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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 microbiotas have been used to assess host health,as well as for disease prevention and treatment, no comparative study of gut microbiotasamong several species of farmed snake is yet available. In this study we characterized andcompared the gut microbiotas of four species of farmed snakes (*Naja atra*, *Ptyas mucosus*,*Elaphe carinata*, and *Deinagkistrodon acutus*) using high-throughput sequencing of the16S rDNA gene in southern China and tested whether there was a relationship betweengut microbiotal composition and host species. **Results.** A total of 629 operationaltaxonomic units (OTUs) across 22 samples were detected. The dominant bacterial phylawere Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria; the dominant bacterialgenera were Bacteroides and Cetobacterium. This was the first report of the dominance ofFusobacteria and Cetobacterium in the snake gut. Our phylogenetic analysis recovered arelatively close relationship between Fusobacteria and Bacteroidetes. Alpha diversityanalysis indicated that species richness and diversity were highest in the gut microbiota ofD. acutus and lowest in that of E. carinata. Significant differences in alpha diversity weredetected among the four farmed snake species. The gut microbiotas of conspecifics weremore similar to each other than to those of heterospecifics. **Conclusion.** This studyprovides the first comparative study of gut microbiotas among several species of farmedsnakes, and provides valuable data for the management of farmed snakes. In farmedsnakes, host species affected the species composition and diversity of the gut microbiota.

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7 Running head: Gut microbiota in farmed snakes

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11 reserve management.

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12 **Background.** The gut microbiota plays an important role in host immunity and metabolic
13 homeostasis. Although analyses of gut microbiotas have been used to assess host health,
14 as well as for disease prevention and treatment, no comparative study of gut microbiotas
15 among several species of farmed snake is yet available. In this study we characterized and
16 compared the gut microbiotas of four species of farmed snakes (*Naja atra*, *Ptyas mucosus*,
17 *Elaphe carinata*, and *Deinagkistrodon acutus*) using high-throughput sequencing of the
18 16S rDNA gene in southern China and tested whether there was a relationship between
19 gut microbiotal composition and host species. **Results.** A total of 629 operational
20 taxonomic units (OTUs) across 22 samples were detected. The dominant bacterial phyla
21 were Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria; the dominant bacterial
22 genera were Bacteroides and Cetobacterium. This was the first report of the dominance of
23 Fusobacteria and Cetobacterium in the snake gut. Our phylogenetic analysis recovered a
24 relatively close relationship between Fusobacteria and Bacteroidetes. Alpha diversity
25 analysis indicated that species richness and diversity were highest in the gut microbiota of
26 D. acutus and lowest in that of E. carinata. Significant differences in alpha diversity were
27 detected among the four farmed snake species. The gut microbiotas of conspecifics were
28 more similar to each other than to those of heterospecifics. **Conclusion.** This study
29 provides the first comparative study of gut microbiotas among several species of farmed
30 snakes, and provides valuable data for the management of farmed snakes. In farmed
31 snakes, host species affected the species composition and diversity of the gut microbiota.

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

33 # INTRODUCTION

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

47 Studies of gut microbiotas are primarily based on host fecal samples, as the collection of these samples is
48 non-invasive (*Costea et al., 2018*). Fecal DNA reflects the composition and structure of the gut microbiota of
49 the host (*Ley et al., 2008*; *Waite & Taylor, 2014*; *Costea et al., 2018*). In mammals, phylogenetic relationships
50 and diet both influence the diversity of the internal microbiota; gut microbial diversity increases as diets
51 change from carnivory to omnivory to herbivory (*Ley et al., 2008*; *Hu et al., 2017*). In birds, the composition
52 of the gut microbiota was often species specific (*Waite & Taylor, 2014*). A thorough characterization of the gut
53 microbiota increases our understanding of gut microbial function, and, consequently, our ability to manipulate

54 the gut microbiota to treat disease (*Kundu et al., 2017*; *Rosshart et al., 2017*; *Hu et al., 2017*). However, there
55 have been few studies of the gut microbiotas of snakes, and the available studies focused on individual species
56 (*Costello et al., 2010*; *Colston, Noonan & Jackson, 2015*; *McLaughlin, Cochran & Dowd, 2015*; *Shi & Sun,*
57 *2017*). Therefore, it remains necessary to comparatively assess the composition, diversity, and phylogeny of
58 snake gut microbiotas.

