



Gut Microbiota enabled Goitered Gazelle (*Gazella subgutturosa*) to Adapt to Seasonal Changes

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ABSTRACT

Goitered gazelle (*Gazella subgutturosa*) mainly live in arid and semi-arid environments. In Qinghai Province, goitered gazelle are found only in the Qaidam Basin, which shows pronounced seasonal changes ranging from cold, dry winters to short, hot summers. The gut microbiota plays a very important role in host health, immunity, and digestion. Therefore, seasonal changes in gut microbiota can affect host digestion and metabolism in ways that help the host adapt to different forage conditions. Understanding the relationship between seasonal variations in the gut microbiota and host adaptability is a good basis for the protection of the goitered gazelle. Here, we present our findings on the diversity of the gut microbiota in fecal samples from wild goitered gazelle from Qaidam Basin, Qinghai-Tibet Plateau, which are the first such data to be reported. A total of 25 phyla were found, of which the dominant phyla are Firmicutes and Bacteroidetes in both summer and winter, representing more than 90% of the overall relative abundance. Diet is a key factor in shaping the gut microbiota. Because of large differences in host diet between summer and winter, there are significant differences in microbiota community structures according to Amova (SS=0.3871; MS=0.387; Fs=6.856; $P<0.001^{***}$). In winter, goitered gazelle must face cold weather as well as shortages in water and forage. To survive in a harsh environment, the relative abundance of Bacteroides, which enhances host metabolism of saccharides, increases significantly. In addition, water reabsorption was also significantly enhanced, which helps the goitered gazelles avoid dehydration in winter.

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Authors' Contribution

WQ and TZ designed and supervised the study. YH, LW, JY and HG analyzed the data. WQ, YH, GL, PS and JZ wrote the manuscript.

Key words

Gut microbiota, Seasonal adaptation, Seasonal microbiota change, *Gazella subgutturosa*, Qaidam Basin.

INTRODUCTION

Gazella subgutturosa (Güldenstaedt, 1780) is also known as the goitered gazelle (IUCN SSC Antelope Specialist Group, 2017). This species generally lives in desert and semiarid habitats (Kingswood and David, 1996) and in Qinghai Province, the goitered gazelle inhabits solely the Qaidam Basin (Cai, 1989), which is an intermountain depression in the Qinghai-Tibet Plateau. The climate characteristics of the Qinghai-Tibet Plateau are cold and dry in winter, but warm and wet in summer (Liu *et al.*, 2017), and seasonal changes in the Qaidam Basin are pronounced, with limited precipitation during the cold, long winter, and substantial rains during the warm, short summer (Shi *et al.*, 2005). According to Xu *et al.* (2008),

goitered gazelle diets vary obviously across seasons, and the goitered gazelles eat noticeably fewer plant species in winter than in summer at the Kalamaili Mountain Nature Reserve. The distribution of high-quality food is scattered in arid environments (Blank *et al.*, 2012a) and a decentralized distribution pattern in food would limit the group size of goitered gazelles to one to three individuals (Blank *et al.*, 2012a); moreover, seasonal changes in food distribution can affect population size (Blank *et al.*, 2012b). In 2006, Ostrowski *et al.* (2006) and Ostrowski and Williams (2006) demonstrated that goitered gazelles can adjust metabolic levels and change the size of their organs to survive in conditions of food and water restriction on the macro level. On the micro level, bacteria play an important role in the balance between health and disease (Lee *et al.*, 2010; Ishaq *et al.*, 2018). It is widely accepted that the gut microbiota can affect the host's metabolism and help meet the energy and nutritional needs of the host by improving host digestion efficiency (Nicholson *et al.*, 2012; Sun *et al.*

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et al., 2016; Tremaroli and Backhed, 2012). With a decrease in edible plant species and food quality, how do goitered gazelles face the problem of food scarcity and respond physiologically and metabolically to seasonal changes in the environment? What is the role of their microbiota in this process?

