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# Age-Related Changes in the Gut Microbiota Composition of Hog Deer (*Axis porcinus*)

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## ABSTRACT

Comprehensive investigation of gut microbiota composition is important for understanding digestive physiology in mammals. The hog deer (*Axis porcinus*) is a small mammal at high risk of extinction in the wild. In the present study, we sequenced the V4 region of 16S rRNA gene and compared the gut microbiota composition among three ages of captive hog deer, including one infant (Z1, six months old), three young (Z2, ten months old) and 12 adult (Z3, 4-5 years old). A total of 26 phyla (15, 22 and 25 in Z1, Z2 and Z3) and 310 genera (153, 254 and 304 in Z1, Z2 and Z3) were identified. In Z1, Z2 and Z3, *Firmicutes* (63.65%, 62.07% and 61.96%) was the predominant phylum, followed by *Bacteroidetes* (29.08%, 29.62% and 30.02%) and *Tenericutes* (3.08%, 3.24% and 2.78%). The alpha diversity (Shannon Index and observed species) of Z2 was significantly higher than that of Z3 ($P < 0.05$). The Shannon Index of Z1 was higher than that of Z2. At the genus level, *Christensenellaceae_R-7_group* and *Ruminiclostridium_5* had higher abundance in Z2 compared with Z3, whereas other genera, such as *Fibrobacter*, *Lachnospiraceae_AC2044_group* and *Oscillibacter* were enriched in Z3. In conclusion, our results reveal significant microbiota composition changes that occur with age in captive hog deer.

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**Authors' Contribution**
**HY and WW performed the procedure, analysed the data, and wrote the manuscript. HY, WW and JY conceived and designed the experiments. HY, WW, LN, YQ and YP extracted data for the analysis. JD, AC, YZ, WC and XY contributed to the sample processing and sequencing.**

**Key words**
**Hog deer, Gut microbiota, Age-related change, Next-generation sequencing, 16S rRNA gene, *Axis porcinus*.**



## INTRODUCTION

*Axis porcinus* is a ruminant in the *Hyelaphus* genus (Cervidae family) and an endemic species of South and Southeast Asia. It is also known as the Indochinese hog deer in China, Cambodia, Thailand, Laos and Vietnam and as the Indian hog deer in Pakistan, Nepal, India, Bangladesh and Burma (Tanushree *et al*., 2000). *Axis porcinus* has nearly been extirpated in many countries including China; therefore, it has been on the Red List at the endangered level by the International Union for the Conservation of Nature (IUCN) since 2008 (Timmins *et al*., 2015). Now, in China, most *Axis porcinus* have been reared in Chengdu Zoo in Sichuan (total number 45) because finding wild *Axis porcinus* is extremely difficult. Captive rearing is increasingly important, as wild hog deer are approaching extinction in the wild. The zoo plays an important role in artificial feeding, enlarging the herd protecting the species *Axis porcinus*. Presently, the urgent and necessary goal is to maintain herd health with artificial feeding. Normal gut physiology and microbiota are important for animal health. Therefore, an investigation of the gut microbiome is important for hog deer conservation.

With the rapid development of next-generation sequencing technology, the gut microbiome has become a hot research topic worldwide. The gut microbiota has become increasingly important to understanding the immunological and physiological functions that maintain the health of both artificially fed and wild animals (Guan *et al*., 2017). Herbivores have higher gut microbial diversity than omnivores and carnivores (Price *et al*., 2012). These microbiota, with approximately 542 genera found throughout cattle gastrointestinal tracts (Mao *et al*., 2015), have multiple functions, such as digesting the cellulose of plant cells, eliminating the anti-nutrition factors in the diet and producing vitamins and microbial protein (Kohl *et al*., 2018; LeBlanc *et al*., 2013). Gut microbiota have been investigated in some species of ruminants, but such investigations are limited in many uncommon species, especially in endangered animals (Guan *et al*., 2017; Li *et al*., 2018). Investigation of the gut microbiota of *Axis porcinus* is important to understanding the gut physiology and digestion of the species, which will be beneficial for maintaining animal health and enlarging the herd population size. However, limited data exist on the gut microbiota

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of *Axis porcinus*. Previous studies on *Axis porcinus* focused on the mitochondrial genome for a genotyping (Hill *et al*., 2017; Wang *et al*., 2017) assessment of a hog deer population (Lwin *et al*., 2016) and for endoparasite assessment (Rana *et al*., 2015; Hussain *et al*., 2022). Although one study investigated the faecal microbiome of six Cervini species, which included *Axis porcinus* (Li *et al*., 2018), only 3 adult hog deer were used to collect the samples. Hence, a thorough investigation of the gut microbiota of *Axis porcinus*, with a determination of the differences between the microbiota community structure of this species and that of other species of Cervidae or ruminants, is necessary.

Many factors, including age and diet, can influence the gut microbial community structure and the dominant bacteria. The ruminal microbial community of goats from 7 days to 2 years old underwent significant changes in response to the shift in age, and a clear age-related pattern was observed in the diversity of the bacterial community with some bacteria (Wang *et al*., 2013). In cows, microbial change with age was also observed (Dill-McFarland *et al*., 2017). The same pattern of GI tract microbiota change with age is expected in *Axis porcinus*, and no microbial data have been collected in other growth stages, such as in the young or the sub-adults. Previous reports confirmed that faeces were used as representations of gastrointestinal tract samples (Tannock *et al*., 2000; Yan *et al*., 2018). Furthermore, faecal sampling is a beneficial method for use on endangered species because it is non-invasive (Hu *et al*., 2017). Therefore, in this study, fresh faecal samples were collected from 1 infant, 3 sub-adults and 12 adults of *Axis porcinus* and evaluated in this study. Their faecal microbiomes were characterized using techniques for sequencing the 16S rRNA gene with the V4 region. Our findings provide new knowledge about this endangered deer's gastrointestinal microbiota structure and composition over age, which will benefit diet formulation for artificial feeding and further assist in health management or species conservation.

## MATERIALS AND METHODS

*Sample collection*

From the herd of *Axis porcinus* captive in Chengdu Zoo, Sichuan province, China, 1 infant, 3 sub-adults and 12 adults were selected. We defined the growth stage as follows: infant, 3-8 months; sub-adult, 8-12 months; and adult, 4-5 years. All animals lived in the same enclosure. The total mixed rations were formulated by the zoo and provided to the animals. The infant animals ingested a different diet consisting of rye, alfalfa pellet, apple, carrot, sophorae leaves, and complete concentrate. The sub-adult and adult *Axis porcinus* both consumed a diet consisting of alfalfa bale, alfalfa pellet, apple, carrot, asparagus lettuce, green grass, and complete concentrate. The ingredients and nutritional composition of the diets are presented in Supplementary Table I. A total of 45 faecal samples were collected from the 1 infant (Z1), 3 sub-adult (Z2) and 12 adult (Z3) *Axis porcinus*. The collector monitored the defecation and animal ID of subjects between 9:00-10:00 am on August 22, 23 and 26, 2017. After observing an animal defecate, the collector immediately transferred the faeces (each faecal sample was larger than 4 g) to a clean sample container using a sterile spoon and then sealed the container in a plastic bag labelled with animal ID and sampling date. During this process, the part of the faeces that touched the ground was removed to guarantee the faeces had not touched anything with bacteria. The fresh faecal samples were stored in a foam cooler with dry ice, and every day after collection, the samples were transported back to the laboratory and stored at -80°C for sequencing. The sequencing information for the samples is provided in Supplementary Table II. All the selected animals lived in same environment and were provided diets by the zoo to meet their requirements. They were healthy and had received no any injection with antimicrobials or other treatments in the previous 6 months. The protocol of this study was approved by the Institution of Animal Care and Ethics Committee. The collection of the *Axis porcinus* faecal samples was approved by the Chengdu Zoo.

*DNA extraction*

According to the kit manual, the total genome DNA from the faecal samples (100 mg) was extracted using QIAamp® Stool Mini Kit (Qiagen, Germany). The concentration and purity of each DNA extract were determined using the Nanodrop One (Thermo Scientific, United States). Then, all DNA was sent to Novogene, Inc. (Chengdu, China) for PCR and sequencing.