59 In recent years, several snake species have been successfully artificially bred on a large scale; such artificial-
60 breeding programs not only satisfy commercial needs, but also reduce pressure on wild snake populations (*Hu*
61 *et al., 2013*; *Hu, Tan & Yang, 2013*; *Li, 2009*). *Naja atra* (Elapidae), *Ptyas mucosus* (Colubridae), *Elaphe*
62 *carinata* (Colubridae), and *Deinagkistrodon acutus* (Viperidae) are the snake species most commonly farmed
63 in southern China (*Li, 2009*); *N. atra* and *P. mucosus* are listed in Appendix II of the Convention on
64 International Trade in Endangered Species of Wild Fauna and Flora (CITES) (1990; https://www.cites.org/).
65 As all four of these snake species are highly edible and have medicinal value, they are being farmed in
66 increasing numbers in southern China (Li, 2009).

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

72 MATERIALS & METHODS

73 Sample collection

74 Fecal samples were collected from specimens of *N. atra*, *P. mucosus*, *E. carinata*, and *D. acutus*. All sampled

75 snakes were healthy adults, hatched in 2014 and reared in similar farm environments. All snakes were kept in
76 farming rooms with a temperature of 28 ± 2°C, and a relative humidity of 80 ± 5%. Snakes were fed farmed
77 chicks (*Gallus domestiaus*) and mice (*Mus musculus*). Fecal samples from *N. atra*, *D. acutus,* and *P. mucosus*
78 were collected at the Gong Xinguo snake farm, Yongzhou City, Hunan Province, China from 8–11 July 2017;
79 fecal samples from *E. carinata* were collected at the Lvdongshan snake farm, Tujia-Miao Autonomous
80 Prefecture of Xiangxi, Hunan Province, China on 26 August 2017. The wildlife operation licenses of the two
81 snake farms were authorized by the Forestry Department of Hunan Province. The work was performed in
82 accordance with the recommendations of the Institution of Animal Care and the Ethics Committee of Central
83 South University of Forestry and Technology (approval number: CSUFT NS # 20175167). The fecal sampling
84 procedures used in this study were non-invasive to the snakes.
85 Fresh fecal samples from different individuals were collected using a sterilized sampling spoon. Samples
86 from the same species were pooled in the same centrifuge tube: *N. atra* pool (group 'Na'; n=6), *P. mucosus*
87 pool (group 'Pmu'; n=4), *E. carinata* pool (group 'Ec'; n=6), and *D. acutus* pool (group 'Da'; n=6). All tubes
88 were frozen for 10 h at -20°C, and then sent within 12 h on dry ice to the Wuhan Sample Center of Beijing
89 Genomics Institute (BGI; Wuhan, China) for DNA extraction.

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

91 Total DNA was extracted from the fecal samples using an E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc.,
92 USA). The V4 hypervariable region of the 16S rDNA gene was amplified using polymerase chain reaction
93 (PCR), with the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-
94 GGACTACHVGGGTWTCTAAT-3'). PCR products were purified with AmpureXP beads (Agencourt,
95 Beckman Coulter, California, USA) to remove any non-specific amplicons. Qualified libraries were pair-end

96 sequenced on a MiSeq System (Illumina, San Diego, CA, USA) with MiSeq reagents using the PE300
97 (PE301+8+8+301) sequencing strategy, following the manufacturer's instructions. All libraries were sequenced
98 on the Illumina MiSeq platform by the BGI (Wuhan, China).
99 The raw sequencing data were filtered, and the low quality reads were removed. The remaining high-quality
100 reads were used for all subsequent analyses (*Fadrosh et al., 2014*). Reads was spliced into tags based on their
101 overlap relationship (Magoč & Salzberg, 2011). Tags were aggregated into OTUs at 97% similarity using
102 USEARCH v7.0.1090 (*Edgar, 2013*). Species annotation was then performed on the OTUs by comparing the
103 OTUs to the 16S database (/RDP_set14/RDP_set14_NCBI_download_20151028) (*Cole et al., 2013*; *Quast et*
104 *al., 2012*) with QIIME v1.80 package (confidence threshold: 0.60; *Caporaso et al., 2010*).
105 **Bioinformatics and statistical analysis**
106 Because niche changes are often reflected at the generic level (*Costea et al., 2018*), and because gut microbes
107 are more frequently studied at the phylum level (*Ley et al., 2008*; *Waite & Taylor, 2014*; *Lyons et al., 2017*;
108 *Costea et al., 2018*), this study focused on genus- and phylum-level analyses of the gut microbiotas of the
109 farmed snakes.
110 The bacterial species corresponding to the recovered OTUs were identified by comparing the OTUs to the
111 species database (/RDP_set14/RDP_set14_NCBI_download_20151028). Profiling area maps and histograms
112 for each sample set at the phylum, class, order, family, and genus levels were created. Heatmap analyses were
113 also performed to compare bacterial community composition among the different host species. A bacterial
114 species was considered dominant when its relative abundance was greater than 10%. All bacterial classes with
115 less than 0.5% relative abundance were combined into an "Others" class.
116 Within each genus, the sequence with the highest abundance was selected as the representative sequence. A

