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Comparison of the Gut Microbiota in the Tibetan Wild Ass (*Equus kiang*) Collected from High and Low Altitude

Honghai Zhang[1,*], Yao Chen[1], Xiaoyang Wu[1], Shuai Shang[2], Jun Chen[2], Jiakuo Yan[1], Qinguo Wei[1], Xibao Wang[1], Yongqiang Lu[3] and Huanxin Zhang[2]

[1]*College of Life Science, Qufu Normal University, Qufu, Shandong, P.R. China*
[2]*College of Marine Life Sciences, Ocean University of China, Qingdao, China*
[3]*Shandong Publishing Group Limited, Jinan, Shandong, P.R. China*

Honghai Zhang and Yao Chen contributed equally to this work.

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YC, XW, SS and HZ conceived and designed the experiments, analyzed the data and contributed reagents/materials/analysis tools. YC performed the experiments, wrote the paper, prepared figures and/or tables and reviewed drafts of the paper. Jun Chen reviewed drafts of the paper. JY, XW, YL and HZ performed the experimental work.

Key words
Tibetan wild ass (*Equus kiang*), Gut microbiota, 16S rRNA, Next-generation sequencing, Altitude.

ABSTRACT

The gut microbiota plays an important role in animal performance and the environment. We collected fresh feces of the Tibetan wild ass (*Equus kiang*) from the Jinan Wildlife World and Qinghai - Tibet Plateau Wild Animal Park. And we divide the sample into Group A (the low altitude area) and Group B (the high altitude area). we studied the basic structure of the gut microbiota by sequencing high throughput sequencing of the 16S rRNA gene V3-V4 hypervariable regions. The differences of gut microbiota in the Tibetan wild ass at different altitudes were compared. We obtained 1474595 16S rRNA gene sequences. The study observed 163genera belonging to 19 phyla in Group A while 210 genera belonging to 19 phyla in Group B with Bacteroidetes and Firmicutes predominating. Additionally, Bacteroidetes and Firmicutes linearly decreased ($P < 0.05$) in Group A and linearly increased ($P < 0.05$) in Group B. The *Ruminococcus_flavefaciens*, *rumen_bacterium_YS2, Acinetobacter_baumannii* and *Bacillus_anthracis* were only found in Group B. All the evidence showed that altitude has a marked impact on the composition of intestinal microflora in the Tibetan wild ass.

INTRODUCTION

There are seven *Equus* Linnaeus species in China, including *Equus kiang, Equus przewalskii, Equus asinus, Equus burchelli, Equus hemionus, Equus grevyi* and *Equus zebra.* The Tibetan wild ass (*Equus kiang*) found in the region Qinghai-Tibet Plateau and is the largest of the wild asses. To protect this species, the Chinese government has classed the Tibetan wild donkeys as a key protected animal and killing of them is banned. This species is also listed in the International Union for Conservation of Nature (IUCN) Red List 2012 of Threatened species. The Tibetan wild ass is a typical large herbivore in the Tibetan Plateau. In summer, these asses mostly live in alpine deserts at an altitude of 5000 meters. During winter, as the temperature drops and food availability decreases, the wild ass migrates to lower elevations. They like to gather in groups and eat sedges and thatches.

The composition and function of the animal gut microbiota plays an important role in the host's physiology and health (Ley *et al*., 2006; Yan *et al*., 2008; Cadwell, 2015). Interactions between microbes and hosts are sufficient to maintain the stability of the gut microbiota (Qin *et al*., 2010). It can ferment undigested substances and produce enzymes to digest polysaccharides that cannot be secreted by human cells (Knight and Girling, 2003; Bridget, 2005). In a healthy gut system, a study found that symbiotic gut microbiota can promote the healthy development of the immune system, improve the body's immunity and maintain the body's health (Chow *et al*., 2010; Ivanov and Honda, 2012).

The gastrointestinal tract is a relatively open system. The diversity and abundance of gut microbes are affected by many factors, such as the organism's age, nutrition, diet, gender and heredity. The gut microbiota of carnivores and herbivores differ in their composition, and there are also considerable differences in amino acid metabolism, with different gut microbiota functioning differently (Muegge *et al*., 2011). The fecal microbiota of dholes (*Cuon alpinus*) might be influenced by the changes in their diet (Wu *et al*., 2016). Animal gut microbes are closely related to the environment in which animals live. Zhang *et al*. (2016) found that the structure and composition of gut microbiota in mammals living in the same area at high altitude and low altitude are different. In addition, habitat is one of the factors that determine the composition of mammalian gut microbiota (He *et al*., 2013).

Up to now, there are many studies on the gut

* Corresponding author: zhanghonghai67@126.com
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microbiota in herbivores (Costa *et al*., 2012; Bian *et al*., 2013; Liu *et al*., 2014). The core feces of healthy horses were dominated by *Firmicutes* (68%) and *Bacteroidetes* (14%) followed by *Proteobacteria* (10%) (Costa *et al*., 2012). The *Firmicutes* (64% males and 64% females) and *Bacteroidetes* (23% males and 21% females), followed by *Verrucomicrobia, Euryarchaeota, Spirochaetes* and *Proteobacteria* (Liu *et al*., 2014). However, the studies on equine animals (*Equus* Linnaeus) are focused on the genetic evolution, behavioral research, habitat suitability assessment, seasonal food habits analysis, population changes and distribution (Kimura *et al*., 2011). Researches on the gut microbiota of Tibetan wild ass have not been reported so far. In present research, using microbial 16S rRNA sequencing technology, we characterized the gut microbiota of the Tibetan wild ass. To explore whether altitude is the main factor affecting gut microbiota, we compared the gut microbiota from high altitude to low altitude.

