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RESEARCH

Post-defecation exposure alters gut microbiota of forest musk deer with implications for conservation metagenomics

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Abstract

In endangered species conservation, fecal samples are a vital non-invasive tool for gut microbiota analysis. Yet, the influence of external exposure time on microbial composition and function remains unclear, constraining data accuracy and reliability. To address this, we investigated the time-gradient effect in the globally endangered forest musk deer (*Moschus berezovskii*). Using non-invasive sampling under standardized captive conditions, fecal samples were collected at six storage times: (0, 1, 2, 4, 6, 8 days). Gut microbiota composition, diversity, enterotypes, and functional differences were assessed through 16S rRNA gene sequencing on the Illumina MiSeq platform. In total, 147,013 valid ASVs (amplicon sequence variants) were obtained showing significant shifts in microbial composition with storage time. Dominant phyla included *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. Increasing storage time led to declining α-diversity, reduced community stability, and more unique genera. PCoA (principal coordinates analysis) and NMDS (non-metric multidimensional scaling) indicated progressive separation of experimental groups from control groups, with Anosim and Adonis confirming progressive separation with storage time. Structurally, *Firmicutes* decreased while *Proteobacteria*, specifically the *Acinetobacter* genus, increased with storage time. Community assembly shifted from deterministic to stochastic processes, reflecting stronger environmental disturbance effects. These results demonstrate that the gut microbiota composition, diversity, and ecological functions in forest musk deer feces are highly sensitive to storage time. Thus, preservation duration must be strictly controlled as a critical variable in microbiome studies. This work establishes methodological standards for non-invasive fecal metagenomics in endangered species, providing theoretical insights and practical guidance for improving scientific rigor in conservation-related microbiome research.

Key points

- *Fecal microbiota diversity and stability decline significantly with longer storage.*
- *Firmicutes decrease while Proteobacteria, especially Acinetobacter, increase over time.*
- *Storage duration strongly impacts microbiome data, requiring strict sampling control.*

Keywords Forest musk deer · 16S rRNA gene sequencing · Fecal storage time gradient · Gut microbiota · Microbial composition

Introduction

In the context of the ongoing global decline in biodiversity, the conservation of endangered species represents a critical component of global biodiversity preservation, ecosystem restoration, and sustainable development (Pereira et al. 2024; Maxwell et al. 2020). Fecal sampling enables the collection of biological information from endangered species when invasive sampling of tissues such as muscle, blood, or stomach contents is often unfeasible due to small population sizes and elusive behavior, providing a non-invasive approach to investigate host genetics, health status, and host and gut microbiota interactions (Grieneisen et al. 2021; Turjeman et al. 2023). Fecal sampling not only serves as an effective non-invasive method but also represents a crucial tool in conservation biology, enabling the collection of biological information from endangered species without direct contact or disruption to their health and behavior, thereby providing

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a low-disturbance alternative particularly suited for rare, dispersed, or highly vigilant taxa (Zhou et al. 2020a, b). Additionally, fecal samples contain abundant biological information, including host DNA, gut microbial communities, hormone levels, parasite species, and metabolic products, which make them uniquely valuable for research in genetics, nutrition, behavioral ecology, and pathology (Hu et al. 2024). The unique value of fecal samples in the conservation of endangered species has been validated in numerous studies (Huang et al. 2023). By extracting host DNA genetic information from feces, the genetic diversity, population dynamics, and potential recovery mechanisms of endangered species can be tracked (Wang et al. 2024). DNA metabarcoding of fecal samples can quantify the dietary characteristics, ecological niche differentiation, and predator-prey food web structure of coexisting endangered species (Shao et al. 2021). Analyzing residual metabolic products and gut microbiota composition in feces can not only assess individual health status, adaptive response mechanisms (Wang et al. 2021), and the microbial response mechanisms to seasonal dietary changes at the species and functional level (Li et al. 2023a, b, c), but also serve as a key indicator for evaluating the success of wild reintroduction programs for captive endangered species (Huang et al. 2024). Hormonal analysis in feces can provide insights into the physiological state, acute and chronic stress levels, and reproductive potential of individuals (Yan et al. 2024; Nie et al. 2015). It is evident that the importance of fecal samples in the conservation of endangered species cannot be overlooked. Therefore, under the current urgent circumstances of endangered species research and conservation, fully exploring and utilizing the research potential of fecal samples will contribute significantly to global biodiversity conservation efforts.

The gut microbiota, as the host's "second genome," has evolved alongside the host to form a complex and powerful microecosystem that plays an irreplaceable role in maintaining host health and ecological adaptation, participating not only in the host's nutritional metabolism and energy acquisition but also in immune regulation, pathogen defense, and behavioral modulation (Foster et al. 2017; iMSMS 2022; Wilde et al. 2024). The composition and diversity of the gut microbiota reflect the complex interactions between the host and its external environment, serving as a key biological indicator of host health status (Schluter et al. 2020; Kennedy et al. 2023). The composition and diversity of the host gut microbiota are shaped by a combination of factors, including diet composition (Gentile and Weir 2018), habitat conditions (Gacesa et al. 2022), social behavior (Wu et al. 2021), and genetic background (Cortes-Ortiz and Amato 2021). With the rapid advancement of sequencing technologies, high-throughput sequencing-based investigations of gut microbial composition, diversity, and key microbial taxa have provided novel approaches for the conservation of endangered species (Wei et al. 2015). Established as an emerging discipline in 2018, conservation metagenomics aims to provide scientific foundations for the conservation and management of endangered species by investigating the genetic composition, structure, and functional attributes of microbial communities (Wei et al. 2019; Hu et al. 2024). With the advent of reference genome resources, the field of conservation genomics has entered a new era, becoming a pivotal approach in global biodiversity conservation efforts (Formenti et al. 2022). A large-scale metagenomic study of the gut microbiota of over 180 wild animal species has led to the development of an extensive annotated bacterial genome database, which includes previously unknown species extracted from the gut of wild animals, highlighting the potential application of wildlife microbiomes in the discovery of industrial enzymes and therapeutic agents (Levin et al. 2021). The metagenomic study on the conservation of giant pandas (*Ailuropoda melanoleuca*) revealed that the species' gut microbiota more efficiently degrades bamboo shoots, facilitating the formation of butyrate, and that the bamboo shoot consumption period is a critical time for energy and nutrient replenishment, offering insights into how giant pandas adapt to high-fiber, low-fat diets from a conservation genomics perspective (Huang et al. 2022). A comparison of gut microbiota differences between humans and primates, such as chimpanzees (*Pan troglodytes verus*, *P. troglodytes troglodytes*, *P. troglodytes schweinfurthii*), bonobos (*Pan paniscus*), and gorillas (*Gorilla gorilla gorilla, G. beringei beringei*), revealed significant microbial community differences between humans and non-human apes, and highlighted the correlation between the loss of human gut microbiota diversity and high human development indices, as well as the important roles of repeated horizontal gene transfer, gene loss, and adaptation to transient hypoxic conditions in the evolution of gut microbiota (Rühlemann et al. 2024). Together, these studies illustrate how microbiome research can support the conservation of endangered species by informing dietary adaptation, monitoring physiological health, and guiding non-invasive sampling strategies such as fecal analysis, which is particularly valuable for species that are difficult to sample directly (Turjeman et al. 2023). By studying the diversity and functional variation of fecal microbiomes, dietary habits, environmental adaptability, and disease resistance of endangered species can be inferred, providing scientific support for habitat conservation and population management. Therefore, integrating gut microbiota research with the multidisciplinary perspective of conservation metagenomics will open new research directions and practical approaches for endangered species conservation. In the analysis of gut microbiota and conservation metagenomics of endangered species, fecal samples, as important non-invasive materials, directly determine the accuracy and scientific validity of research data (Song et al.

2016; Zhou et al. 2020a, b). In addition to the changes in fecal quality caused by different preservation methods (Li et al. 2023a, b, c), the quality of feces changes continuously after excretion, and the exposure time significantly affects the microbial composition and functional characteristics of the samples (Moreno-Torres et al. 2018). Even in frozen environments, the microbial composition and activity in feces alter over time, as microbial metabolism decomposes organic components, further altering the microbial community structure and function (Costello et al. 2015), highlighting the critical importance of exposure duration for understanding the complex host-microbiome relationships, especially in endangered species, where it may lead to misjudgments about population health and habitat adaptability.

The forest musk deer (*Moschus berezovskii*), a typical endangered species, plays a crucial role in ecosystems and, as both an endangered species and the most successfully bred species within the *Moschidae* family, has become an important subject in conservation biology research. This species is globally endangered, with its wild population experiencing a sharp decline, and China, as the country with the richest resources of musk deer, holds over 70% of the global musk deer population (Yang et al. 2003; Jiang et al. 2020). However, due to the economic value of musk deer, habitat fragmentation, and long-term hunting, musk deer populations have decreased by over 97% since the 1950 s, with fewer than 31,800 wild individuals remaining, classified as endangered and critically endangered by the IUCN (International Union for Conservation of Nature) and China's Red List of Vertebrates (Jiang et al. 2022), highlighting the urgency of their conservation. To effectively mitigate the sharp decline of musk deer resources and sustainably utilize musk, China began artificial breeding research on various musk deer species in 1958, with the breeding scale and population of the forest musk deer significantly surpassing that of other musk species, making it the largest and most widely farmed musk deer species in the country. The stability of this captive population provides numerous advantages for experimental design. First, the captive environment allows precise control over variables such as feed composition and temperature and humidity, reducing interference from uncontrollable factors in the wild; second, the continuous collection of fecal samples ensures the integrity of time-series data, facilitating the tracking of microbiome dynamics; moreover, the forest musk deer, as a successful example of captive breeding for an endangered species, provides a reference model for ex situ conservation of other endangered species. In summary, the forest musk deer, as a successfully bred endangered species and a species with high economic value due to musk production, combines the advantages of conservation significance and controlled captive conditions, making it a valuable non-model animal for investigating the relationship between fecal excretion time and gut microbiota composition, and providing insights for conservation genomics research.

This study focuses on the globally endangered forest musk deer as the research subject, employing non-invasive sampling methods to collect fecal samples. By combining 16S rRNA gene amplicon sequencing with the Illumina Miseq high-throughput sequencing platform (Illumina, San Diego, California, USA), the research aims to systematically analyze the dynamic changes in gut microbiota composition, diversity, enterotype, and function across different storage time gradients in captive forest musk deer. The study addresses the following key scientific questions: (i) the succession patterns of gut microbiome composition and dominant microbiota under different storage time series; (ii) the dynamic changes in microbiome diversity, functional characteristics, and enterotype differentiation under different storage time series; (iii) the identification of the post-excretion time window during which fecal microbiota most accurately represent the internal gut microbiome, thereby determining the optimal sample storage time for microbiome analysis. By elucidating the responses of the gut microbiota in captive forest musk deer to fecal storage time, this study provides practical evidence for optimizing fecal sample collection protocols and supporting microbiome-based health assessment and management in captive musk deer. Additionally, this research will contribute to the development of a fecal storage time-based model for studying the gut microbiota of endangered species, offering theoretical references and technical support for the collection, preservation, and subsequent metagenomic research of fecal samples from other endangered species, thereby advancing the field of conservation biology.

Materials and methods

Sample collection and processing

This study was conducted at a forest musk deer breeding facility located in Arou Township, Qilian County, Haibei Tibetan Autonomous Prefecture, on the northeastern edge of the Qinghai-Tibet Plateau. At the breeding facility, the diet of the forest musk deer consisted of both concentrate and roughage feeds, with the concentrate feed mainly comprising carrots, potatoes, maize, and pumpkins, and the roughage primarily consisting of leaves from various fruit tree species. In addition, the facility features standardized enclosed pens, each physically isolated to prevent inter-individual interference. To ensure precise correspondence between fecal samples and individual deer, as well as to avoid repeated sampling, the selected enclosures were thoroughly cleaned 24 h prior to sampling, and the target individuals were isolated overnight in separate pens. Fresh fecal samples were

systematically collected from each pen the following morning. During sampling, to prevent cross-contamination, each fecal sample was collected as soon as possible after defecation using disposable polyethylene gloves and immediately sealed in sterile zip-lock bags.