The gut microbiota can vary across seasons (Zhang *et al.*, 2014; Maurice *et al.*, 2015; Sun *et al.*, 2016), and this variation may contribute to host energy metabolism according to a study on hibernating brown bears (Sommer *et al.*, 2016). However, no reports on the seasonal variations in the gut microbiota of wild goitered gazelles. This paper presents the first profile of the gut microbiota of goitered gazelles from Qaidam Basin, Qinghai-Tibet Plateau. Significant differences in the structures of the gut microbiota were found between winter and summer, which we believe to be closely related to seasonal changes in diet. To survive in cold and dry winter, the relative abundance of *Bacteroides* increased significantly, moreover, the functions of saccharides metabolism enhanced significantly. Water reabsorption was also enhanced significantly in winter. We attempt to figure out the seasonal changing laws of the intestinal microbiota of wild goitered gazelles and how they enable the host to adapt to seasonal changes, including environmental and dietary changes.

The number of goitered gazelles is estimated at 120,000-140,000 but populations throughout the range have decreased mostly because of continued illegal hunting and habitat loss. The goitered gazelle is listed as Vulnerable (IUCN SSC Antelope Specialist Group, 2017). Our data may play an important role in understanding the gut microbiota diversity and its seasonal variability, on which the viability of wild populations of goitered gazelle depend. Analysis of the changes in their intestinal microbiota can help us understand the health and nutritional status of the wild populations of goitered gazelle, which in turn will provide targeted information for their conservation.

MATERIALS AND METHODS

Sample collection

We collected 40 fresh fecal samples belonging to goitered gazelles from the same area, namely Nuomuhong Township, Qaidam Basin, in Qinghai Province. In addition, 20 samples were collected randomly in August 2016, labelled SGA1 to SGA20; and another 20 samples were collected randomly in December 2016, labelled WGA1 to WGA20. All samples were collected after natural defecation, the animals were not frightened by researchers, and no drugs were used to promote defecation. For each sample, we use disposable PE (polyethylene) gloves to avoid contamination. All samples were stored at -20°C

for one week after field work, then transferred to -80°C storage before DNA extraction.

DNA extraction and PCR amplification

DNA from all samples was extracted using the CTAB (cetyltrimethylammonium bromide) method. To obtain 16S V3 and V4 regions, target genes were amplified and using a specific barcode primer set (341F, 806R) (Berg *et al.*, 2012; Michelsen *et al.*, 2014). All PCR reactions were performed in a 30 µL reaction mixture (15 µL Phusion Master Mix 2X, New England Biolabs; 3 µL (6 µM) Primer (2 µM); 10 µL (5 - 10 ng) DNA (1 ng/µL); 2 µL dd H₂O). Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, a final extension was performed at the end of the run at 72°C for 5 min. We mixed an identical volume of 1X loading buffer with the PCR products, then performed electrophoresis on 2% agarose gel for PCR product detection. We chose 400–450 bp bright bands for further experiments. PCR products were mixed in equidensity ratios, then extracted with GeneJET Gel Extraction Kit (Thermo Scientific).

Library preparation and sequencing

Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) was used to generate sequence libraries according to the manufacturer's recommendations and to add index codes. We used a Qubit@ 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system to assess the quality of the libraries. Once they were qualified, the library was sequenced on an Illumina HiSeq 2500 platform.

Data analysis

We used FLASH (Magoč *et al.*, 2011) to merge the paired-end reads from the original DNA fragments according to their unique barcodes, the paired-end reads can be assigned to each sample. QIIME (Caporaso *et al.*, 2010) was used to analyze sequences. Sequences with ≥97% similarity were assigned to the same OTUs. First, the reads were filtered using QIIME quality filters, then we picked a representative sequence for each OTU (Operational Taxonomic Unit) and used the RDP classifier (Wang *et al.*, 2007) to annotate taxonomic information. We discarded all singletons whose OTUs only appeared once with Usearch and clustered the remaining OTUs by UPARSE64. Simultaneously, we were able to count the relative abundance of OTUs. Using the Mothur software, we acquired every taxonomic category information of all samples using the SILVA database. Unweighted unifrac were calculated by QIIME for Principal Coordinate Analysis (PCoA).