Table I.- Information on the sample of the Tibetan wild ass researched in this study.

Group	Sex group	Tibetan wild ass	Sex	Place
A	EK.M	EK1	Male	Jinan wildlife world
		EK2	Male	
		EK3	Male	
	EK.F	EK4	Female	
		EK6	Female	
		EK7	Female	
B	E.M	E1	Male	Qinghai - Tibet Plateau Wild Animal Park
		E5	Male	
	E.F	E2	Female	
		E3	Female	
		E4	Female	
		E6	Female	
		E7	Female	

MATERIALS AND METHODS

Sample collection

In this study, we collected thirteen fresh fecal samples of Tibetan wild ass from Jinan Wildlife World and Qinghai-Tibet Plateau Wild Animal Park. All procedures performed on animals were conducted in accordance with the ethical standards of the Qufu Normal University Animal Care and Use Committee. None of the animals were harmed during the collection of fecal samples. None of the animals had received anti-inflammatory drugs or antimicrobials within the last 4 months, and none of them had any disease. Group E.M and group E.K represented the male group and the female group feces samples (Table I). Among them, four sterile collected fecal samples (Group A) were obtained from the Jinan Wildlife World in May 2016. Other sterile feces samples (Group B) were collected from the Qinghai-Tibet Plateau Wild Animal Park in September 2016. All of samples were fed about three months which can exclude the influence of domestication. The Jinan wildlife world is located in Shandong Province with an average elevation of 200 meters. Its climate type is continental monsoon climate, which is hot and rainy in summer and cold and dry in winter. The Qinghai-Tibet Plateau Wild Animal Park is located in Qinghai Province with an average altitude of 2300 meters. Its climate type is alpine climate. According to our zoo feeding record, we know that Group A and Group B were similar with 80% of the hay. All of samples were stored at −80 °C for further analysis. All sample collection processes were performed in accordance with the ethics committee's requirements. The experiment was approved by the Qufu Normal University Animal Care and Use Committee.

DNA extraction, 16S rRNA gene amplicons and purification

Total genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Germany) after reading the manufacturer's instructions. We checked the DNA concentration and quality using a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, USA) and adjusted the concentration to 20 ng/µl. We amplified the 16S rRNA V3-V4 hypervariable regions using 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) universal primers (Bolnick *et al*., 2014). The PCR reactions system (25 µl). contained: 2× KAPA HiFi Hot Start Ready Mix 12.5 µl; microbial DNA (5 ng/µl) 2.5 µl; amplicon PCR reverse primer (1 µM) 5 µl; and amplicon PCR forward primer (1 µM) 5µl. The PCR method followed the following conditions: 95 °C for initial denaturation for 3 min, denaturation with 25 cycles at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

High-throughput sequencing of 16S rRNA gene amplicons

Each PCR product was analyzed by 2% agarose gel electrophoresis and samples containing a bright strip between 400 and 450 bp. According to the manufacturer's recommendations, we used a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) to construct the DNA library, then, the index codes was added to the library. The quality of sequencing library was assessed with a Qubit@2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Finally, An Illumina

HiSeq 2500 platform was employed to sequence the DNA library, which generated 250-bp paired-end reads.

Bioinformatics analysis

Truncating the barcode and primer sequences, we joined the reads for each sample by using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Mago and Salzberg, 2011). So, we gained the Raw Tags. Then, using the QIIME (V1.7.0) (Caporaso *et al*., 2010) quality control process, the Raw Tags were requested to filter processing to get high quality Clean Tags. Compared with the reference database (Gold database), the tags were used the UCHIME algorithm (UCHIME Algorithm) (Edgar *et al*., 2011) and the Gold database (http://drive5.com/uchime/uchime_download.html) to detect chimeric sequences, which were then removed. Finally we obtained effective tags. Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) (Edgar, 2013) is used on the samples of effective tags, and then a clustering is proceeded. Sequences obtained greater than or equal to 97% similarity were distributed to the same operational taxonomic unit (OTU). With regard to each representative sequence, we use the Mothur method and the SSUrRNA database (Quast *et al*., 2013) of SILVA (http://www.arb-silva.de/) (Wang *et al*., 2007) to get species annotation and taxonomic information and. conducted species annotation analysis. The "Core Set" data information of GreenGene Database and the PyNAST software (Version 1.2) (Yilmaz *et al*., 2014) were employed to study the phylogenetic relationships between all representative OTUs. Alpha Diversity was evaluated by six indices containing the Observed-species, Chao1, Shannon, Simpson, ACE and Good-coverage indices. The six indices were calculated with QIIME (V1.7.0) and demonstrated by R software (Version 2.15.3). About Beta Diversity, We use Qiime software (Version 1.7.0) to calculate the Unifrac distance and build UPGMA sample cluster tree. We use R software (Version 2.15.3) to draw PCA, PCoA diagrams. The PCA analysis was used the ade4 package and ggplot2 package of R software (Version 2.15.3). The PCoA analysis was used the WGCNA, stats and ggplot2 software packages of R software (Version 2.15.3). Anosim analysis and MRPP analysis respectively used the mrpp function and anosim function of R vegan package.

