Differences in gut microbial composition and characteristics among three populations of the bamboo pitviper (Viridovipera stejnegeri)

Jiaqi Zhang[1], Songwen Tan[1], Bing Lyu[1], Min Yu[2], Yue Lan[3], Ruixiang Tang[3], Zhenxin Fan[3], Lei Shi[4], and Peng Guo[1]

[1]Yibin University
[2]Southwest Minzu University
[3]Sichuan University
[4]Xinjiang Agricultural University

August 29, 2024

Abstract

The gut microbiota contributes to host health by facilitating nutrient uptake, digestion, energy metabolism, intestinal development, vitamin synthesis, and immunomodulation, and plays an important role in the growth and reproduction of the animal itself. Considering the paucity of research on the gut microbiota of wild snakes, this study focused on bamboo pitviper (Viridovipera stejnegeri) populations from Anhui, Guizhou, and Hunan, with multiple fecal samples collected from each population (six, five, and three, respectively). Total microbial DNA was extracted from the fecal samples using metagenomic next-generation sequencing and differences in gut microbial composition, abundance, and carbohydrate-active enzymes (CAZymes) were analyzed among the three populations. Results showed no significant variance in the α-diversity of the gut microbes across the three populations, while principal coordinate analysis revealed significant differences in gut microbe composition. The four most abundant phyla in the gut microbiota of V. stejnegeri were Pseudomonadota, Bacteroidota, Actinomycetota, and Bacillota, while the four most abundant genera were Salmonella, Citrobacter, Bacteroides, and Yokenella. Linear discriminant analysis effect size demonstrated notable differences in gut microbial abundance among the three populations. Marked differences in CAZyme abundance were also observed across the microbial communities. Future studies should incorporate diverse ecological factors to evaluate their influence on the composition and function of gut microbiota. This integrated approach, alongside detailed functional analysis of microbiota, should deepen our understanding of gut microbial dynamics in wild snakes.

Differences in gut microbial composition and characteristics among three populations of the bamboo pitviper (*Viridovipera stejnegeri*)

Jiaqi Zhang[1, 2], Songwen Tan[2], Bing Lyu[2], Min Yu[2], Yue Lan[3], Ruixiang Tang[3], Zhenxin Fan[3], Peng Guo[2*], Lei Shi[1*]

[1] Xinjiang Key Laboratory for Ecological Adaptation and Evolution of Extreme Environment Biology, College of Life Sciences, Xinjiang Agricultural University, Urumqi 830052, China

[2] Faculty of Agriculture, Forestry and Food Engineering, Yibin University, Yibin 644007, China

[3] Key Laboratory of Bioresources and Ecoenvironment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu 610065, China

Correspondence

Peng Guo, Faculty of Agriculture, Forest and Food Engineering, Yibin University, Yibin 644000, Sichuan, China.

Lei Shi, Xinjiang Key Laboratory for Ecological Adaptation and Evolution of Extreme Environment Biology, College of Life Sciences, Xinjiang Agricultural University, Urumqi 830052, Xinjiang, China.

* Corresponding authors: ybguop@163.com (PG), leis@xjau.edu.cn (LS)

Abstract

The gut microbiota contributes to host health by facilitating nutrient uptake, digestion, energy metabolism, intestinal development, vitamin synthesis, and immunomodulation, and plays an important role in the growth and reproduction of the animal itself. Considering the paucity of research on the gut microbiota of wild snakes, this study focused on bamboo pitviper (*Viridovipera stejnegeri*) populations from Anhui, Guizhou, and Hunan, with multiple fecal samples collected from each population (six, five, and three, respectively). Total microbial DNA was extracted from the fecal samples using metagenomic next-generation sequencing and differences in gut microbial composition, abundance, and carbohydrate-active enzymes (CAZymes) were analyzed among the three populations. Results showed no significant variance in the α-diversity of the gut microbes across the three populations, while principal coordinate analysis revealed significant differences in gut microbe composition. The four most abundant phyla in the gut microbiota of *V. stejnegeri* were Pseudomonadota, Bacteroidota, Actinomycetota, and Bacillota, while the four most abundant genera were *Salmonella* , *Citrobacter* , *Bacteroides* , and *Yokenella* . Linear discriminant analysis effect size demonstrated notable differences in gut microbial abundance among the three populations. Marked differences in CAZyme abundance were also observed across the microbial communities. Future studies should incorporate diverse ecological factors to evaluate their influence on the composition and function of gut microbiota. This integrated approach, alongside detailed functional analysis of microbiota, should deepen our understanding of gut microbial dynamics in wild snakes.

KEYWORDS

Gut microbiota; Metagenomic next-generation sequencing; *Viridovipera stejnegeri* ; Microbial diversity; Carbohydrate-active enzymes

INTRODUCTION

The gut provides a rich and favorable ecological environment for microbial communities, facilitating a complex and dynamic equilibrium among microorganisms. This balance is subject to continuous modifications across different hosts or developmental stages, contributing to a variety of physiological (*Hertli & Zimmermann, 2022*) and pathological processes, including metabolism (*Lindsay, Metcalfe & Llewellyn, 2020; Wei et al., 2023*), nutrient absorption (*Flint et al., 2012*), immune regulation (*Siddiqui, Maciver & Khan, 2022; Wei et al., 2023*), and host ecological behavior (*O'Donnell et al., 2020*). The composition and structure of normal gut microbiota serve as indicators for assessing animal health as well as diagnosing or preventing disease (*Hu et al., 2017; Kundu et al., 2017; Rosshart et al., 2017*). Gut microbes are shaped by a multitude of factors, such as the evolutionary status (*Kartzinel et al., 2019*), feeding habits (*Jiang et al., 2017*), distribution (*Qin et al., 2014*), seasonal variation (*Sun et al., 2016; Gao, Yang & Shi, 2023*), and climate (*Greenspan et al., 2020; Li et al., 2020*). However, the primary determinants affecting the composition of the gut microbiota are the genetic background and dietary habits of the host (*Kovacs et al., 2011; Doré & Blottière, 2015*). Understanding these factors is crucial for studying the ecology and evolution of animal gut microbes.