*16S rRNA gene PCR and Illumina HiSeq sequencing*

The library preparation and Illumina HiSeq sequencing were performed at Novogene, Inc. The V4 hypervariable region of the 16S rRNA gene was amplified by 16S universal amplicon PCR primers: 515F 5'-GTGYCAGCMGCCGCGGTAA and 806R 5'-GGACTACNVGGGTWTCTAAT (Tamaki *et al*., 2011). A primer barcode was designed for incorporation into the differentiate samples. A final mixture volume of 50 µL was used for the polymerase chain reaction: 6 µL of template faecal DNA, 2 µL of forward and reverse primer (10 µM), 25 µL of 2 × Taq PCR Master Mix (0.1 U/µL), and 15 µL $ddH_2O$. The PCR was carried out under the following conditions: 3 min at 95°C for initial denaturation, 30 cycles of steps 2 - 4 (95°C for 30 s, 55°C for 30 s and 72°C for 30 s) and the final extension at 72°C for 5 min.

Each plate of PCR contained a positive and negative control for amplification confirmation. The positive control was mock community DNA that also was used in the sequencing. The negative control contains no template. The PCR products were measured in electrophoresis using 1.0% agarose gel and purified using a SanPrep DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China). Then, the PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany) for library preparation and sequencing. A TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to build the sequencing libraries according to the manufacturer's instructions. After being established by Qubit and qPCR, the library with achievement of quality parameters was sequenced on an Illumina HiSeq 2500 PE250 platform according to the manufacturer's recommendations.

*Bioinformatics*

After the raw sequences were obtained, the barcodes and original primers from the HiSeq platform were removed. The sequence assembly and quality filtering were conducted using FLASH (Version 1.2.7) (Magoč *et al*., 2011) and QIIME (Version 1.7.0) (Bokulich *et al*., 2013). To eliminate the chimeric sequences, the Gold database was used for comparison with the filtered tags using the UCHIME algorithm (Edgar *et al*., 2011). Then, effective tags were assigned into the operational taxonomic units (OTU) with a similarity greater than or equal to 97%, and species annotation based on RDP classifier algorithm (Edgar *et al*., 2011) was performed using the Greengene Database (DeSantis *et al*., 2006). The datasets generated and analysed during the current study are available in the NCBI BioProject database with the BioProject ID PRJNA533948.

The QIIME was performed to calculate alpha diversities (Shannon Index and observed species) and beta diversity (unweighted and weighted UniFrac distance). The figure of the UPFMA (unweighted pair-group method with arithmetic mean) tree was built in QIIME based on the UniFrac distance. The phylogenetic tree for the genera was generated by using GraPhlAn software (http://segatalab.cibio.unitn.it/tools/graphlan/). The analysis of similarities (ANOSIM) was used to test statistically whether a significant difference existed between the two treatments. The heatmap were made in R using the top 35 genera across all samples. The PCA, PCoA and figures were displayed using R software. The T-test for identification of the bacteria between the sub-adult and adult used non-parametric tests that were archived in R. The R packages used in our analysis contained ggplot2 v.2.2.1, ade4 v.1.7-13, WGCNA v.1.64, stats v. 3.6.0, vegan v.2.5-2 and agricolae v.1.2-8.

## RESULTS

*Overview of the sequencing data*

A total of 2,758,076 high-quality reads with an average length of 371±80 bp were obtained after the quality control. These sequences from 45 faeces samples



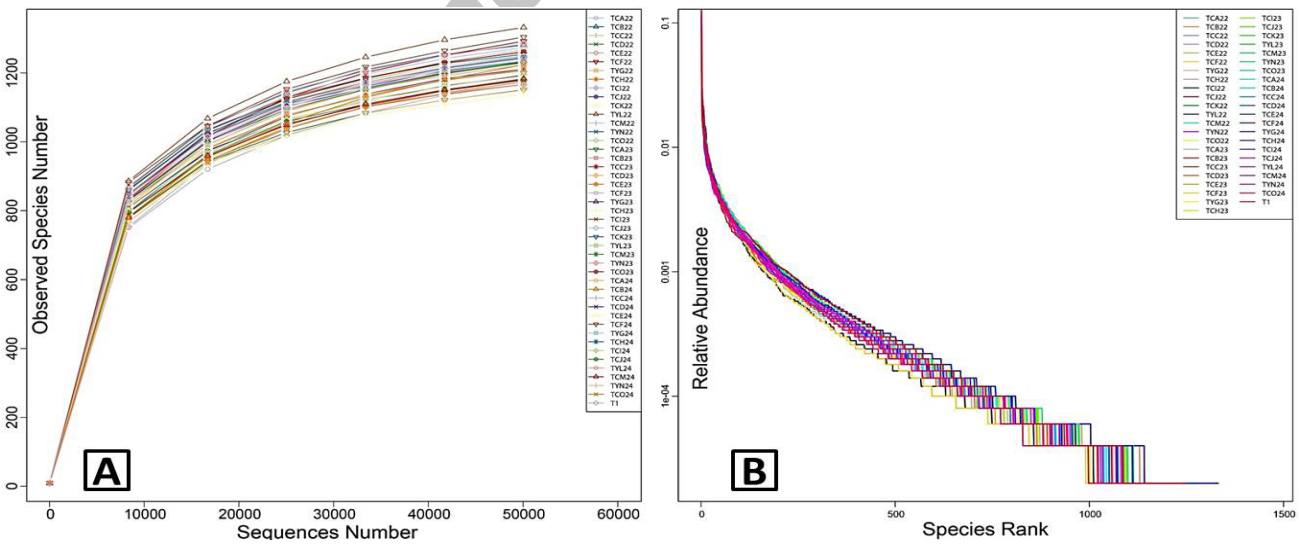


Fig. 1. Species diversity curves. A, rarefaction curves; B, rank abundance curves. Plot A reflects indirectly the rationality of sequencing data size and richness of species in the faecal samples. When rarefaction curves tend to flatten, rational sequencing data is indicated. Plot B represents the richness and evenness of the species. The wider span of the curves illustrates the higher relative abundance of taxa in the x axis direction and the smoothness of the curves showed the evenness of bacterial species in the y axis direction. The figure legend at the top-right corner showed identification of each sample. TC, adult; TY, sub-adults, T1, infant.