RESULTS

We utilized Illumina HiSeq sequencing platform to get the Raw PE of the sample. Then, a total of 1,443,240 effective tags, with average length of 410bp per sample were retrieved from the 13 fecal samples through splicing, quality control and chimaera filtrationed. Based on a genetic distance of 3%, between Group A and Group B (p<0.05), we observed the significant differences using ANOSIM analysis. We used R software (Version 2.15.3) to draw the rarefaction curves and rank abundance curves .The rarefaction curve tended to be flat, indicating that the sequencing data of sequencing are reasonable, while the Rank Abundance curve can visually reflect the richness and uniformity of the sample (Fig. 1). The alpha diversity index and box-plot intuitively reflected the median, degree of dispersion, maximum, minimum and outlier of species diversity between Groups A and B. In Table II, it showed the statistical estimates of species richness from the total number of sequences, the coverage, and the number of OTUs from the 13 samples. The number of OUTs in Group A was higher than that in Group B (Fig. 2), and this difference was significant (Wilcox, P= 0.04< 0.05).

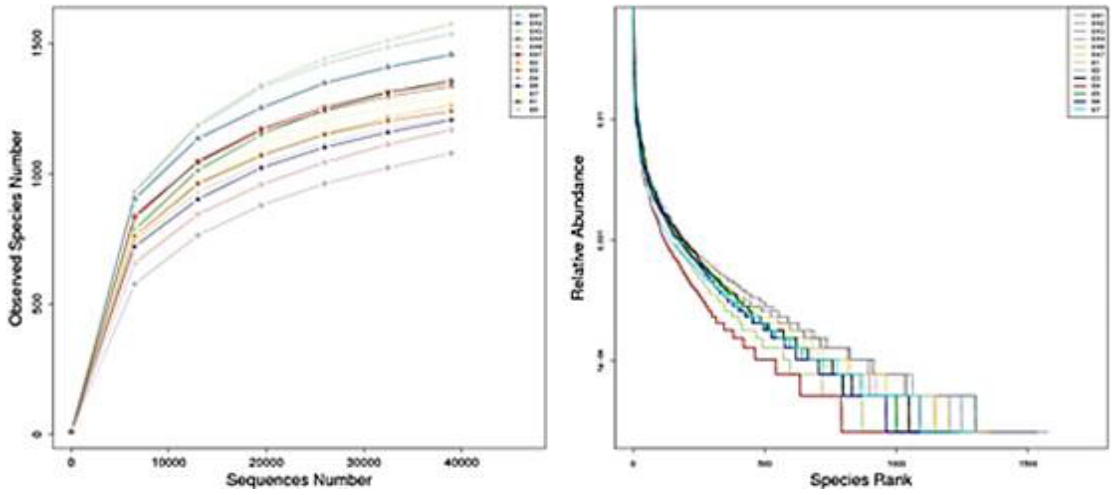

Fig. 1. The rarefaction curves and rank abundance curves of the thirteen Tibetan wild asses.

Table II.- Alpha-diversity of the thirteen Tibetan wild asses in our report.

d	Observed species	OUT (0.03)	Goods coverage (%)	Community diversity		Community richness	
				Shannon	Simpson	Chao1	ACE
EK1	1535	1773	0.994	8.126	0.986	1639.943	1678.386
EK2	1457	1706	0.993	8.299	0.989	1648.256	1633.322
EK3	1575	1795	0.992	8.58	0.994	1863.535	1826.086
EK4	1361	1604	0.993	7.688	0.983	1558.251	1566.456
EK6	1168	1212	0.992	7.446	0.98	1453.503	1446.286
EK7	1350	1580	0.995	8.163	0.99	1455.236	1471.472
E1	1336	1535	0.995	8.284	0.992	1419.085	1461.033
E2	1262	1538	0.993	7.995	0.991	1490.641	1447.119
E3	1240	1432	0.995	7.935	0.986	1324.11	1363.547
E4	1079	1304	0.993	6.518	0.96	1340.57	1334.257
E5	1216	1400	0.994	7.849	0.987	1330.82	1372.455
E6	1206	1408	0.994	7.611	0.982	1390.877	1396.292
E7	1296	1550	0.995	7.248	0.959	1389.767	1426.437

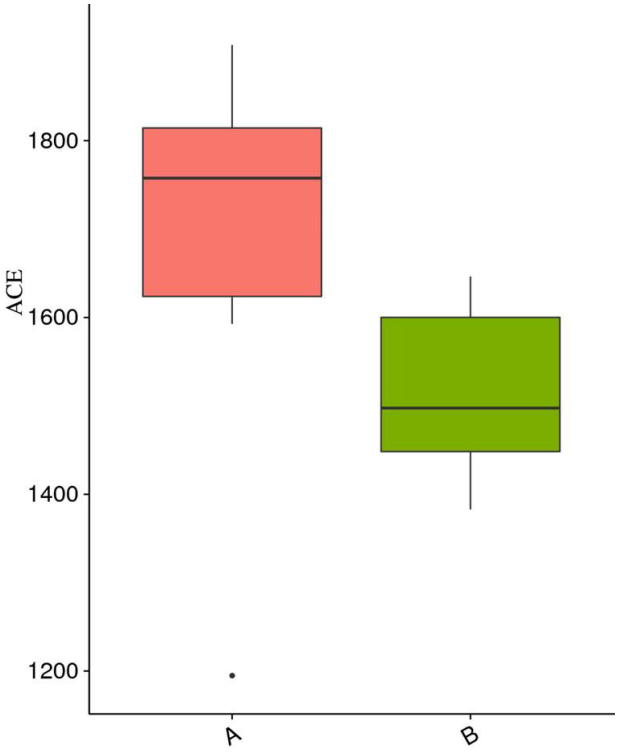

Fig. 2. Comparison of community alpha diversities between high altitude and low altitude samples in the Tibetan wild ass. Diversity was measured by calculating the ACE index.