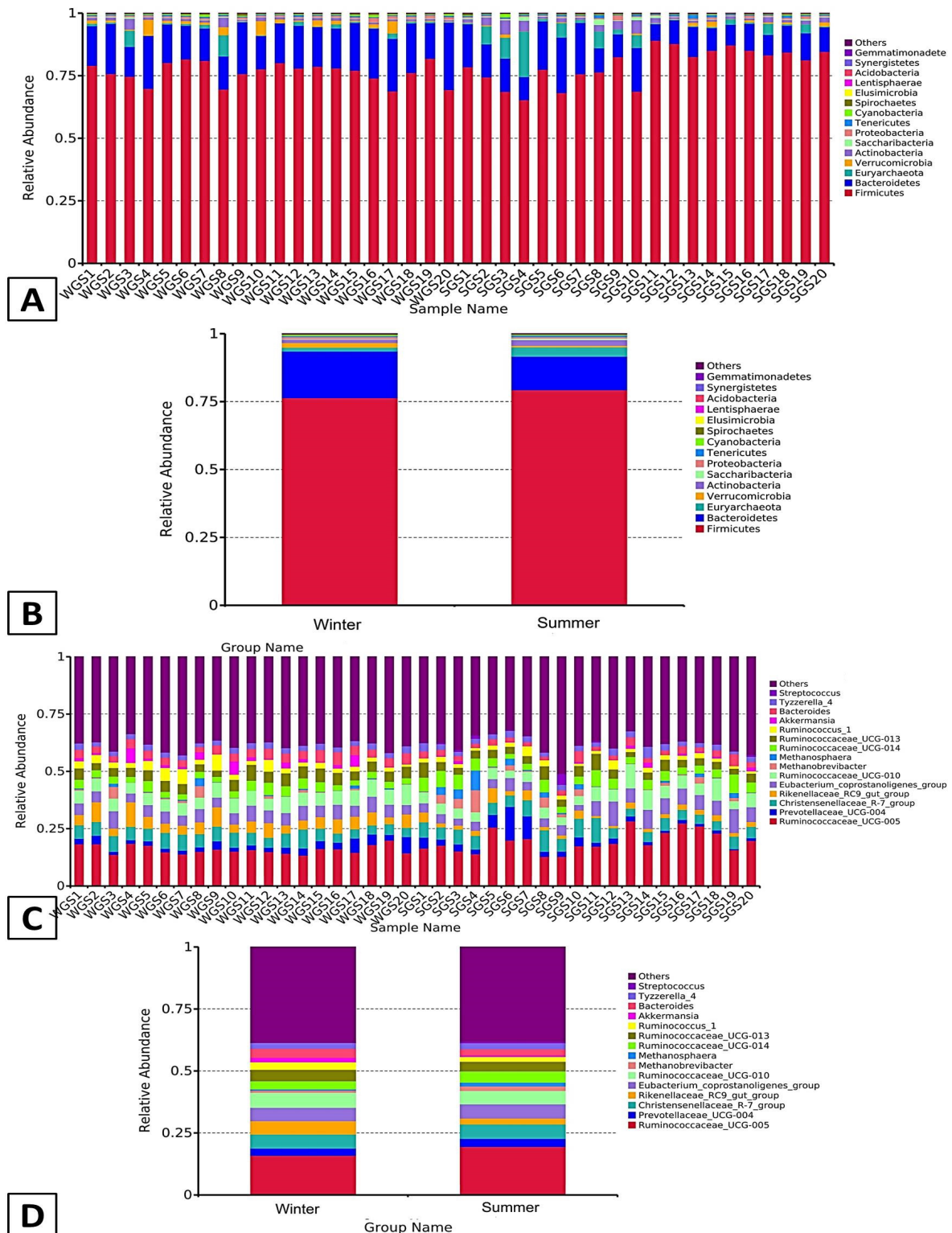


Fig. 1. **A**, the relative abundance of the top 15 phyla in 40 samples; **B**, the relative abundance of the top 15 phyla in two groups, summer and winter; **C**, the relative abundance of the top 15 genera in 40 samples; **D**, the relative abundance of the top 15 genera in two groups, summer and winter.