A variety of factors, including sex, geographical population, breeding status, health condition, local altitude, and temperature, influence the composition and characteristics of gut microbes. For example, significant sex-based differences in microbial community structure have been reported in wild *Calotes versicolor* , with the *Bacteroide* and *Ochrobactrum* genera found to be dominant in wild females and males, respectively (*Zhang et al., 2022*). Differences in fecal microbial abundance and gene functional types have also been noted between the Guilin and Xiangyang populations of *Ptyas dhumnades* (*Li, Sun & Xu, 2021*). Additionally, the relative abundance of *Shigella* species in fecal samples of *Elaphe carinata* , noted for its lack of intestinal diseases, is considerably higher than that of two congeneric species, suggesting a potential link to intestinal

health (*Lu et al., 2019*). Research has also shown that conditional or potentially pathogenic bacteria are often present in the animal gut microbiota. For example, potential pathogenic bacteria such as *Citrobacter* , *Trichococcus* , and*Erysipelothrix* have been detected in the intestines of wild*Rhabdophis tigrinus* (*Tang et al., 2019*) and*Rhabdophis subminiatu* s (*Tang et al., 2019*). Significant differences have also been found in the composition of gut microbiota among populations of *Phrynocephalus vlangalii* living at different altitudes, with changes corresponding to the altitudinal gradient (*Zhang et al., 2018*). Furthermore, based on semi-natural experiments, *Bestion et al . (2017*) demonstrated that increasing temperature can lead to a decrease in microbial diversity in wild lizards, potentially negatively impacting host survival. Conversely, gut microbiota can be regulated to enhance immune capacity in lizards, thus facilitating adaptation to climate change (*Yang et al., 2024*).

The bamboo pitviper (*Viridovipera stejnegeri*) is a common venomous snake with wide distribution across China and Vietnam (*David et al., 2001; 2002; Guo et al., 2022*). It typically inhabits the rocks of streams, grassy and bushy areas, roadsides, vegetable fields, and rock crevices in mountainous areas at elevations ranging from 150 to 2 200 m. These snakes primarily feed on mice, frogs, lizards, and birds (*Zhao, 1998; Guo et al., 2022*). Research by *Guo et al* . (*2016*) on mitochondrial gene fragments and nuclear genes revealed significant population differentiation within this species.

Snakes, lacking the ability to chew, possess a robust intestinal digestion capacity and remarkably strong hunger tolerance. In the current study, we analyzed the composition, abundance, and carbohydrate-active enzymes (CAZymes) of gut microbes in different populations of *V. stejnegeri* using metagenomic next-generation sequencing. Our main goal was to compare the gut microbiota in the three different populations and investigate the potential relationship between habitat factors and gut microbes as well as the crucial roles played by gut microbes during carbohydrate metabolism within populations of*V. stejnegeri* .

MATERIALS AND METHODS

Overview of collection sites

Samples were collected from three different localities during 2022–2023, including Huangshan City, Anhui Province (118deg0'–118deg2'E and 30deg14'N, altitude 197–220 m), Guiyang City, Guizhou Province (106deg9'–106deg49'E and 26deg77'–27deg11'N, altitude 941–1 232 m), and Yongxing County, Hunan Province (112deg22'–112deg23'E, 26deg14'–26deg15'N, altitude 318–319 m) in China. The three locations feature subtropical monsoon climates. The habitats of the Anhui and Hunan populations are primarily composed of scrub and bamboo forests adjacent to streams, while the habitats of the Guizhou population predominantly consist of wooded areas adjacent to streams (Fig. 1).

Sample collection and DNA extraction

In total, 14 samples were collected, including six (three males and three females) from Huangshan, five (three males and two females) from Guiyang City, and three (two males and one female) from Yongxing County. All individuals were healthy adults. Upon capture, the snakes were immediately placed in sterilized plastic collection boxes overnight to collect feces using sterile disposable gloves. The feces were collected in 2-mL collection tubes containing DNA preservation solution and placed in liquid nitrogen for rapid freezing, with subsequent preservation in a -80 degC refrigerator at the Yibin Key Laboratory of Zoological Diversity and Ecological Conservation, Yibin University (YBU). The snakes were released at the collection sites after obtaining fecal samples. Genomic DNA was extracted from the gut contents by a commercial company (Novogene, Beijing, China) utilizing a DNA extraction kit (Catalog number: DP328) following the provided instructions. The Institutional Animal Care and Use Committee at Yibin University (YBU2020007).

Library construction, quality control (QC), and sequencing

Approximately 0.2 μg of DNA per sample was used as input material for the DNA library preparations. In brief, the genomic DNA samples were fragmented by sonication to a size of 350 bp, then polished, A-tailed, and ligated with the full-length adapter for Illumina sequencing, followed by polymerase chain reaction (PCR) amplification. The resulting PCR products were purified using the AMPure XP system (Beverly,

USA). Subsequently, library quality was assessed using the Agilent 5400 system (Agilent, USA) and quantified by quantitative PCR (QPCR) (1.5 nM). The qualified libraries, each with an effective concentration over 2 nM, were pooled and sequenced on the Illumina HiSeq 2500/MiSeq platform using the PE150 strategy by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The original fluorescence image files obtained were transformed to short reads (raw reads) by base calling and BclToFastq, and recorded in FASTQ format (*Cock et al., 2010*).

Data analyses

The quality of the raw reads was assessed using Fastp v0.23.1 (*Chen et al., 2018*), with read pairs discarded if: (1) adapter contamination was present in either read; (2) more than 10% of bases were uncertain in either read; and (3) the proportion of low-quality (Phred quality < 5) bases exceeded 50% in either read.

Due to the unavailability of the whole-genome sequence for *V. stejnegeri* , the genome sequence of its close relative,*Protobothrops mucrosquamatus* , was used as a host reference genome, downloaded from the NCBI database (https://ftp.ncbi.nlm.nih.gov/genomes/al/GCA/001/527/595/GCA001527695.3_P.Mucros_-1.0/). Sequence alignment was performed using Bowtie2 v2.4.1 (*Langmead & Salzberg, 2012; Langmead et al., 2019*) to remove the host sequence. After host removal, the sequences were aligned to standard archaea, bacteria, human, UniVec_Core, and viral databases using Kraken2 v2.0.7 to obtain annotation information and abundance tables (*Wood, Lu & Langmead, 2019*). Target sequences were then partitioned into smaller k-mer segments for assembly using Megahit v1.2.9 (*Li et al., 2015, 2016*) with the parameters (-t 8 -m 0.95 –min-contig-len 300 –k-min 51 –k-max 127 –k-step 20). The assembled sequences were clustered using Cd-hit v4.8.1 (*Fu et al., 2012*) to obtain de-redundant sequences with the parameters (-c 0.95 -aS 0.9 -g 1 -sc 1 -sf 1 -T 8 -M 8000). Protein sequences were predicted using Prodigal v2.6.3 (*Hyatt et al., 2010*). Finally, the annotated protein sequences were obtained by comparison against the carbohydrate database v3.0.5 with Run_dbcan (*Zhang et al., 2018*), with quantification performed using Salmon v0.14.1 (*Patro et al., 2017*).

