

RESEARCH ARTICLE

Gut microbiota dynamics of adult and sub-adult sika deer during reintroduction

Wentao Zhang¹ | Feifei Yang¹ | Heng Bao¹ | Jiale Sun¹ | Wannian Cheng¹ |
Shiyu Chen¹ | Nathan J. Roberts¹  | Yanhui Guan² | Shixian Guo² |
Jixu Sun² | Guangshun Jiang^{1,3} 

¹Feline Research Center of National Forestry and Grassland Administration, College of Wildlife and Protected Area, Northeast Forestry University, Harbin, China

²Administration of Heilongjiang Xiaobeihu National Nature Reserve, Mudanjiang, China

³Northeast Asia Biodiversity Research Center, Northeast Forestry University, Harbin, China

Correspondence

Heng Bao and Guangshun Jiang, Feline Research Center of National Forestry and Grassland Administration, College of Wildlife and Protected Area, NE Forestry University, Harbin 150040, China.
Email: baoheng1990@163.com and jgshun@126.com

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Abstract

Prey populations significantly influence the distribution of top predators. The sika deer (*Cervus nippon*), a key prey species for the Amur tiger in Northeast China, plays a critical role in the recovery and dispersal of Amur tiger populations. Reintroduction is a pivotal strategy for restoring prey populations, but it presents challenges, especially in terms of adaptation to the natural environment during the natural training process before animals are released. In this study, we sampled six adult and six sub-adult sika deer and employed high-throughput sequencing of the 16S ribosomal RNA gene to investigate changes in gut microbial diversity, composition, and function during natural training. The results showed that adult sika deer had higher gut microbiota diversity compared to sub-adults. However, as natural training progressed, the gut microbial diversity in sub-adults approached that of adults. Additionally, beneficial, fiber-digesting bacteria associated with adaptation to the natural environment tended to increase during nature training in both adult and sub-adult sika deer, while pathogenic bacteria tended to decrease. We also compared the metabolic function of the gut microbiota in adult and sub-adult sika deer, which showed that the carbohydrate metabolic function of both adults and sub-adults decreased significantly with natural training, declining more rapidly in sub-adults. Conversely, the lipid metabolic function in sub-adults increased significantly with natural training. Overall, a period of nature training is necessary before reintroducing animals to their natural habitats, and sub-adult sika deer, in particular, exhibit greater adaptability to environmental changes as reflected by their gut microbiota dynamics. These findings offer valuable insights for the reintroduction of sika deer and other ungulates. We recommend incorporating natural training in reintroduction programs and prioritizing sub-adult animals for reintroduction.

KEYWORDS

adults, gut microbiota, natural training, sika deer, sub-adults

Plain language summary

The sika deer is a primary prey species of the Amur tiger, and the availability of prey is an important factor affecting the distribution of top predators. Reintroduction programs are an effective way to restore

Wentao Zhang and Feifei Yang contributed equally to this study.

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prey populations, but a significant challenge is ensuring the re-introduced animals can adapt to their natural environment. In this study, we examined the changes and differences in gut microbiota structure and function in six adult and six sub-adult sika deer during natural training before their reintroduction. The results showed that gut microbiota diversity was higher in adult sika deer than in sub-adults, but as training progressed, sub-adults approached adult levels. In addition, beneficial fiber-digesting bacteria, which are associated with better adaptation to the natural environment, tended to increase during training in both adult and sub-adult sika deer, while pathogenic bacteria tended to decrease. A comparison of gut microbiota metabolic functions revealed that carbohydrate metabolism declined significantly in both groups during training, with a more rapid decline observed in sub-adults. Conversely, lipid metabolism functions of sub-adults increased significantly during the natural training period. These findings suggest that natural training before reintroduction is key and that sub-adult individuals may be better suited to reintroduction programs due to their adaptability.

1 | INTRODUCTION

Reintroduction is an effective strategy for protecting endangered species. Numerous reintroduction projects involving endangered species have been implemented worldwide, including those involving red deer (*Cervus canadensis*) (Guo et al., 2022), Javanian bison (*Bos javanicus*) (Chaiyarat et al., 2020), black bears (*Ursus thibetanus*) (Clark et al., 2002), and giant pandas (*Ailuropoda melanoleuca*) (Shan et al., 2014; Yang et al., 2018). However, the average success rate of these reintroductions remains low, indicating a need for further research and analysis to improve outcomes (Dallas & Warne, 2023; Fischer & Lindenmayer, 2000; Seddon et al., 2012; Tang et al., 2020). One contributing factor to these low success rates may be conditions associated with captivity: limited space to move around, high population densities, and suboptimal hygiene can lead to increased levels of pathogenic bacteria in the gut microbiota of captive animals, potentially compromising their health and adaptability upon release (Kedia et al., 2021; Lecorps et al., 2021; Li, Hu, et al., 2018; Mosca et al., 2016).

