

Seasonal Variation in Gut Microbiota Composition: Comparative Analysis of Siberian Musk Deer (*Moschus moschiferus*) and Forest Musk Deer (*Moschus berezovskii*)

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Abstract

Background: Musk deer is famous for its secretion of musk, but due to various factors that the musk deer population has dropped sharply and it is difficult to expand herd sizes. In light of the rapid development of animal husbandry and scientific technology, as well as the urgent need for wildlife conservation, research on the gut microbiota of musk deer is warranted. The gut microbiota is a complex ecosystem that is regulated by many factors, such as dietary, environmental, host factors, and seasonal changes.

Results: We studied the gut microbiota in Siberian and Forest musk deer using high-throughput sequencing of the V3–V4 region of the 16S rRNA gene. Fecal samples were collected during two seasons to determine associations between community diversity, composition, and seasonal factors. The results showed that the Firmicutes and Bacteroidetes were the predominant phyla in Siberian and Forest musk deer, their relative abundance was significantly different with species and seasonal factors. The Siberian musk deer population sampled in this study had a wider feeding range and no dietary limitations. Affected by the type of food sources and the feed intake, the microbial α -diversity is higher in summer than winter.

Conclusions: The gut bacterial composition of these two musk deer is significantly different, and the abundance between core bacteria is affected by seasonal factors. This study is the first to analyze the composition of the gut microflora in Siberian musk deer, and these results provide a foundation for improving feeding for musk deer populations and further studies investigating the environmental impact of musk deer.

Background

Both Siberian musk deer (*Moschus moschiferus*, SMD) and Forest musk deer (*Moschus berezovskii*, FMD) belong to the family *Moschidae* and genus *Moschus*. They are generally solitary, as well as have a stocky body with short, thin front legs and longer, more powerful hind legs. Siberian musk deer is a medium-sized musk deer (80 ~ 95 cm head-body length and 8 ~ 13 kg in weight), while forest musk deer is one of the smaller musk deer (70 cm head-body length and 7 ~ 9 kg in weight). The musk deer are native to Russia, Kazakhstan, China, Mongolia, and the Koreas, however currently there are only 4000-6000 siberian musk deer existing in Mongolia [1, 2]. More than 5,000 years of history, the musk has been widely used in the production of Chinese traditional medicine and perfume. In the past, most of the musk were obtained by killing active animals to acquire the entire sac [3, 4]. So *Moschus* *genus* spp. populations are declining due to hunting, who makes encountered grim situation, the number of existing less in urgent need of protection. The species are listed under CITES Appendix II, and is protected (Very Rare) under part 7.1 of the Law of the Mongolian Animal Kingdom (2000). It is also on the 2014 Chinese Red List as Critically Endangered A1acd+B1ab (i, ii, iii), and is contained on the China Key List-II [5, 6].

Gut microbial composition and function in wild animals has been widely studied in recent years [7, 8]. A number of vital host functions depend on the gut microbiota, which is involved in the synthesis of

nutrients and systemic immune regulation [9]. The gut microbiota is a complex ecosystem that is regulated by many factors. The composition of gut microbiota is altered by the host, feed, lifestyle, antibiotics and seasonal changes [10- 13]. Studies in horses[14], mice [15], pandas [16], humans [17], and forest musk deer [18] have shown seasonal shifts in gut microbiome structure. These alterations could be caused by the diet. In summer, the rainfalls increased, and the density of plants will be improved. It means that musk deer have sufficient food. However, the food supply will be decreased in winter because of severe weather. Some gut bacteria are opportunistic pathogens, and bacterial overgrowth or an altered community structure are associated with multiple diseases [11, 19]. Siberian musk deer and Forest musk deer have a high rate of disease incidence, especially diseases caused by a bacterial infection, and the mortality rate of diseased animals is high. These infections can be mediated by environmental or host-derived bacteria [20, 21]. The precise role of the gut microbiota on animal health and disease remains unclear and more foundational work is required. The Siberian musk and forest musk deer are herbivorous ruminants. Although the microbiota can be an important tool for assessing animal health, no reports to date have studied these factors in Siberian musk deer.

We characterized the seasonal variation in the gastrointestinal microbiota of Siberian musk deer and forest musk deer using high-throughput sequencing of the 16S rRNA gene in fecal samples. According to the research results of Hu et al [18] that the influence of seasonal factors on the gut microbiota of forest musk deer, and daily food of the siberian musk deer have obvious seasonal changes, we selected representative winter and summer seasons for this research. The main objectives of present study are: (1) to compare the composition and diversity gut microbiota of two species; (2) to determine seasonally (i.e., summer and winter) variations in the microbial community composition and disease of Siberian musk deer; (3) to examine the changes in signaling pathways and functional genes, and; (4) to discuss their potential functions in the diagnosing diseases of the digestive system and for improving feeding methods.

Results

Quality filtering and validation of the dataset

There was an average of 909,993 reads (average length = 428.67 bp; Table S1) obtained from each sample after quality filtering. There were 1,693 OTUs detected, and each sample had between 396 and 865 OTUs (Table S2). Individually rarefaction curves showed that the sequencing depth was adequate for the study (Fig S1). Good's coverage showed that 28 samples had between 99.28 to 99.64% coverage (Table S2). The results showed that the number of OTUs increased rapidly until it gradually reached a plateau. It shows that the amount of sequencing data is large enough, and the increase of sequencing data can not find more OTU, which can reflect the information of most microbial species in the sample.

Core bacterial communities and composition difference in the two species

There were 21 phyla, 34 classes, 82 orders, 142 families, and 310 genera identified in the two musk deer species. There were 658 OTUs that were present in all groups. There were 100, 52, 128, and 10 OTUs that

appeared in each sample of the FS, FW, SS, and SW groups, respectively (Fig 1A; FMD in summer and winter: FS and FW; SMD in summer and winter: SS and SW).