Grouped percentage stacked column charts representing species abundance were produced using the Wekemo Bioincloud platform (https://bioincloud.tech/task-meta). Several diversity indices were calculated in R v4.3.1 (R Development Core Team 2020), including α-diversity indices such as abundance-based coverage estimation, Simpson's diversity index, Chao1 estimator, Shannon-Weiner index, observed species, and Goods's coverage index. The Kruskal-Wallis test was completed to compare α-diversity indices among the three populations.

Principal coordinate analysis (PCoA) using Bray-Curtis distance was performed to detect differences in the composition of gut microbiota across the three populations of *V. stejnegeri* . Adonis analysis of variance (ANOVA) was employed to assess significant differences among the three populations using R v4.3.1. Additionally, linear discriminant analysis (LDA) effect size (LEfSe) was used to examine significant disparities in the abundance of gut bacteria among the three populations at the phylum to genus levels and identify components with notable differences. LDA was then applied to assess the impact of each component on differences in abundance (*Segata et al. 2011*), with the results visualized using R v4.3.1. The linear discriminant analysis criterion was set to a log-transformed value greater than or equal to 2 with a base of 10.

RESULTS

Metagenomic next-generation sequencing

In total, 76.38 gigabytes (GB) of raw data, averaging 5.46 GB per sample, and 509 209 214 sequences (Anhui: 214 833 000; Guizhou: 179 543 666; Hunan: 114 832 548) were obtained from the 14 samples of *V. stejnegeri* across three sampling localities. After QC filtering, 75.16 GB of clean data, averaging 5.38 GB per sample, and 500 924 546 sequences (Anhui: 212 806 018; Guizhou: 178 779 316; Hunan: 109 399 212) were obtained. The base percentages of Q20 and Q30 exceeded 90%, and the GC content was above 40%, indicating high sequencing accuracy. The effective data from all samples exceeded 95%, indicating that most sequences

could be annotated and sampled samples were sufficient and representative for subsequent analyses (Table 1). Sequence length, TPM (transcripts per kilobase of exon model per million mapped reads) values, and number of reads are detailed in Table 2.

Composition and characteristics of gut microbiota in *V. stejnegeri*

Results indicated that the gut microbiota of *V. stejnegeri* predominantly consisted of Bacteria (82.19%), Eukaryota (17.44%), Viruses (0.22%), and Archaea (0.15%). A total of 9 435 bacterial species, belonging to 47 phyla, 101 classes, 214 orders, 514 families, and 1 951 genera, were detected. At the bacterial phylum level, the four most abundant groups, with a relative abundance [?] 3%, were Pseudomonadota (72.12% ± 20.72%), Bacteroidota (14.11% ± 19.00%), Actinomycetota (6.58% ± 8.74%), and Bacillota (4.58% ± 2.79%) (Fig. 2). The dominant phyla in the three different populations are listed in Table 3. At the genus level, the four most abundant genera, with a relative abundance [?] 3%, were *Salmonella* (28.76% +- 23.53%),*Citrobacter* (14.36% +- 13.56%), *Bacteroides* (10.78% +- 20.23%), and *Yokenella* (3.65% +- 6.08%) (Fig. 3). Variations in dominant genera were observed across the different populations (Table 4).

Population differences in gut microbiota of *V. stejnegeri*

The α-diversity analyses revealed no significant differences in the intestinal flora among the three populations of *V. stejnegeri* (Table 5). The PCoA results indicated that 64.3% of the variance was explained by PCoA 1 and PCoA 2, with significant differences in gut microbial diversity among the three populations (Fig. 4, $R2 = 0.48$, $P = 0.01$). PCoA results also indicated that all samples from Anhui clustered with the H2 and H3 samples from Hunan, while all samples from Guizhou clustered with the H1 sample from Hunan. Based on LEfSe, gut microbial abundance among the three populations differed significantly, with 38 microbial taxa from Anhui, three from Guizhou, and five from Hunan explaining the differences among the three populations (Fig. 5). Notably, the Anhui population was characterized by taxa in the phyla Pseudomonadota, Actinomycetota, Myxococcota, Thermodesulfobacteriota, Bacteroidota, and Planctomycetota. In contrast, the Guizhou population was characterized by taxa in the phylum Pseudomonadota, and the Hunan population by taxa in the phylum Bacteroidota (Fig. 5).

Prediction of CAZymes in gut microbiota of *V. stejnegeri*

The top four CAZymes in the gut microbiota of *V. stejnegeri* were GT2 (9.28% ± 1.57%), GT4 (6.16% ± 1.79%), GH23 (4.06% ± 1.30%), and GT51 (3.02% ± 1.00%) (relative abundance [?] 3%) (Fig. 6). No significant differences in α-diversity were detected in the gut microbiota among the three populations (Kruskal-Wallis test, $P > 0.05$). PCoA also showed no significant differences in the diversity of CAZymes among the three populations (Adonis $R2 = 0.18$, $P = 0.26$). However, LEfSe showed that CAZyme abundance differed significantly among the three populations. Key CAZymes and their categories for each population are provided in Fig. 7.

DISCUSSION

In this study, we conducted the first metagenomic next-generation sequencing analysis of the gut microbiome of the common Asian bamboo pitviper. Results identified Pseudomonadota, Bacteroidota, Actinomycetota, and Bacillota as the most dominant phyla, contrasting with previous studies on snakes that identified Proteobacteria, Bacteroidetes, and Firmicutes (see Appendix 1), demonstrating that dominant microbes differ among different snake species. Compared to other snakes, our findings indicated that *V. stejnegeri* possessed a unique gut microbiota, predominantly comprised of Actinobacteria. In contrast, lizards have been shown to harbor a gut microbiota primarily composed of Firmicutes, Bacteroidetes, and Proteobacteria (*Hong et al., 2011; Kohl et al., 2017; Zhang et al., 2018; Zhu et al., 2020; Baldo et al., 2023; Gao, Yang & Shi, 2023*). Thus, while the dominant bacterial communities in snakes and lizards appear to be largely influenced by Proteobacteria and Bacteroidetes, their composition and abundance at the phylum level exhibit distinct differences.

Molecular phylogeny analyses indicated that the Guizhou and Hunan populations exhibited a closer relationship within the three populations (*Guo et al., 2016*). Based on the PCoA results, however, the bacterial

diversity among the three populations was not consistent with the phylogenetic relationships (Fig. 4), suggesting that bacterial diversity is not influenced by population relatedness. Our findings demonstrated that the three populations shared similar dominant microbial phyla. At the genus level with relative abundance greater than 3%, except for *Salmonella* and *Citrobacter* , distinct dominant genera were observed, including *Yokenella* ,*Enterobacter* , and *Serratia* in the Guizhou population,*Bacteroides* in the Hunan population and *Yokenella* in the Anhui population.