Gut microbiota play a crucial role in maintaining the health and development of animal organisms (Tremaroli & Bäckhed, 2012). The host provides the stable environment necessary for the survival of these microbiota while relying on them to break down complex carbohydrates and other nutrients that cannot be directly absorbed, establishing a complex and dynamic balance (Yeoman et al., 2011). There are many factors that influence the diversity of the intestinal flora, such as diet (De Filippo et al., 2010; Turnbaugh et al., 2006), age (Mariat et al., 2009; Zhang, Liu, et al., 2018), seasonal changes (Xue et al., 2015), and habitat environment (Clayton et al., 2016; Kohl, Skopec, et al., 2014; McKenzie et al., 2017). When animals transition from captivity to the wild, they must adapt to intense changes, such as increased food variety,

Practitioner points

- During nature training, the structure and function of the gut microbiota in sub-adult sika deer can quickly align with those of adults, indicating their adaptability to the natural environment.
- Nature training is necessary before animals are reintroduced into the wild, with a recommendation to prioritize sub-adult individuals for reintroduction due to their enhanced adaptability.

expanded range of activity, complex social networks, and the absence of veterinary intervention. These changes can result in significant differences in the diversity, composition, and function of their gut microbiota (Guo et al., 2022; Trevelline et al., 2019).

The sika deer (*Cervus nippon*), once abundant in the forested areas of China, has experienced a dramatic decline in its wild population due to increased human disturbance and illegal poaching. As a result, it is now classified as a Class I protected species in China and serves as one of the primary prey species of the Amur tiger (*Panthera tigris altaica*) (Gu et al., 2018). In captivity, sika deer are typically fed a diet high in carbohydrates and proteins, whereas in the wild, they rely on fiber-rich plants to meet their nutritional needs. If these deer are released directly into the wild without preparation, their gut microbiota may struggle to adapt in time to the sudden shift in environmental conditions and nutritional changes, which could ultimately lead to mortality (Trevelline et al., 2019). Therefore, natural training is essential before reintroduction into the wild. This training allows the deer to gradually adjust to the wild environment,

develop a gut microbial system capable of digesting new foods, detoxify plant secondary metabolites accumulated from plants (Kohl, Weiss, et al., 2014; Miller et al., 2014; Pratchett and Jones, 1991), reduce the number of pathogenic bacteria, and ultimately increase their chances of successful adaptation.

Previous studies have focused on the analysis of differences in gut microbiota between captive and wild populations (Borbón-García et al., 2017; Clayton et al., 2016; Ning et al., 2020; Trevelline et al., 2019), as well as between herbivores of different ages, such as sika deer (Li, Wang, et al., 2018), foals (Costa et al., 2016), goats (Jiao et al., 2016), and cattle (Uyeno et al., 2010). These studies provide a solid theoretical foundation for the success of reintroduction projects. However, there is limited research focused on designing a period of natural training to assess how animals adapt to natural environments before reintroduction. For wild sika deer, the high abundance of fiber-digesting bacteria in the gut is an adaptation to the high-fiber diet typical in their natural environment (Guan et al., 2017). Therefore, during the natural training of captive populations, particular attention should be paid to promoting the growth of fiber-digesting bacteria. In this context, we define fiber-digesting bacteria and beneficial bacteria, which support the health of sika deer, as favorable for adaptation to the natural environment, while pathogenic bacteria, which are detrimental to their health, are considered unfavorable. To test these concepts, we conducted natural training by reducing the amount of dried food and adjusting the size of the rearing environment. During this process, we explored changes and differences in the diversity, composition, and function of the intestinal microbes of sika deer of different ages. We proposed three hypotheses: (1) Sub-adult sika deer exhibit lower gut microbiota diversity than adult sika deer but gradually approach adult levels with natural training; (2) Natural training reduces the abundance of pathogenic bacteria detrimental to adaptation while increasing the abundance of beneficial/fiber-degrading bacteria conducive to adaptation to the natural environment; (3) In the early stages of natural training, adult sika deer have higher levels of functional metabolic pathways than sub-adults, but sub-adults are expected to rapidly approach adult levels.

2 | MATERIALS AND METHODS

2.1 | Sample materials

In August 2021, we selected 12 captive sika deer (*C. nippon hortuloru*) for reintroduction, consisting of six adults and six sub-adults. After conducting disease screenings and genetic purity tests, the deer were transported from a breeding facility in Ning'an, Mudanjiang City, Heilongjiang

Province, to the training ground at Heilongjiang Xiaobeihu National Nature Reserve for natural training.