We commissioned Novogene Co., Ltd. to complete all experiments described in DNA extraction and PCR amplification, Library preparation and sequencing, and Data analysis.

RESULTS

Data qualification

Goods' coverage shows the sequencing depth, and indicated that our reads covered over 99% of every sample. According to effective ratio, all the above data indicate that the all the clean data generated from samples can be qualified as good data, and they were adequate to continue subsequent analysis. The details are shown in [Supplementary Table I](#).

Using a genetic distance of 3%, all 16S rRNA sequence reads were assigned to OTUs and we deleted singletons to guarantee the qualification. We identified 2906 OTUs, and after removing singletons, 2754 OTUs remained. In total, 25 phyla were detected, including those that were unclassified. We classified the 25 phyla into 48 classes, 88 orders, 163 families, and 289 genera.

Diversity analysis of summer and winter microbiota

No OTUs are shared by all samples. The winter group and the summer group shared 2281 OTUs. Only 261 OTUs were specific to the summer group, while 243 OTUs were specific to the winter group. That means that the winter group and summer group are similar in OTUs composition.

At alpha diversity, the numbers of observed species, Shannon index, Simpson index, and chao 1 of the two sets of groups are shown in [Table I](#), as estimated with R (<https://www.r-project.org/>). These data indicate that gut microbiota communities in the summer group are more plentiful in diversity. At the OTU level, the relative abundance of gut microbiota in the summer group is better as the chao1 index shows.

Table I.- The numbers of observed species, Shannon index, Simpson index and Chao 1 between two groups.

Group name	Observed sp.	Shannon	Simpson	Chao1
Winter group	1242.9	8.062777	0.988895	1318.886
Summer group	1298.3	7.927496	0.987097	1445.793

To assess the composition of winter and summer groups, we counted the number of groups at different taxonomic levels. At the phylum level, we found a total of 25 phyla in the winter group including those that were unclassified. In the summer group, only 22 phyla were identified. Firmicutes and Bacteroidetes were the dominant phyla with relative abundance in both groups, contributing approximately 76.394% and 17.177% to the

relative abundance in the winter group, and approximately 79.330% and 12.341% to the relative abundance in the summer group. Thaumarchaeota (0.0007%), SHA-109 (0.0006%), and TM6 (0.0002%) were found only in the winter group, but their relative abundances are extremely low. The top 15 phyla from both groups are shown in [Figure 1](#). In summer and winter, Firmicutes and Bacteroidetes were the dominant phyla in the gut microbiota communities, representing over 90% of the identical microbes. Ten phyla showed significant differences ($P < 0.05^*$) between the two groups according to Metastat analysis with R (<https://CRAN.R-project.org/package=optparse>). These are Bacteroidetes, Chlorobi, Saccharibacteria, Verrucomicrobia, Synergistetes, Tenericutes, Cyanobacteria, Gemmatimonadetes, Thaumarchaeota and Actinobacteria.

At the genus level, *Ruminococcaceae* UCG-005 and *Ruminococcaceae* UCG-010 are the dominant genera in the winter group except the unclassified cases, contributing 15.93% and 6.09% respectively to the overall relative abundance. The dominant genera are *Ruminococcaceae* UCG-005 and *Christensenellaceae* R-7_group in the summer group except the unclassified cases, contributing 19.49% and 5.80% respectively to the overall relative abundance. The relative abundance of *Ruminococcaceae* UCG-005 decreased significantly ($P < 0.05^*$) in winter according to Metastat analysis, while no significant differences was found in *Christensenellaceae* R-7_group. The top 15 genera of two groups are shown in [Figure 1](#).

