxonomic units (OTUs) across 22 samples were detected. The dominant bacterial phyla were Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria; the dominant bacterial genera were Bacteroides and Cetobacterium. This was the first report of the dominance of Fusobacteria and Cetobacterium in the snake gut. Our phylogenetic analysis recovered a relatively close relationship between Fusobacteria and Bacteroidetes. Alpha diversity analysis indicated that species richness and diversity were highest in the gut microbiota of *D. acutus* and lowest in that of *E. carinata*. Significant differences in alpha diversity were detected among the four farmed snake species. The gut microorganisms of conspecifics were more similar to each other than to those of heterospecifics. **Conclusion.** This study provides the first comparative study of gut microorganisms among several species of farmed snakes, and provides valuable data for the management of farmed snakes. In farmed snakes, host species affected the species composition and diversity of the gut microbiota.

**Key words** High-throughput sequencing, Gut microbiota, Host species, Microbial diversity, Farmed snakes
INTRODUCTION

Most animals, including snakes, have symbiotic relationships with their internal microbes, especially those that reside in the host gut (Gao, Wu & Wang, 2010). Studies of these symbiotic relationships have fundamentally increased our understanding of evolution, health, disease, and aging (Kundu et al., 2017). Gut microbiotas are extremely diverse, have unique functional characteristics, and may strongly affect the physiological functions of the host (Costea et al., 2018). For example, the gut microbiota may regulate the immune response, thereby affecting energy homeostasis (Spiljar, Merkler & Trajkovski, 2017) and nutrient metabolism (Shibata, Kunisawa & Kiyono, 2017). Changes in the gut microbiota may influence the functions of the brain and nerves (Kundu et al., 2017). Therefore, the gut microbiota may be an important factor determining the growth, immunity, and survival rate of farmed animals (Hu et al., 2017; Rosshart et al., 2017). The characterization of the gut microbiotas of farmed animals provides a scientific basis for disease diagnosis and health management (Kohl, Skopec & Dearing, 2014; Jiang et al., 2017; Lyons et al., 2017). Such characterizations are also essential for the commercial production of economically important animals and the conservation management of endangered species (Larsen, Mohammed & Arias, 2014).

Studies of gut microbiotas are primarily based on host fecal samples, as the collection of these samples is non-invasive (Costea et al., 2018). Fecal DNA reflects the composition and structure of the gut microbiota of the host (Ley et al., 2008; Waite & Taylor, 2014; Costea et al., 2018). In mammals, phylogenetic relationships and diet both influence the diversity of the internal microbiota; gut microbial diversity increases as diets change from carnivory to omnivory to herbivory (Ley et al., 2008; Hu et al., 2017). In birds, the composition of the gut microbiota was often species specific (Waite & Taylor, 2014). A thorough characterization of the gut microbiota increases our understanding of gut microbial function, and, consequently, our ability to manipulate
the gut microbiota to treat disease (Kundu et al., 2017; Rosshart et al., 2017; Hu et al., 2017). However, there
have been few studies of the gut microbiotas of snakes, and the available studies focused on individual species
(Costello et al., 2010; Colston, Noonan & Jackson, 2015; McLaughlin, Cochran & Dowd, 2015; Shi & Sun,
2017). Therefore, it remains necessary to comparatively assess the composition, diversity, and phylogeny of
snake gut microbiotas.

In recent years, several snake species have been successfully artificially bred on a large scale; such artificial-
breeding programs not only satisfy commercial needs, but also reduce pressure on wild snake populations (Hu
et al., 2013; Hu, Tan & Yang, 2013; Li, 2009). Naja atra (Elapidae), Ptyas mucosus (Colubridae), Elaphe
carinata (Colubridae), and Deinagkistrodon acutus (Viperidae) are the snake species most commonly farmed
in southern China (Li, 2009); N. atra and P. mucosus are listed in Appendix II of the Convention on
As all four of these snake species are highly edible and have medicinal value, they are being farmed in
increasing numbers in southern China (Li, 2009).