Taxonomic composition

Twenty-five prokaryotic phyla were found in thirteen Tibetan wild ass gut microbiota samples (Fig. 3A). According to the results, the most majority of their gut flora belonged to two phyla: Bacteroidetes (44.92± 6.32%), Firmicutes (33.57 ± 5.80%) and Spirochaetes (8.45±2.58%), Fibrobacteres (8.33±5.90%). Other majority phyla were Proteobacteria (1.31 ± 0.42%), Cyanobacteria (0.84±0.70%). The main gut microbiota of the sample was also highly distributed in typical high-altitude mammals, so we hypothesized that the intestinal microbes of Tibetan wild ass may be related to sea-level adaptation. In addition, variations occurred in the microbiota community among samples, *e.g.,* Armatimonadetes was identified only in samples EK4, E1, E3 and E6; Thermomicrobia was occurred only in samples E2 and E7; and Deinococcus-Thermus was occurred in samples EK2 and EK6. Fusobacteria was identified only in samples E4 and E7; Latescibacteria and Gemmatimonadetes were observed only in samples E7. E2, EK7 were not detected Deferribacteres.

At the family level, the abundance of unclassified bacteria in the samples was 11.28%. We identified 126 families. We used the largest abundance of the top 10 species to generate species relative abundance column cumulative plot (Fig. 3B) in order to visually view the samples at different levels of classification, the relative abundance of species and their proportion. Furthermore, at the genus level, we identified 218 genera and the abundance of unclassified bacteria in the samples was 34.86%. The species relative abundance column cumulative plot at the genus level was shown in Figure 3C. To describe the distribution and proportion of gut microbiota in each animal, we classified each sample species, and then classified the species of particular statistics concern (the top 10 abundance) statistics (Fig. 4).

At the species level, the *Ruminococcus_flavefaciens, rumen_bacterium_YS2, Acinetobacter_baumanni*i and *Bacillus_anthracis* are only found in Group B.

