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Gut Microbial Community Structure 肠道微生物群落结构

Diversity and sex-specific differences in the intestinal microbiota of cheetah (*Acinonyx jubatus*)

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Abstract: [Objective] This study aimed to investigate the diversity and sex-specific differences in the intestinal microbiota of cheetah (*Acinonyx jubatus*). **[Methods]** Fecal samples were collected from 9 healthy adult cheetahs (4 male and 5 female), and intestinal microbiota were assessed through high-throughput sequencing of the V3–V4 region of 16S rRNA gene. **[Results]** In total, 599349 effective tags were obtained, with an average length of 405 bp. At 97% sequence similarity, 268 operational classification units (OTUs) were obtained and classified into the bacterial domain, including 10 phyla, 21 classes, 35 orders, 72 families, and 144 genera. Based on relative abundance, the 5 most abundant bacterial phyla were *Firmicutes* (accounting for approximately 42.29% of the total OTUs), *Actinobacteria* (31.54%), *Fusobacteria* (16.66%), *Proteobacteria* (5.30%), and *Bacteroidetes* (4.19%). The low abundance of phylum *Bacteroidetes* was the primary feature of the intestinal microbial community of the cheetah. The 5 most abundant families were *Coriobacteriaceae* (31.28%), *Peptostreptococcaceae* (17.66%), *Fusobacteriaceae* (15.46%), *Lachnospiraceae* (12.40%), and *Clostridiaceae_1* (6.93%). The 5 most abundant genera were *Collinsella* (30.16%), *Fusobacterium* (15.46%), *Peptoclostridium* (11.46%), *Blautia* (8.28%), and *Clostridium_sensu_stricto_1* (6.39%). Approximately 2.32% of the total OTUs were unclassified strains at the genus level. Alpha diversity analysis indicated that the Shannon indices of the intestinal microbiota ranged 2.93–4.41; Simpson indices, 0.72–0.91. The observed species and Shannon indices of the intestinal microbiota of male cheetahs were slightly greater than those of female cheetah. Beta diversity analysis revealed that the difference between sexes was greater than that within groups. However, cluster analysis revealed that intestinal microbes of cheetahs of the same sex were not clustered. **[Conclusion]** The present results show the intestinal microbial diversity of cheetah, and indicate that intestinal microbiota of cheetah do not differ significantly on the basis of sex, thus providing a scientific basis for studies on the conservation of cheetahs and their digestive physiology and for rescuing and feeding cheetahs.

Keywords: cheetah (*Acinonyx jubatus*), intestinal microbiota, 16S rRNA gene, high-throughput sequencing

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Intestinal microbes significantly contribute to numerous aspects including nutrient digestion and absorption, intestinal health and immunity, etc., and are essential for the survival and environmental adaptation of wild animals[1–2]. Cheetahs are fast-running mammals of family *Felidae*. Owing to a loss and fragmentation of their habitat, their population has sharply declined. Cheetahs are included in Appendix I of the CITES and are protected by national legislation in most of their existing and previous habitats[3]. Analysis of the characteristics of intestinal microbial diversity of cheetahs would greatly facilitate studies on their feeding habits and intestinal health status. In recent years, with advancements related to the intestinal microbes of members of family *Felidae*[4], studies on the intestinal microbiota of cheetahs have gradually emerged[5–7]. Becker *et al*. (2014) characterized the fecal microbiota of captive cheetahs in a Belgian zoo via shotgun sequencing and reported a pronounced underrepresentation of members of families *Bifidobacteriaceae* and *Bacteroidetes* in cheetahs in comparison with those in domestic cats[6]. Another study reported the long-term temporal stability of the predominant fecal microbiota in captive cheetahs via PCR coupled with denaturing gradient gel electrophoresis (DGGE) and real-time PCR analysis[7]. High-throughput sequencing would provide deeper insights into the abundance and diversity of the intestinal microbiota of cheetahs. In this study, high-throughput sequencing was performed to investigate the diversity of the intestinal microbial community of cheetahs. Furthermore, the diversity of intestinal microbial diversity among different individuals and sexes was analyzed. The present results would potentially further the current understanding of the cheetah's gut ecosystem and provide basic information for rescuing and feeding cheetahs and to assess their intestinal health and treat diseases in cheetahs.

1 Materials and methods

1.1 Sample collection

Fecal samples were harvested from 9 healthy adult cheetahs (4 male, named AJM01–AJM04; 5 female, named AJF01–AJF05) on December 31, 2017. These animals were approximately 4–6 years old, weight 45–55 kg, with a trunk length of 1.0–1.5 m, and a shoulder height of 0.7–0.9 m. The cheetahs were half-scattered at Jinan Wildlife Park (36° 36′ N 117° 27′ E) in Zhangqiu in Shandong province, P. R. China, feeding on raw beef and rabbit and with ad libitum access to potable tap water. Four months before sampling, these animals were not administered anti-inflammatory or antibacterial drugs and did not have any gastrointestinal disease. Fresh fecal samples were harvested at approximately 5:00 to 6:00 am, stored in a sterilized plastic centrifuge tube, and sent to the laboratory in a portable refrigerator and stored at –80 °C until DNA was extracted. The experiment was approved by the Animal Protection and Utilization Committee of Qufu Normal University.

1.2 DNA extraction, 16S rRNA amplification, and sequence processing

Genomic DNA of the samples was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were used for amplification of the 16S rRNA V3–V4 region. Each reaction was of 30 μL and comprised 15 μL of Phurs Mix (2er), 1.5 μL of each primer, 10 μL of template DNA, and 2 μL of ddH_2O. The cycling conditions were as follows: pre-denaturation at 98 °C for 1 min, denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension 72 °C for 30 s, 35 cycles, and final extension at 72 °C for 5 min. The PCR product was mixed with the same volume of 1×loading buffer (containing Gel green) and detected via

electrophoresis using a 2% agarose gel. Samples at a bright band of 400 bp and 450 bp were excised and mixed at an equal density ratio and purified using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). A library was constructed by using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, California, USA) and quantified using Qubit® 3.0 (Life Technologies, Grand Island, NY, USA). The quantified library was sequenced using Illumina HiSeq 2500 (Illumina, San Diego, California, USA).