The Heilongjiang Xiaobeihu National Nature Reserve, located in the middle of Zhang Guang Cai Ling, Ning'an City, Heilongjiang Province, serves as the historical habitat of the sika deer. The reserve is geographically positioned between 128°33'07"-128°45'48"E and 44°03'16"-44°18'59"N. It stretches 28 km from north to south and 17 km from east to west, covering a total area of 20,834 hectares. A training ground, approximately 12 ha in size, was established within the reserve in an area with abundant natural water sources and minimal human interference. The area was secured with a metal fence featuring a 5 × 5 cm mesh and standing 3–3.5 m tall, further reinforced with a layer of nylon rope netting ranging from 1 to 1.5 m in height. Trees were used to support the fence, with deeply buried tree poles filling larger gaps between the outer trees. The training ground was divided into two sections: an isolation area and a semi-freedom enclosure area. All the sika deer were initially kept together. From Day 1 to 20, the deer were kept in the isolation area to manage potential emergencies caused by environmental changes; this period was defined as isolation time, during which clean river water was artificially provided for drinking. From Day 21 to 68, the deer were kept in the semi-free area, with access to natural river water; this period was defined as semi-free time. The diet consisted mainly of corn grain, wheat bran, and soybean meal, with the nutrient composition following the guidelines of Xiong et al. (2020) (detailed in Supporting Information S1: Table 1). To prevent chronic acidosis, Mongolian oak (*Quercus mongolica*) branches and leaves were also provided twice daily. Detailed information about the natural training design and other related aspects are illustrated in Figure 1.

A total of 94 fresh fecal samples were collected during the natural training process. Each sika deer was individually numbered, and efforts were made to collect fecal samples from each deer at different feeding stages. The groups and the number of samples collected are shown in Supporting Information S1: Table 9. Samples were collected within 2 min of defecation, ensuring minimal delay and contamination. During the collection process, researchers directly observed the deer, recorded the number and date of the sample, and ensured that each sample was accurately linked to the corresponding individual. Disposable gloves were used for sample collection, with gloves being changed between each collection to prevent cross-contamination. After collection, the samples were stored in an ultra-low temperature freezer (−80°C), and experiments were performed as soon as possible. Detailed information about the age structure, sample numbers, and sex of the deer are presented in Supporting Information S1: Table 2–3.

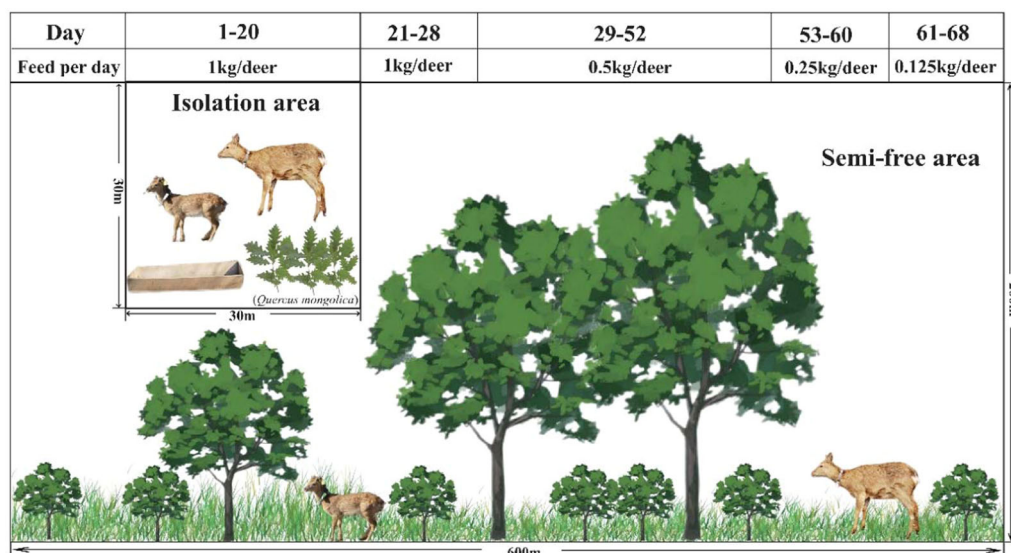


FIGURE 1 Natural training enclosure design schematic. We selected 12 sika deer comprising six adults and six sub-adults. Key considerations in the design of the natural training were to divide the training spatial ground into two parts (small areas for isolation of new individuals measuring 30 × 30 m and large areas measuring 200 × 600 m for free movement), and the importance of gradually reducing concentrated feed quantity. Details about the individual deer, sample numbers, feeding environment, and sampling time are depicted in Supporting Information S1: Table 2–3.