Using a non-invasive sampling method, fresh fecal samples were collected from 24 healthy forest musk deer individuals at the breeding facility to serve as the control group ($n = 24$). Eight individuals were randomly selected from this cohort, and their fecal samples were homogenized and equally divided into three portions, which were then placed into sterile paper cups and exposed to ambient room temperature. Fecal subsamples were placed into individually covered sterile paper cups for the temporal gradient experiment to minimize microbial dispersal between samples, ensuring that observed changes over time reflected intrinsic microbial dynamics rather than cross-contamination. Subsamples were collected at five distinct post-defecation time points: 1 day (T1 group), 2 days (T2 group), 4 days (T3 group), 6 days (T4 group), and 8 days (T5 group), resulting in 24 samples per experimental group (8 individuals × 3 replicates). In total, 144 fecal samples were obtained across the control and all time-gradient treatment groups. All samples were labeled and temporarily stored at − 20 °C in a portable vehicle refrigerator or on dry ice. Upon return to the laboratory, samples were promptly transferred to a − 80 °C ultra-low temperature freezer for long-term storage prior to DNA extraction and downstream microbial analyses.

Fecal microbial DNA extraction, amplification, and high-throughput sequencing for experimental and control groups

Microbial DNA was extracted from forest musk deer fecal samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA). DNA concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and DNA integrity and purity were assessed by 1% agarose gel electrophoresis (5 V/cm, 20 min). The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using universal primers 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), with extracted DNA as the template. The PCR reaction volume was 20 µL, comprising 4 µL of 5 × TransStart FastPfu buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL each of forward and reverse primers (5 µM), 0.4 µL of TransStart FastPfu DNA polymerase (Omega Bio-Tek, Norcross, Georgia, USA), approximately 10 ng of template DNA, and sterile ddH_2O to reach a final volume of 20 µL; ddH_2O was used as a negative control. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 min; 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min. PCR products were stored at 4 °C until further use.

Each fecal sample was subjected to three independent PCR amplifications, and the resulting amplicons were pooled, from which 3 µL was selected for subsequent analysis. The pooled products were separated by 2% agarose gel electrophoresis, and the target bands were excised and purified using an AxyPrep DNA Gel Extraction Kit (Axygen, Union City, California, USA). The concentration of purified DNA was quantified using a Qubit 4.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sequencing libraries were constructed using the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, Texas, USA), and libraries that passed quality control were sequenced on the Illumina MiSeq platform (Illumina, San Diego, California, USA) with paired-end reads of 250 base pairs. Library preparation and high-throughput sequencing were primarily performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Sequencing data processing and annotation

The raw sequencing reads were subjected to quality control using fastp (version 0.19.6, https://github.com/OpenGene/fastp) to remove barcode and primer sequences (Chen et al. 2018). Paired-end reads were then merged based on their overlapping regions using FLASH (Fast Length Adjustment of Short reads, version 1.2.11, http://www.cbcb.umd.edu/software/flash) (Magoč and Salzberg 2011). Subsequently, the merged and quality-filtered reads were denoised using the DADA2 plugin (Callahan et al. 2016) implemented in the QIIME2 analysis pipeline (Version 2019.07) (Bolyen et al. 2019). This denoising process yielded high-resolution amplicon sequence variants (ASVs), which represent distinct biological sequences inferred from the raw data.

Based on the Silva 16S rRNA gene database (version 138), the Naive Bayes classifier within QIIME2 was used for taxonomic classification of the ASVs (https://forum.qiime2.org/t/processing-filtering-and-evaluating-the-silva database-and-other-reference-sequence-data-with-rescript/15494). Spike-in ASVs were identified and extracted from each sample, and a standard curve equation was constructed using their sequencing read counts to calculate the absolute copy numbers of taxonomic units in each sample. The 16S rRNA gene copy numbers for different taxa were then normalized using the rrnDB (https://rrndb.umms.med.umich.edu/) database (Stoddard et al. 2015). The raw ASV representative sequences and abundance tables were processed using QIIME2 (Version 2019.07) (Bolyen et al. 2019), and reference-based clustering was performed using the Greengenes 13_5 database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_5_otus.tar.gz). The resulting clustering data were transformed and uploaded to

the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) platform for functional prediction analysis (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=PICRUSt_normalize). The platform first standardized the data, then generated a functional prediction table, which was used for further analysis of the microbial community's functional potential.

Data statistical analysis

After taxonomic annotation of ASVs, the abundance of each ASV was quantified for each sample. To ensure comparability among samples, all samples were rarefied to the minimum sequencing depth observed across the dataset. The relative abundance of taxa at the phylum and genus levels was calculated and visualized using bar plots for the musk deer samples (using the "stats" package). Venn diagrams were created to depict the shared and unique bacterial phyla and genera across different groups. A clustering heatmap (using the "pheatmap" package) was employed to compare the similarities and differences in the gut microbiota composition between the groups. Alpha diversity (α-diversity) reflects the diversity of gut microbiota composition. In this study, the Sobs (the actual observed ASVs), Shannon, Chao1, and Pielou_e indices were selected to analyze the diversity of musk deer gut microbiota composition. The Sobs and Chao1 indices reflect the richness of the gut microbiota, the Shannon index reflects the diversity of the gut microbiota, and the Pielou_e index reflects the evenness of the gut microbiota. The four α-diversity indices were calculated using QIIME2 software, and the Wilcoxon rank-sum test was used to analyze significant differences in the four α-diversity indices between groups.

Beta diversity (β-diversity) compares the species diversity between different microbial communities and can reflect the differences in the gut microbiota composition between different groups of musk deer. In this study, multiple similarity distance algorithms, such as Bray-Curtis, abundance Jaccard, binary Euclidean, Euclidean, and Hellinger, were used to calculate the distances between samples at the ASV level. Principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) were used to analyze the beta diversity between the groups (using the "vegan" package). PCoA is an unconstrained data reduction method that is used to investigate the similarity and dissimilarity of microbial communities between samples. NMDS analysis maps the species information from samples into a multidimensional space, with the points representing the samples, where the distance between points reflects the degree of difference between samples, generating a spatial map of the samples. Inter-group differences were tested using both Anosim (analysis of similarities) and Adonis (permutational MANOVA, permutational multivariate analysis of variance) analyses (packages "vegan," anosim function). ANOSIM quantified group separation using the R statistic, whereas Adonis assessed the proportion of variance explained by grouping factors through the R^2 statistic with permutation-based significance testing. Deterministic and stochastic contributions to gut microbiota assembly were evaluated using normalized stochasticity ratio (NST) and beta nearest taxon index (βNTI) analyses. Deterministic processes (homogeneous and heterogeneous selection) and stochastic processes (dispersal limitation, homogeneous dispersal, and drift) were inferred from ASV-level taxonomic and phylogenetic data, providing insight into the mechanisms shaping community structure.

At the phylum and genus levels, this study used the Wilcoxon rank-sum test to analyze the differences in the gut microbiota of musk deer across different groups. In the Wilcoxon rank-sum test, a two-tailed test was selected, and the *P*-values were adjusted for multiple testing using the false discovery rate (FDR), with a confidence level set at 0.95. To identify differentially abundant species between groups, the LEfSe software (version 1.0, http://galaxy.biobakery.org/) was used to analyze significant species differences between the groups at the genus level. linear discriminant analysis (LDA) scores were employed to quantify the contribution of the differential species to group differences. The LDA analysis threshold was set from 0 to 5.0, with a 0.5 increment, resulting in 11 different threshold settings.

The Jensen-Shannon divergence (JSD) distance was selected due to its ability to effectively capture compositional differences in microbiome data, while the Calinski-Harabasz (CH) index was applied to objectively evaluate the compactness and separation of clusters. Therefore, the JSD distance algorithm was used to calculate the distances between fecal samples of musk deer at different storage time gradients based on the ASV relative abundance matrix. The CH index was employed to assess the quality of clustering. The optimal number of clusters was determined by selecting the cluster count corresponding to the maximum CH index value, which was then used to analyze the enterotypes of the gut microbiota across different groups and their temporal variations. Additionally, the study conducted a comprehensive analysis of the α-diversity, β-diversity, and community assembly process of the gut microbiota for different enterotypes. Neutral Community Model (NCM) analysis was applied to assess the influence of stochastic processes on gut microbiota assembly by relating species occurrence frequency to their relative abundance. The model's goodness of fit (R^2) indicates the extent to which community assembly is driven by random dispersal and ecological drift, with higher R^2 values reflecting stronger stochastic influence. Furthermore, PICRUSt was used to normalize the ASV abundance table, and functional abundance information at multiple levels, including KEGG Orthology (KO), pathway, and Clusters of Orthologous Groups (COG) functions, was obtained by

comparison with the KEGG (Kyoto Encyclopedia of Genes and Genomes) and EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) databases. In addition, this study employed the BugBase analysis tool (https://bugbase.cs.umn.edu/index.html) to systematically predict and comparatively analyze high-level phenotypes of the gut microbial communities in the control and experimental groups of forest musk deer. The Wilcoxon rank-sum test (for two-group comparisons) or the Kruskal-Wallis rank-sum test (for multiple group comparisons) was applied to analyze the inter-group differences in metabolic-related functions. In the Kruskal-Wallis rank-sum test, FDR adjustment was also applied to the *P*-values, and post hoc testing was performed using the Tukey-Kramer method.

Results

Analysis of changes in gut microbiota composition and α-diversity of captive forest musk deer across different fecal storage time gradients

A total of 144 raw fecal samples from captive forest musk deer were processed through quality filtering and optimization, resulting in high-quality, effective sequences. After rarefaction based on the minimum sequence depth, a total of 147,013 effective ASVs (amplicon sequence variants) were identified across all storage time gradients. Taxonomic annotation of these ASVs revealed that they were classified into 31 phyla, 85 classes, 200 orders, 366 families, and 909 genera.

Taxonomic annotation and relative abundance analysis of all fecal sequencing samples from forest musk deer revealed that, at the phylum level, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were the dominant phyla (relative abundance > 1%) commonly shared across all storage time gradients, collectively accounting for 97.69% of the gut microbiota (Fig. 1a). At the genus level, the dominant genera consistently present across all groups included *Acinetobacter*, *UCG-005*, *Christensenellaceae R-7 group*, *Bacteroides*, *Arthrobacter*, *Rikenellaceae RC9 gut group*, and *Alistipes*. These genera were primarily affiliated with the four dominant phyla mentioned above (Fig. 1b).

Venn diagram analysis revealed that fecal samples of forest musk deer from the six storage time gradient groups shared 20 bacterial phyla. Two unique phyla, *Dependentiae* and *Latescibacterota*, were detected exclusively in the T3 group. However, further analysis showed that *Dependentiae* and *Latescibacterota* originated from only two fecal samples and one fecal sample, respectively, indicating a lack of representativeness. Therefore, it can be concluded that no truly unique bacterial phyla were present across the six groups. In contrast, a total of 399 bacterial genera were shared among all six experimental groups, while each group also harbored unique genera. From T1 to T5, the number of unique genera exhibited an increasing trend with prolonged storage time (Fig. 1c).

The results of the α-diversity of gut microbiota in captive forest musk deer showed that α-diversity exhibited an overall decreasing trend with increasing storage time, and the dispersion among samples gradually increased as well (Fig. 1d). The community stability of the gut microbiota in different groups was assessed using the average variation degree (AVD), where a lower AVD value indicates higher microbial stability. The analysis revealed that the microbial stability of forest musk deer also declined as storage time increased (Fig. 1e).