1.3 Bioinformatics analysis

In accordance with previously reported methods, we removed barcodes and primer sequences and performed sequence quality control analysis and obtained the effective tags[8–12]. The UPARSE software (Version 7.0.1001) was used to cluster the effective tags from all samples and cluster the sequences into operational classification units (OTUs) with 97% similarity[13]. Species were annotated on the basis of representative OTU sequences, using Mothur software (Version 1.35.1) and SILVA's SSUrRNA database (set threshold of 0.8–1.0)[14]. We obtained taxonomic information and statistically analyzed the diversity of bacterial communities in each sample at each rank of classification (kingdom, phylum, class, order, family, and genus). The MUSCLE software (Version 3.8.31) was used for fast multi-sequence alignment[15–16] and the Qiime software (Version 1.9.1) was used to determine the observed-species, chao1, Shannon, Simpson, ACE, goods-coverage, and PD_whole_tree indices[12]. The rarefaction curve and the rank abundance curve were plotted using the R software (Version 2.15.3).

1.4 Analysis of inter-sample and inter-group differences

Based on the sex, we divided the cheetah samples into a male group (AJM) and a female group (AJF). The R software was used to analyze differences between the Alpha diversity index between groups and for Beta diversity analysis, using parametric and non-parametric tests, respectively. The box plot was used to visually reflect the median, dispersion, maximum, minimum, and outlier values of species diversity between samples.

The Unifrac distance was calculated using Qiime software, and the UPGMA sample clustering tree was constructed. Anosim, MRDP, and ADOMIS analysis were performed using R software and AMOVA analysis was performed using Mothur software to assess whether intergroup differences were significantly greater than intragroup differences. We used the R software for principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) analysis. We used LEfSe software (Galaxy Version 1.0) for LEfSe analysis (LDA score=4)[17]. Metastats analysis was performed using R software, and the *q* values of each rank of classification (phylum, class, order, family, and genus) were determined.

2 Results

2.1 Sequencing data and OTU clusters

In total, 599349 effective tags were obtained from 9 cheetah fecal samples, with an average length of 405 bp. Sequences are curated in the SRA database with the accession number SRR7299437. Classification based on 97% sequence similarity, 268 OTUs with 122 OTUs on average were obtained. Based on OTU data, rarefaction and rank abundance curves were plotted. The end of the rarefaction curve tending to be flat, indicating that the sequencing data effectively reflected the diversity of the intestinal microbial community in cheetahs. The rank abundance curve indicated that richness of samples AJM01, AJM02, and AJF04 was greater than that of other samples (Figure 1).

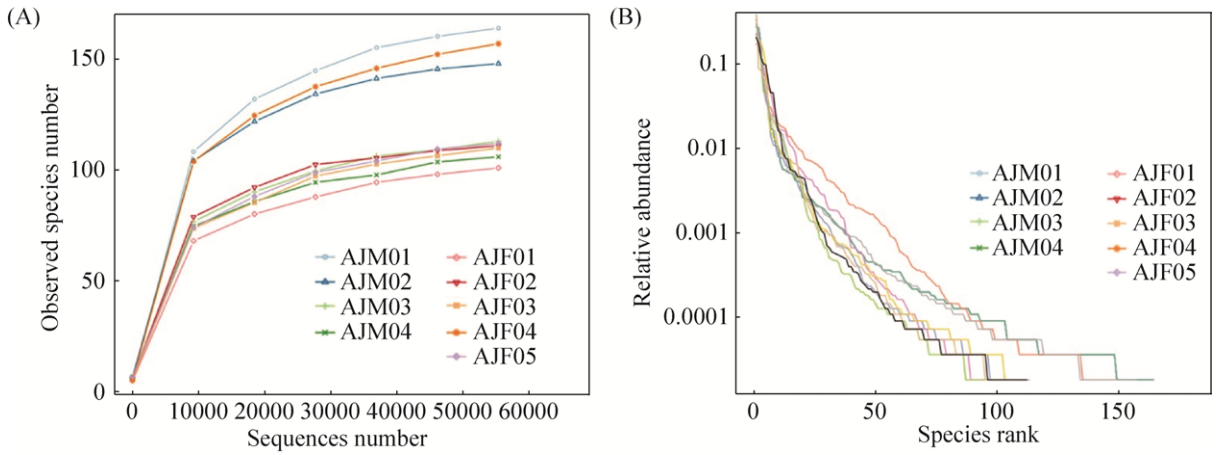

Figure 1. Rarefaction (a) and rank abundance curve (b) analyses of nine *Acinonyx jubatus* fecal samples. In the dilution curve, the abscissa represents the number of sequencing samples randomly extracted from a certain sample, and the ordinate represents the number of operational taxonomic units (OTUs) constructed on the basis of the number of sequences, which reflected the sequencing depth, and different samples are represented by differently colored curves. When the curve tended to flatten, the amount of sequencing data were progressively reasonable. In the Rank Abundance curve, the abscissa represents the serial number sorted on the basis of OTU abundance and the ordinate represents the relative abundance of the corresponding OTUs, and different samples are represented by differently colored polylines. The higher the species richness, the greater the span of the curve along the horizontal axis. The smoothness of the curve reflects the uniformity of the species in the sample. The smoother the curve, the more uniform the species distribution.