2.2 | DNA extraction

Total community genomic DNA extraction was performed using the E.Z.N.A.[™] MagBind Soil DNA Kit (Omega, M5635-02), following the manufacturer's instructions. To verify the quality and concentration of the extracted DNA, we used a Qubit 4.0 (Thermo), ensuring that the extracted DNA met the necessary criteria for subsequent analysis.

2.3 | 16S ribosomal RNA (rRNA) gene amplification by PCR

Our target was the V3–V4 hypervariable region of the bacterial 16S rRNA gene. Polymerase chain reaction (PCR) was started immediately after the DNA was extracted. The 16S rRNA V3–V4 amplicon was amplified using 2 × Hieff[®] Robust PCR Master Mix (Yeasen, 10105ES03). Two universal bacterial 16S rRNA gene amplicon PCR primers (polyacrylamide gel electrophoresis purified) were used: the amplicon PCR forward primer (CCTACGGGNGGCWGCAG) and the amplicon PCR reverse primer (GACTACHVGGGTATCTAATCC). The reaction was set up as follows: template DNA (10 ng/μL) 2 μL; amplicon PCR forward primer (10 μM) 1 μL; amplicon PCR reverse primer (10 μM) 1 μL; 2 × Hieff[®] Robust PCR Master Mix (Yeasen, 10105ES03) (total 30 μL). The plate was sealed, and PCR was performed in a thermal instrument (Applied Biosystems 9700) using the following program: one cycle of denaturing at 95°C for 3 min, first five cycles of denaturing at 95°C for 30 s, annealing at 45°C for 30 s, elongation at 72°C for 30 s, then 20 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final extension at 72°C for 5 min. The PCR products were checked using electrophoresis in 2% (w/v) agarose gels

prepared in TBE buffer (tris, boric acid, ethylene diamine tetraacetic acid) and stained with ethidium bromide. Visualization was carried out under UV light.

2.4 | 16S gene library construction, quantification, and sequencing

We used Hieff next generation sequencing ribosomal database project[™] DNA Selection Beads (Yeasen, 10105ES03) to purify the free primers and primer dimer species in the amplicon product. Samples were delivered to Sangon BioTech for library construction using universal Illumina adaptors and indices. Before sequencing, the DNA concentration of each PCR product was determined using a Qubit[®] 4.0 Green double-stranded DNA assay and it was quality-controlled using a bioanalyzer (Agilent 2100). Depending on coverage needs, all libraries can be pooled for one run. The amplicons from each reaction mixture were pooled in equimolar ratios based on their concentration. Sequencing was performed using the Illumina MiSeq system (Illumina MiSeq), according to the manufacturer's instructions.

2.5 | Sequence processing, operational taxonomic unit (OTU) clustering, representative tags alignment, and biological classification

After sequencing, the paired-end reads were assembled using PEAR software (version 0.9.8) based on the overlap and fastq files were processed to generate individual fasta and qual files, which could then be analyzed by standard methods. The resulting effective tags were clustered into OTUs at a similarity threshold of ≥97% using Usearch

software (version 11.0.667). Chimeric sequences and singleton OTUs (with only one read) were removed, after which the remaining sequences were sorted into their respective samples based on OTU assignments. The tag sequence with the highest abundance was selected as a representative sequence within each cluster. Bacterial OTU representative sequences were classified by blasting from the ribosomal database project.

2.6 | Statistical analysis

Species accumulation curves were plotted using the R package “vegan” to assess the adequacy of sampling. Bacterial diversity was determined through sampling-based OTU analyses and expressed using Shannon and Chao1 indices, both calculated with the “vegan” package (Oksanen et al., 2020). The Wilcoxon rank sum test was used to compare the alpha diversity of adult and sub-adult sika deer at each stage of forage change. Constrained principal coordinates analysis (CPCoA) based on Bray-Cruttis distances was conducted, with statistical significance assessed via permutational multivariate analysis of variance (PERMANOVA) ($p < 0.05$) (Oksanen et al., 2015). For each stage of feed change, the distribution of the gut microbial phyla and genera in adult and sub-adult sika deer was mapped using the R package “ggplot2.” A linear mixed model (LMM) was used to evaluate changes in gut microbial diversity, composition, and metabolic function over time. In this model, gut microbial diversity, composition, and metabolic function served as response variables, with rearing date as the explanatory variable. The individual ID of each sika deer was included as a random effect to account for individual variation (Van Doormaal et al., 2015). The LMM was fitted using the R package “YawMMF” (Zhang et al., 2020). Model testing was performed using the R package “MuMIn” to calculate the marginal and conditional R-squared values of the model. All analyses were conducted in R version 4.3.2.