The classification of sequences from the samples resulted in five different phyla was identified, including *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, *Verrucomicrobia*, and *Proteobacteria* (Fig 1B). We considered these taxa that were shared by all individuals in a group to be the core bacterial community for that group. *Firmicutes* (FMD: 53±8.31 to 64.95±11.73, SMD: 86.2±5.713 to 90.21±3.265) and *Bacteroidetes* (FMD: 38.06±7.431 to 29.03±9.044, SMD:12.75±6.008 to 8.876±2.31) were core and dominant community members for both musk deer species. The relative abundances of *Firmicutes* were significantly higher in SMD than in FMD, and this corresponded to decreases in the relative abundances of *Bacteroidetes* (Fig 1B; Table 2). *Spirochaetes* and *Verrucomicrobia* were only found in forest musk deer (Fig 1B). Also, the difference in gut microbiota composition between the two musk deer can be clearly observed according to the Heatmap . Our results indicated the existence of two core taxa at the class level (*Clostridiales* and *Bacteroidales*) (Fig S2). At the order level, core gut bacterial included *Bacteroidaceae*, *Lachnospiraceae*, *Oscillospirales*, and *Christensenellaceae* (Fig S3), in addition to this 11 core taxa at the family level (Fig S4). These results indicated that the composition of the microbial community may be related to different physiological characteristics in the gut of musk deer.

The alpha diversity of forest musk deer was higher than that of Siberian musk deer based on the Shannon and Chao indices (Shannon index: FMD=4.682±0.428, SMD=4.54±0.56, $p=0.431$; Chao index: FMD=826.46±133.04, SMD=764.18±144.89, $p=0.347$; Table S3; Fig 2A-2B), it is concluded that the community abundance and community diversity of the forest musk deer are higher than the siberian musk deer, but there is no significant difference. There were differences in the beta diversity of the SMD and FMD communities PCoA analysis (Fig 3). ANOSIM analysis reveals intergroup differences in bacterial communities between two musk deer species ($R = 0.6655$, $p = 0.001$). The results of ANOSIM analysis and PCoA analysis mutually verified the differences of bacterial communities between the two musk deer species.

Seasonal differences in bacterial communities

The alpha diversity of the bacterial community in the summer was higher than in the winter for the two musk deer species (Shannon index; FS:4.913±0.249, SS: 4.857±0.517, FW: 4.451±0.454, SW: 4.222±0.443). There was a significant difference in Shannon diversity index between the two seasons for FMD ($p = 0.026$, $Q=0.031$), but not for SMD ($p = 0.194$, $Q=0.233$; Fig 2C; Table S3). The Chao index have significant different in SMD (Chao: $p = 0.03$, $Q=0.061$; Fig 2D). At the phyla level, there were 9 taxa (*Firmicutes*, *Bacteroidota*, *Verrucomicrobiota*, *Actinobacteriota*, *Cyanobacteria*, *Patescibacteria*, *Elusimicrobiota*, *Myxococcota*, and *Deferribacterota*) whose relative abundance was significantly different between seasons in FMD; at the same time, and only one taxon (*unclassified_k_norank_d_Bacteria*) whose relative abundance was significantly different between seasons in SMD (Table 2; Fig S5). At the genus level, there were 14 taxa (*UCG-005*, *norank_f_norank_o_RF39*, *Phascolarctobacterium*, *NK4A214_group*, *Papillibacter*, *UCG-002*,

Eubacterium_siraeum_group, Arthrobacter, unclassified_k_norank_d_Bacteria, norank_f_norank_o_Veillonellales-Selenomonadales, Akkermansia, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Brevundimonas, and unclassified_f_Comamonadacea) whose relative abundance was significantly different between seasons in SMD (Fig 4A). It is worth mentioning that the *Firmicutes/Bacteroidetes* ratio changed with the season in two musk deer.

We are much interested in musk deer gut microbiota is to determine whether specific microbiota could be the change with different season factors. To identify specific microbial candidates present in two specise seasons, LEFse (LDA score ≥ 4) analysis were used to determine specific biomarker belonged to different taxonomy (Fig 4B) from phylum to genus. The result revealed that twelve families (*Bacteroidia, Bacteroidales, Bacteroidetes, Prevotellaceae, Prevotellaceae_UCG_004, Bacteroides, Bacteroidaceae, Verrucomicrobia, Verrucomicrobiae, Verrucomicrobiales, Akkermansia, and Akkermansiaceae*) were significantly enriched in the FS group, three families (*Lachnospirales, Lachnospiraceae, and unclassified Lachnospiraceae*) were significantly enriched in the FW group, five families (*UCG_010, Monoglobus, Monoglobales and Monoglobaceae*) were significantly enriched in the SS group, and five families (*Firmicutes, Clostridia, Christensenellaceae_R_7_group, Christensenellaceae, and Christensenellales*) were significantly enriched in the SW group (Fig 4B).

Predicted functions of the microbial communities

There were significant differences in the functional capacity of SMD and FMD microbial communities. Specifically, three KEGG pathways (Environmental Information Processing, Cellular Processes, and None) were enriched in SMD samples, and five KEGG pathways (Human Diseases, Organismal Systems, Metabolism, Genetic Information Processing, and Unclassified) were enriched in FMD samples at Level 1 (Fig 5A). Also, two groups of samples were significant differences in thirty-three KEGG pathways (Level 2; $p < 0.005$). Among them, seven KEGG pathways (Transcription, Membrane Transport, Cell Motility, Signal Transduction, Cellular Processes and Signaling, Environmental Adaptation, and Carbohydrate Metabolism) were significantly enriched in SMD samples. Twenty-six KEGG pathways (Translation, Replication and Repair, Nucleotide Metabolism, "Folding, Sorting and Degradation", Enzyme Families, and Metabolic Diseases, *et al*) were significantly enriched in FMD samples (Fig 5B). At level 3, a functional comparison of 143 extremely significant differences ($p < 0.001$) was performed. Among them, the SMD group enriches thirty-five functions, and the FMD group enriches other functions. The results also reflect differences in transcription and metabolism between the two groups of animals (Fig S6).