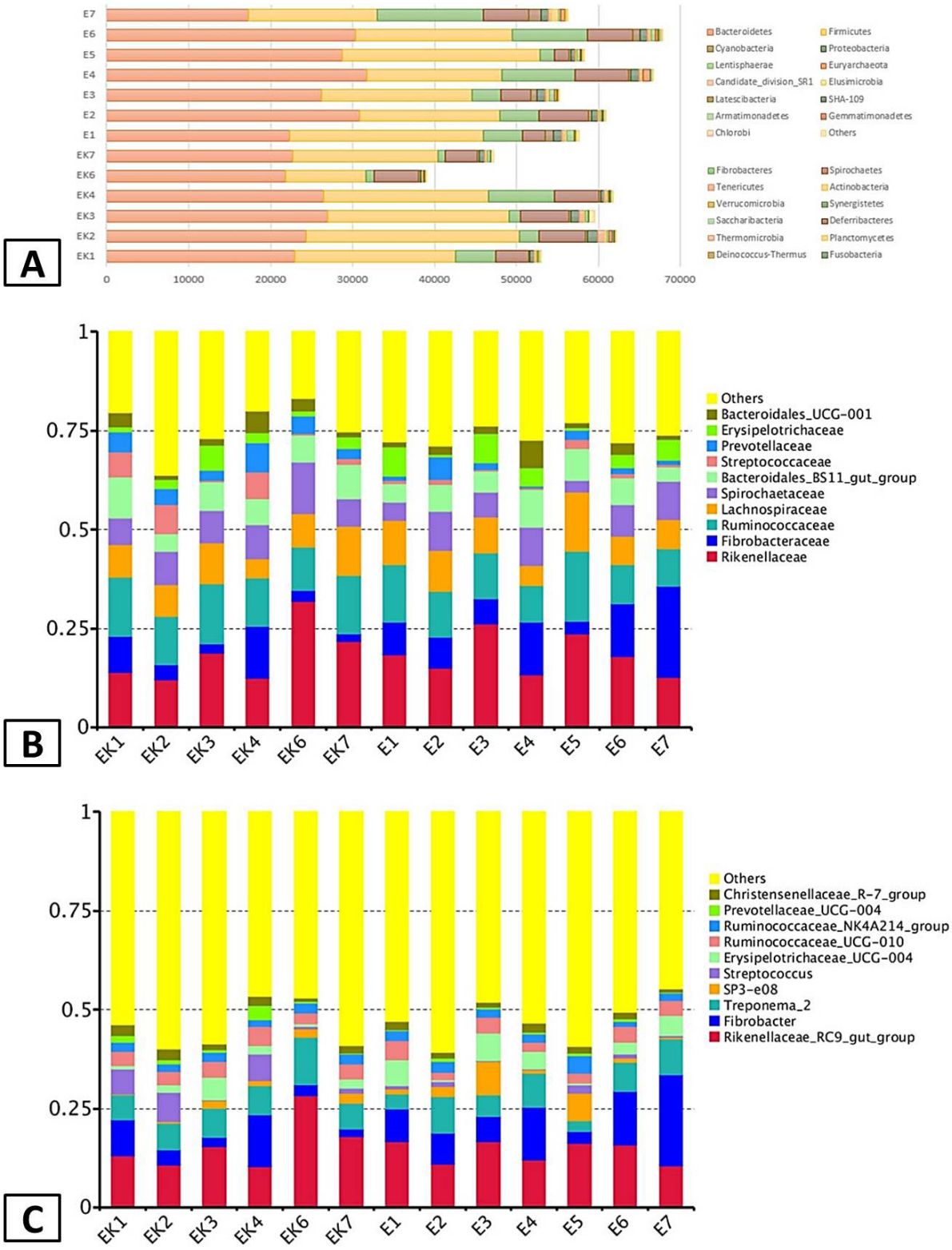

Fig. 3. Gut bacterial composition at the phylum level per sample (A), the relative abundance of the top ten biological species at the family level (B) and the genus level (C).

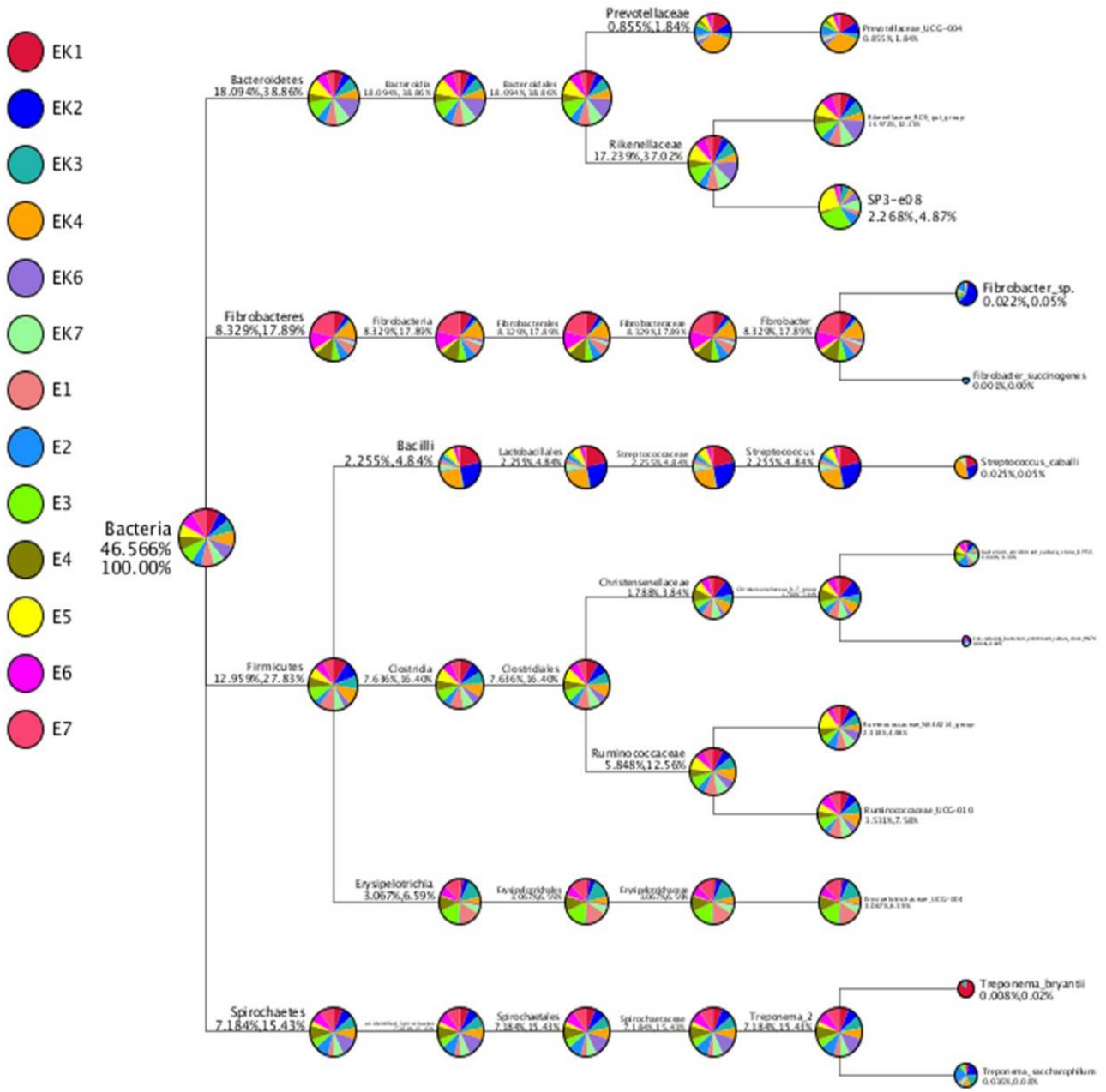

Fig. 4. Tree species classification of samples in the genus level.