2.7 | Function prediction

Functional prediction analysis of bacterial communities was conducted using PICRUSt (v1.1.4) software, by comparing the existing 16S rRNA gene sequencing data with a microbial reference genome database (Douglas et al., 2019) of known metabolic functions, enabling the prediction of bacterial metabolic functions based on known metabolic pathways.

3 | RESULTS

3.1 | Overview of the sequencing data

A total of 6,551,447 high-quality reads were obtained after quality control of 94 sika deer fecal samples

(Mean \pm SD: 69,696 \pm 15,698), with an average sequence length of 412 \pm 1 (Mean \pm SD). We used these reads for subsequent bacterial community diversity analyses, including Alpha-diversity indices such as Shannon and Chao1 (Supporting Information S1: Table 4). Rarefaction analysis was performed based on the Shannon diversity index, and the results showed that with the increase of the sequencing amount, the curve gradually plateaued, indicating that the sequencing depth basically met the requirements for subsequent analysis (Supporting Information S1: Figure 1).

3.2 | Gut microbial diversity of adult and sub-adult sika deer

Our results showed that the Alpha-diversity of gut microbiota in adult and sub-adult sika deer fluctuated with the progression of natural training. On Days 1–20, adult sika deer had significantly higher Shannon and Chao1 indices than sub-adults. In the semi-free stage, adult sika deer continued to show significantly higher Shannon indices than sub-adults on Days 29–52, and significantly higher Chao1 indices on Days 21–52. However, after Day 53, no significant differences were observed between adults and sub-adults across all indices (Figure 2a,b). We employed LMMs to analyze the trends in Shannon and Chao1 indices, using sika deer ID as a random factor and training days as a fixed factor. The results showed an increasing trend in the Shannon and Chao1 indices of sub-adult sika deer, while no significant changes were observed in the adult sika deer. The results of the model are presented in Table 1, and the results of the model validation are shown in Supporting Information S1: Table 5. To explore differences in gut microbiota composition between adult and sub-adult sika deer at various stages of natural training, we performed CPCoA analysis based on Bray-Cruttis distance. The analysis revealed that CPCoA1 accounted for 30.05% and CPCoA2 accounted for 21.39% of the total gut microbiota variation between adult and sub-adult sika deer. The large degree of separation between adult and sub-adult sika deer from Day 1–20 of natural training indicated that their gut microbiota compositions differed considerably, but with natural training, the gut microbiota compositions of adult and sub-adult sika deer showed a trend toward convergence (Figure 3; PERMANOVA: $p < 0.05$).

3.3 | Gut microbial composition and differences between adult and sub-adult sika deer

We identified a total of 31 phyla, 56 classes, 69 orders, 159 families, and 383 genera within the gut microbiota community from the 94 fecal samples of sika deer. The relative abundance of gut microbiota

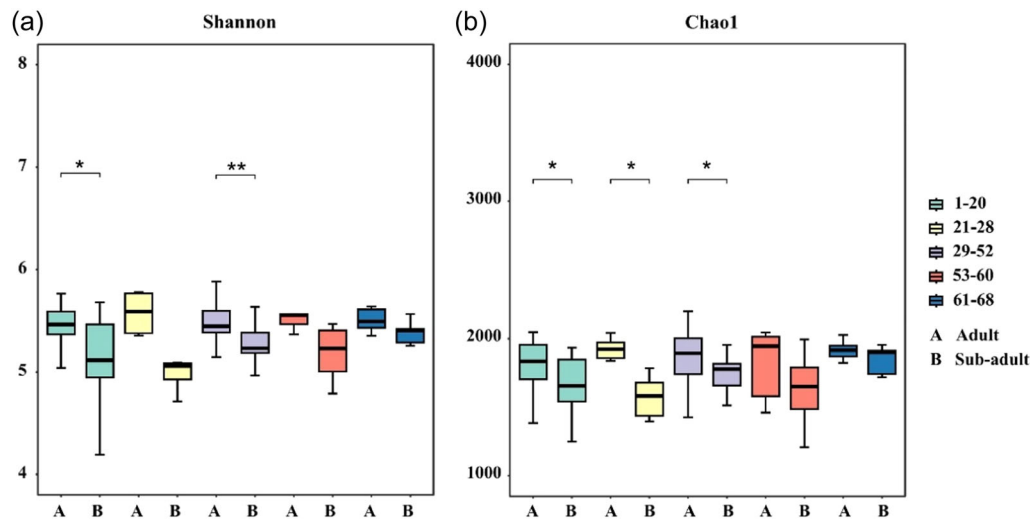


FIGURE 2 Changes and differences in the Shannon and Chao1 indices of the gut microbiome of adult and sub-adult sika deer during natural training. (a) Boxplots showing the Shannon index for the gut microbiome of adult and sub-adult sika deer. (b) Boxplots showing the Chao1 index for the gut microbiome of adult and sub-adult sika deer. Boxes represent the interquartile range, with the horizontal line inside the box indicating the median. Statistical significance is denoted by * $p < 0.05$ and ** $p < 0.01$.