2.2 Microbiota composition and abundance

Based on the results of species annotation, all OTUs in the fecal samples from cheetahs were classified into their corresponding bacterial domain, including 10 phyla, 21 classes, 35 orders, 72 families, and 144 genera. Based on average abundance, the top 10 bacterial phyla were *Firmicutes* (13.33%–65.49%, at an average of 42.29% of the total number of OTUs), *Actinobacteria* (7.66%–54.65%; average, 31.54%), *Fusobacteria* (3.10%–42.32%; average, 16.66%), *Proteobacteria* (0.70%–21.50%; average, 5.30%), *Bacteroidetes* (0.17%–17.77%; average, 4.19%), *Saccharibacteria* (average, 0.012%), *Chloroflexi* (average, 2×10^{-5}), Cyanobacteria (average, 1.8×10^{-5}), *Thermomicrobia* (average, 8×10^{-6}), and *Deferribacteres* (average, 6×10^{-6}). Among these phyla, the abundance varied greatly. For example, *Firmicutes* accounted for only 13.33% of the total number of OTUs in sample AJM04 but 54.65% in sample AJF04; *Actinobacteria* accounted for only 7.66% in AJM04 but 54.67% in AJF03. *Proteobacteria* accounted for only 0.70% in AJF05 but 21.50% in AJM04. Furthermore, *Cyanobacteria* and *Deferribacteres* were present only in AJM02 and AJM03; *Thermomicrobia* were present only in AJM01 and AJF02.

At the family level, among the 72 bacterial families present in the fecal samples of cheetahs, the top 10 were *Coriobacteriaceae* (average, 31.28% of the total number of OTUs), *Peptostreptococcaceae* (17.66%), *Fusobacteriaceae* (15.46%), *Lachnospiraceae* (12.40%), *Clostridiaceae_1* (6.93%), *Bacteroidaceae* (4.15%), *Erysipelotrichaceae* (3.08%), *Campylobacteraceae* (2.19%), *Enterobacteriaceae* (1.40%), and *Helicobacteraceae* (0.92%), among which *Peptostreptococcaceae*, *Lachnospiraceae*, *Clostridiaceae_1*, and *Erysipelotrichaceae* belong to phylum *Firmicutes*, and *Campylobacteraceae*, *Enterobacteriaceae*, and *Helicobacteraceae* belong to phylum *Proteobacteria*. Microbial abundance among samples differed

markedly at the family level. Notably, *SAR86_clade* and *Desulfomicrobiaceae* were present only in AJM01; *Mycobacteriaceae*, AJM02; *Chromatiaceae* and *Hyphomicrobiaceae*, AJM03; *Family_XIV* and *Family I*, AJM02 and AJM03; *Halomonadaceae*, AJF04 and AJF05; *Pasteurellaceae*, AJF04; *Bacteroidales_S24-7_group*, AJF05.

At the genus level, unclassified strains accounted for 2.32% of the total number of OTUs. The top 10 genera of relative abundance were *Collinsella* (average, 30.16% of the total number of OTUs), *Fusobacterium* (15.46%), *Peptoclostridium* (11.46%), *Blautia* (8.28%), *Clostridium_sensu_stricto_1* (6.39%), *Paeniclostridium* (5.40%), *Bacteroides* (4.15%), *Campylobacter* (2.19%), *Ruminococcus_gnavus_group* (1.86%), and *Escherichia-Shigella* (0.92%). Genera belonging to phyla *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Bacteroidetes*, and *Proteobacteria* accounted for 35.86%, 30.16%, 15.46%, 4.15%, and 2.15% of the total number of OTUs, respectively. Among these genera, *Agromyces* was only present in sample AJF05; *Pelistega*, AJF04; *Exiguobacterium* and *Turicibacter*, AJF01; *Advenella*, AJM04; *Paraeggerthella*, AJM01; *Butyricicoccus* and *Erysipelothrix*, AJM02 (Figure 2).

2.3 Analysis of similarities and differences among samples

Analysis of sample similarity revealed that the number of core microbes in the male and female groups were similar (female, 58; male, 65). The Venn diagram (Figure 3-A) shows that the number of OTUs shared by the two sexes was 167. The petal plot shows that 50 OTUs were shared by all samples (Figure 3-B). The core microbes shared by all samples were primarily the following: *Coriobacteriia*, phylum *Actinobacteria*; *Bacilli*, phylum *Clostridia*; *Erysipelotrichia*, phylum *Firmicute*; *Fusobacteriia*, phylum *Fusobacteria*; *Betaproteobacteria*, *Epsilonproteobacteria*, and *Gammaproteobacteria*, phylum *Proteobacteria*; *Bacteroidia*, phylum *Bacteroidete*. The Alpha Diversity indices (including Shannon index, Simpson index, chao1, ACE, goods_coverage, and PD_whole_tree) of the two sexes at the 97% consistency threshold are enlisted in Table 1. Box analysis of the Alpha diversity indices between the two groups revealed that the OTUs and the Shannon index of the AJM group were slightly but not significantly greater than those of the AJF group (P>0.05) (Figure 4). The histogram shows that at the phylum level, both the AJM and the AJF groups displayed a greater abundance of phyla *Firmicutes* and *Actinobacteria*, and the relative abundances of the two phyla did not differ significantly between male and female cheetahs (*Firmicutes*: P=0.9007, *Actinobacteria*: P=0.3073). The abundances of phyla *Cyanobacteria* and *Deferribacteres* were slightly but not significantly greater in the AJM than in the AJF group (P=0.2151; P=0.2149). The histogram shows that the proportion of phylum *Fusobacteria* was greater in sample AJM04 than in other samples, while that of phylum *Actinobacteria* was lower in sample AJM04 than in other samples; however, these differences were not significant between male and female cheetahs (P=0.9059, P=0.3073, respectively) (Figure 5).