Variation of gut microbiota in plateau and plain

To explore gut microbial community differences among different seasons and the potential influence of the sex of a Tibetan wild ass, the distribution of beta diversity measures (weighted and unweighted UniFrac distances) was compared at different altitudes. The result of PCoA was used to show that altitude is the main factor affecting the differences in gut microbiota of the Tibetan wild ass. The results of PCA were similar to those of PcoA (Fig. 5).

Table III.- The ANOSIM analysis of different groups.

Group	R-value	P-value
B-A	0.6839	0.001
EK.M-E.F	0.6923	0.01
EK.F-E.F	0.6821	0.015
EK.F-EK.M	-0.03704	0.5

Statistical analysis

To study the differences between the groups, we used an analysis of ANOSIIM (Table III) and Multi Response Permutation Procedure (MRPP) (Table IV). The results were similar: high altitude is the main factor that affects the gut diversity of Kiang, while gender is not the main factor. This is consistent with the results of PCA and PcoA.

In addition, we also conducted a T-test between Group A and Group B (Fig. 6). From the Figure 6, at class level, Melainabacteria was significantly different between Group A and Group B, and the content of Group B was higher than that of Group A. At order level, Gastranaerophilales was significantly different between Group A and Group B, and higher than that of Group A. At genus level, there are four bacterias were significantly different between Group A and Group B.

Table IV.- The MAPP analysis of different groups.

Group	A	Observed-delta	Expected-delta	Significance
A-B	0.1045	0.5205	0.5813	0.002
E.F-EK.M	0.1071	0.5149	0.5767	0.032
E.F-EK.F	0.1084	0.5325	0.5972	0.035
EK.F-EK.M	0.007474	0.5077	0.5115	0.4

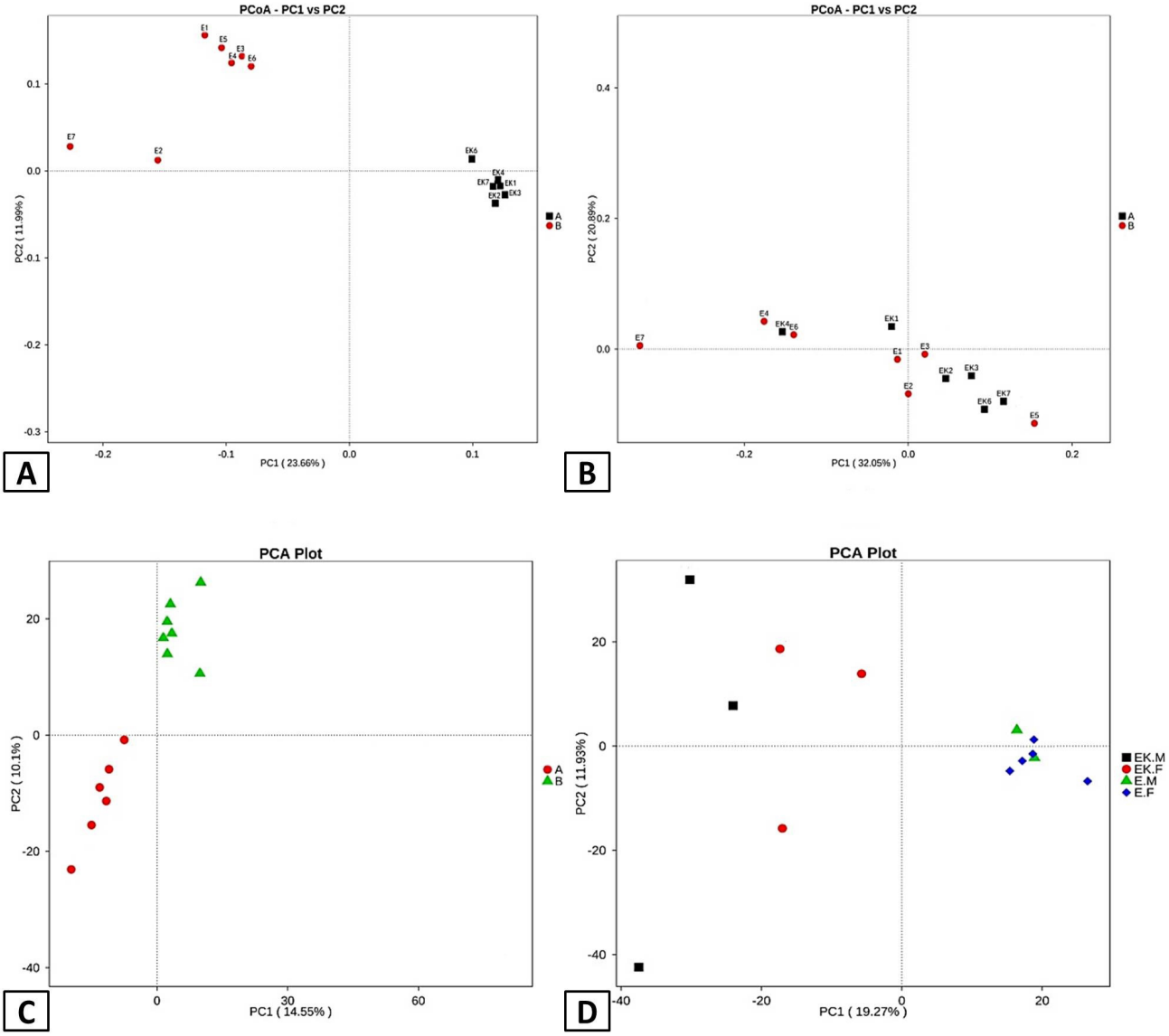

Fig. 5. The principal coordinate analysis of different groups (unweighted, UniFrac, distances).