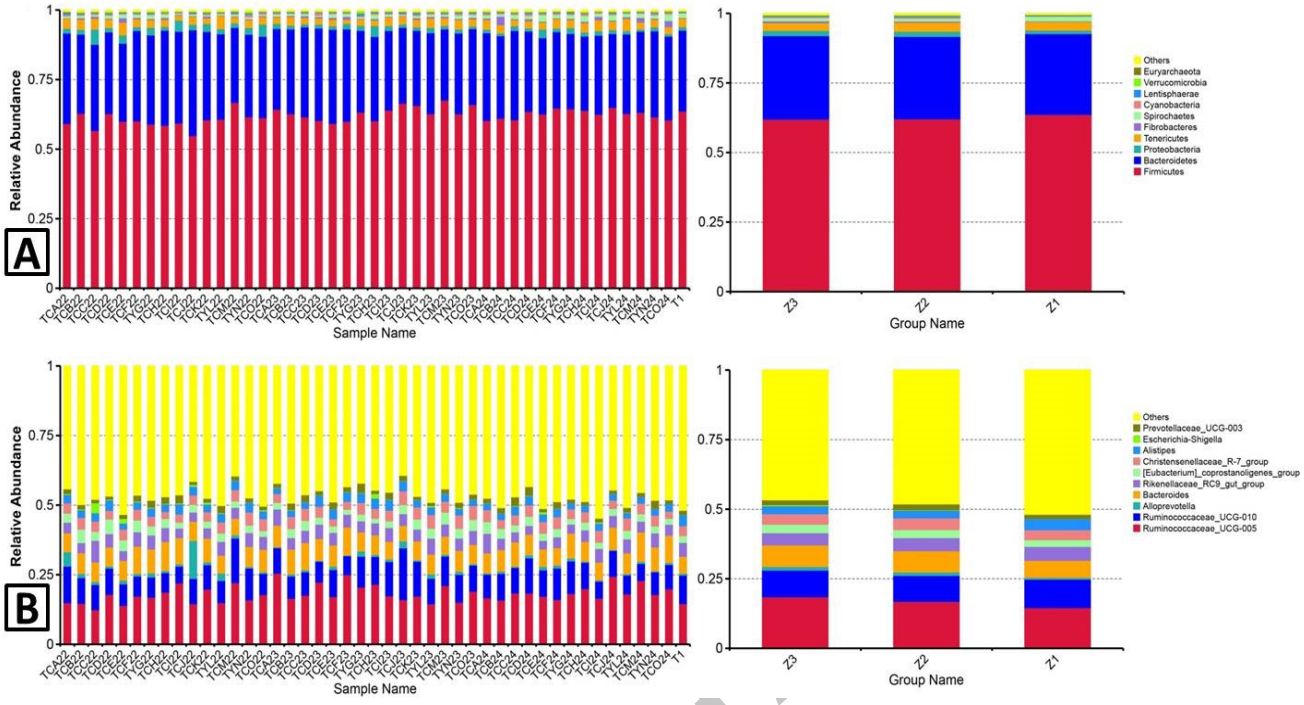


Fig. 2. Gut microbiota composition. Faecal microbial composition of *Axis porcinus* at the phylum (A) and genus (B) level. Each bar represents the top 10 bacterial species ranked by the relative abundance in each individual sample or group. Z3, adult; Z2, sub-adults; Z1, infant; TC, adult; TY, sub-adults; T1, infant.



of *Axis porcinus* were classified into 2215 OTUs, with 97% similarity. The rarefaction curves (Fig. 1A), which are plots of the number of species as a function of the number of samples, flattened gradually, with more species yielding more plateaus. This result indicated that the sequencing depth was sufficient and rational. At the same time, the rank abundance curves that displayed relative species abundance in the faecal samples are presented in Figure 1B. The rank abundance curves visually illustrate both richness and evenness of species.

*The core microbiome of Axis porcinus*

The top 10 species of each sample or growth stage were selected, and the relative abundance of these were used to generate a stacked histogram at the phylum level (Fig. 2A). In Z1, Z2 and Z3, 15, 22 and 25 phyla were identified. At the phylum level of Z1, Z2 and Z3, *Firmicutes* (63.65%, 62.07% and 61.96%) was the predominant phylum, followed by *Bacteroidetes* (29.08%, 29.62% and 30.02%) and *Tenericutes* (3.08%, 3.24% and 2.78%). *Proteobacteria* (1.24%, 1.87% and 1.88%) and *Spirochaetes* (1.63%, 0.98% and 1.06%) were next in Z1, Z2 and Z3.

The genus levels of the gut microbiome of *Axis porcinus* are shown in Figure 2B. In Z1, Z2 and Z3, a total of 153, 254 and 304 genera were identified. At the genus level, the dominant bacteria of Z1, Z2 and Z3 were *Ruminococcaceae_UCG-005* (24.63%, 23.26% and 22.59%), *Ruminococcaceae_UCG-010* (14.58%, 16.87% and 18.52%), *Bacteroides* (6.13%, 7.63% and 7.74%), *Rikenellaceae_RC9_gut_group* (4.97%, 4.71% and 4.41%), *Alistipes* (4.02%, 2.75% and 2.90%), *Christensenellaceae_R-7_group* (3.58%, 4.22% and 3.74%) and *[Eubacterium]_coprostanoligenes_group* (2.44%, 2.89% and 3.04%). Nine other genera with a relative abundance higher than 1% were identified: *Prevotella_1* (2.82%, 1.06% and 1.02%), *Ruminococcaceae_UCG-013* (2.20%, 2.24% and 2.00%), *Prevotellaceae_UCG-003* (1.51%, 2.10% and 1.77%), *Phocaeicola* (0.86%, 1.52% and 1.46%), *Prevotellaceae_UCG-004* (1.00%, 1.62% and 1.43%), *Alloprevotella* (0.67%, 1.25% and 1.31%), *Ruminococcaceae_UCG-014* (1.23%, 1.35% and 1.28%), *Ruminococcus_1* (0.89%, 1.15% and 1.25%) and *Treponema_2* (1.62%, 0.96% and 1.04%).

*Visual representation of the microbial composition*

To better understand the microbial community composition, a heatmap for clustering with relative abundance of genera is shown in Figure 3A. Based on the clustered heatmap, the microbes from Z1 were

grouped together, while those microbes from Z2 and Z3, which were more similar, were grouped together. The species that accounted for different proportions were also marked by different colours and positions of crowding in the heatmap. A significant difference in the microbial composition could be observed. Regarding the weighted UniFrac and unweighted UniFrac distance matrix, we made the unweighted pair-group method with arithmetic mean (UPGMA) clustering analysis to study the similarity between samples in Figure 3B. The dendrograms of UPGMA was similar to the result in the clustered heatmap.

*The relationships of each genus*

To investigate the systematic phylogenetic relationship of the species at the genus level, a combination of genus relative abundance and relations among the top 100 genera is represented. The inside components of the phylogenetic tree comprise the representative sequences of the genera, and the colour of the branches represent the corresponding phylum (Fig. 4). The outside cycle indicates the relative abundance of genera in each group, and the colour of the abundance is represented by the different groups. The core genera of *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, *Ruminococcaceae_UCG-013* and *Ruminococcaceae_UCG-014* were in same phylum (*Firmicutes*), but they are evolutionarily distantly related. The genera of *Bacteroides*, Alloprevotella, Prevotellaceae_*UCG-004*, *Prevotella_1*, *Phocaeicola, Prevotellaceae_UCG-003, Alloprevotella*, *Rikenellaceae_RC9_gut_*group and *Alistipes* belong to the phylum of *Bacteroidetes* and show a close genetic distance. Additionally, these genera under *Bacteroidetes* have a close distance with *Treponema_2* (phylum *Spirochaetes*) rather than with the genera under *Firmicutes*. The *[Eubacterium]_coprostanoligenes_group* and *Ruminococcus_1* belonging to *Firmicutes* shows similar evolutionary relationships.