117 phylogenetic tree was constructed based on these representative sequences using the make_phylogeny.py script
118 in QIIME v1.80 (*Caporaso et al., 2010*). The phylogenetic tree was graphed in the R v3.1.1 (R Development
119 Core Team 2014 [http://www.R-project.org/]).

120 Within each sample, sequences were considered part of the same OTUs at a 97% similarity threshold. A
121 Venn diagram was constructed based on these OTUs with the VennDiagram package (Chen and Boutros 2011)
122 in R v3.1.1 (R Development Core Team 2014 [http://www.R-project.org/]), showing the number of OTUs
123 shared and unique among the different host species. A principal components analysis (PCA) was used to
124 quantify the differences in OTUs composition among samples and the distances between OTUs on a two-
125 dimensional coordinate map. PCA was performed with the ade4 package (*Dray and Dufour, 2007*) in R v3.1.1
126 (R Development Core Team 2014 [http://www.R-project.org/]).

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

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

138 were identified.

139 RESULTS

140 Data quality evaluation

141 Across all samples, 727,310 sequences with an average length of 252 bp were obtained, yielding 629 OTUs
142 (Table S1). The OTUs rank curve indicated that bacterial abundance and evenness differed among samples
143 (Fig. S1). Here, 504 OTUs in the Da group, 192 OTUs in the Ec group, 285 OTUs in the Na group, and 236
144 OTUs in the Pmu group were identified (Fig. S2). The species corresponding to the recovered OTUs were
145 identified by comparing the OTUs to the species database
146 (/RDP_set14/RDP_set14_NCBI_download_20151028). Bacterial species were profiled and histograms were
147 constructed for each pooled sample. On average, 0.10% of all OTUs were unclassified at the phylum level (Fig.
148 1A), and 12.79% were unclassified at the genus level (Fig. 1B).

149 Dominant bacterial taxa across all snake hosts

150 Based on comparisons with the database (/RDP_set14/RDP_set14_NCBI_download_ 20151028), the gut
151 microbiotas of the four farmed snake species fell into 15 phyla, 18 classes, 22 orders, 35 families, and 58
152 genera (Table 1; Fig. 1A,B; Fig. S3–5). Across all samples, the dominant bacterial phyla were Bacteroidetes
153 (30.98%), Proteobacteria (24.80%), Firmicutes (20.96%), and Fusobacteria (20.20%), while the most abundant
154 genera were *Bacteroides* (26.63%) and *Cetobacterium* (19.06%). Phylogenetic analysis indicated that most
155 genera fell into Bacteroidetes, Firmicutes, and Proteobacteria; only two genera fell into Fusobacteria (Fig. 2).
156 The dominant bacterial genera *Bacteroides* and *Cetobacterium* fall into the phyla Bacteroidetes and
157 Fusobacteria, respectively (Fig. 2).

158 Comparisons of gut microbiotas among groups

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159 **(1) Relative abundance**

160 Statistical analysis suggested that the relative abundance of gut microbiota in 8 phyla and 44 genera differed
161 significantly among groups (Fig. 1A,B; Table S2). There were significant differences in the relative
162 abundances of the dominant bacterial phyla (Bacteroidetes, Firmicutes, and Fusobacteria) among the samples
163 (Table 2). However, there was no difference in the relative abundance of Proteobacteria among groups
164 (p=0.115; FDR=0.164; Table 2). There were significant differences in the relative abundances of the dominant
165 bacterial genera *Bacteroides* and *Cetobacterium* among the samples (p=0.002, FDR=0.011; p=0.006,
166 FDR=0.022; Table S2): *Bacteroides* was most abundant in the Na group (40.17%) and in the Ec group
167 (42.09%), while *Cetobacterium* was most abundant in the Pmu group (37.46%; Fig. 1B; Table S2).