Intergroup analysis of Alpha diversity indices revealed no significant difference between male and female groups with respect to OTUs (P=0.4279), the Shannon index (P=0.357), and Simpson index (P=0.3826). Furthermore, analysis of Beta diversity indices revealed no significant difference in diversity between male and female groups (unweighted Beta diversity t-test, P=0.2619, weighted Beta diversity t-test, P=0.1342). Metastats analysis revealed no significant differences (q>0.05) between male and female groups at each rank of classification (Phylum, Class, Order, Family, and Genus). LEfSe analysis did not reveal significantly different biomarkers between male and female groups. To further verify the aforementioned results, intragroup and intergroup differences were compared via Anosim, MRDP, ADOMIS, and AMOVA and consequently, intergroup differences

were slightly but not significantly greater than intragroup differences (ANOSIM: R=0.0125, P=0.418; ADONIS: R=0.098, low resolution; AMOVA: P=0.424) (Figure 6).

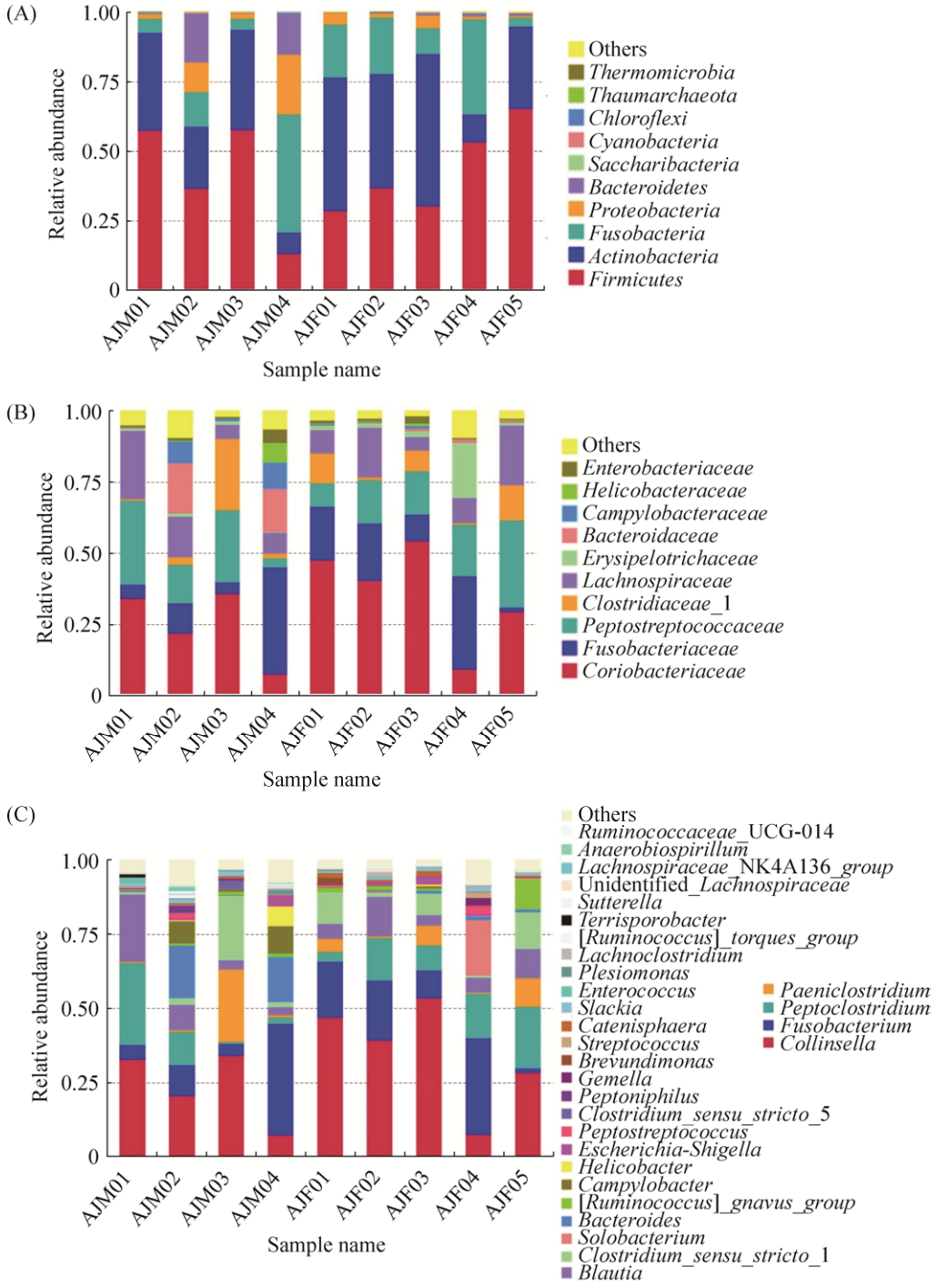

Figure 2. Gut bacterial composition at the phylum (A), family (B), and genus (C) levels in each sample. Based on the results of the species noted, gut bacterial species and phyla were selected in each sample (A) and the relative abundance of the top ten or top thirty biological species at the family level (B) and the genus level are shown (C) along with product column maps.