Previous studies have indicated that reptiles, such as iguanas, turtles, crocodiles, and snakes, can act as vectors for transmitting bacteria to humans, potentially causing paratyphoid fever (*Cohen et al., 1980; Mermin, Hoar & Angulo, 1997; Waterman et al., 1990; Schröter et al., 2004; Grupka et al., 2006; McLaughlin, Cochran & Dowd, 2015*). In the current study, *Salmonella* was the most abundant genus in the gut microbiota of *V. stejnegeri* , with *S. enterica* , the most prevalent species within the genus (32.01% ± 23.73%), constituting 98.42% of the *Salmonella* detected. In addition, the conditionally pathogenic bacterium *Citrobacter* , a facultative anaerobe known to cause enteritis in animals (*Mundy, Macdonald & Dougan, 2010*), was also found at a relatively high abundance, second only to *Salmonella* . The significant presence of*Salmonella* , *Citrobacter* , and *S. enterica* in the gut of *V. stejnegeri* underscores the need for careful and considered conservation and management of wild snake populations.

The composition and abundance of the host gut microbiota are influenced by multiple factors (*Zhu et al., 2022; Zhang et al., 2018*). The three geographic populations of *V. stejnegeri* were generally consistent in latitude, climate, and prey but varied in altitude and habitat. Notably, the Anhui, Hunan, and Guizhou populations are located at altitudes of 197–220 m, 318–319 m, and 941–1 232 m, respectively. Furthermore, the Anhui and Hunan population habitats are mainly characterized by shrub and bamboo forests adjacent to streams, while the Guizhou population habitat primarily features wooded areas alongside streams. Here, PCoA revealed significant differences in the intestinal microflora among the three populations (Fig. 4, $R2 = 0.48$,$P = 0.01$), suggesting that altitude and habitat likely influence the composition and abundance of gut microbes.

CAZymes play a key role in carbon source metabolism. The CAZy database categorizes enzyme families that catalyze the degradation, modification, and biosynthesis of carbohydrates into five major classes and one related module. For example, glycoside hydrolases (GHs) are involved in the hydrolysis and rearrangement of glycosidic bonds, glycosyl transferases (GTs) participate in the formation of glycosidic bonds, polysaccharide lyases (PLs) function in the non-hydrolytic cleavage of glycosidic bonds, carbohydrate esterases (CEs) are involved in the hydrolysis of carbohydrate esters and auxiliary activities (AAs) is a redox enzymes that act in conjunction with CAZymes, and carbohydrate-binding modules (CBMs) facilitate adhesion to carbohydrates (*Lombard et al., 2014; Wardman et al., 2022*). Previous Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of *P. dhumnades* has shown that genes related to metabolism are most prominent at the first level, encompassing six types of biometabolic pathways, with carbohydrate metabolism being the most abundant at the second level, involving 43 pathways (*Li, Sun & Xu, 2021*). Metabolic profiling has also shown that carbohydrate metabolism is highly dominant in*Crotalus horridus* and is the main metabolic pathway in *R. subminiatus* (*McLaughlin, Cochran & Dowd, 2015; Tang et al., 2019*). The gut microbiota CAZyme annotations for the three *V. stejnegeri* populations showed that the Anhui and Guizhou populations were mainly composed of GT2 (8.93% +- 1.68%, 8.82% +- 1.29%), GT4 (6.97% +- 2.25%, 5.04% +- 0.90%), GH23 (4.14% +- 1.32%, 4.79% +- 0.98%), and GT51 (3.31% +- 0.74%, 3.35% +- 0.40%), while the Hunan population was primarily composed of GT2 (10.73% +- 0.57%), GT4 (6.39% +- 0.28%), and GH2 (3.05% +- 0.14%) (relative abundance [?] 3%). These findings show that the composition and abundance of dominant CAZymes differed among the three populations. The LEfSe results further highlighted marked disparities in CAZyme abundance across the three populations. Additionally, certain Proteobacteria strains (Gammaproteobacteria, Enterobacterales, Enterobacteriaceae,*Salmonella*) and a higher proportion of certain Bacteroidetes strains (Bacteroidia, Bacteroidales, Bacteroidaceae, *Bacteroides*) (relative abundance [?] 40%) were noted in the Hunan population.*Bacteroides* species, which metabolize polysaccharides and oligosaccharides, provide nutrition and vitamins to the host and other intestinal microbial residents (*Zafar & Saier, 2021*). It was speculated that this difference ultimately led to differences in CAZymes composition

and abundance among the three populations. However, the specific substrates and chemical mechanisms acted upon by these CAZymes in snake intestines remain unclear. Further research is needed to elucidate how the gut microbiota affects specific carbohydrate metabolism mechanisms.

CONCLUSIONS

The composition and abundance of gut microbiota in *V. stejnegeri* differed from those of other examined snake species. Notably, the gut bacteria of *V. stejnegeri* encompassed 47 phyla, 101 classes, 214 orders, 514 families, and 1 951 genera. The top four most abundant phyla were Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes, respectively, while the four most abundant genera were*Salmonella* , *Citrobacter* , *Bacteroides* , and*Yokenella* , with *S. enterica* , a pathogenic intestinal bacterium, showing the highest relative abundance. The four most abundant CAZymes in the gut microbiota of *V. stejnegeri* were GT2, GT4, GH23, and GT51. Significant differences were observed in the PCoA and LEfSe analyses of the gut microbiota among the three populations, as well as in the composition and abundance of CAZymes. Our study indicated that these population differences in gut microbial composition and abundance were not related to genetic differentiation but may be associated with differences in altitude and habitat. We hypothesized that variations in *Bacteroides* contributed to differences in CAZyme composition and abundance among the three populations. Future research should expand the host range to further explore the relationship between more geographic populations and gut microbiota, and to analyze the specific functions of the gut microbiota in conjunction with gut contents.

ACKNOWLEDGMENTS

We would like to thank He Zhang, Fan Liu, Yuhao Wen, and Yihua Xiang at Yibin Key Laboratory of Zoological Diversity and Ecological Conservation, Yibin University (YBU), and Ke Sun at Guizhou Normal University (GNU), for their assitant in samples collection. We extend our thanks to Jiyang Jiang and Limin Du at Sichuan University (SU) for their help in data analyses.

FUNDING INFORMATION

We gratefully acknowledge funding from the National Natural Science Foundation of China (NSFC 31372152).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are publicly available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1108923 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1108898, reference number are PRJNA1108923 and PRJNA1108898.