168 **(2) Alpha diversity analysis**

169 Alpha diversity indices (Sobs, p= 0.001; Chao, p= 0.0004; ACE, p= 0.0004; Shannon, p= 0.002; and Simpson,
170 p= 0.003) differed significantly among groups, indicating substantial differences in the species richness and
171 diversity of the gut microbiota among groups (Fig. 3; Table S3). In the Da group, the Sobs (288.67), Chao
172 (327.09), ACE (326.61), and Shannon (3.74) indices were significantly higher than those of the other three
173 groups, while the Simpson index (0.05) was significantly lower (Fig. 3; Table S3). This indicated that the
174 community richness and diversity of the gut microbiota in the Da group were higher than those of the other
175 three groups. The Chao (121.18) and ACE (123.41) indices in the Ec group were significantly lower than those
176 of the other three groups, indicating that the community richness and species diversity of the gut microbiota in
177 the Ec group were lower than those of the other three groups (Fig. 3; Table S3).

178 **(3) Similarity analysis**

179 The PCA showed that the gut microbiotas from the same group were more similar to each other than to the gut

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180 microbiotas from different groups, indicating that gut microbiotas were most similar within same snake species.
181 Among the different snake species, the Ec and the Na group were closest, indicating that the gut microbiotas of
182 these two species were similar. In contrast, the Da group was widely separated from the other three groups,
183 indicating that the gut microbiota of the Da group was dissimilar to those of the other three groups (Fig. S6).
184 Beta diversity analyses (Bray-Curtis dissimilarity and the unweighted UniFrac metric) were used to compare
185 species diversity between pairs of pooled samples. The Bray-Curtis distance suggested that the differences
186 within each sample group were small; samples from the same group clustered together (with the exception of
187 samples Na4 and Na5, which clustered with the Ec group; Fig. 4A). The UniFrac metric uses phylogenetic
188 information to compare species-level community composition among samples, and controls for evolutionary
189 distance among sequences (*Lozupone & Knight, 2005*). Here, the unweighted UniFrac metric was consistent
190 with the results of the Bray-Curtis dissimilarity analysis (with the exception of sample Na2, which clustered
191 with the Ec group; Fig. 4B).
192 Heatmap vertical clustering at the genus level showed that samples from the same snake were tightly
193 grouped on short branches, indicating that the composition and abundance of gut bacteria in the same sample
194 were similar (with the exception of Na2 and Pmu3, which clustered with the Ec group; Fig. 5). These results
195 were consistent with the beta diversity analysis. Interestingly, the heatmap analysis showed that the gut
196 microbiota from Ec group and from the Pmu group clustered together along one branch, while the beta
197 diversity analysis suggested that the Ec group clustered with the Na group (Fig. 4,5). This indicated that the
198 clusters generated by different analytical methods differed slightly.

199 DISCUSSION

200 Tens of billions of bacterial species have colonized various animals, typically in the gut (*Costea et al., 2018*).

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

209 **Dominant gut microbes**

210 Bacteroidetes, Proteobacteria, Firmicutes, and Fusobacteria were the dominant phyla in the gut microbiota of
211 the four farmed snake species (Fig. 2). This differed from mammals (*Ley et al., 2008*), birds (*Waite & Taylor,*
212 *2014*), and other reptiles (*Colston, Noonan & Jackson, 2015*; *Keenan, Engel & Elsey, 2013*; *McLaughlin,*
213 *Cochran & Dowd, 2015*; *Jiang et al., 2017*). In previous studies of vertebrates, the gut microbiota have been
214 dominated by the phyla Bacteroidetes and Firmicutes, which influence the physiological functions of the host
215 with respect to metabolism and immunity (*Thomas et al., 2011*). Bacteroidetes species degrade high polymer
216 organic compounds (proteins and carbohydrates); Firmicutes species degrade cellulose into volatile fatty acids,
217 improving cellulose utilization (*Li et al., 2013*).