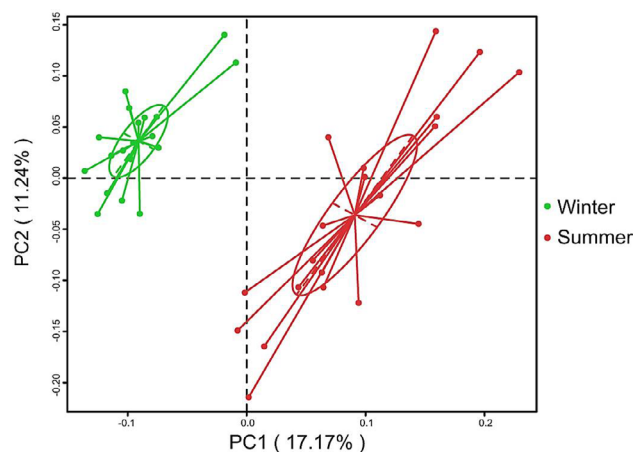


Fig. 2. Cluster analysis result of winter group and summer group according to PCoA analysis.

Seasonal variations of microbiota communities between summer and winter

We discovered a significant difference between the winter group and the summer group by Amova (SS=0.3871; MS=0.387; Fs=6.856; $P < 0.001^{**}$) with

Mothur software. Anosim analysis with R (<https://CRAN.Rproject.org/package=vegan>) revealed a significant difference in the microbiota community at the OTU level between the winter group and the summer group ($R=0.474$; $P=0.001$), and our P value confirmed the reliability of the data. The R -value is 0.474, which is above 0, indicating

that inter-group differences are greater than intra-group differences. According to PCoA analysis, the two groups are well separated (Fig. 2). Based on Lefse analysis (<http://huttenhower.sph.harvard.edu/galaxy/>) (LDA score is 4), we found three biomarkers (Fig. 3) showing significant differences between summer and winter groups.