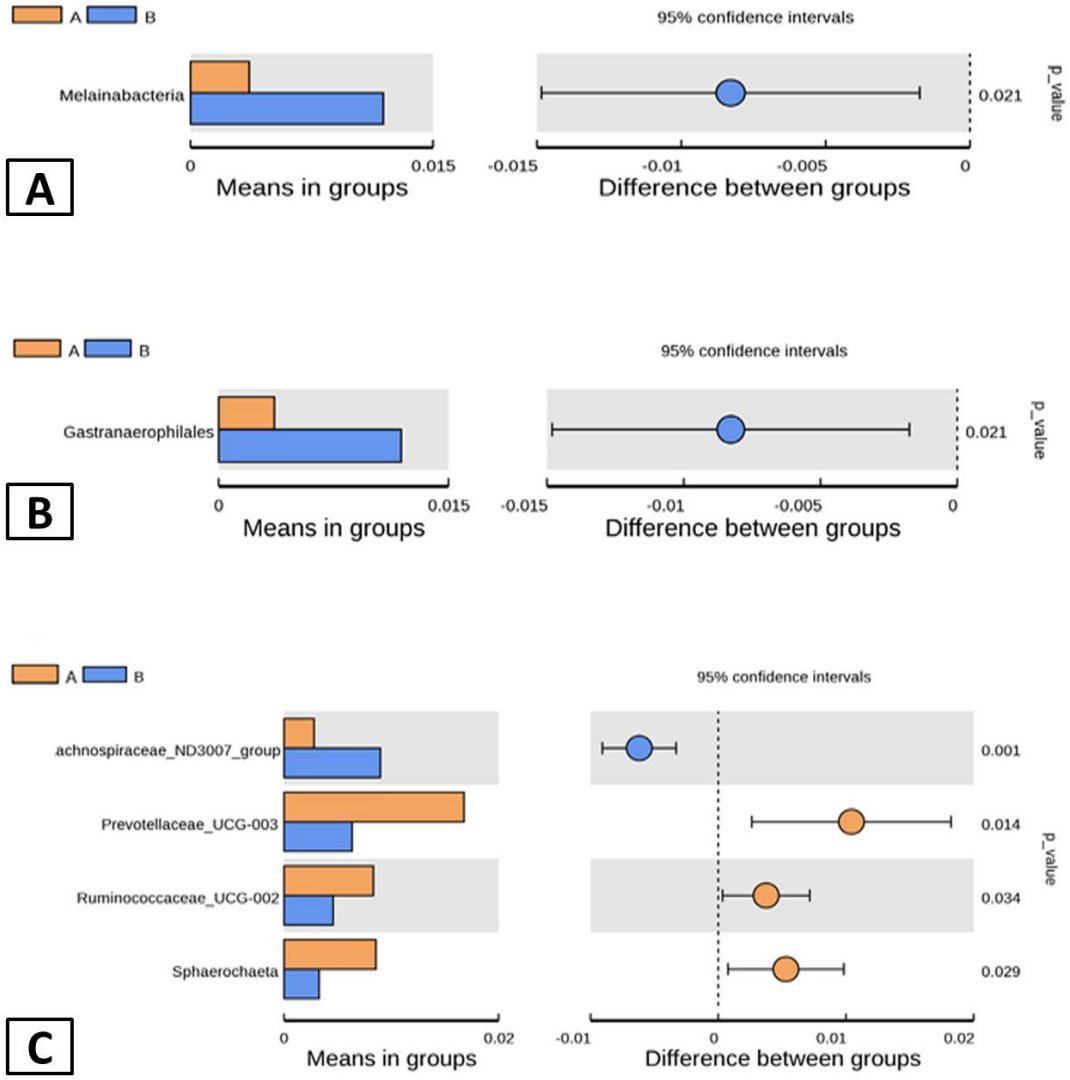

Fig. 6. T-test difference group analysis diagram and at the class level (A), at the order level (B) and at the genus (C).

DISCUSSION

Gut microbiota is indispensable in the process of food assimilation and energy absorption (Ghosh *et al*., 2014). In our study, we performed high-throughput sequencing to characterize the microbial community in the Tibetan wild ass. Similar to previous researches, a study found that the gut microbiota were dominated by Firmicutes (68%) followed by Bacteroidetes (14%) and Proteobacteria (10%) (Costa *et al*., 2012). Chen *et al*. (2017) identified 18 prokaryotic phyla; the two most prevalent phyla were Firmicutes (42.81-55.29%) and Bacteroidetes (21.26-27.82%). Steelman *et al*. (2012) reported that Firmicutes predominated (69.21%) and Verrucmicrobia (18.13%) followed by Bacteroidetes, Proteobacteria, and Spirochaetes. 25 prokaryotic phyla were found in the Tibetan wild ass gut microbiota which was dominated by Bacteriodetes (44.92± 6.32%), Firmicutes (33.57 ± 5.80%) and Spirochaetes (8.45±2.58%). Other typical phyla were Fibrobacteres (8.33±5.90%), Proteobacteria (1.31 ± 0.42%) and Cyanobacteria (0.84±0.70%). From the results, it indicated that the proportion of Firmicutes wad similar in *Equus Linnaeu*s but different in other phyla.