218 Proteobacteria account for less than 10% of all gut microbes in mammals (*Ley et al., 2008*). For example,
219 Proteobacteria enrichment in the human gut was an indicator of gut microbiota imbalance and was associated
220 with host disease (*Shin, Whon & Bae, 2015*). The proportion of Proteobacteria in the gut microbiota of other
221 snakes was relatively high, although this proportion varied greatly by species. For example, the gut microbiota

222 of the Burmese python (*Python bivittatus*) was 10.1% Proteobacteria (*Costello et al., 2010*), while that of the
223 Timber rattlesnake (*Crotalus horridus*) was 85.0% Proteobacteria (*McLaughlin, Cochran & Dowd, 2015*).
224 Similar results were observed in the farmed snake species analyzed here (16.4–36.9%) (Table 2).
225 The proportion of Fusobacteria in the gut microbiotas of mammals, birds, and other snakes was relatively
226 small (*Ley et al., 2008*; *Costello et al., 2010*; *Waite & Taylor, 2014*; *Colston, Noonan & Jackson, 2015*;
227 *McLaughlin, Cochran & Dowd, 2015*). However, Fusobacteria was the dominant bacterial phylum in the guts
228 of five eastern African cichlid fish (*Baldo et al., 2015*) and in the American alligator (*Alligator*
229 *mississippiensis*) (*Keenan, Engel & Elsey, 2013*). Here, Fusobacteria dominated the gut microbiotas of the
230 farmed snakes; this is the first report of the dominance of Fusobacteria in the snake gut microbiota.
231 *Bacteroides* and *Cetobacterium* were the dominant bacterial genera in gut microbiota of the farmed snakes
232 (Fig. 2). *Bacteroides* maintain a complex and beneficial relationship in the host gut, and the symbiotic
233 relationships between these bacteria and their hosts have been widely studied (*Thomas et al., 2011*). For
234 example, *Bacteroides* species have complex systems for sensing nutrient utilization, regulating nutrient
235 metabolism, and acquiring and hydrolyzing otherwise indigestible dietary polysaccharides (*Xu et al., 2003*).
236 *Bacteroides* species control host gut homeostasis by interacting with the host immune system (*Wexler, 2007*).
237 Here, the gut microbiotas of the farmed snakes were dominated by *Bacteroides*, especially in the Ec group
238 (42.09%) and the Na group (40.17%) (Fig. 3), indicating that the gut microbiota in snakes are species
239 dependent. All *Cetobacterium* species are obligate anaerobes in phylum Fusobacteria (Fig. 2). *Cetobacterium*
240 was the dominant genus in the gut microbiotas of all the farmed snakes analyzed herein; this is the first report
241 of the dominance of this genius in the gut microbiotas of snakes.

242 **Fusobacteria in gut microbiotas of farmed snakes**

243 Fusobacteria is a little-studied bacterial phylum, with a somewhat uncertain phylogenetic position (*Keenan,*
244 *Engel & Elsey, 2013*). The results of the present study indicated that Fusobacteria contains only two genera,
245 *Cetobacterium* and *Fusobacterium* (Fig. 2). However, it is possible that Fusobacteria includes additional
246 unclassified genera, and/or that the Fusobacteria have been undersampled in previous studies of gut
247 microbiotas (*Keenan, Engel & Elsey, 2013*). Previous studies have suggested that Fusobacteria have a core
248 genome dissimilar to that of other bacterial lineages (*Mira et al., 2004*). Phylogenetic and comparative
249 genomics analyses indicate that this phylum is closely affiliated with Bacteroidetes and Firmicutes, and may be
250 derived from the Firmicutes (*Mira et al., 2004*). Phylogenetic analysis recovered a close relationship between
251 Fusobacteria and Bacteroidetes, indicating a relatively close evolutionary relationship (Fig. 2). Bacteroidetes is
252 one of the major lineages of bacteria, arising early in bacterial evolution (*Wexler, 2007*). Therefore, the
253 evolutionary relationship between Fusobacteria and Bacteroidetes should be further investigated.
254 Fusobacteria species play a critical role in initial biofilm development (*Mira et al., 2004*), suggesting that
255 the presence of these species in the guts of the farmed snakes may affect the development of the lumen
256 membrane (*Keenan, Engel & Elsey, 2013*). *Cetobacterium* was first isolated from the intestinal contents of a
257 porpoise and from the mouth lesion of a minke whale (*Balaenoptera acutorostrata*) (*Foster et al., 1995*).
258 Species in this genus transform peptones and carbohydrate into acetic acid (*Edwards, Logan & Gharbia, 2015*).
259 Because Fusobacteria and *Cetobacterium* dominated the gut microbiotas of the farmed snakes, species in these
260 taxa were likely commensal inhabitants of snake guts. It is therefore possible to speculate that, in snakes,
261 Fusobacteria and *Cetobacterium* play important roles in digestive organ development and in nutritional
262 metabolism.
263 **The relationship between gut microbiota and host species**