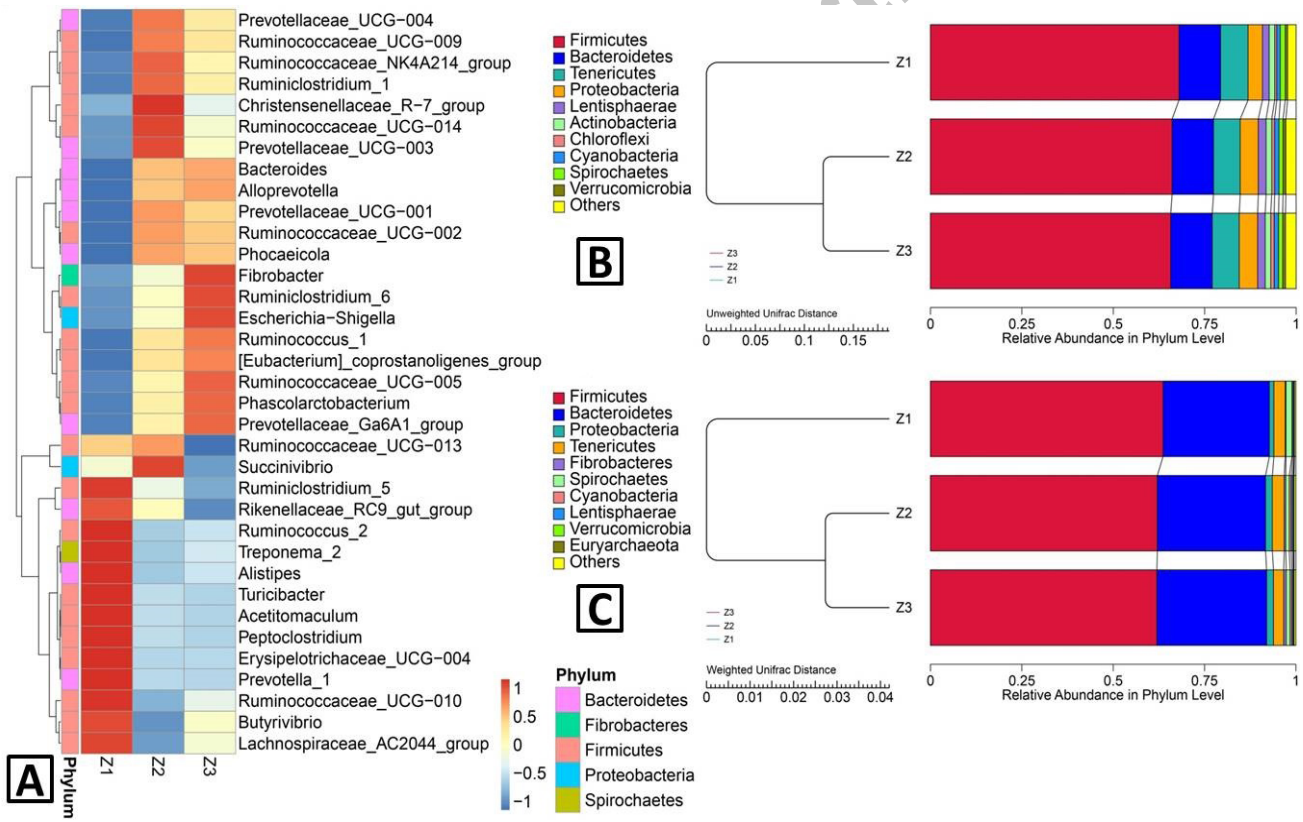


Fig. 3. Heatmap and UPFMA (unweighted pair-group method with arithmetic mean) tree. A, The heatmap of clustering for species abundance. The information for the groups (Z1, Z2 and Z3) and the species mark were revealed along X-axis and Y-axis, respectively. The clustering tree was generated based on the relative abundance of the genera in the top 35. The relative abundance in the heatmap, which was described by colours after normalization, indicate the aggregation degree or content of the bacterial species among the samples at the genus level; B. UPMGA clustering trees—based on unweighted UniFrac distance; C, weighted UniFrac distance. The results of clustering using two distance matrices were combined with the overall percentages of relative abundance among all samples at the phylum level. Z3, adult; Z2, sub-adults; Z1, infant.

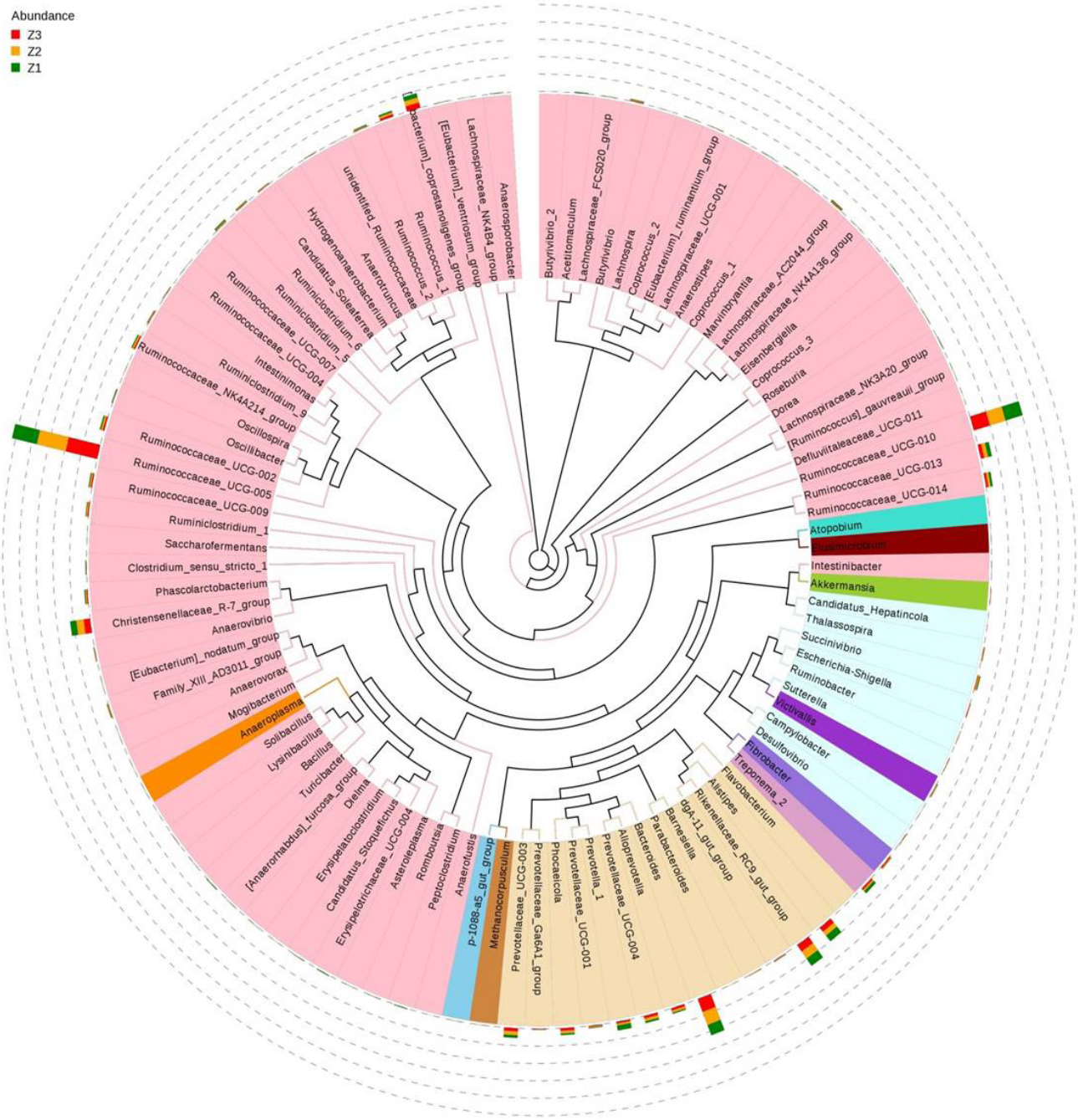


Fig. 4. Genus phylogenetic tree. To investigate the systematic phylogenetic relationship of species at the genus level, the combination of genus relative abundance and relations among the top 100 genera is represented. The inside components of the phylogenetic tree were built with the representative sequences of the genera, and the colour of the branches represent the corresponding phyla. The outside cycle indicates the relative abundance of genera under each group, and the colour of the abundance is represented by the different groups. Z3, adult; Z2, sub-adults; Z1, infant.

*Alpha and beta diversities of* Axis porcinus *in different growth stages*

Alpha diversities between the different growth stages are reported in Figure 5. The Shannon Index and observed species of Z2 were significantly higher than for Z3 (Wilcoxon test: p value= 0.028, p value = 0.025).