β-Diversity and its variation trends in the gut microbiota composition of forest musk deer under different fecal storage time gradients

Principal coordinates analysis (PCoA) based on the Bray-Curtis distance revealed that the degree of separation between the fecal samples from the experimental groups and the control group increased with prolonged storage time. ANOSIM results showed significant differences in gut microbial composition between each of the five experimental time points and the control group ($P < 0.05$). The R values calculated by ANOSIM quantified the degree of dissimilarity, and the results indicated a general upward trend in R values with increasing storage time, suggesting progressively greater divergence in gut microbiota between the experimental and control groups (Fig. 2a). Similarly, non-metric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distance revealed that the stress values for both the control and experimental groups were below 0.2, indicating a good representation of the actual distribution of the data and an accurate reflection of microbial composition differences across time points. Moreover, consistent with the PCoA results, the degree of separation between the experimental groups and the control group increased with longer storage times. Adonis test results, quantified by the R^2 values, also demonstrated an upward trend with increasing storage time, indicating that the significant differences in gut microbiota between the experimental and control groups became progressively more pronounced (Fig. 2b).

In addition to Bray-Curtis, four supplementary metrics were used to perform the ANOSIM and Adonis tests, calculating R and R^2 values along with their corresponding *P*-values for significance testing, which showed an increasing trend at both the ASV and genus levels with prolonged storage time. This consistently indicated that the significant differences in gut microbiota between the experimental and control groups of forest musk deer increased over time, suggesting that the longer the storage duration, the greater the

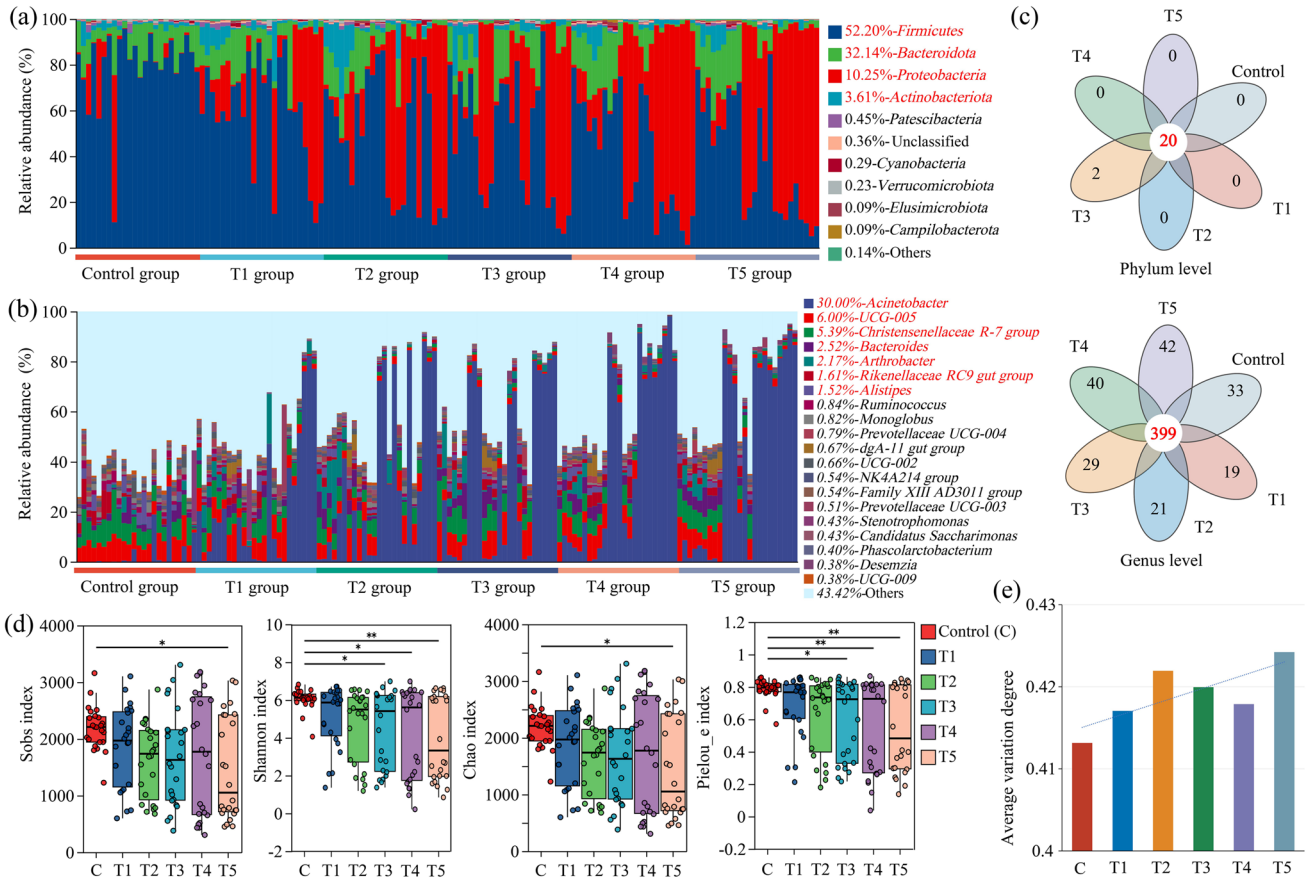

Fig. 1 Composition and diversity analysis of gut microbiota in captive forest musk deer under different fecal storage time gradients. **a**, **b** Relative abundance of gut microbiota at the phylum (**a**) and genus (**b**) levels across different time groups. Red labels indicate dominant phyla or genera (relative abundance > 1%). **c** Venn diagram showing shared and unique bacterial phyla and genera among different time groups. **d** Analysis of α-diversity differences in gut microbiota across different time groups. **e** Microbial community stability index (average variation degree, AVD) across different time groups

divergence in gut microbial composition (Fig. 2c). At the ASV level, β-diversity analysis between groups based on the Bray-Curtis distance algorithm showed that, with increasing storage time, the degree of dispersion among forest musk deer fecal samples within each group also increased. This implies that the variability in gut microbial composition among individual samples became more pronounced as the storage time extended (Fig. 2d).

To assess the differences in the assembly processes of gut microbiota among different groups of forest musk deer, normalized stochasticity ratio (NST) analysis revealed that the NST values for both the control and experimental groups were below 50%, indicating that the gut microbial community assembly was predominantly governed by deterministic processes (Fig. 2e). Combined with βNTI (beta nearest taxon index) analysis, it was found that among deterministic processes, homogeneous selection (HoS) first exhibited a decreasing trend followed by an increase, while heterogeneous selection (HeS) showed a general decreasing trend. Within stochastic processes, both dispersal limitation (DL) and homogeneous dispersal (HD) showed an overall increasing trend, whereas drift and other ecological processes (DR) exhibited a decreasing trend (Fig. 2f). Furthermore, the absolute values of βNTI across all groups were less than 2, indicating that stochastic processes also contributed to gut microbiota assembly, although deterministic processes remained predominant (Fig. 2g).

Analysis of differences in dominant gut microbiota and their temporal variation trends in forest musk deer across different fecal storage time gradients

At the phylum level, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were identified as the dominant bacterial phyla commonly present in the gut microbiota of forest musk deer across different fecal storage time gradients, with their relative abundances varying considerably across time points (Fig. 3a). Among these dominant phyla, *Firmicutes* and *Proteobacteria* exhibited significant differences between groups over time. Specifically, the relative abundance of

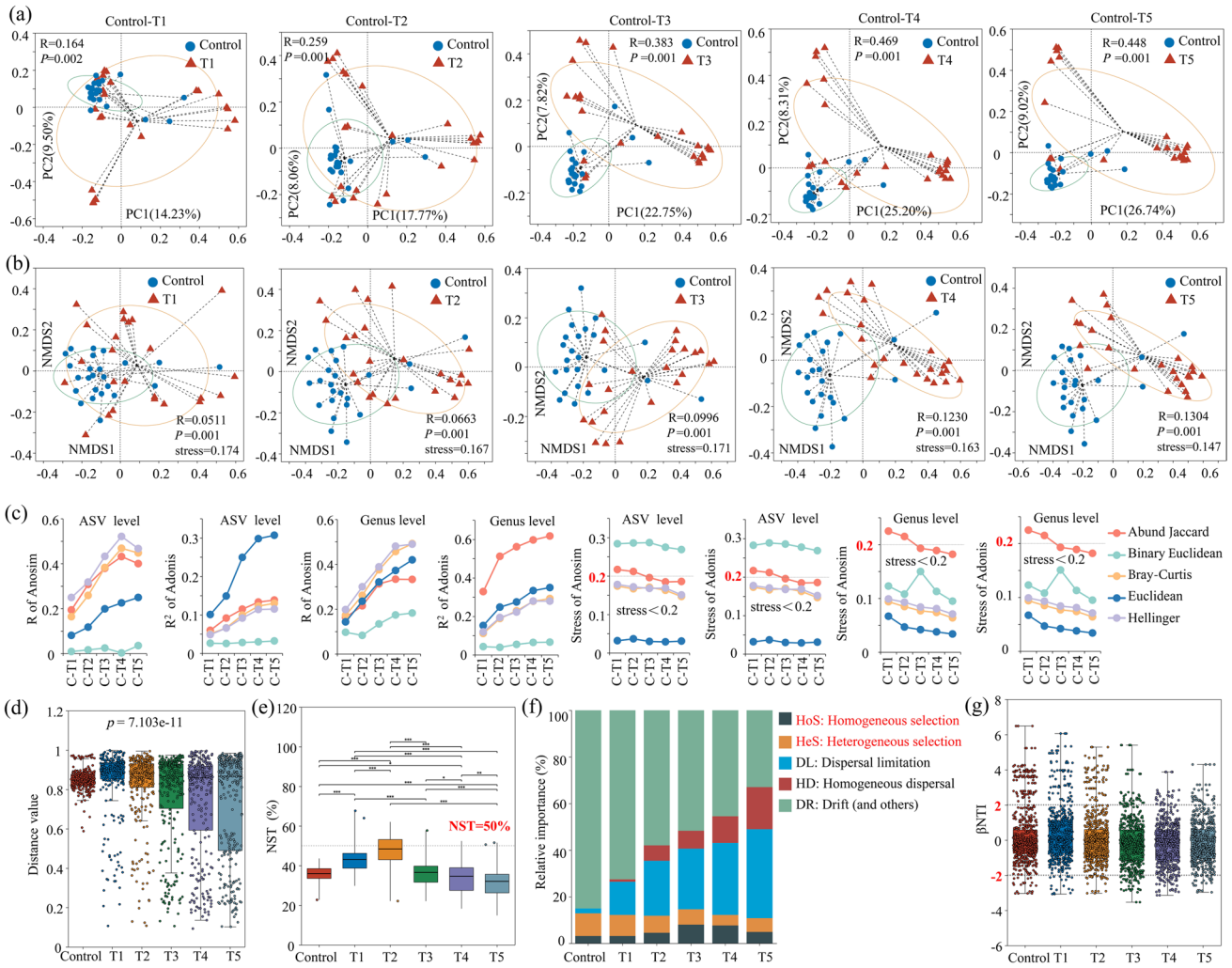

Fig. 2 Analysis of β-diversity and its temporal variation in the gut microbiota composition of forest musk deer across different fecal storage time gradients. **a** PCoA analysis based on Bray-Curtis distance and ANOSIM test between experimental and control groups. **b** NMDS analysis based on Bray-Curtis distance and Adonis test between experimental and control groups. **c** ANOSIM and Adonis tests between experimental and control groups based on four additional distance metrics (Abundance Jaccard, Binary Euclidean, Euclidean, and Hellinger). **d** Intra-group dispersion analysis of forest musk deer fecal samples at the ASV level. **e** NST (normalized stochasticity ratio) analysis of fecal samples from experimental and control groups. **f** Relative importance of different community assembly processes in fecal samples from experimental and control groups. **g** βNTI (beta nearest taxon index) analysis of fecal samples from experimental and control groups

Firmicutes showed a significant overall decreasing trend, while *Proteobacteria* demonstrated a significant increasing trend. In contrast, no significant differences were observed between groups for *Bacteroidetes* and *Actinobacteria*, although *Bacteroidetes* also exhibited a general decreasing trend in relative abundance, and *Actinobacteria* showed no clear pattern of change (Fig. 3a).