The aim of this study was to characterize the fecal microbiotas of four different species of farmed snakes in
southern China and test that host species affected the composition and diversity of the gut microbiota. This
work serves as the first high-throughput sequencing analysis that compares the gut microbiotas of several
farmed snake species. It is beneficial to study the gut microbiotas of snakes to improve the management of
farmed snake populations.

MATERIALS & METHODS

Sample collection

Fecal samples were collected from specimens of N. atra, P. mucosus, E. carinata, and D. acutus. All sampled
snakes were healthy adults, hatched in 2014 and reared in similar farm environments. All snakes were kept in farming rooms with a temperature of 28 ± 2°C, and a relative humidity of 80 ± 5%. Snakes were fed farmed chicks (*Gallus domesticus*) and mice (*Mus musculus*). Fecal samples from *N. atra*, *D. acutus*, and *P. mucosus* were collected at the Gong Xinguo snake farm, Yongzhou City, Hunan Province, China from 8–11 July 2017; fecal samples from *E. carinata* were collected at the Lvdongshan snake farm, Tujia-Miao Autonomous Prefecture of Xiangxi, Hunan Province, China on 26 August 2017. The wildlife operation licenses of the two snake farms were authorized by the Forestry Department of Hunan Province. The work was performed in accordance with the recommendations of the Institution of Animal Care and the Ethics Committee of Central South University of Forestry and Technology (approval number: CSUFT NS # 20175167). The fecal sampling procedures used in this study were non-invasive to the snakes.

Fresh fecal samples from different individuals were collected using a sterilized sampling spoon. Samples from the same species were pooled in the same centrifuge tube: *N. atra* pool (group 'Na'; n=6), *P. mucosus* pool (group 'Pmu'; n=4), *E. carinata* pool (group 'Ec'; n=6), and *D. acutus* pool (group 'Da'; n=6). All tubes were frozen for 10 h at -20°C, and then sent within 12 h on dry ice to the Wuhan Sample Center of Beijing Genomics Institute (BGI; Wuhan, China) for DNA extraction.

**DNA extraction, sequencing, and operational taxonomic units (OTUs) annotation**

Total DNA was extracted from the fecal samples using an E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc., USA). The V4 hypervariable region of the 16S rDNA gene was amplified using polymerase chain reaction (PCR), with the primers 515F (5’-GTGCCAGCMGGCGGTA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’). PCR products were purified with AmpureXP beads (Agencourt, Beckman Coulter, California, USA) to remove any non-specific amplicons. Qualified libraries were pair-end
sequenced on a MiSeq System (Illumina, San Diego, CA, USA) with MiSeq reagents using the PE300 (PE301+8+8+301) sequencing strategy, following the manufacturer's instructions. All libraries were sequenced on the Illumina MiSeq platform by the BGI (Wuhan, China).

The raw sequencing data were filtered, and the low quality reads were removed. The remaining high-quality reads were used for all subsequent analyses (Fadrosh et al., 2014). Reads was spliced into tags based on their overlap relationship (Magoč & Salzberg, 2011). Tags were aggregated into OTUs at 97% similarity using USEARCH v7.0.1090 (Edgar, 2013). Species annotation was then performed on the OTUs by comparing the OTUs to the 16S database (/RDP_set14/RDP_set14_NCBI_download_20151028) (Cole et al., 2013; Quast et al., 2012) with QIIME v1.80 package (confidence threshold: 0.60; Caporaso et al., 2010).

**Bioinformatics and statistical analysis**

Because niche changes are often reflected at the generic level (Costea et al., 2018), and because gut microbes are more frequently studied at the phylum level (Ley et al., 2008; Waite & Taylor, 2014; Lyons et al., 2017; Costea et al., 2018), this study focused on genus- and phylum-level analyses of the gut microbiotas of the farmed snakes.