ORCID

Jiaqi Zhang https://orcid.org/0009-0008-2107-163X

Song-Wen Tan https://orcid.org/0009-0009-1461-6328

Bing Lyu https://orcid.org/ 0000-0001-5594-1543

Min Yu https://orcid. org/0000-0002-7014-9869

Yue Lan https://orcid.org/0009-0009-2868-3740

Ruixiang Tang https://orcid.org/0000-0001-6265-9167

Zhenxin Fan https://orcid.org/0000-0003-0422-9497

Peng Guo https://orcid. org/000-000I-5585-292X

Lei Shi https://orcid. org/0000-0001-7227-7728

REFERENCES

Baldo L, Tavecchia G, Rotger A, Igual JM, Riera JL. (2023). Insular holobionts: persistence and seasonal plasticity of the Balearic wall lizard (*Podarcis lilfordi*) gut microbiota. PeerJ 11:e14511. DOI 10.7717/peerj.14511.

Bestion E, Jacob S, Zinger L, Di Gesu L, Richard M, White J, Cote J. (2017). Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. Nature Ecology & Evolution 1(6):161. DOI 10.1038/s41559-017-0161.

Chen S, Zhou Y, Chen Y, Gu J. (2018). Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17):i884-i890. DOI 10.1093/bioinformatics/bty560.

Cock PJ, Fields CJ, Goto N, Heuer ML, Rice PM. (2010). The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Research 38(6):1767-1771. DOI 10.1093/nar/gkp1137.

Cohen ML, Potter ME, Pollard RA, Feldman RA. (1980). Turtle-associated*salmonellosis* in the United States, effect of public health action,1970 to 1976. JAMA 243 (12):1247-9. DOI 10.1001/jama.1980.03300380027016.

Colston TJ, Noonan BP, Jackson CR. (2015). Phylogenetic analysis of bacterial communities in different regions of the gastrointestinal tract of *Agkistrodon piscivorus* , the cottonmouth snake. PLoS One 10(6):e0128793. DOI 10.1371/journal.pone.0128793.

Costello EK, Gordon JI, Secor SM, Knight R. (2010). Postprandial remodeling of the gut microbiota in *Burmese pythons* . The ISME Journal 4(11):1375-1385. DOI https://doi.org/10.1038/ismej.2010.71.

David P. (2001). On the occurrence of the snake genus *Ovophis* (Serpentfs: Viperidae: Crotalinae) on Hainan island, China. Acta Zootaxonomica Sinica 26(3): 388-393.

Dore J, Blottiere H. (2015). The influence of diet on the gut microbiota and its consequences for health. Current Opinion in Biotechnology 32:195-199. DOI 10.1016/j.copbio.2015.01.002.

Flint HJ, Scott KP, Louis P, Duncan SH. (2012). The role of the gut microbiota in nutrition and health. Nature Reviews Gastroenterology Hepatology 9(10): 577-589. DOI https://doi.org/10.1038/nrgastro.2012.156.

Fu L, Niu B, Zhu Z, Wu S, Li W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28(23):3150-2. DOI 10.1093/bioinformatics/bts565.

Gao WZ, Yang Y, Shi L. (2023). Seasonal dietary shifts alter the gut microbiota of a frugivorous lizard *Teratoscincus roborowskii (* Squamata, Sphaerodactylida*e)* . Ecology and Evolution 13*(* 8*)* : e10363. DOI 10.22541/au.167611328.84712536/v1.

Greenspan SE, Migliorini GH, Lyra ML, Pontes MR, Carvalho T, Ribeiro LP, Moura-Campos D, Haddad CF, Toledo LF, Romero GQ, Becker CG. (2020). Warming drives ecological community changes linked to host-associa ted microbiome dysbiosis. Nature Climate Change 10, 1057-1061. DOI https://doi.org/10.1038/s41558-020-0899-5.

Grupka LM, Ramsay EC, Bemis DA. (2006). *Salmonella* surveillance in a collection of *rattlesnakes* (*Crotalus spp.*). Journal of Zoo and Wildlife Medicine 37(3):306-312. DOI: 10.1638/05-059.1.

Guo P, Liu Q, Wu YY, Zhu F, Zhong GH. (2022). Pitvipers of China. Beijing: Science Press 212-220.

Guo P, Liu Q, Zhu F, Zhong GH, Chen X, Myers EA, Che J, Zhang L, Ziegler T, Nguyen T Q, Burbrink FT. (2016). Complex longitudinal diversification across South China and Vietnam in *Stejneger's* pit viper, *Viridovipera stejnegeri* (Schmidt, 1925) (Reptilia: Serpentes: Viperidae). Molecular ecology 25(12): 2920-2936. DOI https://doi.org/10.1111/mec.13658.

Hertli S, Zimmermann P. (2022). Molecular interactions between the intestinal microbiota and the host. Molecular microbiology 117*(6)* :1297-1307. DOI https://doi.org/10.1111/mmi.14905.

Hong, PY, Wheeler E, Cann Isaac KO, Mackie Roderick I. (2011). Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galapagos Islands using 16S rRNA-based pyrosequencing. ISME Journal 5(9): 1461-1470. DOI https://doi.org/10.1038/ismej.2011.33.

Hu X, Liu G, Shafer ABA, Wei Y, Zhou J, Lin S, Wu H, Zhou M, Hu D, Liu S. (2017). Comparative analysis of the gut microbial communities in forest and alpine musk deerusing high-throughput sequencing. Frontiers in Microbiology 8(e2836):572. DOI 10.3389/fmicb.2017.00572.

Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119. DOI 10.1186/1471-2105-11-119.

Jiang HY, Ma JE, Li J, Zhang XJ, Li LM, He N, Liu HY, Luo SY, Wu ZJ, Han RC, Chen JP. (2017). Diets alter the gut microbiome of crocodile lizards. Frontiers in Microbiology 8: 2073. DOI 10.3389/fmicb.2017.02073.

Kartzinel TR, Hsing JC, Musili PM, Brown BR, Pringle RM. (2019). Covariation of diet and gut microbiome in African megafauna. Proceedings of the National Academy of Sciences of the United States of America 116(47):23588-23593. DOI 10.1073/pnas.1905666116.

Kohl KD, Brun A, Magallanes M, Brinkerhoff J, Laspiur A, Acosta JC, Caviedes-Vidal E, Bordenstein SR. (2017). Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions and transmission. Molecular Ecology 26(4):1175-1189. DOI 10.1111/mec.13921.

Kovacs A, Ben-Jacob N, Tayem H, Halperin E, Iraqi FA, Gophna U. (2011). Genotype is a stronger determinant than sex of the mouse gut microbiota. Microbial Ecology 61(2):423-8. DOI 10.1007/s00248-010-9787-2.

Kundu P, Blacher E, ElinavE, Pettersson S. (2017). Our gut microbiome: the evolving inner self. Cell 171(7):1481-1493 DOI 10.1016/j.cell.2017.11.024.

Langmead B, Salzberg SL. (2012). Fast gapped-read alignment with Bowtie2. Nature Methods 9(4):357-9. DOI 10.1038/nmeth.1923.