At the genus level, among the 17 dominant bacterial genera identified, *Acinetobacter*, *Christensenellaceae R-7 group*, *Ruminococcus*, *Monoglobus*, and *NK4A214 group* exhibited significant intergroup differences across the different storage time gradients. Specifically, the relative abundance of *Acinetobacter* showed a significant overall increasing trend, while the relative abundances of the other four dominant genera displayed significant overall decreasing trends (Fig. 3b). The remaining 12 dominant genera did not show significant differences among groups (Fig. 3b).

LEfSe analysis with a total of 11 LDA score thresholds showed that as the LDA threshold increased, the number of significantly different taxa gradually decreased. Within the same LDA threshold, the number of significantly different taxa between the experimental and control groups increased progressively with extended storage time, indicating a continuous accumulation of indicator microbial taxa (Fig. 3c).

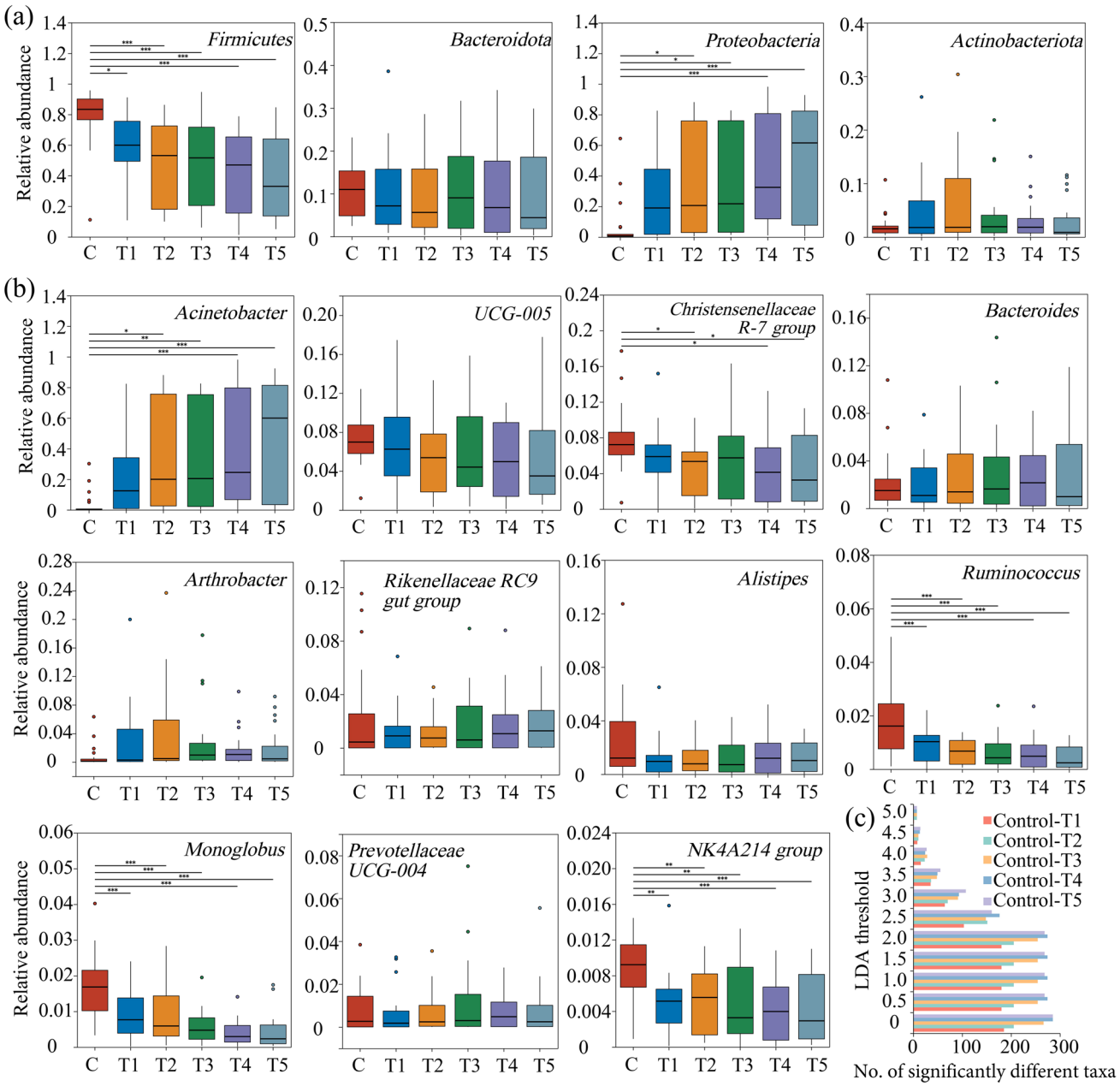

Fig. 3 Analysis of dominant gut microbial taxa and their variation trends in forest musk deer across different storage time gradients. **a**, **b** Intergroup comparison of dominant bacterial phyla (**a**) and dominant bacterial genera (**b**) in the gut microbiota under different storage time gradients. **c** LEfSe analysis based on different LDA score thresholds to identify significantly different taxa among forest musk deer groups

Analysis of enterotype differences and their variation trends in the gut microbiota of forest musk deer across different storage time gradients

The Jensen-Shannon divergence (JSD) distance algorithm was used to calculate the distances between forest musk deer fecal samples across different storage time gradients based on the relative abundance matrix of ASVs. The larger the Calinski-Harabasz (CH) index, the more tightly the samples are clustered within each type, and the more dispersed the clusters are between different types, indicating better clustering results.

Clustering of ASV relative abundances using Jensen-Shannon divergence and evaluation with the Calinski-Harabasz index showed that the optimal number of clusters was determined to be two, as the clustering result was best when the CH index was maximized. The analysis revealed that the fecal samples from forest musk deer could be divided into two enterotypes: Enterotype 1 (ET1) and Enterotype 2 (ET2) (Fig. 4a). Of the 144 captive forest musk deer samples, 81

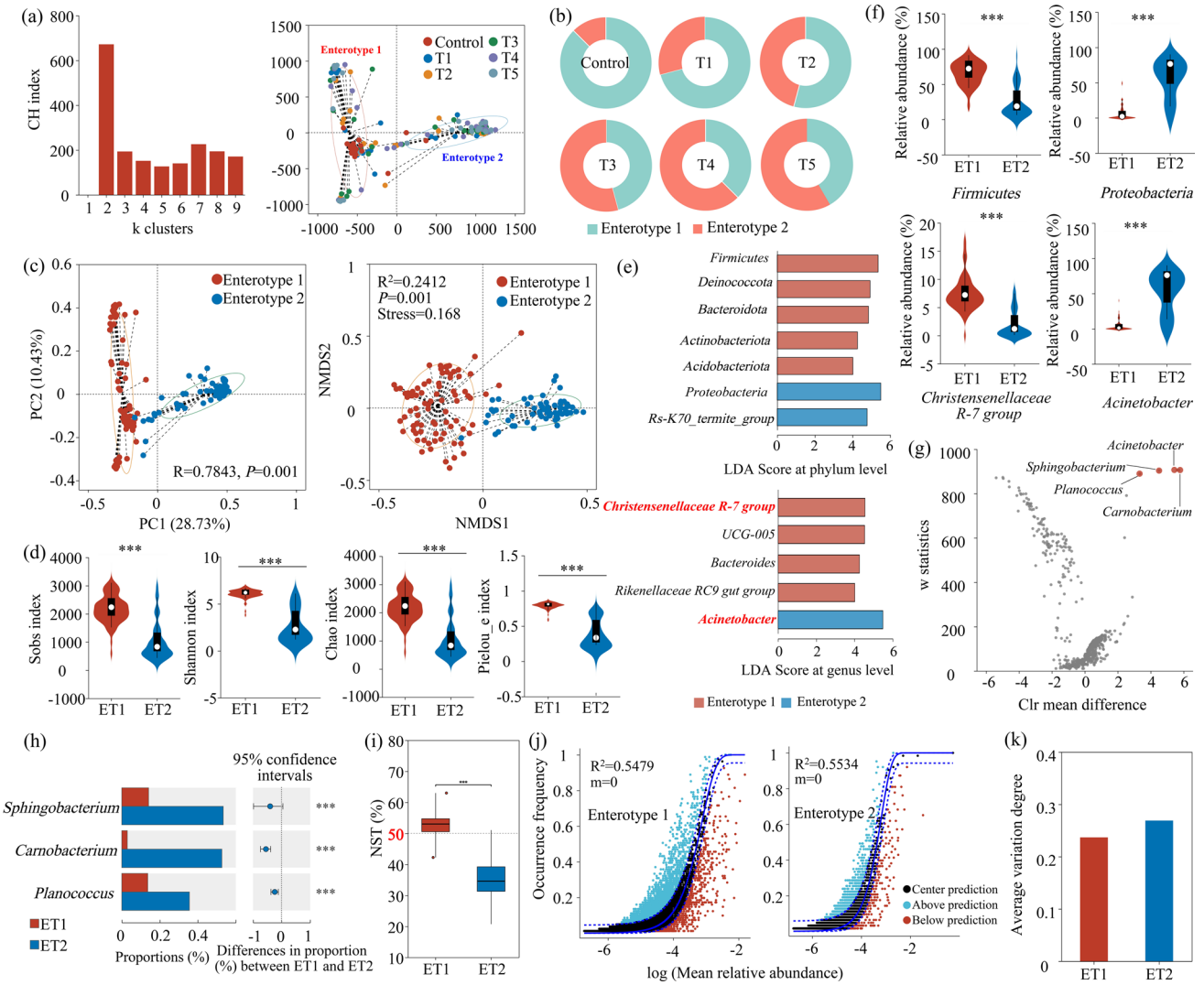

Fig. 4 Analysis of gut microbial enterotype differences and their variation trends in forest musk deer under different storage time gradients. **a** Optimal clustering number determination and enterotype classification analysis of gut microbiota in the experimental and control groups. **b**–**d** Enterotype distribution analysis (**b**), β-diversity analysis of different enterotypes based on PCoA and NMDS (**c**), and α-diversity analysis based on four diversity indices (**d**) in the experimental and control groups. **e**, **f** Identification of biomarkers at the phylum and genus levels across different enterotypes (**e**) and intergroup comparison of their relative abundances (**f**). **g**, **h** Differential genus identification with the highest variation between enterotypes based on ANCOM analysis (**g**) and intergroup comparison of these genera (**h**). **i**, **j** Analysis of community assembly processes between enterotypes based on NST (**i**) and NCM (**j**) analysis. **k** Average variation degree (AVD) analysis reflecting community stability across different enterotypes

samples belonged to Enterotype 1, accounting for 56.25%, while 63 samples belonged to Enterotype 2, accounting for 43.75%. In the control group, Enterotype 1 was dominant, accounting for 87.50%. As the storage time increased, the proportion of Enterotype 1 in the five different time periods was 70.83%, 54.17%, 45.83%, 37.50%, and 41.67%, showing a downward trend, while Enterotype 2 exhibited an overall upward trend (Fig. 4b).