The bacterial species corresponding to the recovered OTUs were identified by comparing the OTUs to the species database (/RDP_set14/RDP_set14_NCBI_download_20151028). Profiling area maps and histograms for each sample set at the phylum, class, order, family, and genus levels were created. Heatmap analyses were also performed to compare bacterial community composition among the different host species. A bacterial species was considered dominant when its relative abundance was greater than 10%. All bacterial classes with less than 0.5% relative abundance were combined into an "Others" class.

Within each genus, the sequence with the highest abundance was selected as the representative sequence. A
phylogenetic tree was constructed based on these representative sequences using the make_phylogeny.py script in QIIME v1.80 (Caporaso et al., 2010). The phylogenetic tree was graphed in the R v3.1.1 (R Development Core Team 2014 [http://www.R-project.org/]). Within each sample, sequences were considered part of the same OTUs at a 97% similarity threshold. A Venn diagram was constructed based on these OTUs with the VennDiagram package (Chen and Boutros 2011) in R v3.1.1 (R Development Core Team 2014 [http://www.R-project.org/]), showing the number of OTUs shared and unique among the different host species. A principal components analysis (PCA) was used to quantify the differences in OTUs composition among samples and the distances between OTUs on a two-dimensional coordinate map. PCA was performed with the ade4 package (Dray and Dufour, 2007) in R v3.1.1 (R Development Core Team 2014 [http://www.R-project.org/]).

Alpha diversity describes species diversity at a single site or within a single sample (Schloss et al., 2009). Alpha diversity was estimated by calculating the number of observed species (Sobs), the Chao index, the abundance-based coverage estimator (ACE), the Shannon index, and the Simpson index using mothur v1.31.2 (http://www.mothur.org/wiki/Calculators). Difference analysis and mapping were performed in R v3.1.1 (White, Nagarajan & Pop, 2009). To compare differences in bacterial diversity between pairs of snake species, beta diversity was analyzed using Bray-Curtis dissimilarity and the weighted and unweighted UniFrac metrics with QIIME v1.80 (Caporaso et al., 2010).

Differences in bacterial species abundance among samples were identified using the kruskal.test package (White, Nagarajan & Pop, 2009) in R v3.1.1 (R Development Core Team 2014 [http://www.R-project.org/]), adjusting for the false discovery rate (FDR) and with the threshold P-value among groups set to 0.05. Based on these results, the bacterial species that most influenced the differences in sample composition among groups...
RESULTS

Data quality evaluation

Across all samples, 727,310 sequences with an average length of 252 bp were obtained, yielding 629 OTUs (Table S1). The OTUs rank curve indicated that bacterial abundance and evenness differed among samples (Fig. S1). Here, 504 OTUs in the Da group, 192 OTUs in the Ec group, 285 OTUs in the Na group, and 236 OTUs in the Pmu group were identified (Fig. S2). The species corresponding to the recovered OTUs were identified by comparing the OTUs to the species database (/RDP_set14/RDP_set14_NCBI_download_20151028). Bacterial species were profiled and histograms were constructed for each pooled sample. On average, 0.10% of all OTUs were unclassified at the phylum level (Fig. 1A), and 12.79% were unclassified at the genus level (Fig. 1B).

Dominant bacterial taxa across all snake hosts

Based on comparisons with the database (/RDP_set14/RDP_set14_NCBI_download_20151028), the gut microbiotas of the four farmed snake species fell into 15 phyla, 18 classes, 22 orders, 35 families, and 58 genera (Table 1; Fig. 1A,B; Fig. S3–5). Across all samples, the dominant bacterial phyla were Bacteroidetes (30.98%), Proteobacteria (24.80%), Firmicutes (20.96%), and Fusobacteria (20.20%), while the most abundant genera were Bacteroides (26.63%) and Cetobacterium (19.06%). Phylogenetic analysis indicated that most genera fell into Bacteroidetes, Firmicutes, and Proteobacteria; only two genera fell into Fusobacteria (Fig. 2). The dominant bacterial genera Bacteroides and Cetobacterium fall into the phyla Bacteroidetes and Fusobacteria, respectively (Fig. 2).