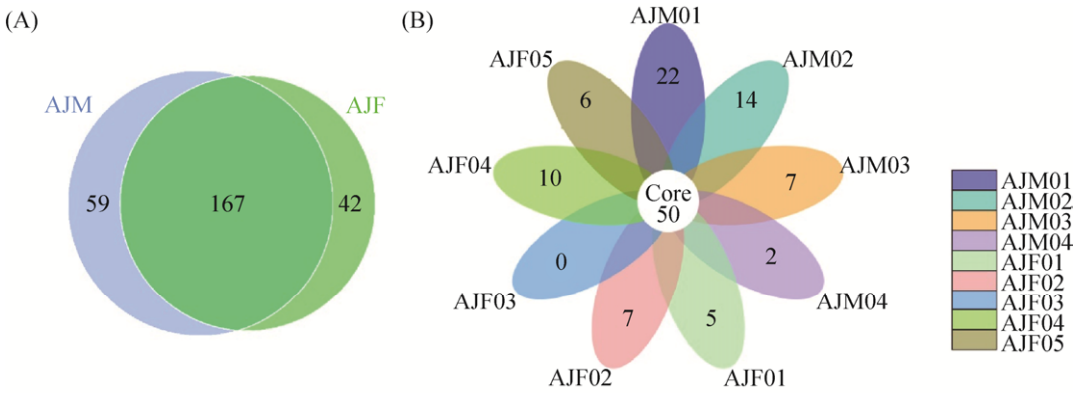

Figure 3. The Venn map of male and female groups (A) and petal map of different samples (B). Based on the analysis of operational taxonomic unit (OTU) clusters, study requirements, and common and unique OTUs among different samples (groups), a Venn diagram and petal diagram were constructed. Each circle in the Venn diagram represents a group, the number of circles and circle overlaps represent the number of OTUs shared between groups, and the number without overlap represents the number of unique OTUs in the group. Each petal in the petal diagram represents a sample, with different colors representing different samples, the middle core number representing the total number of OTUs shared by all samples, and the number on the petals representing samples with unique OTU numbers.

Table 1. Alpha diversity indices

Alpha diversity index	ACE	chao1	OTUs	Shannon	Simpson	PD Whole tree
AJM	144.56±24.76	140.812±22.650	132.75±27.78	3.65±0.62	0.85±0.06	13.13±2.31
AJF	129.15±25.48	126.65±27.03	118.20±22.13	3.30±0.30	0.81±0.07	12.50±2.18
AJM-AJF p value	0.3917	0.4210	0.4279	0.3570	0.3826	0.6917

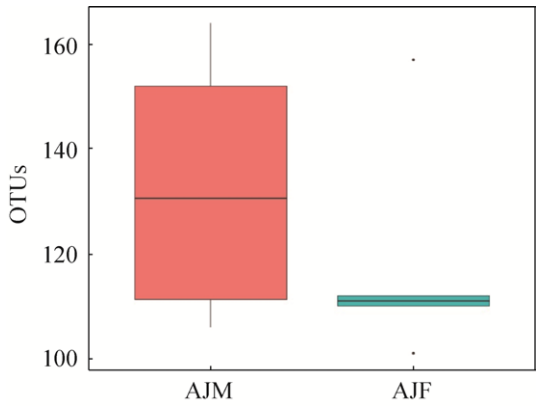

Figure 4. Comparison of the alpha diversity of microbial communities between sexes. The box chart reflects the median, standard deviation, maximum, minimum, and outlier values of species diversity in the group. Alpha diversity was determined on the basis of the observed species indices. *T*-test and Wilcox rank sum test were performed to analyze differences in species diversity between groups.

To further elucidate intergroup and intragroup differences, the Beta diversity was determined on the basis of the weighted and unweighted Unifrac distance to determine the difference coefficient among samples and to construct a heat map. Consequently, intragroup differences were slightly but not significantly greater in the AJM group than in the AJF group (unweighted unifrac P=0.2619, weighted unifrac P=0.1342). To assess differences between groups, PCoA, PCA, and NMDS analysis were performed to cluster samples of cheetahs in accordance with the diversity of fecal bacteria. No complete separation was observed between the two sexes, indicating that there is no significant sex-based correlation in the intestinal microbiota diversity in cheetahs (Figure 7). UPGMA cluster analysis based on the weighted and unweighted Unifrac distance matrix further verified that the gut microbiota diversity in cheetahs was not clustered by sex (Figure 8).

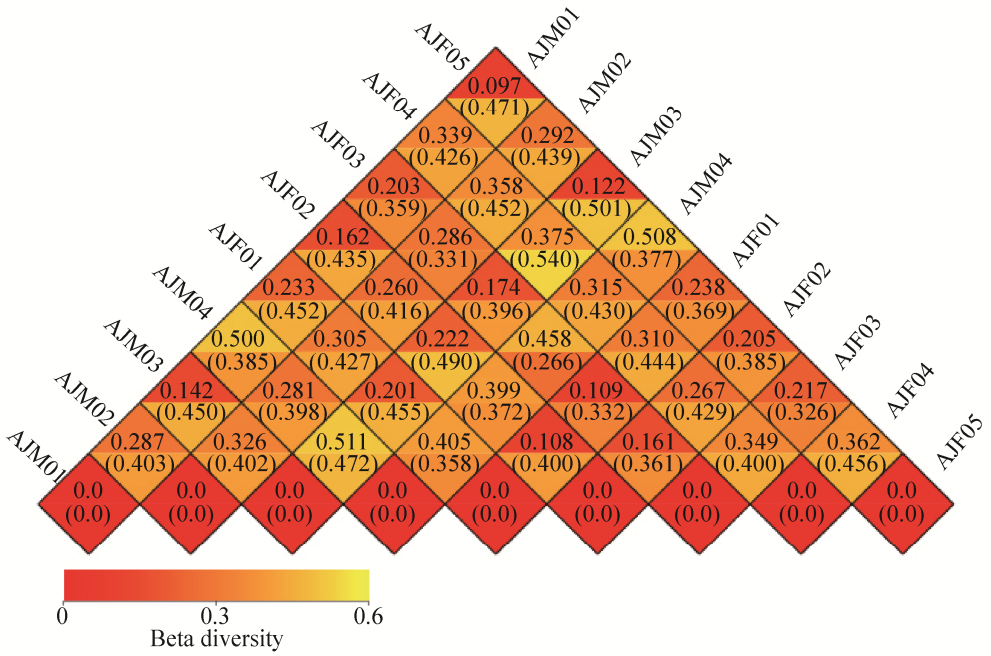

Figure 5. Heatmap of weighted and unweighted UniFrac distances. The weighted and unweighted Unifrac distances were considered to evaluate the difference coefficient between the two samples. The upper and lower values in the same box represent the weighted and unweighted Unifrac distance, respectively. The smaller the value, the smaller the difference in species diversity between the two samples.