PCoA clustering analysis indicated that the samples within Enterotype 1 and Enterotype 2 were relatively concentrated, with a clear separation between the two groups. The ANOSIM test revealed an R-value of 0.7843 ($P = 0.001$). Additionally, NMDS analysis showed a stress value of 0.168, which is less than 0.2, and the Adonis test returned an R^2 value of 0.2412 ($P = 0.001$), effectively reflecting the true arrangement of the data and accurately capturing the differences in microbial composition at the genus level between the two enterotypes. These results were consistent with the PCoA analysis (Fig. 4c). Furthermore, the distribution of Enterotype 2 samples was relatively concentrated. The Sobs, Shannon, Chao1, and Pielou_e indices were used to reflect the α-diversity of

microorganisms in different enterotypes. The results showed that all four α-diversity indices for Enterotype 1 were significantly higher than those for Enterotype 2 ($P < 0.001$) (Fig. 4d), indicating that the community richness, diversity, and evenness of Enterotype 1 were significantly greater than those of Enterotype 2.

LEfSe analysis with the LDA threshold set to 4.0 revealed that, at the phylum level, *Firmicutes* had the highest LDA score in Enterotype 1, while *Proteobacteria* had the highest in Enterotype 2. At the genus level, *Christensenellaceae R-7 group* in Enterotype 1 and *Acinetobacter* in Enterotype 2 showed the highest LDA scores. These findings suggest that Enterotype 1 is primarily dominated by *Christensenellaceae R-7 group* within the *Firmicutes* phylum, while Enterotype 2 is mainly dominated by *Acinetobacter* within the *Proteobacteria* phylum (Fig. 4e). Inter-group difference analysis of these four dominant phyla and genera further showed that the relative abundances of *Firmicutes* and *Christensenellaceae R-7 group* were significantly higher in Enterotype 1 compared to Enterotype 2, whereas *Proteobacteria* and *Acinetobacter* were significantly more abundant in Enterotype 2 than in Enterotype 1 (Fig. 4f). According to the ANCOM (analysis of composition of microbiomes) differential abundance test, *Acinetobacter*, *Sphingobacterium*, *Carnobacterium*, and *Planococcus* exhibited the greatest differences between Enterotype 1 and Enterotype 2 at the genus level (Fig. 4g). Among them, *Acinetobacter* showed a significantly higher relative abundance in Enterotype 2 (Fig. 4e), while the other three genera had significantly higher relative abundances in Enterotype 1 compared to Enterotype 2 (Fig. 4h).

To evaluate the differences in the assembly processes of gut microbiota between Enterotype 1 and Enterotype 2 in the forest musk deer, the NST analysis revealed that the NST values for Enterotype 1 were all above 50%, indicating that the gut microbial community assembly was predominantly governed by stochastic processes. In contrast, the NST values for Enterotype 2 were all below 50%, suggesting that its community assembly was primarily driven by deterministic processes (Fig. 4i). Furthermore, NCM analysis showed that the overall community variance (represented by the R^2 value, which indicates the goodness of fit to the neutral model) for Enterotype 1 and Enterotype 2 was 54.79% and 55.34%, respectively. These relatively high R^2 values suggest that both enterotypes were well-fitted to the neutral model, indicating that deterministic influences were relatively minor and that stochastic processes played a more dominant role in shaping the community (Fig. 4j). In addition, the community stability index analysis indicated that the average variation degree (AVD) of Enterotype 2 was higher than that of Enterotype 1. Since lower AVD values represent higher gut microbiota stability, this suggests that Enterotype 1 exhibited greater community stability than Enterotype 2 (Fig. 4k).

Functional differences and variation trends of gut microbiota in forest musk deer under different storage time gradients

Functional annotation of the gut microbiota genes in forest musk deer was conducted based on the eggNOG database. At level 2, representing the second hierarchical tier of functional annotation in the eggNOG database, 23 functional categories were annotated. Excluding "Function unknown," eight functions exhibited a relative abundance greater than 5%, primarily associated with various metabolic processes (Fig. 5a). Inter-group comparison revealed significant differences in the following functions: Translation, ribosomal structure and biogenesis (J); Transcription (K); Carbohydrate transport and metabolism (G); and Replication, recombination and repair (L). The abundance of these four functions showed a decreasing trend with the prolongation of storage time. The other four less significant functions also exhibited an overall declining trend (Fig. 5b). At level 3, representing the third hierarchical tier of functional annotation in the eggNOG database, a total of 4469 COG functions were identified. Analysis of inter-group comparisons between the control and experimental groups showed that the proportion of functions with significant *P*-values exhibited a general upward trend, indicating that functional differences between groups increased with prolonged storage durations (Fig. 5c).

Functional annotation of the gut microbiota genes in the forest musk deer was performed using the KEGG database. At level 1, six functional categories were annotated, with metabolism-related functions showing the highest relative abundance. No significant differences were observed among the groups for this category. However, genetic information processing (GIP), environmental information processing (EIP), and cellular processes (CP) exhibited significant inter-group differences. The relative abundance of these three functions showed an overall decreasing trend with increasing storage time, although not all pairwise differences between time points were statistically significant. The remaining three non-significant functions also showed an overall decreasing trend (Fig. 5d). Among the 46 annotated functions at level 2, 11 functions with a relative abundance greater than 2% were selected for inter-group comparison. Only translation (TR), replication and repair (RR), and membrane transport (MT) showed significant differences among the groups. The abundance of these three functions also decreased progressively with prolonged storage time, while the other eight non-significant functions followed a similar overall downward trend (Fig. 5e). In addition, a statistical analysis of inter-group differences in *P*-values was conducted across multiple functional levels, including level 2, level 3, Enzyme functions, KO

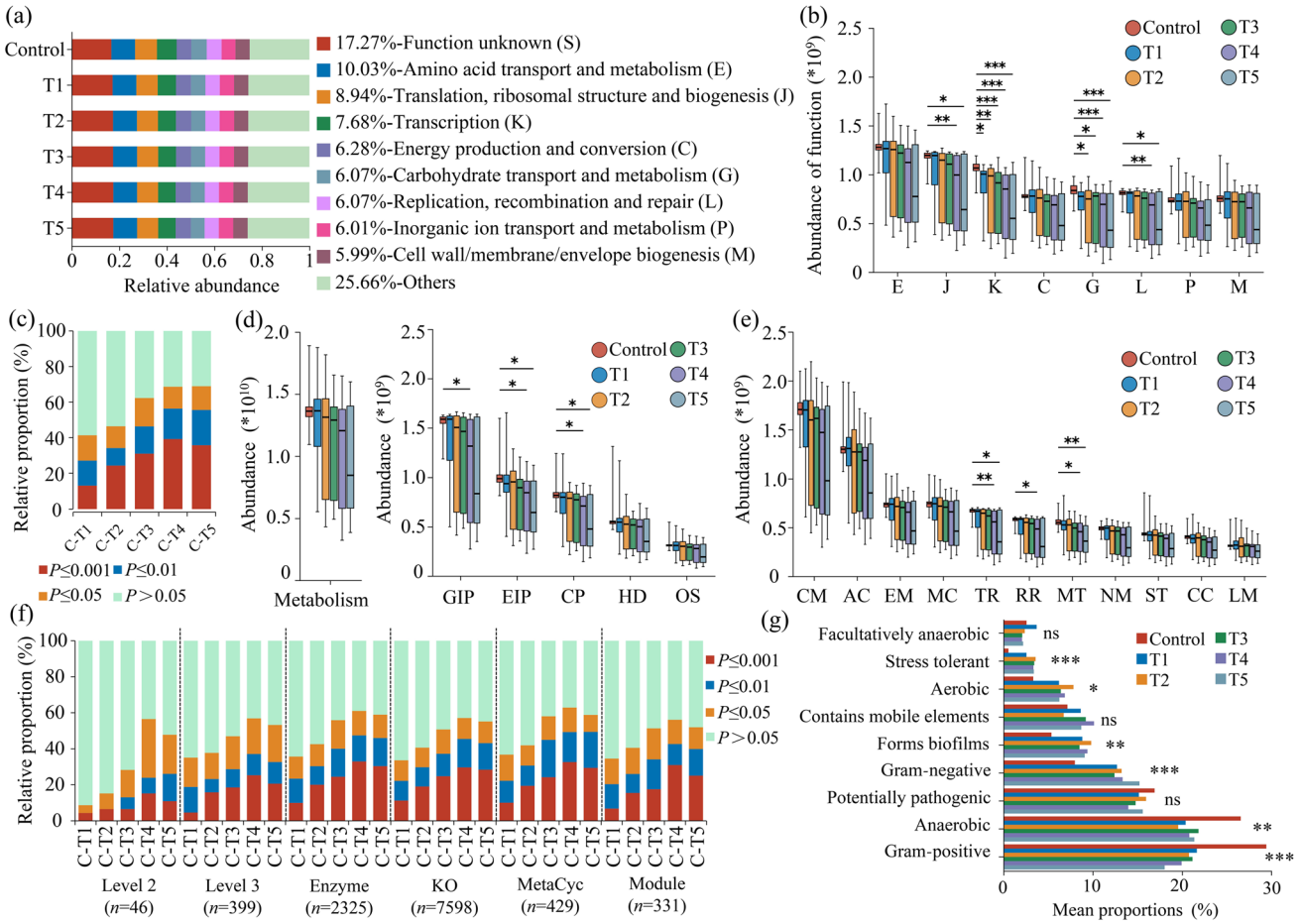

Fig. 5 Functional differences and variation trends of the forest musk deer gut microbiota under different storage time gradients. **a**–**c** Functional annotation based on the eggNOG database (**a**), inter-group differences at the level 2 functional category (**b**), and statistical analysis of *P*-value differences at level 2 (**c**). **d**–**f** Functional annotation based on the KEGG database and inter-group comparisons at level 1 (**d**) and level 2 (**e**), along with statistical analysis of *P*-value differences at level 3 (**f**). **g** Phenotypic prediction analysis of the gut microbiota in experimental and control groups using BugBase

functions, MetaCyc functions, and Module functions, comparing the control group with the five experimental groups at different storage time points. The proportion of significant *P*-values increased overall, indicating that functional differences between the control and the experimental groups became more pronounced with increasing storage duration (Fig. 5f).

Phenotypic prediction analysis using BugBase tools revealed significant inter-group differences in six microbial phenotypes: Gram-positive, anaerobic, Gram-negative, biofilm formation, aerobic, and stress-tolerant. Among these, the relative abundance of the first two phenotypes (Gram-positive and anaerobic) showed a decreasing trend, while the remaining four phenotypes (Gram-negative, forms biofilms, aerobic, and stress-tolerant) exhibited an increasing trend (Fig. 5g).

Discussions

Temporal dynamics of gut microbiota composition and diversity in response to fecal sample storage duration

Currently, global biodiversity is undergoing an unprecedented crisis, and the conservation of endangered species urgently requires support from innovative technological approaches. In recent years, the rapid development of metagenomics and gut microbiome research has provided novel perspectives for conservation biology. As a key regulator of host physiological functions, the gut microbiota participates in processes such as nutrient metabolism, immune defense, and disease resistance, and its

composition and stability are directly related to the host's ability to survive and adapt (Trevelline et al. 2019). Compared with traditional invasive sampling methods (such as blood or tissue collection), fecal samples have become an ideal material for metagenomic studies of endangered species due to their non-invasive nature, ease of collection, and abundance of host-derived microbial genetic information. However, once excreted, fecal samples are exposed to environmental conditions that can induce dynamic changes in microbial communities, which may significantly affect the accuracy of experimental results (Song et al. 2016). This potential bias is particularly critical in time-sensitive field studies, yet the underlying mechanisms remain insufficiently explored in endangered species. The forest musk deer, a first-class protected species in China, holds the dual identity of being both globally endangered and subject to large-scale captive management, making it an ideal model for investigating gut microbiota dynamics in endangered animals. In this study, we employed 16S rRNA gene high-throughput sequencing technology to systematically characterize the changes in gut microbial composition, diversity, and function in fecal samples from captive forest musk deer across different post-defecation time intervals. The findings provide a methodological foundation for enhancing the scientific rigor and applied value of metagenomic research in endangered species.