Comparisons of gut microbiotas among groups
(1) Relative abundance

Statistical analysis suggested that the relative abundance of gut microbiota in 8 phyla and 44 genera differed significantly among groups (Fig. 1A,B; Table S2). There were significant differences in the relative abundances of the dominant bacterial phyla (Bacteroidetes, Firmicutes, and Fusobacteria) among the samples (Table 2). However, there was no difference in the relative abundance of Proteobacteria among groups ($p=0.115; \text{FDR}=0.164; \text{Table 2}$). There were significant differences in the relative abundances of the dominant bacterial genera *Bacteroides* and *Cetobacterium* among the samples ($p=0.002, \text{FDR}=0.011, p=0.006, \text{FDR}=0.022; \text{Table S2}$): *Bacteroides* was most abundant in the Na group (40.17%) and in the Ec group (42.09%), while *Cetobacterium* was most abundant in the Pmu group (37.46%; Fig. 1B; Table S2).

(2) Alpha diversity analysis

Alpha diversity indices (Sobs, $p=0.001$; Chao, $p=0.0004$; ACE, $p=0.0004$; Shannon, $p=0.002$; and Simpson, $p=0.003$) differed significantly among groups, indicating substantial differences in the species richness and diversity of the gut microbiota among groups (Fig. 3; Table S3). In the Da group, the Sobs (288.67), Chao (327.09), ACE (326.61), and Shannon (3.74) indices were significantly higher than those of the other three groups, while the Simpson index (0.05) was significantly lower (Fig. 3; Table S3). This indicated that the community richness and diversity of the gut microbiota in the Da group were higher than those of the other three groups. The Chao (121.18) and ACE (123.41) indices in the Ec group were significantly lower than those of the other three groups, indicating that the community richness and species diversity of the gut microbiota in the Ec group were lower than those of the other three groups (Fig. 3; Table S3).

(3) Similarity analysis

The PCA showed that the gut microbiotas from the same group were more similar to each other than to the gut
microbiotas from different groups, indicating that gut microbiotas were most similar within same snake species. Among the different snake species, the Ec and the Na group were closest, indicating that the gut microbiotas of these two species were similar. In contrast, the Da group was widely separated from the other three groups, indicating that the gut microbiota of the Da group was dissimilar to those of the other three groups (Fig. S6).

Beta diversity analyses (Bray-Curtis dissimilarity and the unweighted UniFrac metric) were used to compare species diversity between pairs of pooled samples. The Bray-Curtis distance suggested that the differences within each sample group were small; samples from the same group clustered together (with the exception of samples Na4 and Na5, which clustered with the Ec group; Fig. 4A). The UniFrac metric uses phylogenetic information to compare species-level community composition among samples, and controls for evolutionary distance among sequences (Lozupone & Knight, 2005). Here, the unweighted UniFrac metric was consistent with the results of the Bray-Curtis dissimilarity analysis (with the exception of sample Na2, which clustered with the Ec group; Fig. 4B).

Heatmap vertical clustering at the genus level showed that samples from the same snake were tightly grouped on short branches, indicating that the composition and abundance of gut bacteria in the same sample were similar (with the exception of Na2 and Pmu3, which clustered with the Ec group; Fig. 5). These results were consistent with the beta diversity analysis. Interestingly, the heatmap analysis showed that the gut microbiota from Ec group and from the Pmu group clustered together along one branch, while the beta diversity analysis suggested that the Ec group clustered with the Na group (Fig. 4,5). This indicated that the clusters generated by different analytical methods differed slightly.

**DISCUSSION**

Tens of billions of bacterial species have colonized various animals, typically in the gut (Costea et al., 2018).
Because the gut microbiotas of healthy adult animals are stable, the composition and structure of the normal
gut microbiota can be used to assess animal health and diagnose or prevent disease (Kundu et al., 2017; Rosshart et al., 2017; Hu et al., 2017). A variety of bacteria have colonized the guts of various snake species, providing nutrition and immune protection to the host (Colston, Noonan & Jackson, 2015; Costello et al., 2010; McLaughlin, Cochran & Dowd, 2015). These results indicated that the composition of gut microbiota was unique to each species of farmed snake. That is, even though the farmed snakes were kept in similar environments, fed similar diets, and were of similar ages, there were interspecific differences in the composition and diversity of the gut microbiotas that depended on host species.