There was a significant difference in predicted microbiota function in SMD between summer and winter. The relative abundance of genes (Level 1) involved in Metabolism, Organismal Systems, and Human Diseases was significantly different in two seasons (Fig 5C). The relative abundance of gene families (Level 2) involved in Xenobiotics Biodegradation and Metabolism, Endocrine System, and Metabolism of Cofactors and Vitamins was significantly higher in the SS samples than in the SW samples (Fig 5C). Besides, the Influenza A, Toxoplasmosis, Colorectal cancer, Viral myocarditis, and Smell cell lung cancer functions (Level 3) were significantly enriched in the SS group (Fig 5C).

Discussion

A high-fiber diet coupled with long-term evolutionary adaptation has resulted in a large number of symbiotic bacteria in the gastrointestinal tract of herbivores to help the body digest and absorb nutrients. Meanwhile, the high-fiber diet makes the variety of ruminants gut microbiota having a distinct feature and similar structure. Our data shows the difference in microbial communities between musk deer species as well as the seasonal variations in the microbial communities of each deer species.

The α -diversity of the gut microbiota in FMD samples was significantly different in two seasons, and the diversity lower in winter than in summer (Shannon index: $p < 0.05$; Chao index: $p < 0.05$); meanwhile, the diversity of the gut microbiota in SMD samples was not significantly different between seasons. Many studies have shown that a number of factors have an impact on gut microbial diversity, such as host healthy, diet structure, and lifestyles [22, 23, 24]. Indeed, survival strategies are most important for wild populations with low energy intake and high energy consumption in winter. To be specific, in terms of food types, there are fewer types of food to choose from in winter, and it is more difficult to eat the herbs on the surface, which results in a single diet for Siberian musk deer. It is speculated that the less variety of food is a factor that causes the lower gut microbiota diversity of Siberian musk deer.

The core bacterial phyla in both musk deer species were *Firmicutes* and *Bacteroidetes*, which is consistent with previous studies of the microbiota of musk deer [18, 25, 26] and other ruminants [27- 29]. The phyla are found in the guts of many mammals, which suggest the importance of these bacteria in the gut ecosystem [30]. *Firmicutes* are predominant cellulolytic bacteria that able to degrade fiber and form short-chain fatty acids (SCFAs), which are utilized by the host [31-33]. Ruminants cannot produce the necessary cellulolytic enzymes to degrade fiber and must rely on bacteria to produce these enzymes [34]. Certain *Bacteroidetes* species are specialists that degrade high molecular weight organic material (i.e., carbohydrates and proteins), and the genomes of these species are known to contain large numbers of CAZymes [35]. The seasonal variations in microbiome composition in our study are likely due to seasonal changes in diets. Diet is an important factor that shapes gut microbial communities [36-38]. Specifically, the ratio of fiber to carbohydrates in diets can impact microbiome composition in ruminants [39]. For example, the content of animal protein and saturated fatty acids are related to the abundance of *Actinomycetes*, while the abundance of carbohydrates, monosaccharides (glucose and fructose) and the abundance of *Pseudomonas* and *Prevotella* are highly correlated. *Bacteroidetes* were more abundant in musk deer consuming a high-starch and high-protein diet during the summer; meanwhile, *Firmicutes* were more abundant in musk deer consuming a high-fiber diet during the winter [31, 40, 41]. Except for various leaves, the food of captive forest musk is mainly carrots and various grains. Compared with the Siberian musk deer, forest musk deer food contains more protein and fat, coupled with a lot of energy to avoid foraging and avoiding natural enemies, it is not difficult to understand that *Bacteroides* is higher in the forest musk deer.

In Siberian musk deer, the *Firmicutes/Bacteroidetes* ratio (F/B ratio: 6.761-10.163) was significantly higher than that of forest musk (F/B ratio: 1.393-2.237). This may be related to the feeding habits of

these animals, and these seasonal changes could increase energy efficiency during the winter [10,42]. Compared to forest musk deer, the Siberian musk deer has a broader diet, a relatively high living altitude, and a colder climate that requires more energy [2, 43]. Recent research reports [44, 45] have confirmed that the ratio of *Firmicutes* and *Bacteroidetes* is related to fat deposition. Especially, the abundance of *Firmicutes* increases could be promoting more fat deposition[46]. Other studies[47] have shown that the genes of intestinal microorganisms with high F/B ratios are rich in encoding lysing polysaccharide start enzymes and eight glycosidic bond hydrolase families involved in sugar metabolism. And the residues of nutrients in mice with a high F/B ratio were significantly lower than those in lean mice. These results indicate that intestinal microorganisms with a high F / B ratio have higher fermentation efficiency and obtain more energy from food, thereby promoting fat deposition. In our experiments, the siberian musk deer, living in the wild, winter cold, and the shortage of food, not only need a lot of energy to find food and evade predators but also consume energy to maintain body temperature. So the *Firmicutes* in their gut microbiota can help them draw energy from food as much as possible to maintain the body's needs. *Spirochaetae* were only found in the FMD group that may be related to feeding conditions and animal habits. In this study, Siberian musk deer lived in a wide range and had a primitive diet, while forest musk deer were raised artificially in captivity and fed by breeders. Similar phenomena have been found in previous studies on sika deer [29] and human living environment [48] – different living environment makes *spirochaetae* only appear in intestinal flora of a certain living environment. In addition, studies have shown that animals fed diets rich in fungi, vitamin C and dietary fiber have lower levels of *spirochaetae* in gut microbiota [49], which is in line with our experimental animal Siberian musk deer similar, but need further validation.