This study first analyzed the compositional changes of the gut microbiota of the forest musk deer under different storage durations. The results revealed that the dominant phyla in the gut microbiota of the forest musk deer were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*, which together accounted for 97.69% of the total composition. These findings are consistent with gut microbiota studies of various large herbivores. Previous research has indicated that *Firmicutes* dominate the degradation of cellulose and lignin in herbivores' guts, capable of fermenting complex polysaccharides to generate short-chain fatty acids (SCFAs), which provide energy for the host (Koyun et al. 2022; Zeng et al. 2022). *Firmicutes* in the rumen of ruminant animals (such as *Ruminococcus*) were directly involved in the breakdown of plant cell walls (Peng et al. 2021). *Bacteroidetes* can degrade dietary fibers and pectic substances, with *Bacteroides* species producing various carbohydrate-active enzymes (CAZymes) that assist the host in utilizing plant polysaccharides (Li et al. 2021). In herbivores, the abundance of *Bacteroidetes* was significantly correlated with high-fiber diets (Zeng et al. 2022). Therefore, *Firmicutes* and *Bacteroidetes* are the dominant phyla in the gut microbiota of herbivores, playing crucial roles in food digestion, nutrient absorption, and immune responses in the host.

Additionally, at the genus level, *Acinetobacter*, *UCG-005*, *Christensenellaceae R-7 group*, *Bacteroides*, *Arthrobacter*, *Rikenellaceae RC9 gut group*, and *Alistipes* were identified as the dominant genera in the gut microbiota of the forest musk deer, which also play significant roles in the gut microbiota of other large herbivores. Previous studies have indicated that the *Christensenellaceae R-7 group*, as a core fiber-degrading microbial group, works synergistically with other fiber-degrading microbes to facilitate the breakdown of plant cell wall polysaccharides (Xia et al. 2021), and in ruminants, *Christensenellaceae* is associated with fiber degradation abilities, potentially promoting nutrient absorption by participating in lignocellulose degradation. *Bacteroides*, recognized as a "keystone genus" in early gut colonization, helps shape a stable intestinal ecosystem (Buzun et al. 2024) and was a dominant genus in the gut microbiota of herbivores such as musk oxen (*Ovibos moschatus*) and horses, efficiently degrading plant polysaccharides (such as cellulose and hemicellulose) to assist the host in obtaining energy from plant-based diets (Aggerbeck et al. 2022; Li et al. 2023a, b, c). *Bacteroides* can break down complex plant polysaccharides by expressing abundant carbohydrate-active enzymes (CAZymes), producing short-chain fatty acids (SCFAs) such as acetate and propionate, which not only provide energy for the host but also regulate intestinal pH and inhibit pathogen growth (Fang et al. 2024). The *Rikenellaceae RC9 gut group*, a dominant genus in the gut microbiota of herbivores, was positively correlated with polyunsaturated fatty acids (PUFA) and lipid metabolic products, potentially influencing nutrient absorption through its involvement in lipid metabolism processes in herbivores like yaks (Guo et al. 2024). *Alistipes*, which is positively correlated with the production of acetic acid and propionic acid (Tang et al. 2024), participates in the synthesis of short-chain fatty acids, which are important metabolic products for digesting plant fibers in herbivores and help the host obtain energy from high-fiber diets (Xu et al. 2024). *Acinetobacter* and *Arthrobacter* were typically not dominant genera in the gut microbiomes of musk deer and alpine musk deer (*Moschus chrysogaster*) (Jiang et al. 2021). However, this study found that with prolonged storage time of fecal samples, the relative abundance of these two genera increased significantly to over 1%, becoming one of the dominant genera. This phenomenon may be related to the selective proliferation of specific microorganisms or environmental adaptation during the sample preservation process.

The Venn diagram analysis revealed that a total of 20 shared bacterial phyla were identified across six groups of fecal samples with different storage time gradients of the forest musk deer. As storage time increased, the number of unique bacterial genera showed a gradual increase, indicating that fecal storage time has a significant impact on the composition and diversity of the gut microbiota. Previous studies have suggested that the impact of fecal storage time and sampling site on the composition and diversity of the gut microbiota in dogs and cats is minimal. However,

storage time and sampling location (such as the fecal surface or core) can reflect subtle changes in microbial composition through β-diversity analysis, and individual differences remain the primary determinant of microbiome structure (Langon 2023). Furthermore, fecal storage time significantly influences the composition and dynamic changes of the gut microbiota, with prolonged storage potentially leading to the collapse of specific bacterial groups and an increase in bacteriophage numbers, even causing shifts in microbiome types (Shkoporov et al. 2023). These findings align with the results of this study, suggesting that the impact of indoor fecal sample storage time on the gut microbiota is a general phenomenon.

This study analyzed the α-diversity of the gut microbiota of captive forest musk deer under different fecal storage time gradients using the Sobs, Shannon, Chao1, and Pielou_e indices, and evaluated differences by performing pairwise comparisons between the Control group and each of the five experimental time points using the Wilcoxon rank-sum test. To ensure statistical robustness and minimize false-positive findings from multiple comparisons, a false discovery rate (FDR) adjustment was applied across all four α-diversity indices. The results showed that, with an increase in fecal sample storage time, the overall α-diversity of the forest musk deer gut microbiota decreased, and the variability between samples gradually increased. These findings were consistent with the analysis of the average variation degree, which indicated that, with the extension of storage time, the stability of the forest musk deer gut microbiota significantly decreased. This phenomenon was likely associated with reduced microbial metabolic activity and the loss of certain microbial groups, together with the proliferation of taxa originally present at low abundance and below the sequencing detection threshold during prolonged storage, rather than explicit contamination by foreign microorganisms. Previous studies have noted that, under non-freezing conditions, the α-diversity and stability of microbial communities in fecal samples significantly declined over time. In fecal samples stored at room temperature, the microbial community structure changed due to ongoing fermentation, leading to an imbalance in the dominant microbial groups (Yang et al. 2020; Gratton et al. 2016). Under room temperature conditions, as storage time increased (from 24–48 h to 7 days), the α-diversity of the microbial community in feces significantly changed, the community stability decreased, and levels of specific metabolites (such as short-chain fatty acids and bile acids) fluctuated significantly (Isokääntä et al. 2024). Metagenomic assessments of the impact of room temperature storage methods on microbial communities found that different storage methods led to changes in microbial composition, indicating that environmental exposure does indeed affect microbial diversity and stability (Pribyl et al. 2021). These results are consistent with those of the present study, indicating that the storage time of fecal samples at room temperature has an irreversible impact on the diversity and stability of the gut microbiota.

This study used β-diversity analysis to reveal the differences and similarities in the gut microbiota composition of captive forest musk deer under different fecal storage time gradients. PCoA and NMDS analyses based on Bray-Curtis distance algorithms showed that, with an increase in fecal sample storage time, the β-diversity between the experimental group and the control group (Control group) gradually increased, and the results of ANOSIM and Adonis tests further confirmed the significance of this difference ($P < 0.05$). This study applied both ANOSIM and Adonis to evaluate overall group-level differences in gut microbial composition, with separate Control-versus-time point comparisons primarily presented for illustrative purposes to highlight progressive changes in microbial communities with increasing storage time. Using permutation-based statistical methods, which are robust to non-independence among groups, this approach captures consistent directional trends across multiple metrics, providing a reliable depiction of storage-time effects on gut microbiota of forest musk deer. As storage time increased, both the R-value from the ANOSIM test and the R^2-value from the Adonis test showed an upward trend, indicating that the microbial community differences between the experimental group and the control group significantly increased. Previous studies have indicated that, under room temperature conditions, storage time had a minimal impact on the α-diversity of infant gut microbiota, but with prolonged storage time (0–2 h), the relative abundance of certain low-abundance microbial groups changed, and β-diversity was primarily influenced by individual differences (Guo et al. 2016). With further extension of fecal sample storage time (1 week, 2 weeks, or 6 months), both the α and β-diversity of the gut microbiota showed significant changes (Ma et al. 2020). These results were consistent with the findings of the present study, suggesting that fecal storage time initially led to increased heterogeneity in gut microbiota composition, whereas the magnitude of compositional change tended to plateau after several days of storage, indicating a potential stabilization of the microbial community following prolonged ambient exposure. Optimizing fecal sample preservation methods could help reduce the interference of storage time on microbial community analysis results.

Temporal characteristics of the effects of fecal storage time on gut morphotype and functional changes

This study used the Jensen-Shannon divergence (JSD) distance algorithm and the Calinski-Harabasz (CH) index to classify fecal samples from captive forest musk deer under

different storage time gradients into two enterotypes. The results indicated that Enterotype 1 predominated in the control group (87.50%), but its proportion gradually decreased with extended storage time, while the proportion of Enterotype 2 significantly increased. This suggests that storage time has a significant impact on the enterotype distribution of the forest musk deer gut microbiota, and the shift from Enterotype 1 to Enterotype 2 may be closely related to environmental changes during the storage process. PCoA and NMDS analyses further confirmed significant differences in the microbiota composition between Enterotype 1 and Enterotype 2. The α-diversity indices of Enterotype 1 were significantly higher than those of Enterotype 2, indicating that Enterotype 1 exhibited higher richness, diversity, and evenness. Previous studies have pointed out that under room temperature conditions (37 °C in an anaerobic environment), with the extension of fecal sample storage time up to 48 h, phage activity could lead to the collapse of specific bacterial groups, such as *Prevotella*, and trigger a shift in enterotype, suggesting that storage time significantly affects gut microbiota composition and enterotype distribution through phage-bacterial interactions (Shkoporov et al. 2023). This finding is consistent with the current study, which also demonstrates that storage time significantly alters the enterotype distribution of gut microbiota, particularly transitioning from a high-diversity, high-stability Enterotype 1 to a low-diversity, low-stability Enterotype 2.

This study conducted annotation analysis of the gut microbiota functions in forest musk deer based on the eggNOG and KEGG databases, revealing the trend of functional changes under different storage time gradients. At the level 2 functional classification, a significant decline was observed in functions related to translation, ribosome structure and biogenesis (J), transcription (K), carbohydrate transport and metabolism (G), and replication, recombination, and repair (L) with increasing storage time. This result suggests that storage time has a significant impact on the core metabolic functions of the forest musk deer gut microbiota. Previous studies have pointed out that with extended fecal sample storage time (from 24–48 h to 7 days), the levels of microbial metabolites such as short-chain fatty acids (SCFAs) significantly increase in untreated fecal samples, while the use of 95% ethanol or specific commercial preservatives effectively maintains the stability of the fecal metabolome, with results comparable to rapid freezing (Isokääntä et al. 2024). Additionally, fecal microbes frozen in −80 °C deoxyglycerol buffer for 12 months retained cultivability and functional metabolic responses to prebiotics (fructo-oligosaccharides, FOS), while those stored on ice in deoxyglycerol buffer maintained functional activity for 48 h (Zhang et al. 2022). These findings indirectly suggest that, in the absence of effective preservation measures, gut microbial functions are more susceptible to alteration during storage, which is consistent with the functional shifts observed in fecal samples stored at room temperature in the present study.

Implications for conservation biology and limitations of the study

This study systematically analyzed the composition, diversity, enterotype, and function of the gut microbiota in captive forest musk deer under different storage time gradients, revealing the significant impact of storage time on gut microbiota. The findings provide valuable insights for the conservation biology research of forest musk deer and other endangered herbivorous species. First, the results indicated that storage time significantly altered the composition and function of the gut microbiota in forest musk deer, particularly the decrease in the abundance of core microbiota and the increase in the abundance of *Proteobacteria*. This phenomenon suggests that in captive environments, the storage conditions of fecal samples may significantly influence the accuracy of microbiota analysis results, which in turn could affect the assessment of animal health. Therefore, future conservation biology studies should optimize fecal sample collection and preservation methods to minimize the interference of storage time on microbiota data. Furthermore, the study found that storage time significantly reduced both the α-diversity and β-diversity of the gut microbiota and led to a shift in enterotype from the high-diversity, high-stability enterotype 1 to the low-diversity, low-stability enterotype 2. This result suggests that prolonged fecal storage time under captive sampling conditions may negatively affect the observed stability of the gut microbiota in forest musk deer, which could introduce bias in the assessment of microbial composition and diversity if sample preservation is not properly controlled.