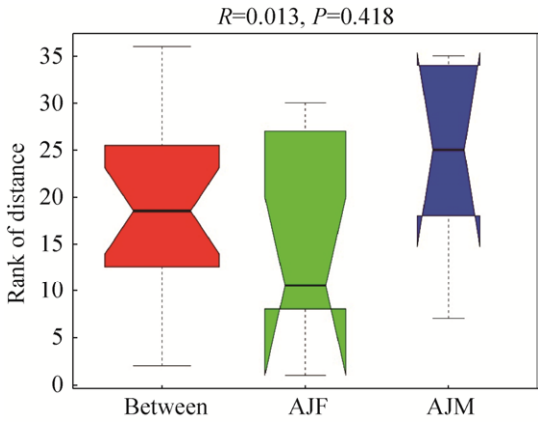

Figure 6. Anosim analysis of intergroup and intragroup differences. Anosim was used to investigate whether the intergroup differences were greater than the intragroup differences. The *R*-value was between (–1, 1). An *R*-value greater than 0 indicates that the intergroup difference is significant; an *R*-value less than 0 indicates that the intragroup difference is greater than the intergroup difference. *P*-values less than 0.05 indicated statistical significance. The ordinate represents the rank of the distance between the samples. The abscissa "Between" reflects intergroup findings and the other two reflect intragroup findings.

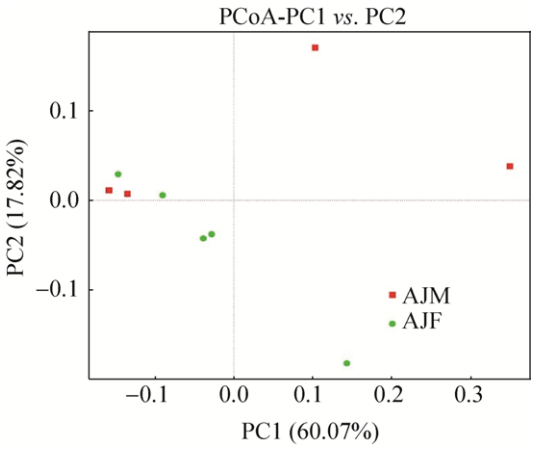

Figure 7. Principal coordinate analysis (PCoA) between groups based on the weighted UniFrac distance. PCoA analysis was performed on the basis of weighted and unweighted Unifrac distances, and the principal coordinate combination with the largest contribution in shown. The horizontal coordinates represent one principal component. The ordinate represents another principal component. The percentage represents the contribution of the principal component to differences among samples. Each point in the diagram represents a sample. The samples of the same sex group are represented by the same color.

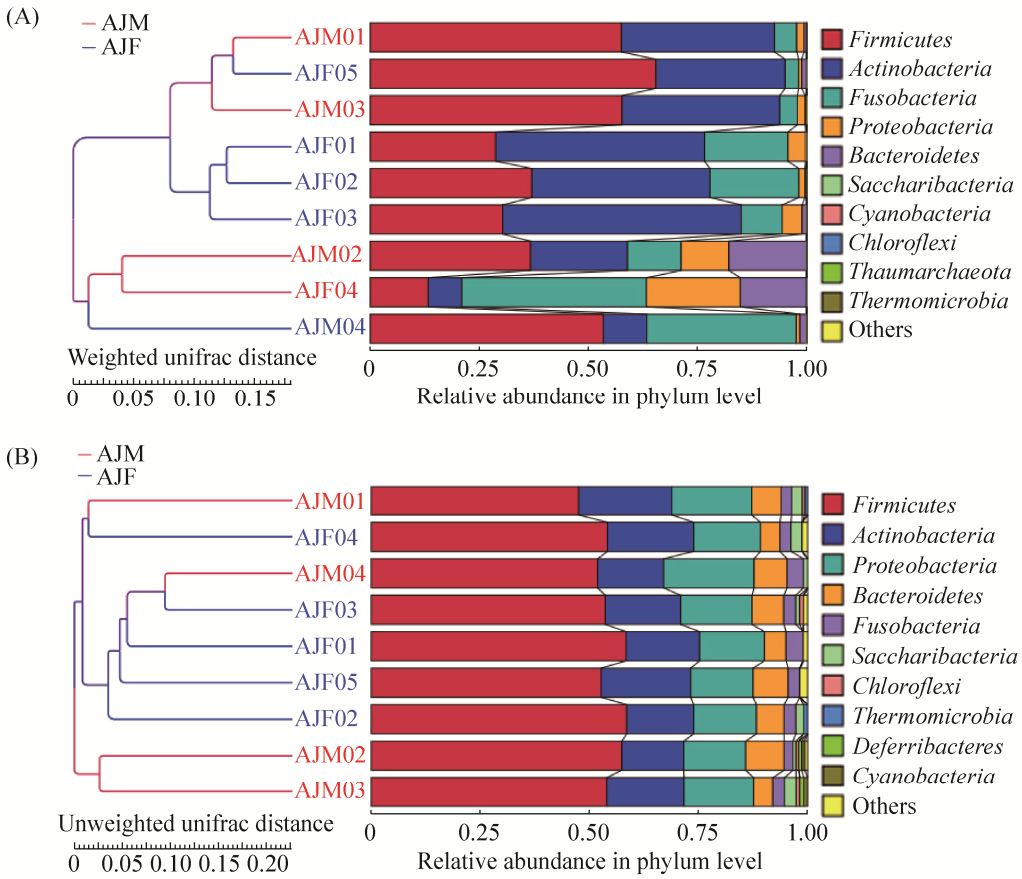

Figure 8. UPGMA clustering analysis based on weighted and unweighted UniFrac distance matrices. To assess the similarity among different samples, samples were clustered to construct a clustering tree of samples, and UPGMA clustering analysis was performed on the basis of the Weighted Unifrac distance matrix (A) and Unweighted Unifrac distance matrix (B).