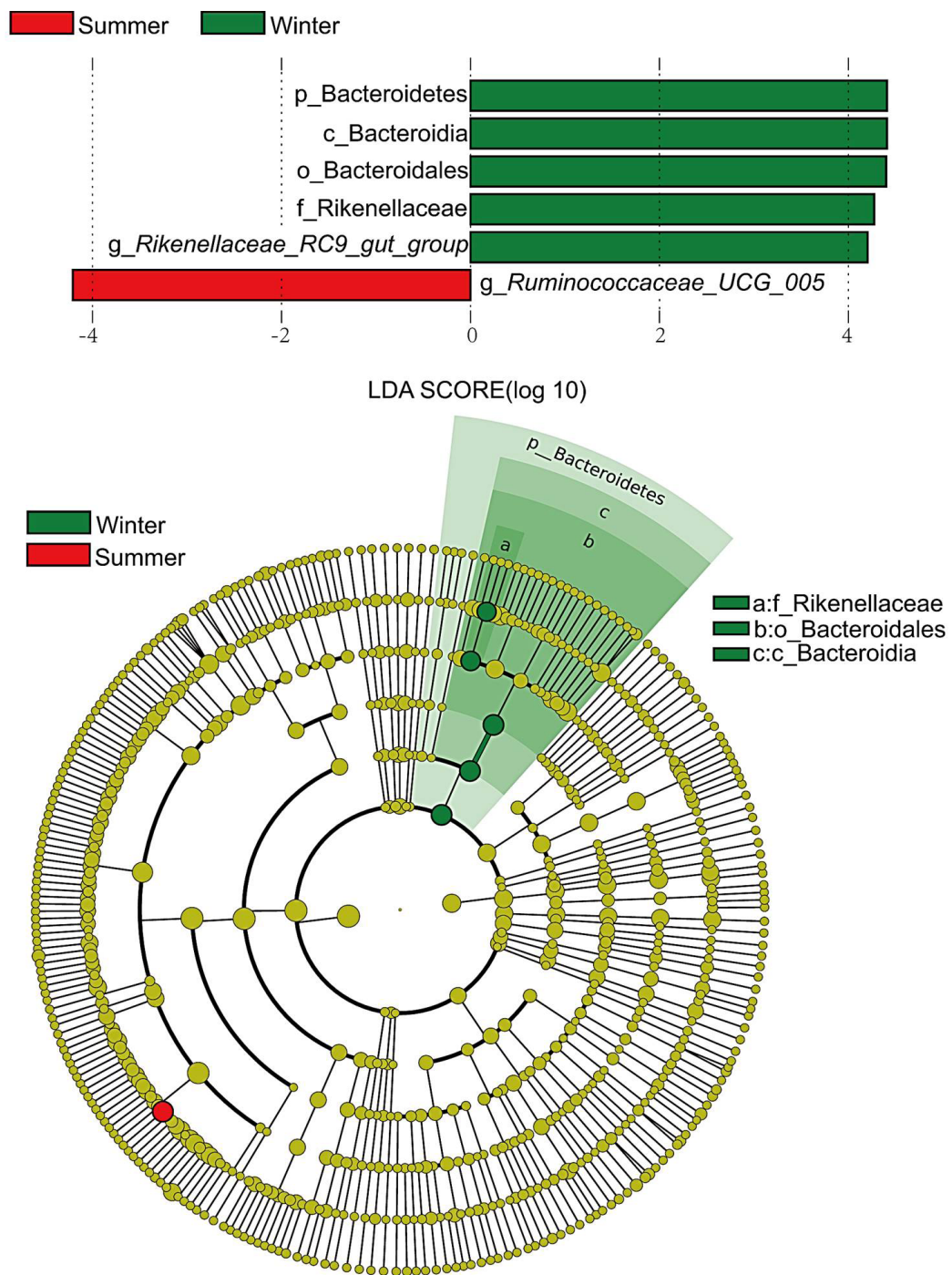


Fig. 3. Lefse analysis results of winter group and summer group (LDA score is 4).

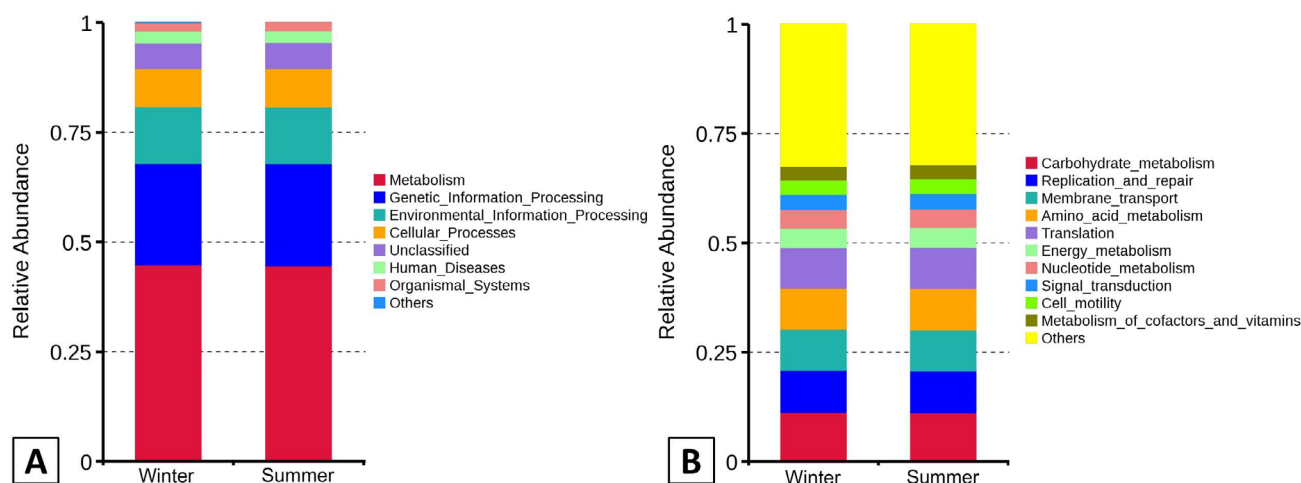


Fig. 4. Microbiota functions based on KEGG database. **A**, microbiota functions on first level; **B**, microbiota functions on second level.