Langmead B, Wilks C, Antonescu V, Charles R. (2019). Scaling read aligners to hundreds of threads on general-purpose processors. Bioinformatics 35(3):421-432. DOI 10.1093/bioinformatics/bty648.

Li D, Liu CM, Luo R, Sadakane K, Lam TW. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31(10):1674-6. DOI 10.1093/bioinformatics/btv033.

Li D, Luo R, Liu CM, Leung CM, Ting HF, Sadakane K, Yamashita H, Lam TW. (2016). MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods 102:3-11. DOI 10.1016/j.ymeth.2016.02.020.

Li DL, Sun Y, Xu FR. (2021). Differences in faeces microbiome composition and characteristics between two populations of *Ptyas dhumnade* . Chinese Journal of Zoology 56(5):11. DOI 10.13859/j.cjz.202105007.

Li G, Yin B, Li J, Wang J, Wei W, Bolnick DI, Wan X, Zhu B, Zhang Z. (2020). Host-microbiota interaction helps to explain the bottom-up effects of climate change on a small rodent species. The ISME journal 14(7):1795-1808. DOI https://doi.org/10.1038/s41396-020-0646-y.

Lindsay EC, Metcalfe NB, Llewellyn MS. (2020). The potential role of the gut microbiota in shaping host energetics and metabolic rate. Journal Of Animal Ecology 89*(11)* : 2415–2426. DOI 10.1111/1365-2656.13327.

Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Research 42(Database issue):D490-5. DOI 10.1093/nar/gkt1178.

Lu SX, Li PP, Lu YY, Yu QL, Zhou ZY. (2019). Fecal microbial diversity of three species of *Elaphe* in captive breeding. Chinese Journal of Zoology 54(2): 270-278. DOI 10.1385.9/j.cjz.201902013.

McLaughlin RW, Cochran PA, Dowd SE. (2015). Metagenomic analysis of the gut microbiota of the Timber Rattlesnake, *Crotalus horridus* . Molecular Biology Reports 42(7):1187-95. DOI 10.1007/s11033-015-3854-1.

Mermin J, Hoar B, Angulo FJ. (1997). Iguanas and Salmonella marina infection in children: a reflection of the increasing incidence of reptile-associated *salmonellosis* in the United States. Pediatrics 99: 399-402. DOI 10.1542/peds.99.3.399.

Mundy R, Macdonald T T, Dougan G. (2010). *Citrobacter rodentium* of mice and man. Cellular Microbiology 7(12): 1697-1706. DOI 10.1111/j.1462-5822.2005.00625.x.

O'Donnell MP, Fox BW, Chao PH, Schroeder FC, Sengupta P. (2020). A neurotransmitter produced by gut bacteria modulates host sensory behaviour. Nature 583(7816):415-420. DOI 10.1038/s41586-020-2395-5.

Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. Nature Methods 14(4):417-419. DOI 10.1038/nmeth.4197.

Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, Guo J, Le Chatelier E, Yao J, Wu L, Zhou J, Ni S, Liu L, Pons N, Batto JM, Kennedy SP, Leonard P, Yuan C, Ding W, Chen Y, Hu X, Zheng B, Qian G, Xu W, Ehrlich SD, Zheng S, Li L. (2014). Alterations of the human gut microbiome in liver cirrhosis. Nature 513(7516):59-64. DOI 10.1038/nature13568.

Qin Z, Wang S, Guo D, Zhu J, Chen H, Bai L, Luo X, Yin Y. (2019). Comparative analysis of intestinal bacteria among venom secretion and non-secrection snakes. Scientific Reports 9(1):6335. DOI 10.1038/s41598-019-42787-6.

R Development Core Team. (2020). R: a language and environment for statistical computing. *Vienna, Austria: R foundation for statistical computing.* Available at http://www.Rproject.org.

Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, Hickman HD, McCulloch JA, Badger JH, Ajami NJ, Trinchieri G, Pardo-Manuel De Villena F, Yewdell JW, Rehermann B. (2017). Wild mouse gut microbiota promotes host fitness and improves disease resistance. Cell 171(5):1015-1028.e13. DOI 10.1016/j.cell.2017.09.016.

Schroter M, Roggentin P, Hofmann J, Speicher A, Laufs R, Mack D. (2004). Pet snakes as a reservoir for *Salmonella* enterica subsp. diarizonae (Serogroup IIIb): a prospective study. Applied and Environmental Microbiology 70(1):613-5. DOI 10.1128/AEM.70.1.613-615.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. (2011). Metagenomic biomarker discovery and explanation. Genome Biology 12(6):R60. DOI 10.1186/gb-2011-12-6-r60.

Shi YD, Sun H. (2017). Characterization of the intestinal microflora of . *Elaphe taeniura* Journal of Hunan Agricultural University (Natural Sciences) 43(03):292-297. DOI 10.13331/j.cnki.jhau.

Siddiqui R, Maciver SK, Khan NA. (2022). Gut microbiome-immune system interaction in reptiles. Journal of Applied Microbiology 132*(4)* :2558-2571. DOI 10.1111/jam.15438.

Sun BH, Wang X, Bernstein S, A. Huffman M, Xia DP, Gu ZY, Chen R, K. Sheeran L, Wagner R. S, Li JH. (2016). Marked variation between winter and spring gut microbiota in free-ranging Tibetan macaques (*Macaca thibetana*). Scientific Reports 6, 26035. DOI 10.1038/srep26035.

Tang WJ, Yang SJ, Cheng YQ, Zhang C, Zhu GX. (2019). Intestine microflora composition and distribution characteristics in*Rhabdophis tigrinus* (Squamata: Colubridae). Chinese Journal of Zoology 54(4):589-598. DOI 10.13859/j.cjz.201904016.

Tang W, Zhu G, Shi Q, Yang S, Ma T, Mishra SK, Wen A, Xu H, Wang Q, Jiang Y, Wu J, Xie M, Yao Y, Li D. (2019). Characterizing the microbiota in gastrointestinal tract segments of *Rhabdophis subminiatus* : dynamic changes and functional predictions. Microbiology Open 8(7): e00789. DOI 10.1002/mbo3.789.

Wardman JF, Bains RK, Rahfeld P, Withers SG. (2022). Carbohydrate-active enzymes (CAZymes) in the gut microbiome. Nature Reviews Microbiology 20(9):542-556. DOI https://doi.org/10.1038/s41579-022-00712-1.

Waterman SH, Juarez G, Carr SJ, Kilman L. (1990). *Salmonella* arizonae infections in Latinos associated with rattlesnake folk medicine. American Journal Of Public health 80(3): 286-289. DOI https://doi.org/10.2105/ajph.80.3.286.