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

278 CONCLUSION

279 The compositions of the gut microbiotas of four farmed snake species in southern China were different to those
280 of other snakes and vertebrates. The gut bacteria of these four species fell into 15 phyla, 18 classes, 22 orders,
281 35 families, and 58 genera. The dominant bacterial phyla were Bacteroidetes, Proteobacteria, Firmicutes, and
282 Fusobacteria, while the dominant bacterial genera were *Bacteroides* and *Cetobacterium*. This was the first
283 report that Fusobacteria and *Cetobacterium* dominated the gut microbiotas of snake species. Gut microbiotal
284 diversity was highest in *D. acutus* and lowest in *E. carinata*. There were interspecific differences in gut

285 microbiota composition, diversity, and the Firmicutes/Bacteroidetes relative abundance ratio among the four
286 farmed snake species. Our results supported our hypothesis that host species was an important factor affecting
287 the gut microbiotas of snakes. Further studies of snake gut microbiotas should investigate the relationship
288 between phylogenetic position and function, as well as the characteristics of dominant bacteria that were
289 unclassifiable. It is important to determine whether the immunity and growth of farmed snake populations can
290 be improved by inoculating fecal suspensions generated by healthy wild snakes into the guts of farmed
291 conspecifics. In addition, it would also be useful to establish an open database of microbial data from the guts
292 of snakes and other reptile groups.

293 ADDITIONAL INFORMATION AND DECLARATIONS

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299 Competing Interests

300 The authors declare there are no competing interests.

301 Author Contributions

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

305 • Jing Ren performed the experiments, analyzed the data and wrote the paper.

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

307 • Xinguo Gong performed the experiments.

308 Animal Ethics

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

311 The work was performed in accordance with the recommendations of the Institution of Animal Care and the
312 Ethics Committee of Central South University of Forestry and Technology (approval number: CSUFT NS #
313 20175167).

314 Data Availability

315 The following information was supplied regarding data availability:

316 The raw data has been supplied as a Supplementary File.

317 Supplemental Information

318 Supplemental files used in this paper have been uploaded to the submission system of PeerJ
319 (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.

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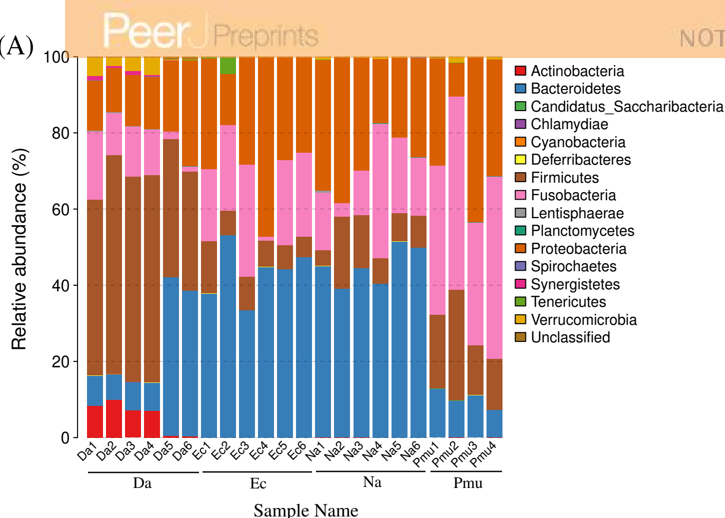