Seasonal variations in gut microbiota functions

We predicted the gut microbiota functions of winter and summer groups with Tax4Fun using the KEGG database (<https://www.kegg.jp/>) and found a significant difference ($p < 0.05^*$) between winter and summer groups. The main functions are transporters and two-component systems, as shown in Figure 4. The main functions that were significantly improved ($p < 0.01^{**}$) in the winter group were galactose metabolism, lipopolysaccharide biosynthesis, carbon fixation in photosynthetic organisms, vasopressin-regulated water reabsorption, fructose and mannose metabolism, amino acid metabolism, protein digestion, and absorption. In the summer group, novobiocin biosynthesis, carbohydrate metabolism, phenylalanine metabolism, and thiamine metabolism were significantly enhanced ($p < 0.01^{**}$).

DISCUSSION

The bacterial microbiota is commonly dominated by Firmicutes and Bacteroidetes in mammals (Bian *et al.*, 2013; Combes *et al.*, 2017; Huang *et al.*, 2018; Rodriguez *et al.*, 2015; Zhang *et al.*, 2018). Our results are consistent with this previously published conclusion. Seasonal variation is the main reason leading to microbiota community fluctuation. Diets play an important role in shaping the microbiota (De Filippo *et al.*, 2010). The composition and diversity of diet can affect the composition of the microbiota (Zmora *et al.*, 2018). Xu *et al.* (2008) used the fecal microscopic method to evaluate the food habits of goitered gazelles (*Gazella subgutturosa sairensis*) from the Kalamaili Mountain Nature Reserve in northern Xinjiang, where diets varied seasonally. In

winter, 17 species from six families are edible; in summer, goitered gazelle consume 30 species from twelve families (Xu *et al.*, 2008). A healthy, diverse diet promotes a more diverse gut microbiota (Zmora *et al.*, 2018). Goitered gazelles consume more plant species in the summer and their main foods are *Ceratoides latens* and some non-graminaceae species (Xu *et al.*, 2008). Therefore, the alpha diversity of the gut microbiota is better in summer, and the seasonal variation in microbiota structures in goitered gazelle between summer and winter may be related to seasonal changes in edible plant species and food quality.

High-fat diets can cause the proportion of *Ruminococcaceae* to decrease and that of *Rikenellaceae* to increase (Daniel *et al.*, 2014). In our research, the proportions of the relative abundance of these bacteria are also changed, *Ruminococcaceae* UCG-005 decreased significantly and *Rikenellaceae* RC9 gut group increased significantly in winter. At the family level, the relative abundance of *Rikenellaceae* increased significantly in winter. These changes may be due to the changes of fat content in the diet. Sacsoul (*Haloxylon ammodendron*) is the main food source for goitered gazelles in winter (Xu *et al.*, 2008), and its crude fat content increases from May to October (Gao and Ji, 1996). The changes in relative abundance of *Ruminococcaceae* UCG-005 and *Rikenellaceae* RC9 gut group are likely in response to the changes in crude fat content in the goitered gazelles' diet. In addition, the content of crude fiber increases from summer to winter in sacsoul, to help the host degrade cellulose, as a response, the relative abundance of *Butyrivibrio* increases significantly in winter.

Thaumarchaeota, SHA-109, and TM6 were found only in winter, and there was a significant difference in the