The PCoA analyses were also showing the distinct separations and reciprocal relationships among musk deer from different areas, it indicated that the composition and structure of the bacterial community were significantly different between forest and siberian musk deer. At the same times, the season factors would also not be negligible effect factors on musk deer gut microbiota. For forest musk deer, have many phyla bacteria were significantly different, and this result is similar to that of Hu *et al* [18,25,26,50]. However, the SMD group was less affected by seasons, and only one phylum bacteria (*unclassified_k_norank_d_Bacteria*) significant difference, which needs further research and discussion. At the genus level, *UCG-005* and *Phascolarctobacterium* were enriched in winter samples and had significant differences, while the other genus with significant differences were enriched in summer samples. As we all know, the gut microbiota can affect the health and metabolism of the host. Among them, the large number of microorganisms and the complexity of the genomes associated with the microbial community limit the study of the metabolic profile of the bacterial community [22,51,52,53]. PICRUSt analysis enables robust assessments regarding the impact of environmental factors and biological processes on the composition of microbial communities. Microbial functional analysis showed that the most abundant biological pathways in all samples were translation, amino acid metabolism, carbohydrate metabolism, replication and repair, and membrane transport; this agrees with previous studies [54- 56]. In the SMD group, some pathways differed between the two seasons. Especially in the disease, the results show a variety of diseases was significantly enriched in the summer sample, the

results of this follow-up study provides a guideline. PICRUSt analysis does have limitations, as other pathways, such as some disease, were identified as significant but are not relevant to microbial communities of musk deer. Transcriptomic and metabolomic analyses would be more applicable to determining bacterial functions.

This is the first characterization of the gut microbiota in SMD, and it is also the first time to compare the structure of the gut microbiota in FMD and SMD. We show that that musk deer gut microbiota is complex and taxonomically plastic, and it varies by species of musk deer. Microbiota composition changes seasonally, likely due to changes in environmental conditions and diet. Limitations of this study include the sample size collected was relatively small, the inability to control for potentially important factors, such as weather and the lack of species-level resolution via 16S rRNA tagged sequencing. Shotgun metagenomic sequencing would provide additional insight into the gut microbial community in musk deer. In light of the rapid development of agriculture and technology, as well as the urgent need for wildlife conservation, further research on the gut microbiota of musk deer is warranted.

Conclusions

We characterized the seasonal variation in the gastrointestinal microbiota of Siberian musk deer and forest musk deer using high-throughput sequencing of the 16S rRNA gene in fecal samples. The results show that the composition and diversity gut microbiota of these two musk deer has significant differences. The *Firmicutes* and *Bacteroidetes* were the predominant phyla in both species, their relative abundance was significantly different with species and seasonal factors. In addition, the microbial α -diversity is higher in summer than winter, and determine seasonal variations in the microbial community composition of Siberian musk deer. This study is the first to analyze the composition of the gut microbiota in Siberian musk deer, and these results provide a foundation for improving feeding for musk deer populations and further studies investigating the environmental impact of musk deer.

Methods

Animals and Sample Collection

Four healthy adult Siberian musk deer were sampled from the musk-deer breeding center in Mongolia (Gachuurt Village, Ulaanbaatar; 47°55'N 107°09'E). The animals used in this experiment were captured from the wild status by the staff of the Mongolian musk-deer breeding center, which is the partner of the project, and kept in pens within a certain range of breeding. The wild habit of the Siberian musk deer is protected to a certain extent, through a wider range of captive breeding (each musk deer is raised in pen of 20 m×40 m) and avoiding the disturbance of the breeders as much as possible (Fig S7). The altitude of the breeding center is about 1783 m, and the annual average precipitation is 200-400 mm. The average summer temperature is 20-30 °C, which is hot and cool. The average temperature in winter is about -30 °C, and the lowest temperature is -40 °C. The siberian musk deer food partly from the leaves of plants grown in the fence, another part of the feed is mainly supplied by keepers such as *Cetrarian* lichens and

mushrooms (Fig S7). They spend 4-5 hours and about 9 hours in summer days and winter days respectively, to feed with a repetition of 5-8 times a day during the dusk and dawn. Stomach filled with food weighs 400-600 gr, on average it is 520 gr. In July, their diet is 80% fresh green plants and 20% moss and lichens. In December, their diet is 90% *Cetrarian* lichens (e.g., *Cetrariahivaeis*, *Cetrariaislanica*, *Cetrariacucuetata*) and 10% green moss. They also consume pine nuts, barberry leaves, pine, and spruce (Table 1; Table S4). During the summer Siberian musk deer eats 2 kg of a green plant, 0.6 kg moss lichens or moss, in winter 3.7 kg lichens, 0.4 kg moss per day. According to the research Musk deer drinks 100 ml of water for once, 3-4 times a day.

Ten forest musk deer were sampled from the Inner Mongolia Ulanbator League in China (40°42'N 113°26'E). The average altitude is 1400 meters and the average rainfall is 400 mm. The hottest month is July, the average temperature is 20.4 °C, the highest temperature is 36.5 °C, the average temperature in winter is -13.5 °C, and the lowest temperature is -37.5°C. Forest musk deer diet consisted mainly of leaves (e.g., mulberry leaves (*Morus alba*), elm leaves (*Ulmus pumila*), *broussonetia papyrifera* leaves) and seasonal vegetables (e.g., pumpkin, carrots, and corn). They were fed with fresh leaves in the summer and dried leaves in the winter. Under captive conditions, due to changes in environmental conditions, the type of feeding is greatly restricted, so a proper supply of local vegetables and minerals is essential. Each kilogram of leaves contains 250 mg of carotene, 1200 mg of calcium, 320 mg of iron, and also contains copper, zinc, phosphorus, manganese and other trace elements and vitamins. The content of crude protein in the dry matter of the foliage feed is 17% to 22%. The content was up to 36% in June, and gradually decreased after July, and fell to 12% in November. In addition, all the forest musk deer were healthy, and not used any antibiotics during the past 3 months.

Ten fecal samples were collected from forest musk deer in both summer (July, FS1–FS10) and winter (December, FW1–FW10). 4 fecal samples were collected from Siberian musk deer in both summer (July, SS6, SS8–SS10) and winter (December, SW1–SW4). Musk deer usually defecate in the early morning (around 5-6 o'clock) with fixed defecation sites. Fresh fecal samples were collected with a sterile disposable glove to avoid human contamination. Samples were stored in sterile centrifuge tubes, sealed, and placed on liquid nitrogen for transportation to the laboratory. Samples were then stored at -80°C until DNA extraction.