**Dominant gut microbes**

Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria were the dominant phyla in the gut microbiota of the four farmed snake species (Fig. 2). This differed from mammals (Ley et al., 2008), birds (Waite & Taylor, 2014), and other reptiles (Colston, Noonan & Jackson, 2015; Keenan, Engel & Elsey, 2013; McLaughlin, Cochran & Dowd, 2015; Jiang et al., 2017). In previous studies of vertebrates, the gut microbiota have been dominated by the phyla Bacteroidetes and Firmicutes, which influence the physiological functions of the host with respect to metabolism and immunity (Thomas et al., 2011). Bacteroidetes species degrade high polymer organic compounds (proteins and carbohydrates); Firmicutes species degrade cellulose into volatile fatty acids, improving cellulose utilization (Li et al., 2013).

Proteobacteria account for less than 10% of all gut microbes in mammals (Ley et al., 2008). For example, Proteobacteria enrichment in the human gut was an indicator of gut microbiota imbalance and was associated with host disease (Shin, Whon & Bae, 2015). The proportion of Proteobacteria in the gut microbiota of other snakes was relatively high, although this proportion varied greatly by species. For example, the gut microbiota
of the Burmese python (*Python bivittatus*) was 10.1% Proteobacteria (*Costello et al., 2010*), while that of the Timber rattlesnake (*Crotalus horridus*) was 85.0% Proteobacteria (*McLaughlin, Cochran & Dowd, 2015*).

Similar results were observed in the farmed snake species analyzed here (16.4–36.9%) (Table 2).

The proportion of Fusobacteria in the gut microorganisms of mammals, birds, and other snakes was relatively small (*Ley et al., 2008; Costello et al., 2010; Waite & Taylor, 2014; Colston, Noonan & Jackson, 2015; McLaughlin, Cochran & Dowd, 2015*). However, Fusobacteria was the dominant bacterial phylum in the guts of five eastern African cichlid fish (*Baldo et al., 2015*) and in the American alligator (*Alligator mississippiensis*) (*Keenan, Engel & Elsey, 2013*). Here, Fusobacteria dominated the gut microbiotas of the farmed snakes; this is the first report of the dominance of Fusobacteria in the snake gut microbiota.

*Bacteroides* and *Cetobacterium* were the dominant bacterial genera in gut microbiota of the farmed snakes (Fig. 2). *Bacteroides* maintain a complex and beneficial relationship in the host gut, and the symbiotic relationships between these bacteria and their hosts have been widely studied (*Thomas et al., 2011*). For example, *Bacteroides* species have complex systems for sensing nutrient utilization, regulating nutrient metabolism, and acquiring and hydrolyzing otherwise indigestible dietary polysaccharides (*Xu et al., 2003*). *Bacteroides* species control host gut homeostasis by interacting with the host immune system (*Wexler, 2007*).

Here, the gut microbiotas of the farmed snakes were dominated by *Bacteroides*, especially in the Ec group (42.09%) and the Na group (40.17%) (Fig. 3), indicating that the gut microbiota in snakes are species dependent. All *Cetobacterium* species are obligate anaerobes in phylum Fusobacteria (Fig. 2). *Cetobacterium* was the dominant genus in the gut microbiotas of all the farmed snakes analyzed herein; this is the first report of the dominance of this genus in the gut microbiotas of snakes.