TABLE 1 Covariate coefficients and p values for linear mixed models (sika deer ID as a random factor) of the Shannon and Chao1 indices of gut microbiota over training days in adult and sub-adult sika deer.

Age structure	Alpha diversity index	Covariates	Coefficients	Confidence interval		<i>p</i> Value
				(2.5%–97.5%)		
Adult	Shannon	Intercept	5.382	5.209	5.553	<0.001
		Training day	0.002	−0.002	0.006	0.220
	Chao1	Intercept	1803.552	1672.739	1935.014	<0.001
		Training day	1.228	−1.017	3.485	0.286
Sub-adult	Shannon	Intercept	4.99	4.753	5.223	<0.001
		Training day	0.005	0.001	0.009	0.017
	Chao1	Intercept	1540.544	1385.284	1692.871	<0.001
		Training day	3.701	0.828	6.5	0.014

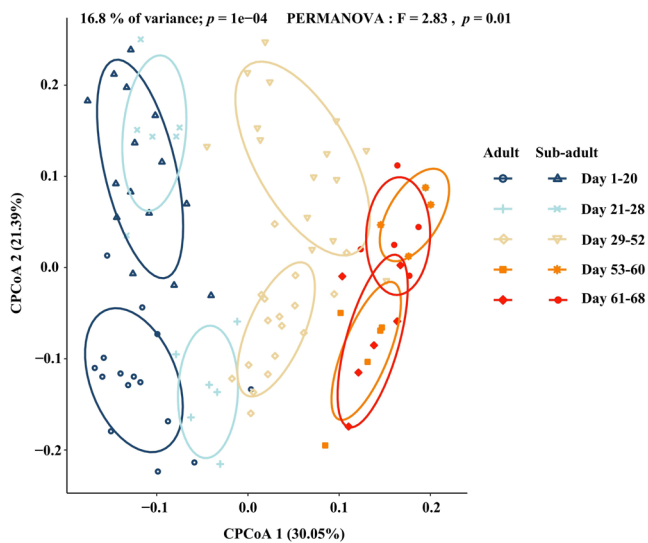


FIGURE 3 Constrained principal coordinate analysis (CPCoA) of gut microbiota in adult and sub-adult sika deer during natural training.

between adult and sub-adult sika deer is shown in Figure 4. Our analysis revealed the core phyla common to both adult and sub-adult sika deer were Firmicutes and Bacteroidetes, collectively accounting for more than 80% of the microbiota. During natural training, gut microbiota that play a crucial role in the adaptation of both adult and sub-adult sika deer to the natural environment include Firmicutes, Verrucomicrobia, and Actinobacteria at the phylum level, and *Roseburia* and *Akkermansia* at the genus level. We applied LMMs to analyze adult and sub-adult sika deer gut microbiota conducive to adaptation to the field environment, using feeding days as a fixed effect and sika deer ID as a random effect. The model results are shown in Table 2, with the model validation detailed in Supporting Information S1: Table 6. Our results showed that, among the gut microbiota favorable for adaptation, the relative abundance of all microbes showed an increasing trend in both adult and sub-adult sika deer, with the exception of Firmicutes in