Wei Y, Zhou M, Fang W, Liu Q, Mao H, Chen B, Zhang T, Xu Y, Zhang W, Zheng Y, Hu X. (2023). Differences in the luminal and mucosal gut microbiomes and metabolomes of oriental rat snake *(Ptyas mucosus)* . Applied Microbiology and Biotechnology 107(10):3257-3271. DOI https://doi.org/10.1007/s00253-023-12524-1.

Wood DE, Lu J, Langmead B. (2019). Improved metagenomic analysis with Kraken2. Genome biology 20(1):257. DOI https://doi.org/10.1186/s13059-019-1891-0.

Yang J, Liu W, Han X, Hao X, Yao Q, Du W. (2024). Gut microbiota modulation enhances the immune capacity of lizards under climate warming. Microbiome 12(1): 37. DOI https://doi.org/10.1186/s40168-023-01736-2.

Zafar H, Saier MH Jr. (2021). Gut Bacteroides species in health and disease. Gut Microbes 13(1):1-20. DOI https://doi.org/10.1080/19490976.2020.1848158.

Zhang B, Ren J, Yang D, Liu S, Gong X. (2019). Comparative analysis and characterization of the gut microbiota of four farmed snakes from southern China. PeerJ 7:e6658. DOI https://doi.org/10.7717/peerj.6658.

Zhang H, Yohe T, Huang L, Entwistle S, Wu P, Yang Z, Busk PK, Xu Y, Yin Y. (2018). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. Nucleic Acids Research 46(W1):W95-W101. DOI https://doi.org/10.1093/nar/gky418.

Zhang L, Yang F, Li T, Dayananda B, Lin L, Lin C. (2022). Lessons from the diet: Captivity and sex shape the gut microbiota in an oviparous lizard (*Calotes versicolor)* . Ecology and Evolution 12(2):e8586. DOI https://doi.org/10.1002/ece3.8586.

Zhang W, Li N, Tang X, Liu N, Zhao W. (2018). Changes in intestinal microbiota across an altitudinal gradient in the lizard*Phrynocephalus vlangalii* . Ecology and Evolution 8(9):4695-4703. DOI https://doi.org/10.1002/ece3.4029.

Zhao EM, Jiang YM, Huang QY, Zhao H, Huang MH, Ma JF, Zheng J, Huang ZJ, Wei G, Yang DT, Li JD, Zong Y. (1998). Fauna Sinica: Reptilia Vol.3 Squamata Serpentes. Beijing: Science Press 453-463.

Zhu W, Shi X, Qi Y, Wang X, Chang L, Zhao C, Zhu L, Jiang J. (2022). Commensal microbiota and host metabolic divergence are associated with the adaptation of *Diploderma vela* to spatially heterogeneous environments. Integrative Zoology 17(3):346-365. DOI https://doi.org/10.1111/1749-4877.12590.

FIGURE LEGENDS

Fig. 1 Habitat of *Viridovipera stejnegeri* (a) in Guizhou (b) and Anhui (c) populations.

Fig. 2 Relative abundance of gut bacteria in three populations of *V. stejnegeri* at the phylum level. Abscissa represents three sampling areas, different colors represent classification of bacteria at the phylum level. Full names of the three sampling areas are provided in Fig. 1.

Fig. 3 Relative abundance of gut bacteria in three populations of *V. stejnegeri* at the genus level. Different colors represent classification of bacteria at the genus level. Full names of the three sampling areas are

provided in Fig. 1.

Fig. 4 PCoA of Bray-Curtis distance matrix for bacterial diversity differences among three populations of *V. stejnegeri.*

Fig. 5 LEfSe of the three populations of *V. stejnegeri* . LDA scores identify significantly different taxa among Anhui, Guizhou, and Hunan populations. Degree of influence of a taxon is expressed by bar length. Only taxa meeting a significant LDA threshold > 2 are shown. Letters p, c, o, f, and g represent phylum, class, order, family, and genus, respectively.

Fig. 6 Relative abundance of CAZymes in *V. stejnegeri* across three populations.

Fig. 7 Differences in CAZymes among three populations of *V. stejnegeri* . LDA scores reflect differences in relative abundance of CAZymes among populations.

TABLES

Table 1 Quality of metagenomic next-generation sequencing data from three populations of *Viridovipera stejnegeri* (average values)

Notes: Q20 and Q30: percentage of bases with Phred values > 20 and > 30, respectively, against total bases from Illumina HiSeq 2500/MiSeq; GC Content: percentage of G and C bases against total number of bases; Effective rate: ratio of valid data retained after filtering original data.

Table 2 Salmon quantitative analysis of gut microbiota samples from three populations of *Viridovipera stejnegeri* (average values)

Notes: Length (bp): actual length of original FASTQ sequence; Effective length (bp): FASTQ sequence effective length; TPM value (transcripts per kilobase of exon model per million mapped reads): transcripts per thousand base transcripts per million mapped reads, summarizing length, expression, and number of genes, used to estimate sample expression; Num reads: estimated number of reads matched to each sample.

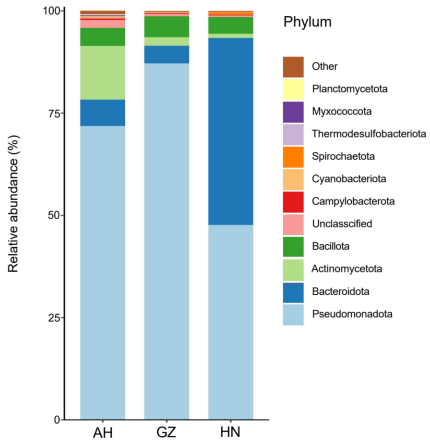
Table 3 Dominant phyla in three populations of *V. stejnegeri* (relative abundance [?] 3%)

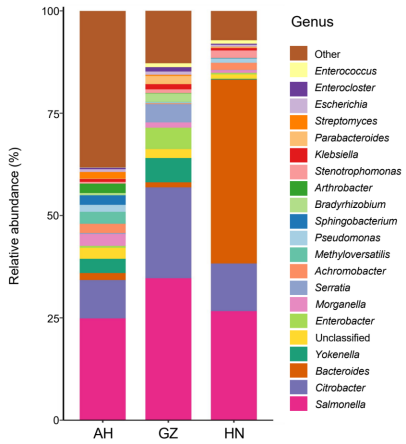
Table 4 Dominant genera in three populations of *V. stejnegeri* (relative abundance [?] 3%)