**Fusobacteria in gut microbiotas of farmed snakes**
Fusobacteria is a little-studied bacterial phylum, with a somewhat uncertain phylogenetic position (Keenan, Engel & Elsey, 2013). The results of the present study indicated that Fusobacteria contains only two genera, Cetobacterium and Fusobacterium (Fig. 2). However, it is possible that Fusobacteria includes additional unclassified genera, and/or that the Fusobacteria have been undersampled in previous studies of gut microbiotas (Keenan, Engel & Elsey, 2013). Previous studies have suggested that Fusobacteria have a core genome dissimilar to that of other bacterial lineages (Mira et al., 2004). Phylogenetic and comparative genomics analyses indicate that this phylum is closely affiliated with Bacteroidetes and Firmicutes, and may be derived from the Firmicutes (Mira et al., 2004). Phylogenetic analysis recovered a close relationship between Fusobacteria and Bacteroidetes, indicating a relatively close evolutionary relationship (Fig. 2). Bacteroidetes is one of the major lineages of bacteria, arising early in bacterial evolution (Wexler, 2007). Therefore, the evolutionary relationship between Fusobacteria and Bacteroidetes should be further investigated.

Fusobacteria species play a critical role in initial biofilm development (Mira et al., 2004), suggesting that the presence of these species in the guts of the farmed snakes may affect the development of the lumen membrane (Keenan, Engel & Elsey, 2013). Cetobacterium was first isolated from the intestinal contents of a porpoise and from the mouth lesion of a minke whale (Balaenoptera acutorostrata) (Foster et al., 1995). Species in this genus transform peptones and carbohydrate into acetic acid (Edwards, Logan & Gharbia, 2015).

Because Fusobacteria and Cetobacterium dominated the gut microbiotas of the farmed snakes, species in these taxa were likely commensal inhabitants of snake guts. It is therefore possible to speculate that, in snakes, Fusobacteria and Cetobacterium play important roles in digestive organ development and in nutritional metabolism.

The relationship between gut microbiota and host species
Many factors affect the vertebrate gut microbiotas, including host species, diet, and age (Ley et al., 2008; Waite & Taylor, 2014; Hu et al., 2017; Jiang et al., 2017). The gut microbiota may also vary in different regions of the gut tract (Ley et al., 2008; Waite & Taylor, 2014). Diet and host species influence the composition of the gut microbiota more than other factors (Waite & Taylor, 2014). The gut microbiota of the Burmese python was dominated by Firmicutes and Bacteroidetes (Costello et al., 2010), while the gut microbiota of the timber rattlesnake was uniquely dominated by Proteobacteria (McLaughlin, Cochran & Dowd, 2015). Bacteroidetes, Firmicutes, and Proteobacteria also dominated the gut microbiota of the cottonmouth snake (Colston, Noonan & Jackson, 2015). Therefore, the dominant bacterial phyla vary based on snake species. However, diet, age, habitat, and research method varied in previous studies of snake microbiotas, possibly affecting the distribution of bacterial species abundance at the phylum level. Here, phylum-based alpha diversity (Fig. 3), PCA (Fig. S6), beta diversity (Fig. 4A,B), and cluster analyses suggested a relationship between the composition of the gut microbiota and the host species. The species studied here were similar with respect to diet, health, habitat, and age. This suggested that host species was the most important factor shaping the microbiota of the snake gut.

CONCLUSION

The compositions of the gut microbiotas of four farmed snake species in southern China were different to those of other snakes and vertebrates. The gut bacteria of these four species fell into 15 phyla, 18 classes, 22 orders, 35 families, and 58 genera. The dominant bacterial phyla were Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria, while the dominant bacterial genera were Bacteroides and Cetobacterium. This was the first report that Fusobacteria and Cetobacterium dominated the gut microbiotas of snake species. Gut microbiotal diversity was highest in D. acutus and lowest in E. carinata. There were interspecific differences in gut
microbiota composition, diversity, and the Firmicutes/Bacteroidetes relative abundance ratio among the four farmed snake species. Our results supported our hypothesis that host species was an important factor affecting the gut microbiotas of snakes. Further studies of snake gut microbiotas should investigate the relationship between phylogenetic position and function, as well as the characteristics of dominant bacteria that were unclassifiable. It is important to determine whether the immunity and growth of farmed snake populations can be improved by inoculating fecal suspensions generated by healthy wild snakes into the guts of farmed conspecifics. In addition, it would also be useful to establish an open database of microbial data from the guts of snakes and other reptile groups.