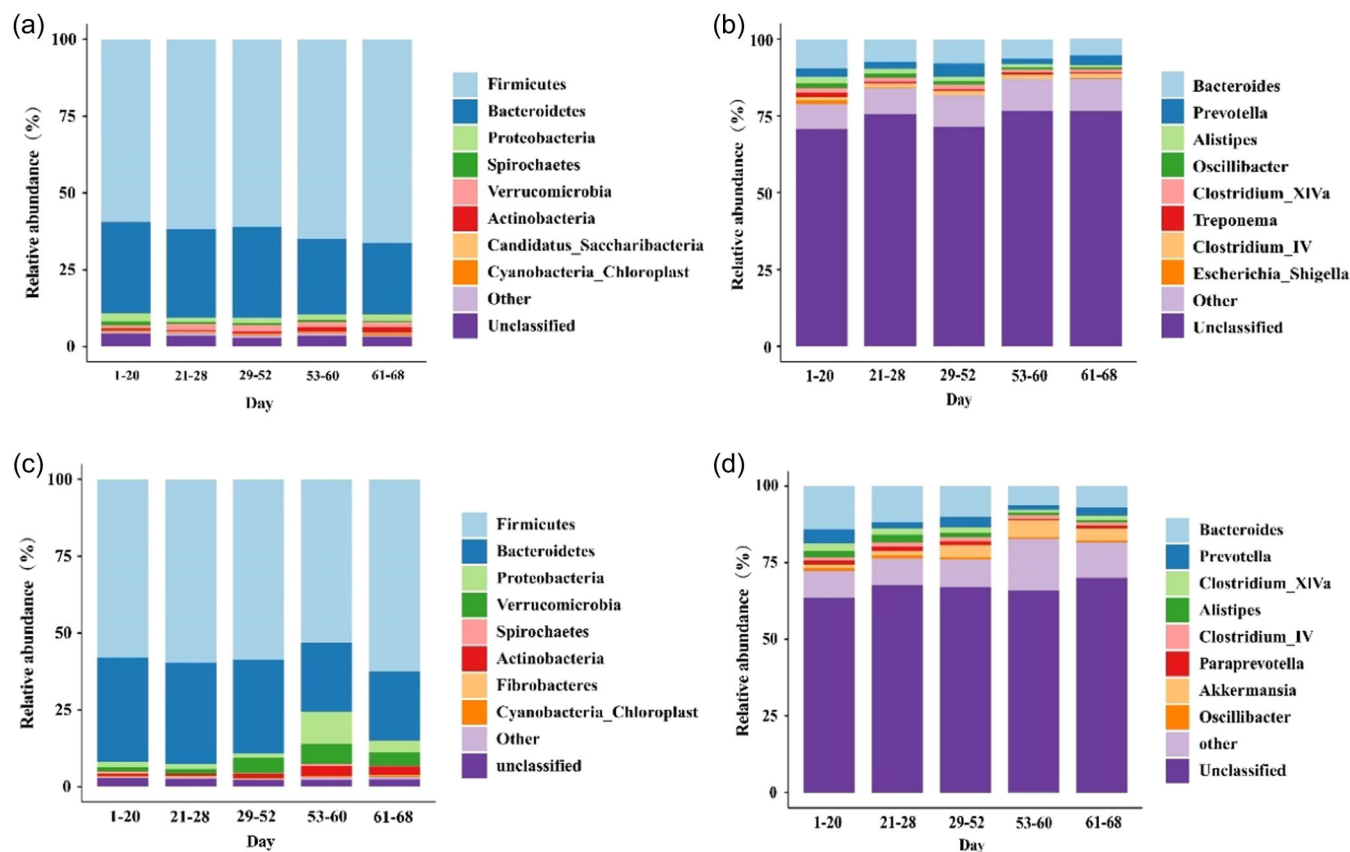


FIGURE 4 Relative abundance of phyla and genera of gut microbiota in adult and sub-adult sika deer. (a) Relative abundance of phyla in the gut microbiota of adult sika deer. (b) Relative abundance of genera in the gut microbiota of adult sika deer. (c) Relative abundance of phyla in the gut microbiota of sub-adult sika deer. (d) Relative abundance of genera in the gut microbiota of sub-adult sika deer. "Other" refers to the number of other bacteria, and "Unclassified" refers to sequences that could not be classified.

sub-adults, which showed a nonsignificant increasing trend. Sub-adult sika deer exhibited a higher rate of increase in these beneficial gut microbiota than adults, except for Firmicutes.

Gut microbiota that are unfavorable to the adaptation of adult and sub-adult sika deer to their natural environment include Spirochaetes at the phylum level, and *Alistipes*, *Treponema*, *Oscillibacter*, and *Clostridium_XIVa* at the genus level. LMMs were used to analyze the gut microbiota of adult and sub-adult sika deer that are unfavorable for adaptation to the field environment, with days of feeding as a fixed effect and sika deer ID as a random effect. The model results are shown in Table 3, and the model validation is shown in Supporting Information S1: Table 7. The results indicated that among the gut microbiota unfavorable for adaptation to the field environment, Spirochaetes, *Treponema*, and *Oscillibacter* declined more rapidly in the gut microbiota of adult sika deer than in sub-adults, with no significant trend of decline observed for Spirochaetes and *Treponema* in sub-adults. *Alistipes* and *Clostridium_XIVa* in the gut microbiota of sub-adult sika deer showed a faster rate of decline than in adults, with no significant decreasing trend in *Clostridium_XIVa* in the adult sika deer.