relative abundance of Thaumarchaeota between summer and winter. Thaumarchaeota are capable of nitrification, reducing body odor, decreasing pH and removing certain nitrogen compounds (Moissl-Eichinger *et al.*, 2017). Approximately 25%-45% of daily urea is degraded in the intestine in humans (Fishbach and Sonnenburg, 2011). We suppose that the main function of Thaumarchaeota in winter is to remove certain nitrogen compounds to avoid poisoning its host. Normal intestinal conditions tend to be low in pH. Therefore, a Thaumarchaeota-induced decrease in pH is overall beneficial for intestinal balance. In summer, no Thaumarchaeota was found in feces, but the relative abundance of *Lactobacillus* increased significantly. *Lactobacillus* is a kind of probiotic bacteria that can also decrease intestinal pH, thereby preventing pathogenic bacteria overgrowth and maintaining the ecological balance in the intestine (Chen and Deng, 2017). Although there is a significant difference in gut microbiota composition between summer and winter, the ultimate purpose of the favored microbes appears to be consistent, as all the changes are devoted to maintaining intestinal balance and normal metabolism.

Metabolites from intestinal microbiota are key determinants of host-microbe mutualism (Trompette *et al.*, 2014). The temperature in the Qaidam Basin is below 0 °C in winter, and goitered gazelles need more energy to maintain a constant body temperature. However, as the edible plants are fewer in number and their quality is poorer, it becomes more of a challenge to take in sufficient energy for survival. In our research, the relative abundance of *Bacteroides* increased significantly in winter, and *B. thetaiotaomicron* was only found in winter. *Bacteroides* enable the host to degrade carbohydrates, especially polysaccharides, as well as proteins, and enhance the nutrient utilization of the host (Li *et al.*, 2017). *Bacteroides* are important members of the gut microbiota and *Bacteroides* species actively catabolize polysaccharides. Intestinal *Bacteroides* have evolved species-specific physical interactions with the host that mediate stable and resilient gut colonization (Wexler and Geffen, 2014). Meanwhile, galactose metabolism, lipopolysaccharide biosynthesis, fructose and mannose metabolism, and glycosaminoglycan degradation were significantly enhanced in winter. We suppose that the increase in the relative abundance of *Bacteroides* improves saccharide metabolism in the goitered gazelle. In winter, the enhancement of saccharide metabolism may be a way of compensating for the reduction of nutrients in foods, to ensure that there is no reduction in the total energy intake by the host. Carbohydrate metabolism in the mammalian gut may take place in a concerted manner with the help of various members of the gut microbiota. Thus, carbohydrates are presumed to be partly responsible

for changes in microbiota in a changeable environment (Milani *et al.*, 2015). It is a strategy for the goitered gazelle to regulate metabolic levels with the help of functional changes in the gut microbiota.

Qaidam Basin is surrounded by mountains that prevent warm, south-westerly air from entering the basin's interior, resulting in minimal precipitation (Wang *et al.*, 2018). In winter, some rivers in the Qaidam Basin dry up, exposing their riverbeds. Gemmatimonadetes prefer drier soils (De Bruyn *et al.*, 2011) and it increased significantly in winter. The changes in Gemmatimonadetes reflect an adaptation to the water shortage problem in winter faced by goitered gazelles. To survive in a water shortage environment, vasopressin-regulated water reabsorption was significantly enhanced.

CONCLUSION

Our results provide an initial view on the gut microbiota diversity of the goitered gazelle and how the microbiota enables the host to adapt to seasonal changes. We wanted to find out how the seasonal changes in the microbiota affects goitered gazelle, as well as to reveal the relationship between gut microbiota diversity and host adaptability. The goitered gazelle is the main ungulate in arid environments and its survival status is closely related to the balance of the arid landscapes. The interplay between the goitered gazelle and its gut microbiota is the result of a long evolutionary process. Understanding the relationship between the gut microbiota and the host is a precondition for conservation. Microbiota variations in the fecal matter of the goitered gazelle can be used as one of the standard tests to assess nutrient status and the animals' adaptability to environmental changes. Based on these data, a predictive diagnosis system can be established and the physical conditions of the goitered gazelle can be estimated according to the composition and functions of the gut microbiota.

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Statement on the welfare of animals

All procedures performed in studies involving animals were approved by the Ethics and Welfare of

Experiment Animals Committee affiliated to Northwest Institute of Plateau Biology.

Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20181031121013>

Statement of conflict of interest

Authors have declared no conflict of interest.

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