From a methodological perspective, this study systematically revealed the impact of storage time on the gut microbiota of forest musk deer using various analytical methods, providing a methodological reference for similar research. Although the 16S rRNA sequencing technology used in this study efficiently analyzed microbiota composition and diversity, it has certain limitations in functional prediction, particularly its inability to directly reflect the specific metabolic activity of the microbiota. Furthermore, although multiple distance algorithms and statistical methods were employed, the relatively limited sample size may affect the generalizability of the results. Therefore, future research should integrate metagenomic sequencing and metabolomics techniques to more comprehensively reveal the functions of the gut microbiota and its relationship with host health.

Author contributions FJ, HFG, and TZZ conceived and designed the study. FJ and HFG performed the data analysis. FJ drafted the initial

version of the manuscript. FJ, HFG, PFS, JJZ, ZYC, CBL, HMG, and RDZ contributed to sample collection and laboratory experiments. TZZ critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Data availability The datasets generated and analyzed in this study on the gut microbiota of forest musk deer are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate A total of 144 fecal samples from captive forest musk deer were collected, with permission from the authorities at the forest musk deer breeding centers in Qilian County, Qinghai Province, China, respectively. All procedures were approved by the Ethical Committee for Experimental Animal Welfare of the Northwest Institute of Plateau Biology.

Competing interests The authors declare no competing interests.

References

Aggerbeck MR, Nielsen TK, Mosbacher JB, Schmidt NM, Hansen LH (2022) Muskoxen homogenise soil microbial communities and affect the abundance of methanogens and methanotrophs. Sci Total Environ 827:153877. https://doi.org/10.1016/j.scitotenv.2022.153877

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857. https://doi.org/10.1038/s41587-019-0209-9

Buzun E, Hsu CY, Sejane K, Oles RE, Vasquez Ayala A, Loomis LR, Zhao J, Rossitto LA, McGrosso DM, Gonzalez DJ, Bode L, Chu H (2024) A bacterial sialidase mediates early-life colonization by a pioneering gut commensal. Cell Host Microbe 32(2):181-190.e9. https://doi.org/10.1016/j.chom.2023.12.014

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13(7):581–583. https://doi.org/10.1038/nmeth.3869

Chen S, Zhou Y, Chen Y, Gu J (2018) Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17):i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Cortes-Ortiz L, Amato KR (2021) Host genetics influence the gut microbiome. Science 373(6551):159–160. https://doi.org/10.1126/science.abj5287

Costello SP, Conlon MA, Vuaran MS, Roberts-Thomson IC, Andrews JM (2015) Faecal microbiota transplant for recurrent *Clostridium* difficile infection using long-term frozen stool is effective: clinical efficacy and bacterial viability data. Aliment Pharmacol Ther 42(8):1011–1018. https://doi.org/10.1111/apt.13366

Fang Z, Ma M, Wang Y, Dai W, Shang Q, Yu G (2024) Degradation and fermentation of hyaluronic acid by *Bacteroides* spp. from the human gut microbiota. Carbohydr Polym 334:122074. https://doi.org/10.1016/j.carbpol.2024.122074

Formenti G, Theissinger K, Fernandes C, Bista I, Bombarely A, Bleidorn C, Ciofi C, Crottini A, Godoy JA, Höglund J, Malukiewicz J, Mouton A, Oomen RA, Paez S, Palsbøll PJ, Pampoulie C, Ruiz-López MJ, Svardal H, Theofanopoulou C, de Vries J, Waldvogel AM, Zhang G, Mazzoni CJ, Jarvis ED, Bálint M, European Reference Genome Atlas (ERGA) Consortium (2022) The era of reference genomes in conservation genomics. Trends Ecol Evol 37(3):197–202. https://doi.org/10.1016/j.tree.2021.11.008

Foster KR, Schluter J, Coyte KZ, Rakoff-Nahoum S (2017) The evolution of the host microbiome as an ecosystem on a leash. Nature 548(7665):43–51. https://doi.org/10.1038/nature23292

Gacesa R, Kurilshikov A, Vich Vila A, Sinha T, Klaassen MAY, Bolte LA, Andreu-Sánchez S, Chen L, Collij V, Hu S, Dekens JAM, Lenters VC, Björk JR, Swarte JC, Swertz MA, Jansen BH, Gelderloos-Arends J, Jankipersadsing S, Hofker M, Vermeulen RCH, Sanna S, Harmsen HJM, Wijmenga C, Fu J, Zhernakova A, Weersma RK (2022) Environmental factors shaping the gut microbiome in a Dutch population. Nature 604(7907):732–739. https://doi.org/10.1038/s41586-022-04567-7

Gentile CL, Weir TL (2018) The gut microbiota at the intersection of diet and human health. Science 362(6416):776–780. https://doi.org/10.1126/science.aau5812

Gratton J, Phetcharaburanin J, Mullish BH, Williams HR, Thursz M, Nicholson JK, Holmes E, Marchesi JR, Li JV (2016) Optimized sample handling strategy for metabolic profiling of human feces. Anal Chem 88(9):4661–4668. https://doi.org/10.1021/acs.analchem.5b04159

Grieneisen L, Dasari M, Gould TJ, Björk JR, Grenier JC, Yotova V, Jansen D, Gottel N, Gordon JB, Learn NH, Gesquiere LR, Wango TL, Mututua RS, Warutere JK, Siodi L, Gilbert JA, Barreiro LB,

Alberts SC, Tung J, Archie EA, Blekhman R (2021) Gut microbiome heritability is nearly universal but environmentally contingent. Science 373(6551):181–186. https://doi.org/10.1126/science.aba5483

Guo Y, Li SH, Kuang YS, He JR, Lu JH, Luo BJ, Jiang FJ, Liu YZ, Papasian CJ, Xia HM, Deng HW, Qiu X (2016) Effect of short-term room temperature storage on the microbial community in infant fecal samples. Sci Rep 6:26648. https://doi.org/10.1038/srep26648

Guo T, Zhang Q, Wang X, Xu X, Wang Y, Wei L, Li N, Liu H, Hu L, Zhao N, Xu S (2024) Targeted and untargeted metabolomics reveals meat quality in grazing yak during different phenology periods on the Qinghai-Tibetan Plateau. Food Chem 447:138855. https://doi.org/10.1016/j.foodchem.2024.138855

Hu Y, Hu Y, Zhou W, Wei F (2024) Conservation genomics and metagenomics of giant and red pandas in the wild. Annu Rev Anim Biosci 12:69–89. https://doi.org/10.1146/annurev-animal-021022-054730

Huang G, Wang L, Li J, Hou R, Wang M, Wang Z, Qu Q, Zhou W, Nie Y, Hu Y, Ma Y, Yan L, Wei H, Wei F (2022) Seasonal shift of the gut microbiome synchronizes host peripheral circadian rhythm for physiological adaptation to a low-fat diet in the giant panda. Cell Rep 38(3):110203. https://doi.org/10.1016/j.celrep.2021.110203

Huang G, Shi W, Wang L, Qu Q, Zuo Z, Wang J, Zhao F, Wei F (2023) PandaGUT provides new insights into bacterial diversity, function, and resistome landscapes with implications for conservation. Microbiome 11(1):221. https://doi.org/10.1186/s40168-023-01657-0

Huang G, Qi D, Yang Z, Hou R, Shi W, Zhao F, Li Z, Yan L, Wei F (2024) Gut microbiome as a key monitoring indicator for reintroductions of captive animals. Conserv Biol 38(1):e14173. https://doi.org/10.1111/cobi.14173

iMSMS Consortium (2022) Gut microbiome of multiple sclerosis patients and paired household healthy controls reveal associations with disease risk and course. Cell 185(19):3467-3486.e16. https://doi.org/10.1016/j.cell.2022.08.021

Isokääntä H, Pinto da Silva L, Karu N, Kallonen T, Aatsinki AK, Hankemeier T, Schimmel L, Diaz E, Hyötyläinen T, Dorrestein PC, Knight R, Orešič M, Kaddurah-Daouk R, Dickens AM, Lamichhane S (2024) Comparative metabolomics and microbiome analysis of ethanol versus OMNImet/gene•GUT fecal stabilization. Anal Chem 96(22):8893–8904. https://doi.org/10.1021/acs.analchem.3c04436

Jiang F, Zhang J, Gao H, Cai Z, Zhou X, Li S, Zhang T (2020) Musk deer (*Moschus* spp.) face redistribution to higher elevations and latitudes under climate change in China. Sci Total Environ 704:135335. https://doi.org/10.1016/j.scitotenv.2019.135335

Jiang F, Gao H, Qin W, Song P, Wang H, Zhang J, Liu D, Wang D, Zhang T (2021) Marked seasonal variation in structure and function of gut microbiota in forest and alpine musk deer. Front Microbiol 12:699797. https://doi.org/10.3389/fmicb.2021.699797

Jiang F, Song P, Wang H, Zhang J, Liu D, Cai Z, Gao H, Chi X, Zhang T (2022) Comparative analysis of gut microbial composition and potential functions in captive forest and alpine musk deer. Appl Microbiol Biotechnol 106(3):1325–1339. https://doi.org/10.1007/s00253-022-11775-8

Kennedy KM, de Goffau MC, Perez-Muñoz ME, Arrieta MC, Bäckhed F, Bork P, Braun T, Bushman FD, Dore J, de Vos WM, Earl AM, Eisen JA, Elovitz MA, Ganal-Vonarburg SC, Gänzle MG, Garrett WS, Hall LJ, Hornef MW, Huttenhower C, Konnikova L, Lebeer S, Macpherson AJ, Massey RC, McHardy AC, Koren O, Lawley TD, Ley RE, O'Mahony L, O'Toole PW, Pamer EG, Parkhill J, Raes J, Rattei T, Salonen A, Segal E, Segata N, Shanahan F, Sloboda DM, Smith GCS, Sokol H, Spector TD, Surette MG, Tannock GW, Walker AW, Yassour M, Walter J (2023) Questioning the fetal microbiome illustrates pitfalls of low-biomass microbial studies. Nature 613(7945):639–649. https://doi.org/10.1038/s41586-022-05546-8

Koyun OY, Callaway TR, Nisbet DJ, Anderson RC (2022) Innovative treatments enhancing the functionality of gut microbiota to improve quality and microbiological safety of foods of animal origin. Annu Rev Food Sci Technol 13:433–461. https://doi.org/10.1146/annurev-food-100121-050244

Langon X (2023) Validation of method for faecal sampling in cats and dogs for faecal microbiome analysis. BMC Vet Res 19(1):274. https://doi.org/10.1186/s12917-023-03842-7

Levin D, Raab N, Pinto Y, Rothschild D, Zanir G, Godneva A, Mellul N, Futorian D, Gal D, Leviatan S, Zeevi D, Bachelet I, Segal E (2021) Diversity and functional landscapes in the microbiota of animals in the wild. Science 372(6539):eabb5352. https://doi.org/10.1126/science.abb5352

Li M, Li S, Guo X, Guo C, Wang Y, Du Z, Zhang Z, Xie C, Ding K (2021) Discrete genetic loci in human gut *Bacteroides thetaiotaomicron* confer pectin metabolism. Carbohydr Polym 272:118534. https://doi.org/10.1016/j.carbpol.2021.118534

Li C, Li X, Guo R, Ni W, Liu K, Liu Z, Dai J, Xu Y, Abduriyim S, Wu Z, Zeng Y, Lei B, Zhang Y, Wang Y, Zeng W, Zhang Q, Chen C, Qiao J, Liu C, Hu S (2023) Expanded catalogue of metagenome-assembled genomes reveals resistome characteristics and athletic performance-associated microbes in horse. Microbiome 11(1):7. https://doi.org/10.1186/s40168-022-01448-z