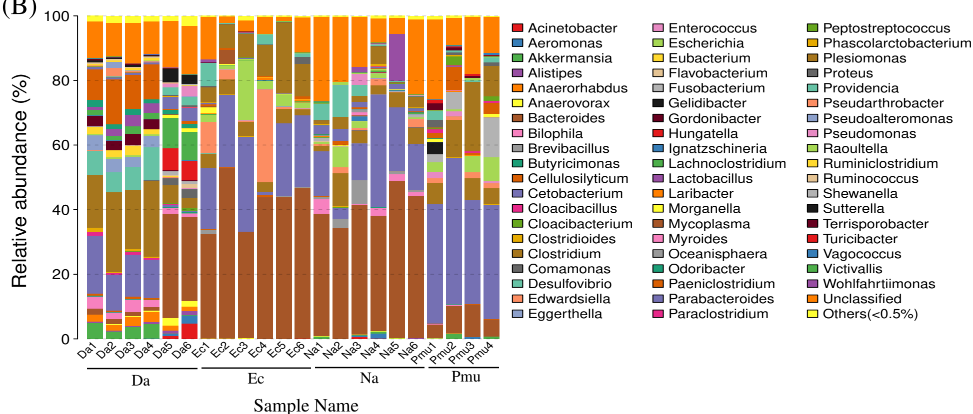

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Figure 2(on next page)

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

Genera are colored by phylum.

- Actinobacteria
- Bacteroidetes
- Candidatus_Saccharibacteria
- Chlamydiae
- Deferribacteres
- Firmicutes
- Fusobacteria
- Lentisphaerae
- Proteobacteria
- Spirochaetes
- Synergistetes
- Tenericutes
- Verrucomicrobia

Figure 3(on next page)

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.

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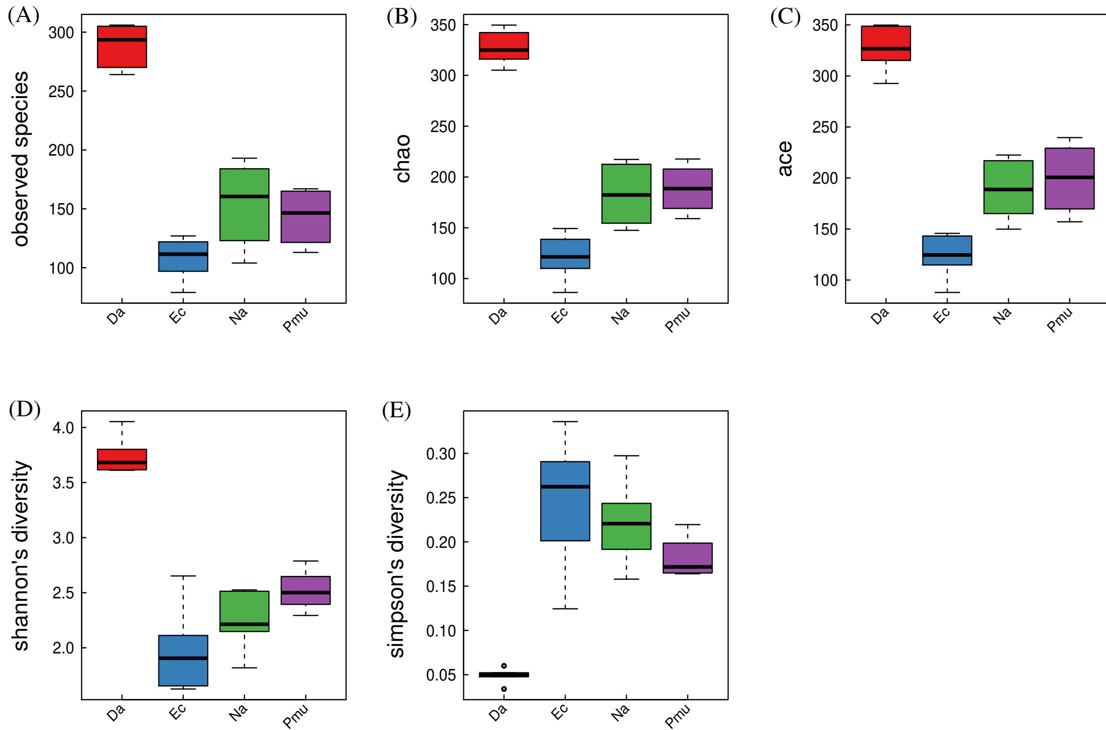

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Figure 4(on next page)

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.