3 Discussion

This study assessed the diversity of the cheetah intestinal microbial community via Illumina-based high-throughput sequencing of 16S rRNA. The present results show that the core bacteria of the intestinal microbiota of cheetahs comprise phyla *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, and *Bacteroidetes*. Becker *et al*. reported that intestinal microbes of cheetahs include phyla *Firmicutes* (94.7%), *Actinobacteria* (4.3%), *Fusobacteria* (0.6%), and *Proteobacteria* (0.4%) on shotgun sequencing[6]. Our results are concurrent with those of Becker *et al*. However, owing to the significant difference in the amount of sequencing data, the composition and proportion of bacterial phyla in the intestinal microbiota of cheetahs determined herein is different from that reported by Becker *et al*.[6]. Several studies have assessed the intestinal microbiota of members of family *Felidae*. For example, Tun *et al*. reported that the primary gut microbes of domestic cats (*Felis catus*) belonged to phyla *Bacteroidetes*/*Chlorobi*, *Firmicutes*, and *Proteobacteria*[4]. Nunez-Diaz *et al*. reported that gut microbes of captive Iberian lynx (*Lynx lynx*) primarily comprised phyla *Proteobacteria* (54.95%–61.76%), *Firmicutes* (33.58%–42.38%), *Bacteroidetes* (0.11%–6.03%), and *Actinobacteria* (0.96%–5.86%)[18]. Zhang *et al*. reported that the gut microbes of snow leopard (*Panthera uncia*)

primarily comprised phyla *Firmicutes* (56.5%), *Actinobacteria* (17.5%), *Bacteroidetes* (13%), and *Proteobacteria* (13%)[19]. At the phylum level, the intestinal microbial community of cheetahs is similar to that of other members of family *Felidae*, being more similar to that of snow leopards. However, compared to that of the other members of family *Felidae*, the intestinal microbial community of cheetahs has certain unique characteristics, especially the relatively low abundance of phylum *Bacteroidetes* (4.19%). Bacteria of phylum *Bacteroidetes* promote polysaccharide decomposition to improve nutrient utilization, accelerate intestinal vascular formation, promote host immune development, improve host immunity, and maintain a balance and stability in the intestinal ecosystem. This phylum accounts for a large proportion (approximately 68% of the total number of OTUs) in the intestinal microbial community of cats[4]; however, its proportion in the digestive tract in wild animals of family *Felidae* is low, especially in lynxes[6]. Owing to obvious differences in food composition and habitat between domestic and wild animals, domestic cats are not the same as wild animals of family *Felidae* with respect to numerous physiological characteristics. Furthermore, exogenous carbohydrate supplementation may increase the proportion of phylum *Bacteroidetes*/*Chlorobi* in the intestinal microbiota of domestic cats[4]. Therefore, our results are concurrent with those of Becker *et al.*, who investigated the significance of the domestic cat as an optimal model of endangered wild cats in an interventional study on animal nutrition[6]. However, in this study, approximately 4.19% of total OTUs were classified into phylum *Bacteroidetes*, which was not reported before[6]. This is probably owing to the use of high-throughput Illumina-based sequencing. High-throughput sequencing technology allows for a greater sequencing depth and provides significantly more data than first-generation sequencing technologies, which better highlight the composition and diversity of intestinal microbiota and reflect low-abundance groups in the intestinal tract.

Proteobacteria were more abundant in sample AJM04 (27.38% of total OTUs) than in other samples in this study. It is reported that the high abundance of phylum *Proteobacteria* in the gut microbiota is associated with diseases including intestinal inflammation[20]. Concurrently, Suchodolski *et al*. reported that domestic dogs with intestinal diseases had a lower abundance of phylum *Bacteroidetes* but a higher abundance of phylum *Proteobacteria* than normal healthy dogs[21]. Numerous bacteria in phylum *Proteobacteria* are pathogenic, including genera *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and *Yersinia* and order *Legionellales*, and a few non-parasitic bacteria such as nitrogen-fixing bacteria. Most members of the phylum are facultative or obligate anaerobes, chemoautotrophs, and heterotrophs. Moreover, numerous strains in phylum *Proteobacteria* are associated with inflammation, and some strains are associated with imbalances in the microbiota of the female reproductive tract[22]. Herein, we carried out health evaluation of cheetahs during sample collection. We did not find that sample AJM04 had intestinal diseases. By tracing the feeding history of sample AJM04, the sample was infected with *Ascaris lumbricoides* approximately 1 year ago, and we speculated that this may have accounted for the high abundance of phylum *Proteobacteria*. However, we speculated that the high abundance of phylum *Proteobacteria* in sample AJM04 may also result from individual differences because phylum *Proteobacteria* constitutes a major component of the intestinal microbiota of numerous carnivores and herbivores, such as wolf (*Canis lupus*, 9.2% of the total number of OTUs)[23], dhole (*Cuon alpinus*, 9.33%–17.60%)[24–25], snow leopard (*Uncia uncia*, 13%)[26], domestic cat (*Felis catus*, approximately 6%)[4], and takin (*Budorcas taxicolor*, approximately 2.37%)[27]. The abundance of phylum *Proteobacteria* is also high in the sable (*Martes zibellina*; 29.1%)[28] and the lynx (*Lynx lynx*; 54.95%–61.76%)[18].

Therefore, it remains unclear whether the high abundance of phylum *Proteobacteria* reflects an intestinal infectious disease.