All experiment and animal care activities conducted according to the animal ethics guidelines of the Bioethics Committee of the College of Veterinary Medicine in Inner Mongolia Agricultural University (12150000460029509N) and Institute of Traditional Medicine and Technology, Ulaanbaatar, Mongolia. And we got a permit of CITES management authority of Mongolia in 2018.

DNA Extraction

Bacterial DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were crushed on dry ice before extraction. The DNA concentration was measured using a NanoDrop 2000 Spectrophotometer and confirmed by 1.0% agarose

gel electrophoresis. The concentration and purity of DNA were tested using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). Extracted DNA was stored at -80°C until PCR.

PCR Amplification and 16S rRNA Gene Sequencing

The hypervariable V3-V4 region of the 16S rRNA gene was amplified using the universal bacterial PCR primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') [57]. The total reaction volume was 20 µL mixture, and consisted of: 4 µL 5× FastPfu Buffer, 2 µL dNTPs (2.5mM), 0.8 µL Forward Primer (5µM), 0.8 µL Reverse Primer (5µM), 0.4 µL FastPfu Polymerase, 0.2 µL BSA, 10 ng Template DNA, and the remaining volume was ddH₂O. The PCR conditions were: 3 min at 95°C for initial denaturation; followed by 27 cycles of 95°C for 30s, 55°C for 30s and 72°C for 45s, and; a final extension step for 10 min at 72°C. PCR products were extracted from 2% agarose gels, purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using the QuantiFluor™-ST Kit (Promega, USA). Samples were sequenced on a MiSeq (300 bp paired-end reads) at the Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China).

Bioinformatics and Statistical Analyses

Sequence assembly and quality filtering were performed using FLASH (version 1.2.11; <https://ccb.jhu.edu/software/FLASH/index.shtml>) and QIIME (version 1.9.1; <http://qiime.org/install/index.html>), respectively. The quality control method was: 1) filter bases with a tail mass value < 20 and set a 50 bp window. If the average mass value in the window was lower than 20, the back base was truncated from the window. Reads less than 50 bp were removed; 2) Based on the overlap between the PE reads, the reads were merged with a minimum overlap length of 10 bp; 3) The maximum mismatch ratio allowed by the overlap area of the splicing sequence was 0.2; 4) Sequences were assigned to samples using barcoded primers. Operational taxonomic units (OTUs) were picked using Usearch (version 7.0.1090, <http://drive5.com/uparse/>).

Taxonomy was assigned to the OTUs using the Ribosomal Database Project (RDP) classifier (version 2.11, <http://sourceforge.net/projects/rdp-classifier/>) against the Silva 138(Release138 <http://www.arb-silva.de>) database with a confidence threshold of 0.97. Alpha diversity indices, including Chao, ACE, Shannon, Simpson, and Good's Coverage, as well as beta diversity analysis, were performed in Mothur (version v.1.30.2, http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity) and visualized using R (version 3.5.0). Wilcoxon rank-sum tests were used to determine statistically significant differences in alpha diversity and taxa relative abundance. Venn diagrams were used to visualize shared OTUs, which were defined as the core microbiome, using the Venn Diagram package in R. The community Bar charts were based on the data table in the tax_summary_a folder, use the R language tool for drawing. Heatmaps were generated using Bray–Curtis dissimilarity with average linkage hierarchical clustering using the vegan package in R. Principal co-ordinates analysis (PCoA) ordination based on Bray- Curtis dissimilarities and an analysis of similarity (ANOSIM) test were used to determine the composition differences and the significance of community compositional differences among groups. Perform PCoA

Statistical analysis and plotting used the vegan package in R. Differences between groups were determined using Kruskal-Wallis H-tests using the stats package in R and the SciPy package in Python (version 2.6.4). Linear discriminant analysis effect size (LEfse) analysis was conducted to indicate the significant factors using the online Galaxy workflow framework (<http://huttenhower.sph.harvard.edu/galaxy/>) and the cutoff value for the linear discriminant analysis score was set to the default level of 4.0 [58]. PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, <http://picrust.github.io/picrust/index.html>) was used to predict the functional gene content of microorganisms (Langille MG *et al.*, 2013)[59]. The predicted function is precalculated for genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) database were used to predict gene function. STAMP analysis was used to assess functional richness between groups. Welch's t-test was used for STAMP (version v2.1.3) software for differential analysis. Differences were considered as statistically significant when $P < 0.05$.

Abbreviations

SMD: Siberian musk deer (*Moschus moschiferus*)

FMD: forest musk deer (*Moschus berezovskii*)

FW: forest musk deer in the winter

FS: forest musk deer in the summer

SS: Siberian musk deer in the summer

SW: Siberian musk deer in the winter

OTUs: Operational taxonomic units

PCoA: Principal co-ordinates analysis

ANOSIM: an analysis of similarity test

LEfse: Linear discriminant analysis effect size analysis

KEGG: the Kyoto Encyclopedia of Genes and Genomes database

Declarations

Ethics approval and consent to participate

All experiment and animal care activities conducted according to the animal ethics guidelines of the Bioethics Committee of the College of Veterinary Medicine in Inner Mongolia Agricultural University

(12150000460029509N) and Institute of Traditional Medicine and Technology in Mongolia, Ulaanbaatar. And we got a permit of CITES management authority of Mongolia in 2018.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequences obtained in this study can be found in the NCBI Sequence Read Archive with the accession number: SRP198443.

Competing interests

All authors and funding bodies declare that they have no competing interests.

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Author contributions

SH and CC designed and led the project, and revised the manuscript; RNS contributed to complete all experiments and analysis of experimental data and wrote the manuscript. MD, ME and BL participated in the sample collection and analysis of experimental data. All authors have read and approved the final manuscript.

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Tables

Table 1. Detailed characteristics of four population groups in this study. FW: forest musk deer in the winter; FS: forest musk deer in the summer; SS: Siberian musk deer in the summer; SW: Siberian musk deer in the winter.