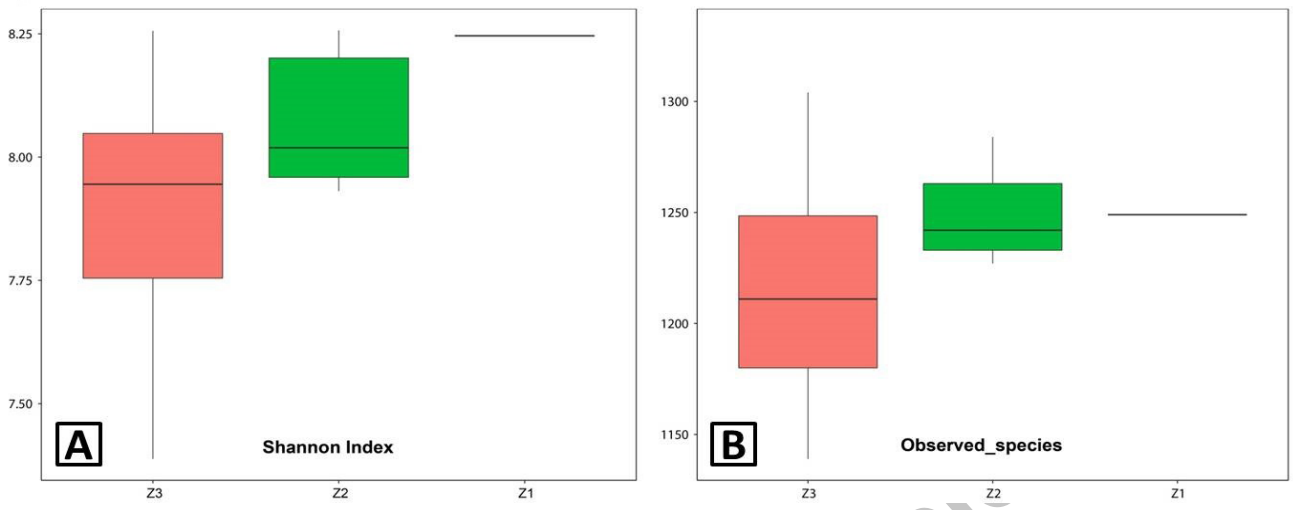


Fig. 5. Alpha diversities. A, Shannon index; B, observed species. The comparisons for Alpha-diversity (Shannon index and observed species) are shown in the different growth stages (Z1, Z2 and Z3). Z3, adult; Z2, sub-adults; Z1, infant.

The Beta-diversity using principle coordinates analysis (PCoA), unweighted UniFrac and principal component analysis (PCA) plot among Z1, Z2 and Z3 are shown in Figure 6. ANOSIM was used to test the differences (Fig. 6B) and showed no difference between Z2 and Z3. From the plot, we can observe that Z1 is separate from Z2 and Z3, in spite of having only 1 sample in Z1. Neither the PCA nor the PCoA plot showed a different cluster pattern between Z2 and Z3, but large variation occurred within groups in the ANOSIM results for these age groups. The PCA plot clustered with groups (Fig. 6D), indicating that Z2 and Z3 exhibited some difference, but this difference was not significant. The results indicate the individual variation observed within the treatment was large compared to that observed between sub-adult and adult *Axis porcinus*.

*Bacterial taxa differentially represented in sub-adult and adult periods*

Based on the results of the alpha and beta diversity, we confirmed that the microbial community structure changed at the different growth stages. Therefore, determining which bacteria caused this change in structure alteration was necessary. To find the different species between Z2 and Z3, a T-test was performed. Figure 7 uses a bar plot to show the species with significantly different relative abundances in Z2 and Z3 at the levels of phylum and genus. At the phylum level, the abundance of *Fibrobacteres* was higher in Z3 than in Z2. At the genus level, while *Christensenellaceae_R-7_group* and *Ruminiclostridium_5* had higher abundance in Z2 compared with Z3, other genera such as *Fibrobacter*, *Lachnospiraceae_AC2044_group* and *Oscillibacter* were enriched in Z3.

## DISCUSSION

A strong association exists between the gut microbiota and health or disease in domestic animals. The community structure of gut microbiota in these animals has already been reported, but data for these endangered animals are limited. The measurement of the gut microbiome of *Axis porcinus* contributes to our understanding of the species' gut physiology and will be helpful for diet formulation, which will have a positive impact on *Axis porcinus* health and conservation. Since *Axis porcinus* is nearly extinct, maintaining their health and enlarging the population is important. In consideration of the few remaining populations of *Axis porcinus*, the first step is to investigate the hog deer gastrointestinal microbiota according to its growth stage. We characterized the composition and structure of the faecal microflora of *Axis porcinus* in 3 growth stages (infant, sub-adult and adult); further, we provided the core gut microbiota in the different stages and demonstrated the dissimilarities of this microbiome with those of other cervids.

According to next-generation sequencing analysis, the predominant phyla detected in the faecal samples were *Firmicutes* (63.65%, 62.07% and 61.96% in Z1, Z2 and Z3) and *Bacteroidetes* (29.08%, 29.62% and 30.02% in Z1, Z2 and Z3). Small differences were observed in the predominant bacteria among the age groups in this study. These differences may be attributed to the single sample obtained from an infant and the small difference that existed between the sub-adult and adult groups. The sub-

adult period of *Axis porcinus* had a similar gut physiological environment to that of the adult due to the age, diet and habitation. In this study, the sub-adult and adult lived in the same pen and consumed the same diet. Since sexual maturity in *Axis porcinus* occurs at 8-12 months of age, the lack of alteration in the gut microbiome composition after maturity is reasonable (Odamaki *et al*., 2016). In terms of the core phyla, Li *et al*. (2018) found that *Firmicutes* (49.61%) was the predominant phylum followed by *Bacteroidetes* (37.74%) in six adult Cervini species (*A. porcinus*, *C. elaphus, C. nippon, R. unicolor, D. dama, and E. davidianus*) and one Muntiacini species (*E. cephalophus*). The relative abundance of *Firmicutes* in a wild and captive sika deer group was 77.62% and 50.71%, respectively, and