**ADDITIONAL INFORMATION AND DECLARATIONS**

**Funding**

This study was funded by the National Natural Science Foundation of China (No.31472021) and the Project for Wildlife Conservation and Management of the State Forestry Administration of China (No.16180617). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests**

The authors declare there are no competing interests.

**Author Contributions**

• Bing Zhang conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables and reviewed drafts of the paper.
Jing Ren performed the experiments, analyzed the data and wrote the paper.

Daode Yang and Shuoran Liu conceived and designed the experiments, reviewed drafts of the paper.

Xinguo Gong performed the experiments.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e. approving body and any reference numbers):

The work was performed in accordance with the recommendations of the Institution of Animal Care and the Ethics Committee of Central South University of Forestry and Technology (approval number: CSUFT NS # 20175167).

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as a Supplementary File.

Supplemental Information

Supplemental files used in this paper have been uploaded to the submission system of PeerJ (https://peerj.com/manuscripts/30756/files/).

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Figure 1 (on next page)

Composition of the gut microbiotas of four snake species by bacterial (A) phylum and (B) genus.

Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group.
Figure 2 (on next page)

Genus-level phylogeny of gut microbiota from four snake species.

Genera are colored by phylum.
Alpha diversity of bacterial communities across four snake species.

(A) Observed species (Sobs) index; (B) chao index; (C) abundance-based coverage estimator (ACE); (D) Shannon's diversity index; (E) Simpson's diversity index. The top and bottom of each box indicate the first and third quartiles, the line inside the box indicates the median, and the ends of the dotted lines represent the minimum and the maximum. Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group.
Beta diversity of the gut microbiotas of four snake species.

(A) Cluster tree generated based on Bray-Curtis distances. (B) Cluster tree generated based on unweighted UniFrac distances. Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group.
Figure 5 (on next page)

Heatmap showing the genus-level bacterial community composition in the gut microorganisms of four snake species.

Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group.
Table 1 (on next page)

Composition of the fecal microbiotas of four snake species.

Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group.
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<th>Group</th>
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<th>Number of classes</th>
<th>Number of orders</th>
<th>Number of families</th>
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<tr>
<td>Pmu</td>
<td>11</td>
<td>16</td>
<td>19</td>
<td>28</td>
<td>44</td>
</tr>
<tr>
<td>Ec</td>
<td>9</td>
<td>15</td>
<td>19</td>
<td>27</td>
<td>44</td>
</tr>
<tr>
<td>Da</td>
<td>12</td>
<td>18</td>
<td>22</td>
<td>34</td>
<td>53</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>18</td>
<td>22</td>
<td>35</td>
<td>58</td>
</tr>
</tbody>
</table>
Table 2 (on next page)

The relative abundance of the dominant bacterial phyla of four snake species (Mean± Standard Deviation).

Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group. Differences in dominant bacterial species abundance among groups were identified using the `kruskal.test` package in R v3.1.1, adjusting for the false discovery rate (FDR) and with the threshold.
<table>
<thead>
<tr>
<th>Dominant phylum</th>
<th>Na group</th>
<th>Pmu group</th>
<th>Ec group</th>
<th>Da group</th>
<th>p</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>45.07±4.92</td>
<td>10.22±2.32</td>
<td>43.54±6.93</td>
<td>18.24±16.89</td>
<td>=0.015</td>
<td>=0.012</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>9.91±5.45</td>
<td>18.71±7.51</td>
<td>7.88±3.04</td>
<td>46.54±10.73</td>
<td>=0.002</td>
<td>=0.011</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>16.81±10.55</td>
<td>42.53±8.38</td>
<td>19.42±9.59</td>
<td>9.57±6.56</td>
<td>=0.008</td>
<td>=0.015</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>27.67±8.10</td>
<td>27.74±14.28</td>
<td>28.31±10.81</td>
<td>16.38±6.08</td>
<td>=0.115</td>
<td>=0.164</td>
</tr>
</tbody>
</table>