Species	Population group	Date	Age (yrs)	Number of samples	Geographic distribution	Diet
Siberian musk deer (SMD)	SW	Dec 2017	2-9	4	Mongolia, Ulaanbaatar	<i>Abietoideae, Usnea, etc.</i>
	SS	July 2018	2-9	4	Mongolia, Ulaanbaatar	<i>Acerginnala, Tricholomamongolicum, etc.</i>
Forest musk deer (FMD)	FS	Aug 2018	2.5-5	10	China, Inner Mongolia	fresh leaves and seasonal vegetables
	FW	Dec 2018	2.5-5	10	China, Inner Mongolia	dry leaves and seasonal vegetables

Table 2. Relative abundances (mean ± SD) of bacterial phyla in summer and winter.

Statistical significance was determined using Wilcoxon Rank-sum tests ($0.01 < p \leq 0.05$ marked as *, $0.001 < p \leq 0.01$ marked as **, $p \leq 0.001$ marked as ***).

Species	Species name	Summer (%)	Winter (%)	P-value
FMD	Firmicutes	53±8.31	64.95±11.73	0.021
	Bacteroidota	38.06±7.431	29.03±9.044	0.026
	Spirochaetota	1.578±2.032	3.405±6.944	0.791
	Verrucomicrobiota	3.752±5.117	1.08±1.821	0.031
	Proteobacteria	1.645±2.527	0.552±1.119	0.076
	Actinobacteriota	0.798±0.67	0.363±0.387	0.034
	Cyanobacteria	0.698±0.342	0.281±0.229	0.011
	Patescibacteria	0.133±0.046	0.138±0.341	0.017
	Desulfobacterota	0.087±0.044	0.093±0.042	0.649
	Synergistota	0.154±0.358	0.021±0.031	0.176
	Campilobacterota	0.019±0.027	0.039±0.044	0.618
	Rs-K70_termite_group	0.02±0.039	0.038±0.07	0.967
	Elusimicrobiota	0.032±0.031	<0.01	<0.01
	Myxococcota	<0.01	<0.01	0.017
	Deferribacterota	0	<0.01	0.034
SMD	Firmicutes	86.2±5.713	90.21±3.265	0.312
	Bacteroidota	12.75±6.008	8.876±2.31	0.665
	Proteobacteria	0.311±0.159	0.669±1.046	0.665
	Cyanobacteria	0.252±0.111	0.137±0.072	0.194
	Actinobacteriota	0.208±0.182	0.012±0.008	0.147
	Desulfobacterota	0.149±0.181	0.069±0.01	0.885
	Patescibacteria	0.029±0.039	<0.01	0.307
	Elusimicrobiota	0.034±0.030	0	0.069
	Verrucomicrobiota	0.026±0.017	<0.01	0.11
	unclassified_k__norank_d__Bacteria	0.019±0.004	<0.01	0.028
	Acidobacteriota	<0.01	0	0.453
	Planctomycetota	<0.01	0	0.453
	Myxococcota	<0.01	0	0.453

Figures

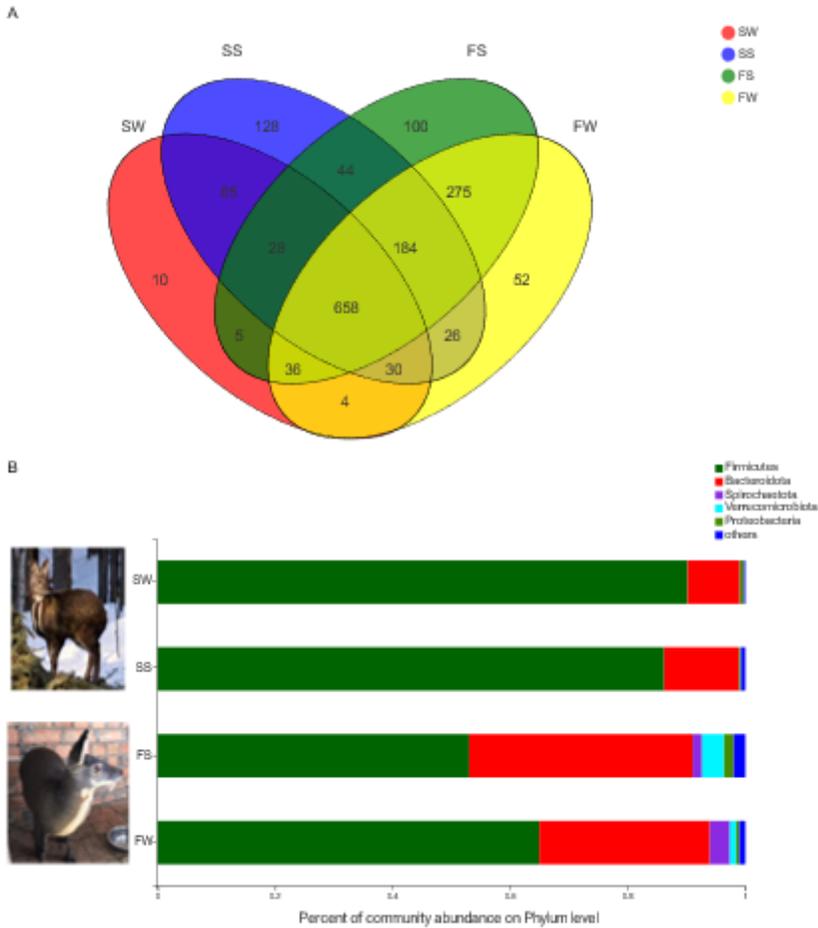


Figure 1

OTUs and bacterial taxa shared between the four groups. Venn diagrams show the number of OTUs shared by forest musk and Siberian musk deer sampled during two seasons (A). Bar charts showing the core bacterial taxa at the phylum level for each group: (B). All taxa with relative abundances less than 1% were grouped as "Other taxa". FW: forest musk deer in the winter; FS: forest musk deer in the summer; SS: Siberian musk deer in the summer; SW: Siberian musk deer in the winter.