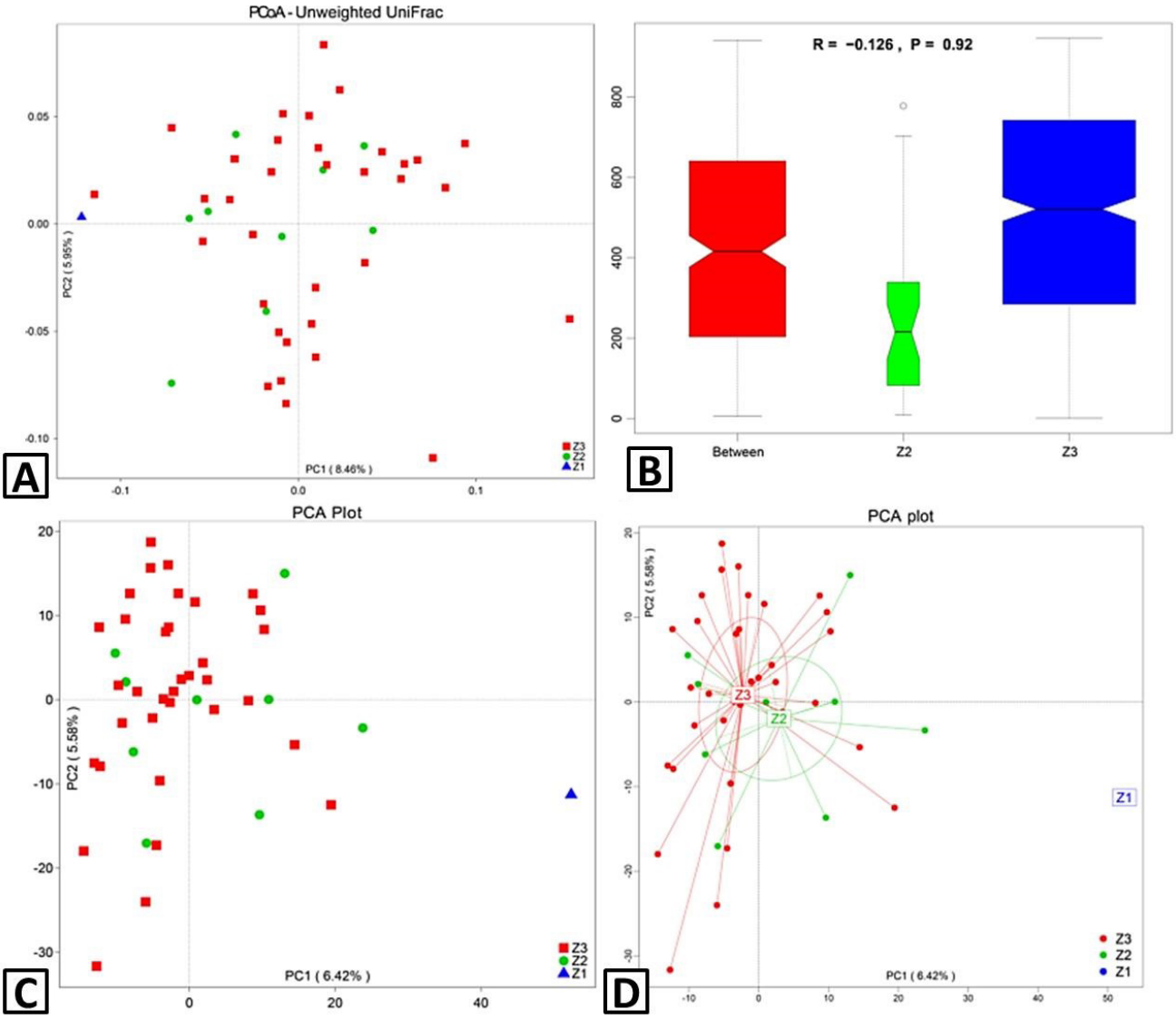


Fig. 6. Beta diversity. Principal coordinates analysis (PCoA) 2D images were developed using unweighted UniFrac (A) and distance matrices. (B) ANOSIM (analysis of similarities) analysis. Principal component analysis (PCA) plot (C) was drawn based on OTUs levels. (D) PCA plot clustering by groups. Each sample is represented by a point with Z1 in blue triangle, Z2 in green cycle and Z3 in red square R-value: R-value range (–1, 1). Actual results are generally between 0 and 1. An R-value close to 0 represents no significant inter-group and intra-group differences, whereas the R-value close to 1 shows that inter-group differences are greater than intra-group differences. P-value: The P-value represents the confidence level of the statistical analysis; $P < 0.05$ reflects a statistically significant difference. The y-axis represents the distance rank between samples, and the x-axis represents the results between both groups. Intra-group results are shown for each group. In the plot, the R-value was negative and close to 0, indicating no differences, and $P > 0.05$ shows that this result was not statistically significant. Z3, adult; Z2, sub-adults; Z1, infant.

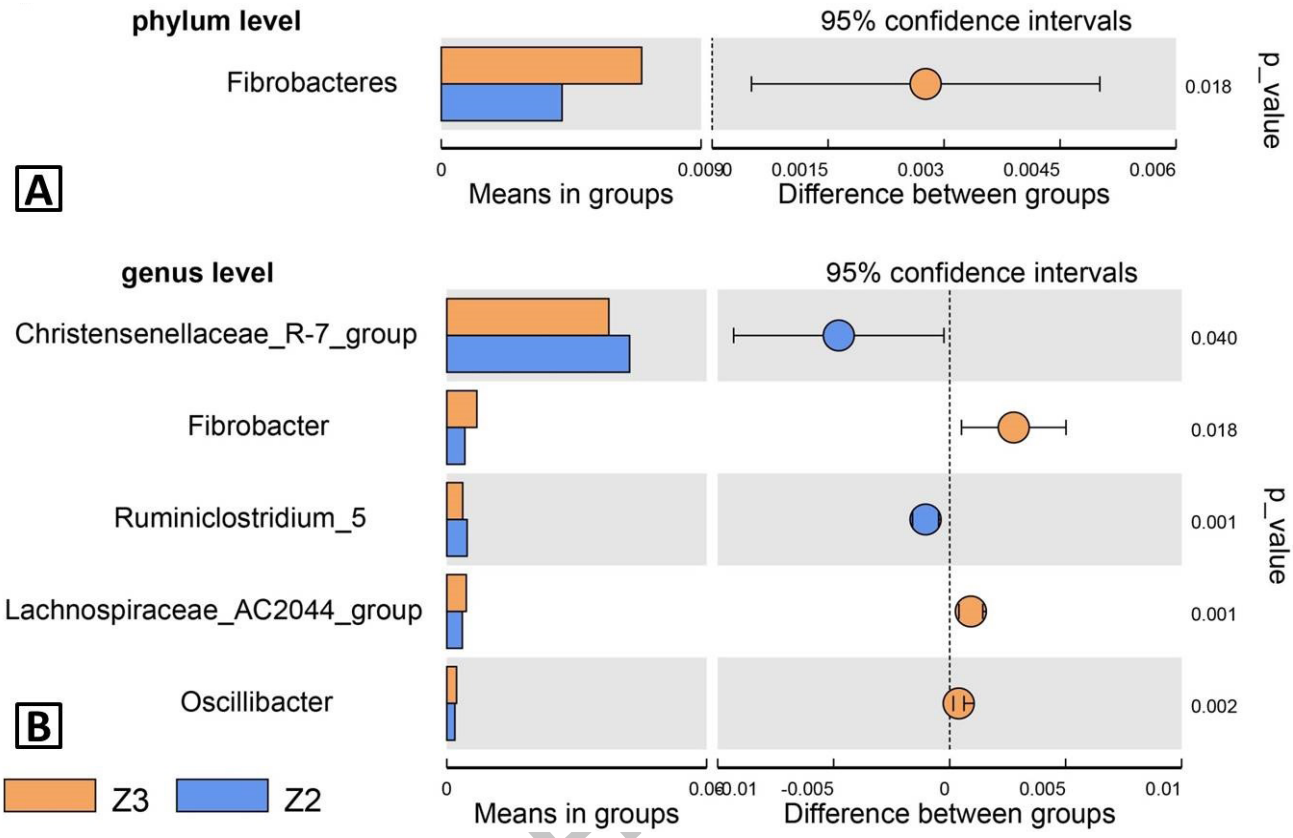


Fig. 7. Species used to differentiate Z2 from Z3 by T-test. A, Bar plot of species with different abundance at phylum level; B, bar plot of species with different abundance at genus level. Each bar represented the mean value of relative abundance of specie. The right plot showed the confidence intervals of 95%, and the cycle whose colour represented the group was the average difference. The best values were p values. Z3, adult; Z2, sub-adults; Z1, infant.

in *Bacteroidetes* was 18.29% and 32.00%, respectively (Guan *et al*., 2017). The phylum of *Firmicutes* might be highly related to deer ingestion or fermentation of a high-fibre diet (Guan *et al*., 2017; Costa *et al*., 2012). *Bacteroidetes* in the faecal microbiota played an important role in the degradation of high fat and protein from intestinal secretions (Flint *et al*., 2015). Bacterial phyla with low abundance, including *Tenericutes*, *Proteobacteria*, *Spirochaetes*, *Fibrobacteres*, *Verrucomicrobia* and *Cyanobacteria,* in this study were also observed in other ruminants (Jami *et al*., 2013; Pitta *et al*., 2010). *Tenericutes* accounted for approximately 3% of the microbiota detected in this study, but only 1.3% was observed in wild sika deer, with *Tenericutes* ranking ahead of *Proteobacteria* (0.540%) (Guan *et al*., 2017). The function of *Tenericutes* in the gut remains unclear but is speculated to be related to artificial rearing. *Proteobacteria* could help degrade lignin in its main food source (Fang *et al*., 2012). These core phyla were highly related to the diet. Based on our data, even though the relative abundance of the core bacteria is different from other species of deer, the dominant gut bacterial species of *Axis porcinus* and other *Cervidae* species were similar.