Li Q, Fei HL, Luo ZH, Gao SM, Wang PD, Lan LY, Zhao XF, Huang LN, Fan PF (2023) Gut microbiome responds compositionally and functionally to the seasonal diet variations in wild gibbons. NPJ Biofilms Microbiomes 9(1):21. https://doi.org/10.1038/s41522-023-00388-2

Li XM, Shi X, Yao Y, Shen YC, Wu XL, Cai T, Liang LX, Wang F (2023) Effects of stool sample preservation methods on gut microbiota biodiversity: new original data and systematic review with meta-analysis. Microbiol Spectr 11(3):e0429722. https://doi.org/10.1128/spectrum.04297-22

Ma J, Sheng L, Hong Y, Xi C, Gu Y, Zheng N, Li M, Chen L, Wu G, Li Y, Yan J, Han R, Li B, Qiu H, Zhong J, Jia W, Li H (2020) Variations of gut microbiome profile under different storage conditions and preservation periods: a multi-dimensional evaluation. Front Microbiol 11:972. https://doi.org/10.3389/fmicb.2020.00972

Magoč T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27(21):2957–2963. https://doi.org/10.1093/bioinformatics/btr507

Maxwell SL, Cazalis V, Dudley N, Hoffmann M, Rodrigues ASL, Stolton S, Visconti P, Woodley S, Kingston N, Lewis E, Maron M, Strassburg BBN, Wenger A, Jonas HD, Venter O, Watson JEM (2020) Area-based conservation in the twenty-first century. Nature 586(7828):217–227. https://doi.org/10.1038/s41586-020-2952-y

Moreno-Torres KI, Sinnott DM, Wolfe BA, Marsh AE, Saville WJA, Moritz M, Garabed RB (2018) Risk of environmental exposure to small coccidia from wild canid feces in rural Ohio. Am J Vet Res 79(11):1179–1187. https://doi.org/10.2460/ajvr.79.11.1179

Nie Y, Speakman JR, Wu Q, Zhang C, Hu Y, Xia M, Yan L, Hambly C, Wang L, Wei W, Zhang J, Wei F (2015) Animal physiology. Exceptionally low daily energy expenditure in the bamboo-eating giant panda. Science 349(6244):171–174. https://doi.org/10.1126/science.aab2413

Peng X, Wilken SE, Lankiewicz TS, Gilmore SP, Brown JL, Henske JK, Swift CL, Salamov A, Barry K, Grigoriev IV, Theodorou MK, Valentine DL, O'Malley MA (2021) Genomic and functional analyses of fungal and bacterial consortia that enable lignocellulose breakdown in goat gut microbiomes. Nat Microbiol 6(4):499–511. https://doi.org/10.1038/s41564-020-00861-0

Pereira HM, Martins IS, Rosa IMD, Kim H, Leadley P, Popp A, van Vuuren DP, Hurtt G, Quoss L, Arneth A, Baisero D, Bakkenes M, Chaplin-Kramer R, Chini L, Di Marco M, Ferrier S, Fujimori

S, Guerra CA, Harfoot M, Harwood TD, Hasegawa T, Haverd V, Havlík P, Hellweg S, Hilbers JP, Hill SLL, Hirata A, Hoskins AJ, Humpenöder F, Janse JH, Jetz W, Johnson JA, Krause A, Leclère D, Matsui T, Meijer JR, Merow C, Obersteiner M, Ohashi H, De Palma A, Poulter B, Purvis A, Quesada B, Rondinini C, Schipper AM, Settele J, Sharp R, Stehfest E, Strassburg BBN, Takahashi K, Talluto L, Thuiller W, Titeux N, Visconti P, Ware C, Wolf F, Alkemade R (2024) Global trends and scenarios for terrestrial biodiversity and ecosystem services from 1900 to 2050. Science 384(6694):458–465. https://doi.org/10.1126/science.adn3441

Pribyl AL, Parks DH, Angel NZ, Boyd JA, Hasson AG, Fang L, MacDonald SL, Wills BA, Wood DLA, Krause L, Tyson GW, Hugenholtz P (2021) Critical evaluation of faecal microbiome preservation using metagenomic analysis. ISME Commun 1(1):14. https://doi.org/10.1038/s43705-021-00014-2

Rühlemann MC, Bang C, Gogarten JF, Hermes BM, Groussin M, Waschina S, Poyet M, Ulrich M, Akoua-Koffi C, Deschner T, Muyembe-Tamfum JJ, Robbins MM, Surbeck M, Wittig RM, Zuberbühler K, Baines JF, Leendertz FH, Franke A (2024) Functional host-specific adaptation of the intestinal microbiome in hominids. Nat Commun 15(1):326. https://doi.org/10.1038/s41467-023-44636-7

Schluter J, Peled JU, Taylor BP, Markey KA, Smith M, Taur Y, Niehus R, Staffas A, Dai A, Fontana E, Amoretti LA, Wright RJ, Morjaria S, Fenelus M, Pessin MS, Chao NJ, Lew M, Bohannon L, Bush A, Sung AD, Hohl TM, Perales MA, van den Brink MRM, Xavier JB (2020) The gut microbiota is associated with immune cell dynamics in humans. Nature 588(7837):303–307. https://doi.org/10.1038/s41586-020-2971-8

Shao X, Lu Q, Xiong M, Bu H, Shi X, Wang D, Zhao J, Li S, Yao M (2021) Prey partitioning and livestock consumption in the world's richest large carnivore assemblage. Curr Biol 31(22):4887-4897.e5. https://doi.org/10.1016/j.cub.2021.08.067

Shkoporov AN, O'Regan O, Smith L, Khokhlova EV, Draper LA, Ross RP, Hill C (2023) Dynamic nature of viral and bacterial communities in human faeces. iScience 27(2):108778. https://doi.org/10.1016/j.isci.2023.108778

Song SJ, Amir A, Metcalf JL, Amato KR, Xu ZZ, Humphrey G, Knight R (2016) Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. mSystems 1(3):e00021-16. https://doi.org/10.1128/mSystems.00021-16

Stoddard SF, Smith BJ, Hein R, Roller BR, Schmidt TM (2015) rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic Acids Res 2015(D1):D593. https://doi.org/10.1093/nar/gku1201

Tang Z, Bao P, Ling X, Qiu Z, Zhang B, Hao T (2024) In vitro digestion under simulated saliva, gastric and small intestinal conditions and fermentation of nicotinamide mononucleotide, and its effects on the gut microbiota. Food Res Int 177:113779. https://doi.org/10.1016/j.foodres.2023.113779

Trevelline BK, Fontaine SS, Hartup BK, Kohl KD (2019) Conservation biology needs a microbial renaissance: a call for the consideration of host-associated microbiota in wildlife management practices. Proc Biol Sci 286(1895):20182448. https://doi.org/10.1098/rspb.2018.2448

Turjeman S, Pekarsky S, Corl A, Kamath PL, Getz WM, Bowie RCK, Markin Y, Nathan R (2023) Comparing invasive and noninvasive faecal sampling in wildlife microbiome studies: a case study on wild common cranes. Mol Ecol Resour 23(2):359–367. https://doi.org/10.1111/1755-0998.13708

Wang L, Huang G, Hou R, Qi D, Wu Q, Nie Y, Zuo Z, Ma R, Zhou W, Ma Y, Hu Y, Yang Z, Yan L, Wei F (2021) Multi-omics reveals the positive leverage of plant secondary metabolites on the gut microbiota in a non-model mammal. Microbiome 9(1):192. https://doi.org/10.1186/s40168-021-01142-6

Wang M, Wang G, Huang G, Kouba A, Swaisgood RR, Zhou W, Hu Y, Nie Y, Wei F (2024) Habitat connectivity drives panda recovery. Curr Biol 34(17):3894-3904.e3. https://doi.org/10.1016/j.cub.2024.07.037

Wei F, Wang X, Wu Q (2015) The giant panda gut microbiome. Trends Microbiol 23(8):450–452. https://doi.org/10.1016/j.tim.2015.06.004

Wei F, Wu Q, Hu Y, Huang G, Nie Y, Yan L (2019) Conservation metagenomics: a new branch of conservation biology. Sci China Life Sci 62(2):168–178. https://doi.org/10.1007/s11427-018-9423-3

Wilde J, Slack E, Foster KR (2024) Host control of the microbiome: mechanisms, evolution, and disease. Science 385(6706):eadi3338. https://doi.org/10.1126/science.adi3338

Wu WL, Adame MD, Liou CW, Barlow JT, Lai TT, Sharon G, Schretter CE, Needham BD, Wang MI, Tang W, Ousey J, Lin YY, Yao TH, Abdel-Haq R, Beadle K, Gradinaru V, Ismagilov RF, Mazmanian SK (2021) Microbiota regulate social behaviour via stress response neurons in the brain. Nature 595(7867):409–414. https://doi.org/10.1038/s41586-021-03669-y

Xia X, Wei H, Hu L, Peng J (2021) Hydratability and improved fermentability in vitro of guar gum by combination of xanthan gum. Carbohydr Polym 258:117625. https://doi.org/10.1016/j.carbpol.2021.117625

Xu B, Song P, Jiang F, Cai Z, Gu H, Gao H, Li B, Liang C, Qin W, Zhang J, Yan J, Liu D, Sun G, Zhang T (2024) Large-scale metagenomic assembly provide new insights into the genetic evolution of gut microbiomes in plateau ungulates. NPJ Biofilms Microbiomes 10(1):120. https://doi.org/10.1038/s41522-024-00597-3

Yan Z, Liu X, Liu H, Xu H, Liu Y, Li C, Wang B, Cui S, Jia T, Yang D, Zhang C, Liu X, Buesching CD, Liu D (2024) Assessment of stress levels and reproductive condition in giant pandas: insights from hair, faecal and saliva samples. Conserv Physiol 12(1):coae044. https://doi.org/10.1093/conphys/coae044

Yang Q, Meng X, Lin X, Feng Z (2003) Conservation status and causes of decline of musk deer (*Moschus* spp.) in China. Biol Conserv 109(3):333–342. https://doi.org/10.1016/S0006-3207(02)00159-3

Yang L, Hou K, Zhang B, Ouyang C, Lin A, Xu S, Ke D, Fang L, Chen Q, Wu J, Yan C, Lian Y, Jiang T, He J, Wang H, Fu Y, Xiao C, Chen Z (2020) Preservation of the fecal samples at ambient temperature for microbiota analysis with a cost-effective and reliable stabilizer EffcGut. Sci Total Environ 741:140423. https://doi.org/10.1016/j.scitotenv.2020

Zeng X, Xing X, Gupta M, Keber FC, Lopez JG, Lee YJ, Roichman A, Wang L, Neinast MD, Donia MS, Wühr M, Jang C, Rabinowitz JD (2022) Gut bacterial nutrient preferences quantified in vivo. Cell 185(18):3441-3456.e19. https://doi.org/10.1016/j.cell.2022.07.020

Zhang X, Walker K, Mayne J, Li L, Ning Z, Stintzi A, Figeys D (2022) Evaluating live microbiota biobanking using an ex vivo microbiome assay and metaproteomics. Gut Microbes 14(1):2035658. https://doi.org/10.1080/19490976.2022.2035658

Zhou W, Yang S, Li B, Nie Y, Luo A, Huang G, Liu X, Lai R, Wei F (2020) Why wild giant pandas frequently roll in horse manure. Proc Natl Acad Sci U S A 117(51):32493–32498. https://doi.org/10.1073/pnas.2004640117

Zhou J, Nelson TM, Rodriguez Lopez C, Sarma RR, Zhou SJ, Rollins LA (2020) A comparison of nonlethal sampling methods for amphibian gut microbiome analyses. Mol Ecol Resour 20(4):844–855. https://doi.org/10.1111/1755-0998.13139

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