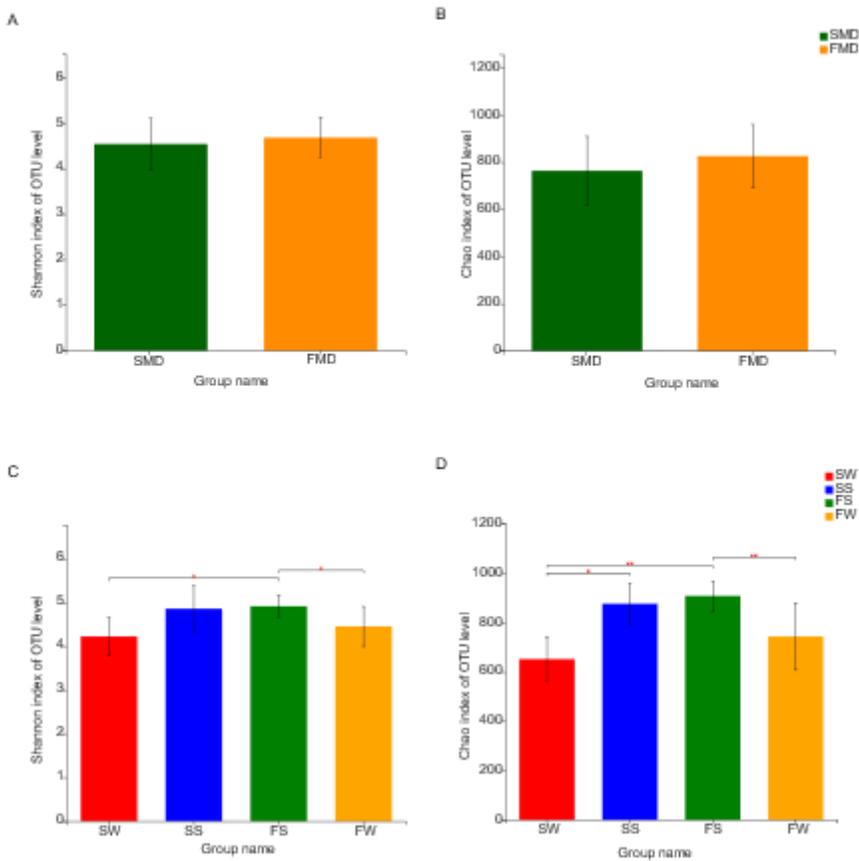


Figure 2

Differences in microbial diversity (Shannon and Chao) between the four groups. Histogram (A) showing the differences in the Shannon index between the SMD and FMD. Histogram (B) showing the differences in the Chao index between the SMD and FMD. Histogram (C) showing the differences in the Shannon index between the four groups. A plot (D) showing the differences in the Chao index between the four groups. Significance was determined using Wilcoxon Rank-sum tests. ($0.01 < p \leq 0.05$ marked as *, $0.001 < p \leq 0.01$ marked as **, $p \leq 0.001$ marked as***).FW: forest musk deer in the winter; FS: forest musk deer in the summer; SS: Siberian musk deer in the summer; SW: Siberian musk deer in the winter.

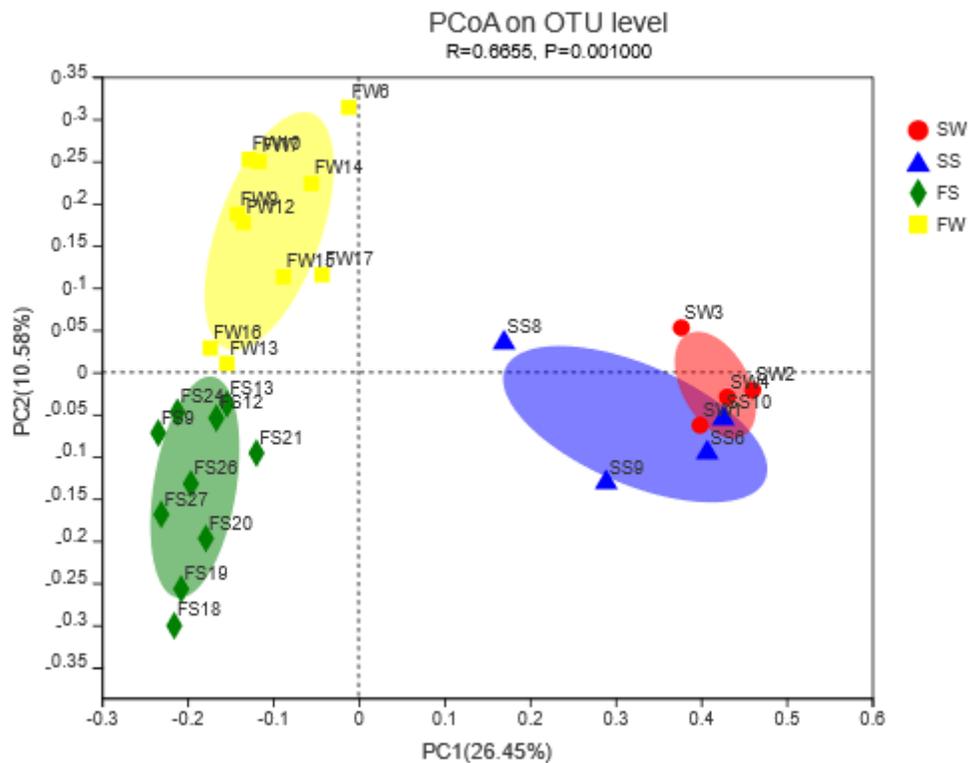


Figure 3

Non-metric multidimensional scaling (PCoA) plots. To show the distances between the samples based on dissimilarity in the OTU composition of each sample, which was calculated using the Bray–Curtis dissimilarity index. Different colors represent different groups. FW: forest musk deer in the winter; FS: forest musk deer in the summer; SS: Siberian musk deer in the summer; SW: Siberian musk deer in the winter.