Regarding the genera represented in the faeces, the *Ruminococcaceae_UCG-005* and *Ruminococcaceae_UCG-010* were 2 main genera found in this study and belonged to the phylum *Firmicutes*, which is consistent with previous research on the golden takin (Li *et al*., 2017), sika deer (Guan *et al*., 2017) and wild forest musk deer (Li *et al*., 2017). *Ruminococcaceae* made contributions to fibre digestion (Marteau *et al*., 2004; Fernando *et al*., 2010), which meant these *Axis porcinus* could adapt to a wild environment even though they were presently feeding in a zoo. Additionally, alfalfa provided a higher fibre concentration in their diet. *Bacteroides*, *Rikenellaceae_RC9_gut_group* and *Alistipes* belonged to the phylum *Bacteroidetes* and were also some of the dominant bacteria. The similar results could also be found in sika deer and musk deer (Guan *et al*., 2017; Li *et al*., 2017). *Bacteroides* not only boosts host immunity but also sustains gut

bacterial ecological balance, which could reflect adaptive changes of microbiota under long-term feeding in the zoo. At the same time, some members of *Bacteroides* are opportunistic pathogens that could cause endogenous infections when the normal microbiome stability is disordered. Previous reports found that some *Bacteroides* species increased while lactic acid bacteria species were reduced in animals with gastrointestinal diseases (Marteau *et al*., 2004). The abundance of *Prevotella_1* decreased with increasing age according to our data. *Prevotella* is the most abundant genus in other ruminant animals, such as dairy cattle, and shows an increased abundance when the cattle are provided a diet with a high ration of grain (Fernando *et al*., 2010); thus, the reduction of *Prevotella_1* was understandable because a high ratio of grain was provided in the diet supplied to the infant *Axis porcinus*, and a higher percent of fibre was present in the diet fed to the adult ruminants. *Christensenellaceae_R-7_group* was also found in lambs (Huang *et al*., 2017). Other dominant genera in our result could also be found in other ruminant (Martínez *et al*., 2010; Cockburn *et al*., 2016).

The alpha diversities showed significant differences among the different ages. The community structure of the gut microbiota changes during the ageing process, and clear differences in the microbial composition were observed among the infants, adults and the elderly (Conlon *et al*., 2014). Preserving adequate microbial richness and diversity is vital for providing gut microbiota with functional redundancy, adaptability and thus systematic robustness against environmental challenges (Odamaki *et al*., 2016). A decreased alpha diversity from sub-adults to adults in this study might be caused by the intake of nutrients. The well-distributed diet of concentrate and forage was consumed for sub-adult *Axis porcinus*, whereas the high-fibre diet might be easily accessible to adult animals. From the PCoA plot, little difference existed between the sub-adult and adult. The distance between the infant *Axis porcinus* and the other age groups could be attributed to the single sample used. Moreover, the PCA analysis also showed the distinct cluster of Z1 separate from the other groups (Z2 and Z3), which is related to the change in core microbiota shown in both the heatmap and the phylogenetic trees. The microbiome of this single infant could be representative for the age, indicating the impact of age on the gut microbiota; however, it could be an error. Either way, this difference provides a direction for future research. Overall, distinct microbiome alpha diversity in the faecal microbiota of *Axis porcinus* at different ages is confirmed, and no difference was observed in the beta diversity between the sub-adults and adults. Wang *et al*. (2016) observed that the beta diversity of goat rumen microbiota showed no clear clustering patterns based on age groups, and alpha diversity did show an age-dependent pattern. Dill-McFarland *et al*. (2017) found the Shannon Index of faecal microbiota in a 2-year-old cow showed little decrease compared with that present when the cow was 1 year old. Possibly, the abundance or diversity of faecal bacteria decreased to a level from birth to adult due to the greater maturity of stomach function, which results in lower nutrition entry to the large intestine (Dill-McFarland *et al*., 2017). Further studies about gut microbiota of *Axis porcinus* need to investigate more age stages and collect more infant samples for analysis.

Identifying the bacteria that are differentially represented among the growth stages is important for a better understanding of gut microbiome function with age, which could provide clues about why the composition of the gut microbiota changes with age even for humans. The T-test was performed to find differences in the species between the sub-adult and adult groups since only 1 infant was included in the youngest group. At the phylum level, only 1 phylum (*Fibrobacteres*) with higher abundance in the adults was found compared with those phyla observed in the other two groups. The phylum *Fibrobacteres* was associated with the concentration of fibre in the diet in previous research and was higher in deer (Guan *et al*., 2017). Our results confirm that the adult *Axis porcinus* has a strong ability to digest fibre in feed compared to the sub-adult, which is rational for ruminants. At the genus level, *Ruminiclostridium_5* and *Christensenellaceae_R-7_group* were lower in adult animals. These 2 genera were positively related with fasting insulin and a decrease of blood glucose (Zhang *et al*., 2017). Regarding glucose metabolism, the young animals are prone to low blood sugar; thus, these 2 genera are enriched in the sub-adult *Axis porcinus*. Other species, including *Fibrobacter*, *Oscillibacter* and *Lachnospiraceae_AC2044_group,* had higher abundance in the adults. Based on taxonomy, we knew that *Fibrobacter* belonged to *Fibrobacteres*, with four other genera are annotated as *Firmicutes*. Thus, the *Fibrobacter* genus caused the change in the relative abundance of the phylum *Fibrobacteres*. *Oscillibacter* and *Lachnospiraceae_AC2044_group* were highly related with volatile fatty acid digestion and fermentation in ruminants (Kumar *et al*., 2012; Lemaire *et al*., 2018). These species play a role in fibre digestion and absorption with increasing age.

This study on *Axis porcinus* characterized the fundamental gut microbial composition and structure in the infant, sub-adult and adult groups using next-generation sequencing technology and illustrated the significant change that occurred in gut microbiota over the different ages. This understanding is important for *Axis porcinus* gut physiology, health, feeding and for better protection.

In *Axis porcinus*, the main phylum was *Firmicutes* and *Bacteroidetes*, and 2 main genera (*Ruminococcaceae_UCG-005* and *Ruminococcaceae_UCG-010*) were found. Significant microbial abundance changes with age were found, and the bacteria included the following: *Christensenellaceae_R-7_group*, *Lachnospiraceae_AC2044_group*, *Ruminiclostridium_5*, *Fibrobacter* and *Oscillibacter*. These microbes are related to diet digestion and nutrient metabolism. Our data could provide insights for animal feeding and/or diet formulation for these captive-reared deer.

A limitation of this study might be the single sample obtained from an infant *Axis porcinus*. However, the gut microbiota of the infant in our data could still show the core bacteria. Considering that age, sex, diet and environmental factors affect gut microbiota (Kozik, 2017; Bergmann, 2017), more detailed studies on *Axis porcinus* should be conducted, including an investigation of diet nutrition levels and source. If possible, the rumen microbial composition that plays an important role in digesting fibre should be determined because *Axis porcinus* is a ruminant. Moreover, since *Axis porcinus* is an endangered animal, investigation of its microbiota when the captive animals are free and in a simulated wild environment is important for better conservation and protection of the species. Of course, these kinds of studies should be performed when enough animals are present. Furthermore, the metabolic pathway of these different bacterial species through the different ages of the deer should be conducted at the metagenomic level to discover the deeper mechanism.

## CONCLUSION

Using next-generation sequencing technology, we first investigated the core phyla and genera of the gut microbiome of *Axis porcinus* in different growth stages (young, sub-adult and adult). Significant microbial composition changes were observed with age. Additionally, 5 significant genera were determined to change as *Axis porcinus* ages. Faecal sample collection can provide a non-invasive method to research the gut microbiome of endangered animals and produce enough information to understand gut physiology.

## ACKNOWLEDGEMENTS

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*Ethics statement*

In the present study, the fresh faecal samples were collected by the keepers while they were cleaning and feeding. The study design and all experimental methods were approved by the Animal Care and Use Committee of the Chengdu Zoo.

*Supplementary material*

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190729100714

*Statement of conflict of interest*

The authors have declared no conflict of interests.