Previous studies have reported that numerous intestinal microbial communities contain a certain proportion of unclassified bacteria. Herbivores tend to have higher proportions of unclassified intestinal bacteria owing to the complexity and indigestibility of food[29–30]. In this study, unclassified bacteria accounted for 2.32% of the intestinal microbiota of cheetahs. In other carnivorous, such as wolves and dholes, unclassified bacteria accounted for 26% and 34.3%, respectively, of the intestinal microbiota[20,22]. Herein, the rarefaction curve shows that sequencing data to analyze the intestinal microbial diversity of cheetahs are adequate. However, herein, we observed a small percentage of unclassified bacteria in the intestinal microbiota of the cheetah, probably owing to the high protein content of the cheetah and the relatively constant food source.

The diversity of the intestinal microbiota of wild animals is associated with their sex[28]. In this study, sex-based difference coefficients were evaluated on the basis of the weighted and the unweighted Unifrac distance. Consequently, the difference between the male and female groups was not significant. Moreover, UPGMA cluster analysis based on the weighted and unweighted Unifrac distance matrix revealed no marked sex-based clustering of the intestinal microbiota of cheetahs, and the results of PCoA, PCA, and NMDS were consistent with those of cluster analysis, indicating no sex-based difference in the diversity of the intestinal microbiota of cheetahs. However, intergroup differences were greater than intragroup differences, indicating slight sex-based differences in the diversity of the intestinal microbiota of cheetahs, the non-significance of this difference probably resulting from the small number of samples and marked intragroup differences in the diversity of the intestinal microbiota of cheetahs. For example, the weighted Unifrac difference in microbial diversity between samples AJM03 and AJM04 was the largest (0.511), while that between AJM01 and AJF05 was the smallest (0.097) (Figure 5). These large individual differences and relatively small sample sizes may have compromised the statistical power of the sex-based differences in the diversity of the intestinal microbiota of cheetahs to a certain degree.

In summary, this study describes high-throughput Illumina-based sequencing of the hypervariable region of bacterial 16S rRNA gene to evaluate the species composition and diversity of the intestinal microbiota of cheetahs. The present results show the core intestinal microbiotal composition of cheetahs and a lower abundance of phylum Bacteroidetes[20,26]. However, it remains unclear whether this characteristic is unique to cheetahs or is shared with other wild felid animals[6,18]. No correlation was observed between intestinal microbial diversity and sex in cheetahs, different from that in other carnivores[28]. Intestinal microbes in cheetahs play important roles in digestion, nutrient absorption, and intestinal health. This study provides novel in-depth insights into the diversity of the intestinal microbiota of cheetahs, thus facilitating studies on the physiology and health of cheetahs and lay the foundation for the breeding of captive cheetahs and for the wild reintroduction of captive animals.

Authors' contributions

Lei Chen conceived, designed, performed the experiments and analyzed the data; Mi Liu wrote the paper; Ying Gao, Weilai Sha contributed materials; Jiaxin Chen and Jing Zhu modify the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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猎豹(*Acinonyx jubatus*)肠道微生物多样性及其性别差异研究

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摘要：【目的】探讨猎豹(*Acinonyx jubatus*)肠道微生物多样性特征。【方法】通过采集新鲜粪便样品，对 9 只健康成年野生猎豹(4 只雄性，5 只雌性)的肠道微生物 16S rRNA 基因 V3–V4 区进行高通量测序，对猎豹肠道微生物多样性进行研究。【结果】测序共获得肠道微生物 16S rRNA 基因 V3–V4 区有效序列 599349 条，序列平均长度 405 bp。通过以 97%的序列相似性进行分类，共获得操作分类单元(OTU) 268 个。经序列比对和分类鉴定，这些 OTU 都属于细菌域，包括 10 个门，21 个纲，35 个目，72 个科，144 个属。其中，丰度最高的 5 个细菌门是厚壁菌门(*Firmicutes*，平均占 OTU 总数的 42.29%%)、放线菌门(*Actinobacteria*，31.54%)、梭杆菌门(*Fusobacteroidetes*，16.66%)、变形菌门(*Proteobacteria*，5.30%)和拟杆菌门(*Bacteroidetes*，4.19%)。拟杆菌门的丰度较低是猎豹肠道微生物的主要特征。丰度最高的 5 个科依次是红蝽杆菌科(*Coriobacteriaceae*，31.28%)、消化链球菌科(*Peptostreptococcaceae*，平均占 17.66%)，梭杆菌科(*Fusobacteriaceae*，15.46%)、毛螺菌科(*Lachnospiraceae*，12.40%)、梭菌科 I (*Clostridiaceae*_I，6.93%)等。丰度最高的 5 个属依次是柯林斯氏菌属(*Collinsella*，30.16%)、梭杆菌属(*Fusobacterium*，15.46%)、艰难梭菌属(*Peptoclostridium*，11.46%)、*Blautia* 属(8.28%)和狭窄梭菌属 1 (*Clostridium_sensu_stricto_1*，6.39%)。约有 2.32%的 OTU 没有归类到属。群落 alpha 多样性分析结果显示，猎豹肠道微生物群落 Shannon 指数为 2.93–4.41，Simpson 指数为 0.72–0.91。通过依据性别进行分组，对雌雄两组之间的 alpha 多样性比较表明，雄性组的物种和 Shannon 指数略高于雌性组。Beta 多样性分析表明，雌雄两组之间的差异高于各组内部不同个体之间的差异。然而，聚类分析显示，相同性别的猎豹的肠道微生物并没有聚在一起。【结论】本文通过高通量测序技术研究了猎豹肠道微生物多样性特征和性别差异，为猎豹的保护、救护饲养和消化生理学研究提供了基础数据。

关键词：猎豹，肠道微生物群落，16S rRNA 基因，高通量测序

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