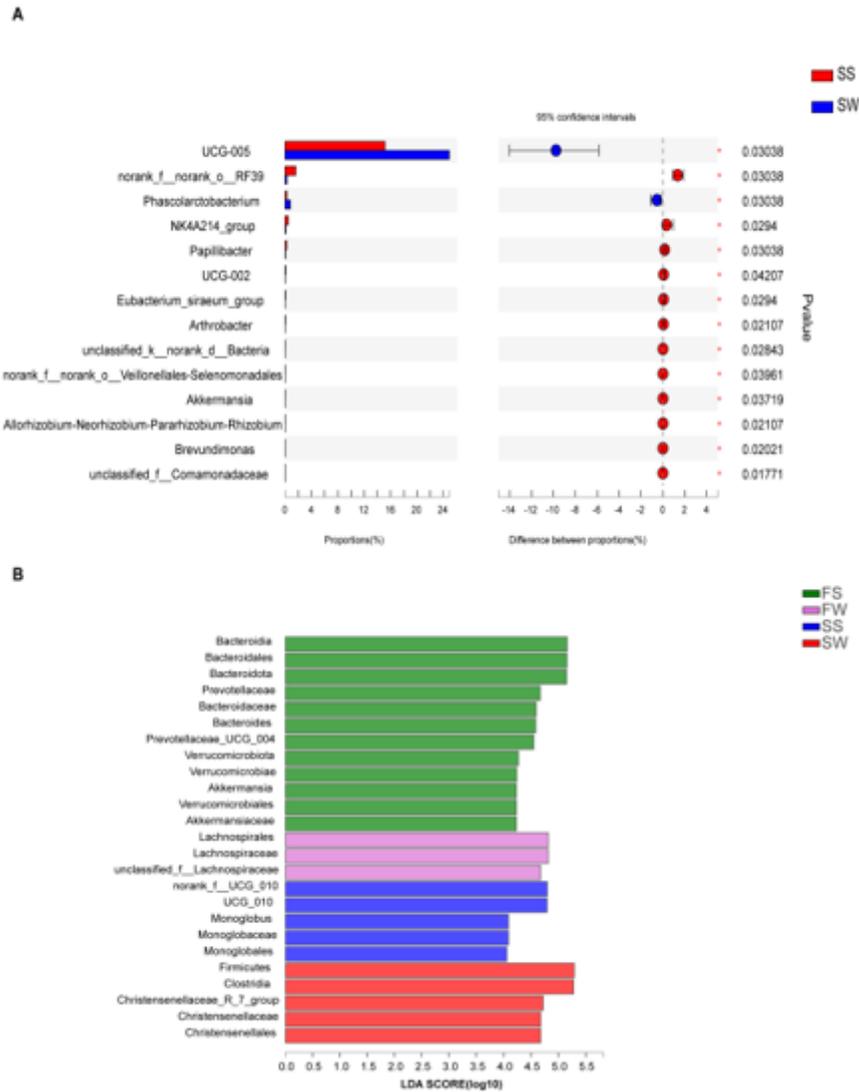


Figure 4

Seasonal variations in bacterial composition in musk deer. Histogram (A) showing differentially abundant taxa at the phylum level in SMD. Significance was determined using Wilcoxon Rank-sum tests. ($0.01 < p \leq 0.05$ marked as *, $0.001 < p \leq 0.01$ marked as **, $p \leq 0.001$ marked as ***). LDA histogram (B) showing the differences in taxa relative abundance. The length of the bar column represents the LDA score ($LDA > 4$). A larger LDA score indicates a higher contribution of that species to the overall differences in community structure. FW: forest musk deer in the winter; FS: forest musk deer in the summer; SS: Siberian musk deer in the summer; SW: Siberian musk deer in the winter.

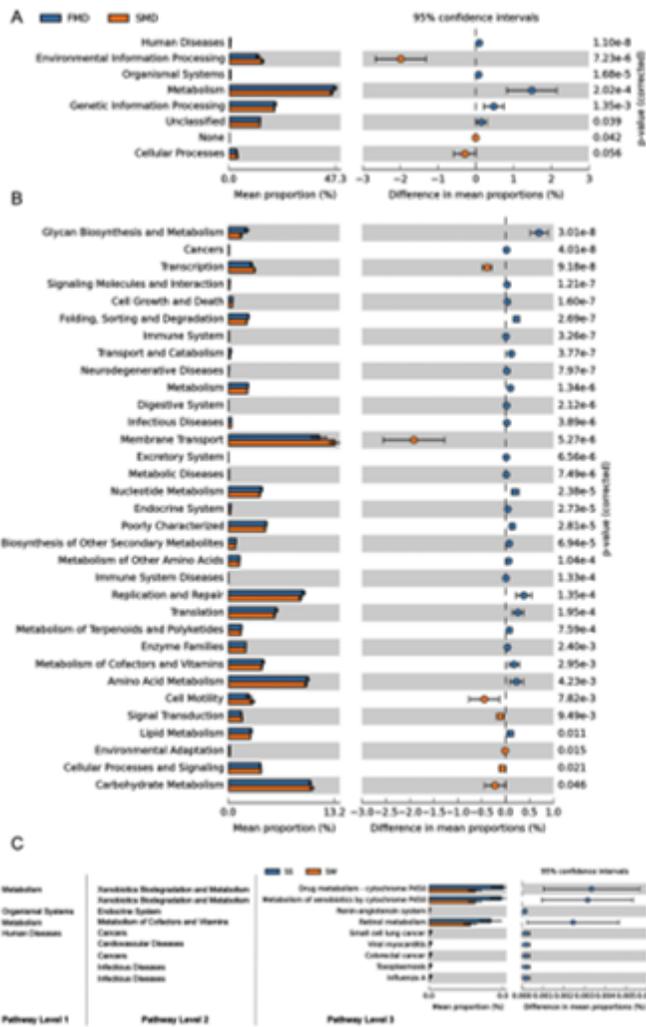


Figure 5

Predication of microbial function. Histogram (A) comparing functional pathways in SMD and FMD (Level 1). Histogram (B) comparing functional pathways in the FMD and SMD groups (Level 2). Histogram (C) showing seasonal differences in functional pathways in SMD.

Supplementary Files

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