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## Supplementary Material

# Age-Related Changes in the Gut Microbiota Composition of Hog Deer (*Axis porcinus*)

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**Supplementary Table SI.- Diet composition (dry-basis).**

| Infant | | Sub-adult and adult | |
|---|---|---|---|
| **Ingredients** | **g/kg** | **Ingredients** | **g/kg** |
| Green rye | 563.4 | Alfalfa pellet | 180 |
| Alfalfa pellet | 140.8 | Alfalfa bale | 120 |
| Apple | 28.2 | Apple | 40 |
| Carrot | 28.2 | Carrot | 40 |
| Sophorae leaves | 56.3 | Asparagus lettuce | 40 |
| Complete Concentrate | 183.1 | Green grass | 400 |
| | | Complete concentrate | 180 |

**Supplementary Table SII.- Sampling collection information.**

| Microbiome No. | Animal ID | Sampling date | Gender | Sample ID | Treatment |
|---|---|---|---|---|---|
| 1 | 1 | 22-Aug | Male | ZTD16126372 | T1 |
| 2 | A | 22-Aug | Female | ZTD16111529 | TCA |
| 3 | B | 22-Aug | Male | ZTD16111530 | TCB |
| 4 | C | 22-Aug | Male | ZTD16111531 | TCC |
| 5 | D | 22-Aug | Female | ZTD16111532 | TCD |
| 6 | E | 22-Aug | Male | ZTD16111533 | TCE |
| 7 | F | 22-Aug | Male | ZTD16111534 | TCF |
| 8 | G | 22-Aug | Male | ZTD16111535 | TYG |
| 9 | H | 22-Aug | Female | ZTD16111536 | TCH |
| 10 | I | 22-Aug | Male | ZTD16111537 | TCI |
| 11 | J | 22-Aug | Male | ZTD16111538 | TCJ |
| 12 | K | 22-Aug | Female | ZTD16111539 | TCK |
| 13 | L | 22-Aug | Female | ZTD16111540 | TYL |

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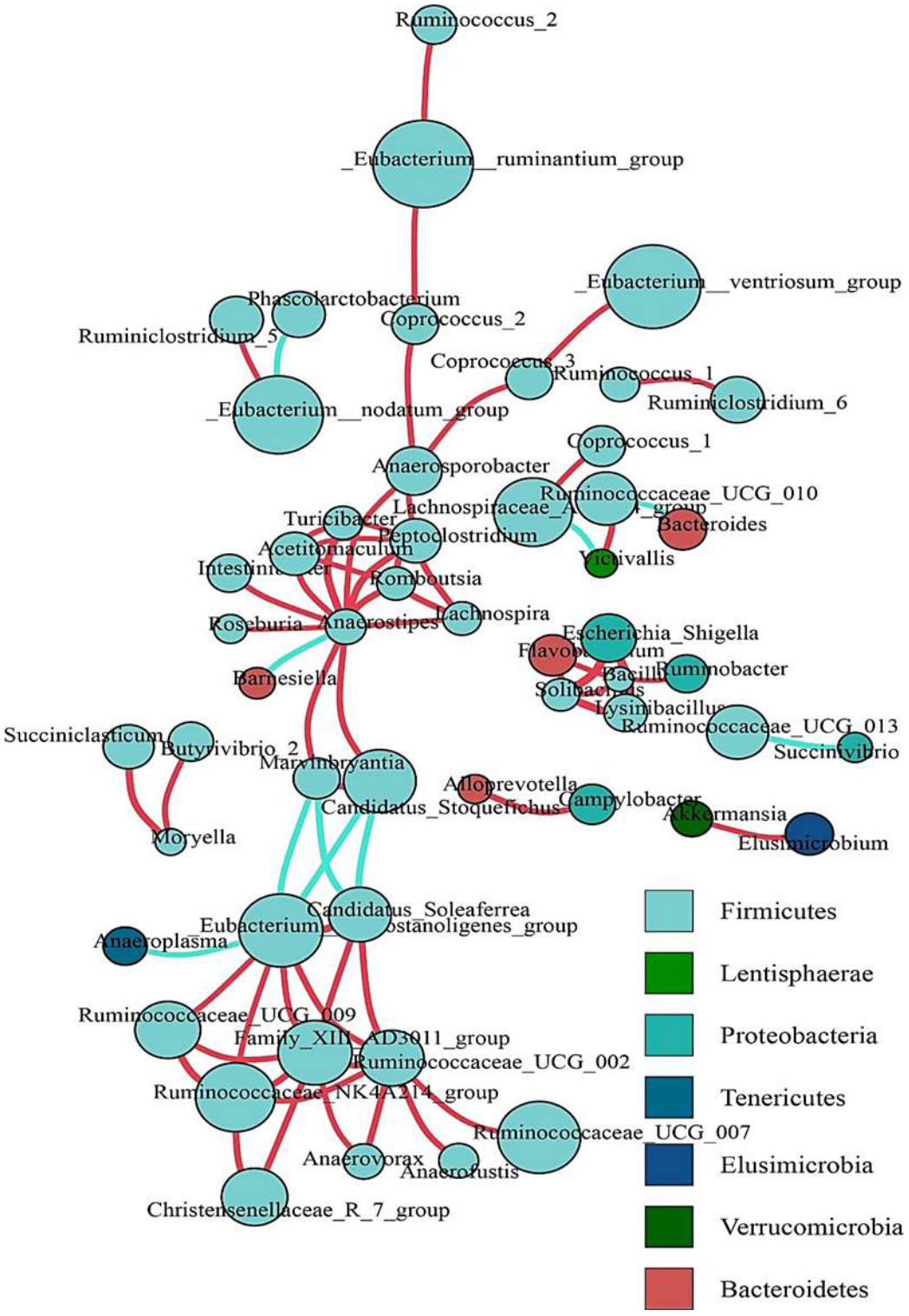
0030-9923/2023/0001-0001 $ 9.00/0

| Microbiome No. | Animal ID | Sampling date | Gender | Sample ID | Treatment |
|---|---|---|---|---|---|
| 14 | M | 22-Aug | Male | ZTD16111541 | TCM |
| 15 | N | 22-Aug | Male | ZTD16111542 | TCN |
| 16 | O | 22-Aug | Female | ZTD16111543 | TCO |
| 17 | A | 23-Aug | Female | ZTD16111544 | TCA |
| 18 | B | 23-Aug | Male | ZTD16111545 | TCB |
| 19 | C | 23-Aug | Male | ZTD16111546 | TCC |
| 20 | D | 23-Aug | Female | ZTD16111547 | TCD |
| 21 | E | 23-Aug | Male | ZTD16111548 | TCE |
| 22 | F | 23-Aug | Male | ZTD16111549 | TCF |
| 23 | G | 23-Aug | Male | ZTD16111550 | TYG |
| 24 | H | 23-Aug | Female | ZTD16111551 | TCH |
| 25 | I | 23-Aug | Male | ZTD16111552 | TCI |
| 26 | J | 23-Aug | Male | ZTD16111553 | TCJ |
| 27 | K | 23-Aug | Female | ZTD16111554 | TCK |
| 28 | L | 23-Aug | Female | ZTD16111555 | TYL |
| 29 | M | 23-Aug | Male | ZTD16111556 | TCM |
| 30 | N | 23-Aug | Male | ZTD16111557 | TCN |
| 31 | O | 23-Aug | Female | ZTD16111558 | TCO |
| 32 | A | 24-Aug | Female | ZTD16111559 | TCA |
| 33 | B | 24-Aug | Male | ZTD16111560 | TCB |
| 34 | C | 24-Aug | Male | ZTD16111561 | TCC |
| 35 | D | 24-Aug | Female | ZTD16111562 | TCD |
| 36 | E | 24-Aug | Male | ZTD16111563 | TCE |
| 37 | F | 24-Aug | Male | ZTD16111564 | TCF |
| 38 | G | 24-Aug | Male | ZTD16111565 | TYG |
| 39 | H | 24-Aug | Female | ZTD16111566 | TCH |
| 40 | I | 24-Aug | Male | ZTD16111567 | TCI |
| 41 | J | 24-Aug | Male | ZTD16111568 | TCJ |
| 42 | L | 24-Aug | Female | ZTD16111569 | TYL |
| 43 | M | 24-Aug | Male | ZTD16111570 | TCM |
| 44 | N | 24-Aug | Male | ZTD16111571 | TYN |
| 45 | O | 24-Aug | Female | ZTD16111572 | TCO |

Z3, adult; Z2, sub-adults; Z1, infant; TC, adult; TY, sub-adults; T1, infant.




Supplementary Fig. S1. Network analysis. Network was used to explore co-occurrence patterns of bacterial taxa. A connection stands for a strong (Spearman's $P > 0.6$) and significant ($P < 0.01$) correlation. The size of each node is proportional to the relative abundance at genus level; the thickness of each connection between two nodes (edge) is proportional to the value of Spearman's correlation coefficients; the color of edge meant positive (red) and negative (blue) relation.