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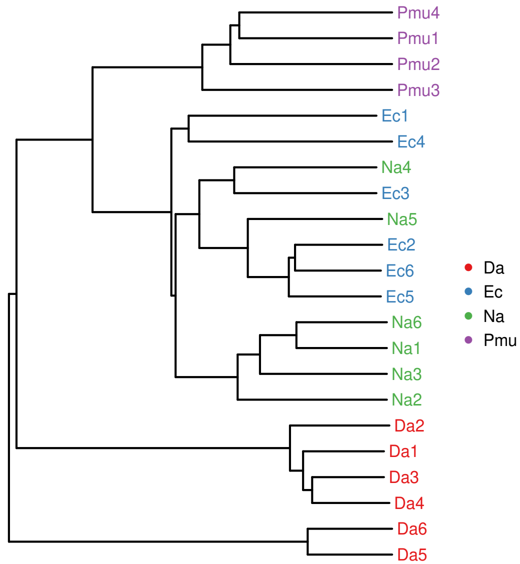

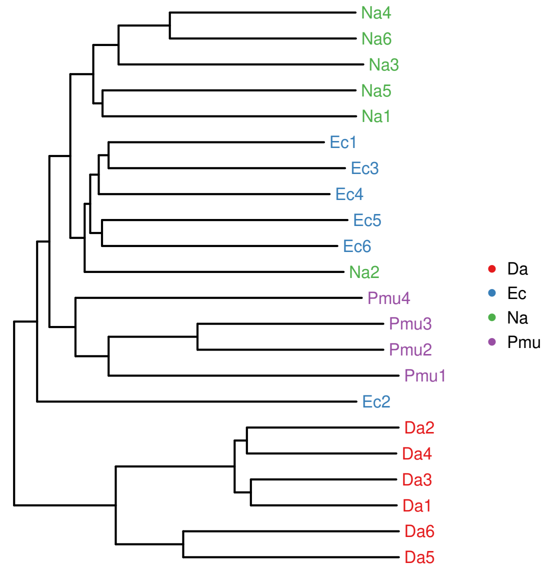

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Figure 5 (on next page)

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

Na: *Naja atra* group, Pmu: *Ptyas mucosus* group, Ec: *Elaphe carinata* group, and Da: *Deinagkistrodon acutus* group.

Relative Abundance

−2 −1 0 1

Log10

Terrisporobacter
Cellulosilyticum
Clostridioides
Proteus
Morganella
Peptostreptococcus
Enterococcus
Fusobacterium
Raoultella
Laribacter
Brevibacillus
Oceanisphaera
Ignatzschineria
Gelidibacter
Mycoplasma
Vagococcus
Paraclostridium
Turicibacter
Pseudomonas
Aeromonas
Myroides
Comamonas
Flavobacterium
Cloacibacterium
Shewanella
Wohlfahrtiimonas
Hungatella
Anaerovorax
Acinetobacter
Victivallis
Phascolarctobacterium
Ruminiclostridium
Anaerorhabdus
Gordonibacter
Eggerthella
Butyricimonas
Alistipes
Sutterella
Ruminococcus
Pseudoalteromonas
Lactobacillus
Cloacibacillus
Pseudarthrobacter
Eubacterium
Desulfovibrio
Odoribacter
Bilophila
Akkermansia
Providencia
Edwardsiella
Plesiomonas
Unclassified
Clostridium
Cetobacterium
Bacteroides
Paeniclostridium
Escherichia
Others(<0.5%)
Parabacteroides
Lachnoclostridium

Na3 Na5 Na1 Na4 Na6 Pmu2 Pmu1 Pmu4 Na2 Ec3 Ec5 Ec4 Ec6 Pmu3 Ec1 Ec2 Da5 Da6 Da2 Da4 Da1 Da3

Sample Name

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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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1

Group	Number of Phyla	Number of classes	Number of orders	Number of families	Number of genera
Na	11	17	20	31	49
Pmu	11	16	19	28	44
Ec	9	15	19	27	44
Da	12	18	22	34	53
Total	15	18	22	35	58

2

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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.

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Dominant phylum	Na group	Pmu group	Ec group	Da group	*p*	FDR
Bacteroidetes	45.07±4.92	10.22±2.32	43.54±6.93	18.24±16.89	=0.015	=0.012
Firmicutes	9.91±5.45	18.71±7.51	7.88±3.04	46.54±10.73	=0.002	=0.011
Fusobacteria	16.81±10.55	42.53±8.38	19.42±9.59	9.57±6.56	=0.008	=0.015
Proteobacteria	27.67±8.10	27.74±14.28	28.31±10.81	16.38±6.08	=0.115	=0.164

1