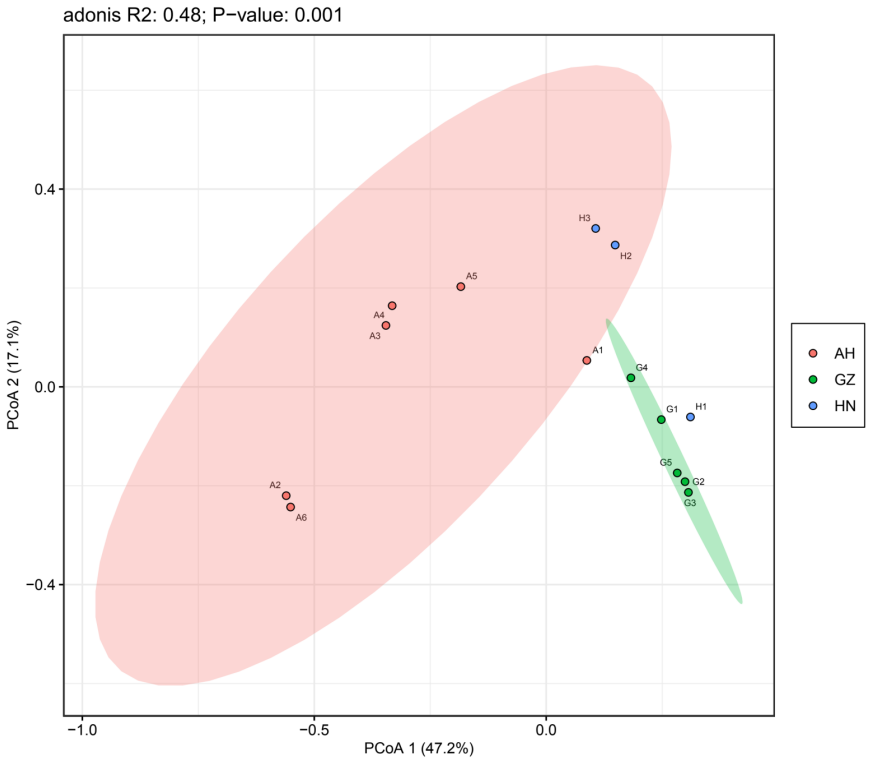
Table 5 Gut microbial α-diversity in *V. stejnegeri* across three populations

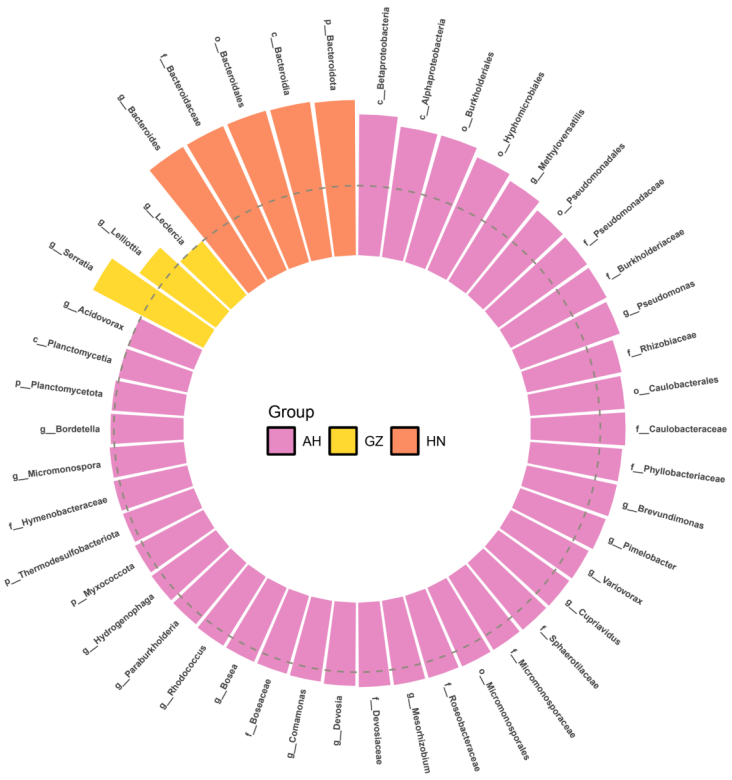
APPENDICES: SUPPLEMENTARY MATERIALS

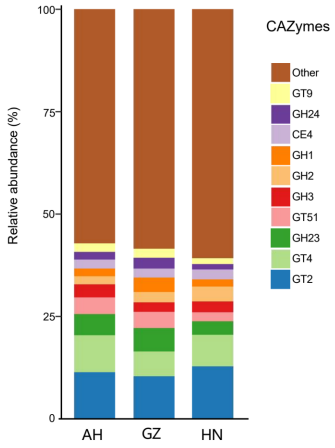
Appendix 1 Dominant bacteria of gut microbiota in different snake species

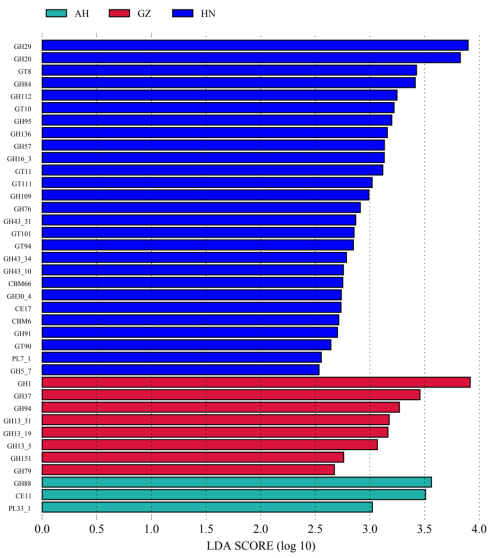












Hosted file

Table 1.docx available at https://authorea.com/users/823591/articles/1220327-differences-in-gut-microbial-composition-and-characteristics-among-three-populations-of-the-bamboo-pitviper-viridovipera-stejnegeri

Hosted file

Table 2.docx available at https://authorea.com/users/823591/articles/1220327-differences-in-gut-microbial-composition-and-characteristics-among-three-populations-of-the-bamboo-pitviper-viridovipera-stejnegeri

Hosted file

Table 3.docx available at https://authorea.com/users/823591/articles/1220327-differences-in-gut-microbial-composition-and-characteristics-among-three-populations-of-the-bamboo-pitviper-viridovipera-stejnegeri

Hosted file

Table 4.docx available at https://authorea.com/users/823591/articles/1220327-differences-in-gut-microbial-composition-and-characteristics-among-three-populations-of-the-bamboo-pitviper-viridovipera-stejnegeri

Hosted file

Table 5.docx available at https://authorea.com/users/823591/articles/1220327-differences-in-gut-microbial-composition-and-characteristics-among-three-populations-of-the-bamboo-pitviper-viridovipera-stejnegeri

Hosted file

Appendix 1.docx available at https://authorea.com/users/823591/articles/1220327-differences-in-gut-microbial-composition-and-characteristics-among-three-populations-of-the-bamboo-pitviper-viridovipera-stejnegeri