In the gut microbiota of mammals, Firmicutes and Bacteroidetes were accounted for >98 % of the 16S rRNA sequences (Lay *et al*., 2006). In our study, data analysis showed that Bacteroidetes is the first rich phylum of Tibetan wild ass gut microbiota, followed by Firmicutes. Several studies have revealed the Bacteroidetes for the normal development of the gastrointestinal tract (Thomas

et al., 2011). The gut Bacteroidetes can produce butyrate which is thought to have antineoplastic properties and thus plays a role in maintaining a healthy gut (Kim and Milner, 2007). Within Firmicutes, Clostridiales is the dominated feces of Tibetan wild ass. Firmicutes and Clostridiales are dominant bacteria in the gut and rumen of many animals (Wang *et al*., 2005; Ley *et al*., 2008). Several researches reported that Firmicutes was associated with obesity (Schwiertz *et al*., 2010; Angelakis *et al*., 2012). In the gastrointestinal tract of ruminants, with the continuous increase of hay fiber content, the proportion of Clostridial abundance of intestinal microorganisms is continuously increases. This means, this order is an important index of intestinal bacterial ecosystem function and metabolic differences. These studies are similar to our findings. Based on the published scientific research, mammals that use plants as the main food source need to face up to 60% of the components of the plant cell walls that are difficult to digest and absorb, such as cellulose and hemicellulose (Lynd *et al*., 1999). Bacteria that can degrade cellulose and other polysaccharides include Bacteroidetes, Bacteroides-Prevotella, Clostridiales and Spirillum orders. In addition, there is a close relation between obesity and the abundance ratio of Firmicutes and *Bacteroidetes,* and the high ratio of Firmicutes and Bacteroidetes will lead to obesity in animals such as pigs and mice (Ley *et al*., 2006). In the high-altitude sample group, the ratio of Firmicutes to Bacteroidetes was significantly higher than that of low-altitude samples groups. Therefore we speculate that the gut microbiota of species from high altitudes tend to evolve a more efficient way for digesting the cellulose and hemicellulose, converting short chain fatty acids to adapt to the high cold, low oxygen and low energy diet (Zhang *et al*., 2016).

From Figure 6, we know the *Melainabacteria* of the high-altitude sample (Group B) was significantly higher than that of the low-altitude sample (Group A). *Melainabacteria* can synthesize several B and K vitamins. We surmise, these bacteria are beneficial to their host. Although they lack linked electron transport chains, they have many methods to create a membrane potential which can generate ATP via ATP synthase. Besides, they can use Fe hydrogenase for H_2 which consumed by other microorganisms (Wikipedia: https://en.wikipedia.org/wiki/Melainabacteria, April 18, 2018). We speculate that the reason for this result may be due to altitude. Because of the harsh environment at high altitude and lack of food, the Tibetan wild ass has to adapt to expand the range of feeding. During the evolutionary process, *Melainabacteria* gradually appear and occupies an advantage to adapt to the high-altitude living environment.

Besides, we found that several rumen bacteria are only present in Group B, which is may directly related to the high-fiber diet in Group B. The *Ruminococcus_flavefaciens* and *rumen_bacterium_YS2* inhabit the rumen of the Tibetan wild ass. We suggest that they may play a role in volatile fatty acid metabolism in the rumen. To investigate the relationship between above bacterial populations and environmental factors, we performed a literature search focusing on specific bacterial species. These bacterial populations allow their hosts to digest cellulose. They are involved in glycolysis and the tricarboxylic acid cycle, so cellulose is decomposed by a piece and eventually produces volatile fatty acids, which are absorbed by ruminants. These findings of *Ruminococcus_flavefaciens* and *rumen_bacterium_YS2* in the microbiota of the Tibetan wild ass may help to explain how this energy conservation manifests itself under adverse conditions.

Previous studies have shown that sex is one of the most important contributors to the structural diversity of mammalian gut microbiota (Bolnick *et al*., 2014; Abdul-Aziz *et al*., 2016). However, in our study, we did not investigate the effect of gender on the gut microbiota in Tibetan wild ass, what we will do in later research.

CONCLUSION

In brief, we described the main fecal bacteria population of the Tibetan wild ass and provided a taxonomic basis for further investigation of the intestinal ecology of the Tibetan wild ass. In this research, we also found altitude have a marked impact on the composition of intestinal microflora in Tibetan wild ass which is similar to the research on human and Tibetan-chicken gut microbiota. We speculate that the altitude influences the intestinal flora of Tibetan antelope in the following aspects. First, the main fecal bacteria population of the Tibetan wild ass adapts to high altitude environment while others do not adapt. Second, the gut microbiota can help the Tibetan wild ass adapt to high cold, low oxygen, and low energy diet and to a high-altitude living environment. In the near future, we still solve all of the questions. Our study may contribute to the management of feed formulations to prevent the disease in these animals. These results also add to our understanding of the gut microbiota in this species.

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Availability of date and materials

The raw data obtained had been deposited in the NCBI Sequence Read Archive (SRA) with the Bio Project ID-PRJNA436598, Accession: SRP133795, ID: SUB3742196.

Statement of conflicts of interest

All authors state that they have no competing interests.

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