3.4 | Analysis of differences in metabolic functions between adult and sub-adult sika deer

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, functional changes in the gut microbiota of adult and sub-adult sika deer during natural training (KEGG pathway level 2) were analyzed to investigate how such alterations in microbiota structure would affect functional pathways (Supporting Information S1: Table 10). Carbohydrate metabolism and lipid metabolism were selected as secondary metabolic pathways contributing to adaptation to the natural environment. LMMs were used to analyze changes in these metabolic functions in adult and sub-adult sika deer, with days of feeding as a fixed effect and sika deer ID as a random effect. The model results are shown in Table 4, and the model validation is shown in Supporting Information S1: Table 8. The model results revealed that the functional pathways of carbohydrate metabolism in the gut microbiota of both adult and sub-adult sika deer decreased significantly. In contrast, the functional pathways of lipid metabolism in the gut microbiota of both adult and sub-adult sika deer showed an increasing trend, although this was not significant in adults ($p = 0.337$).

TABLE 2 Covariate coefficients and p values for a linear mixed model (sika deer ID as a random factor) of beneficial microbes/fiber-digesting bacteria in the gut microbiota with training days in adult and sub-adult sika deer.

Age structure	Microbes	Covariates	Coefficients	Confidence interval		p Value
				(2.5%–97.5%)		
Adult	Firmicutes	Intercept	57.996	52.404	63.636	<0.001
		Training day	0.106	0.024	0.189	0.015
	Verrucomicrobia	Intercept	1.083	0.428	1.735	0.003
		Training day	0.016	0.002	0.031	0.029
	Actinobacteria	Intercept	0.256	−0.093	0.606	0.167
		Training day	0.017	0.009	0.025	<0.001
	<i>Roseburia</i>	Intercept	0.426	0.105	0.757	0.023
		Training day	0.011	0.006	0.017	<0.001
	<i>Akkermansia</i>	Intercept	0.584	0.097	1.068	0.022
		Training day	0.014	0.003	0.026	0.016
Sub-adult	Firmicutes	Intercept	57.564	54.152	61.003	<0.001
		Training day	0.025	−0.052	0.106	0.529
	Verrucomicrobia	Intercept	0.451	−1.807	2.717	0.694
		Training day	0.083	0.026	0.136	0.004
	Actinobacteria	Intercept	−0.225	−0.937	0.479	0.535
		Training day	0.042	0.025	0.059	<0.001
	<i>Roseburia</i>	Intercept	0.756	0.255	1.256	0.005
		Training day	0.016	0.004	0.029	0.014
	<i>Akkermansia</i>	Intercept	0.231	−1.86	2.275	0.826
		Training day	0.071	0.021	0.122	0.009

4 | DISCUSSION

An important reason for the low success rate of reintroduction efforts is the inability of the animals' gut microbiota to quickly adapt to the drastic changes in diet and other environmental factors when transitioning from captivity to the wild (Guo et al., 2022; Lecorps et al., 2021; Trevelline et al., 2019). In response to this challenge, our study implemented a natural training regimen for sika deer before their reintroduction in Heilongjiang Xiaobeihu Nature Reserve. The study analyzed changes in gut microbial diversity, composition, and function in both adult and sub-adult sika deer during this training, demonstrating the existence of distinct adaptation processes. Natural training, in effect, altered the gut microbial structure of adult and sub-adult sika deer in a way that favored their adaptation to the natural environment.

4.1 | Gut microbial diversity in adult and sub-adult sika deer

Most studies on gut microbiota rely on fecal samples, which reflect the overall status of microbial communities in the intestinal tract (De La Torre et al., 2019; Su et al., 2018; Zhang, Shi, et al., 2018). Research has

shown that the primary factors influencing gut microbiota diversity in mammals are their living environment and diet (Henriques et al., 2020; Ley et al., 2008; Youngblut et al., 2019). Increased microbial diversity is associated with improved host health, function, and stability (Bestion et al., 2017; Dallas & Warne, 2023; Lozupone et al., 2012; Mckenney et al., 2018; Tap et al., 2015).

Beta diversity reflects the differences between sample groups (Knights et al., 2011). PCoA revealed significant differences in the gut microbiota between adult and sub-adult sika deer (Figure 3; PERMANOVA: $p < 0.05$). However, as natural training progressed, the gut microbiota of adult and sub-adult sika deer exhibited converging trends, suggesting that natural training indeed influences gut microbiota diversity, allowing sub-adult gut microbiomes to rapidly approach those of adults.

Alpha diversity indices were significantly higher in adult sika deer compared to sub-adults from Days 1 to 52, but these differences diminished as natural training progressed. The model results showed that the Alpha diversity of gut microbiota in sub-adults tended to increase significantly with training, while there was no significant change in adults. These results suggest that in natural environments, where most plants have not been previously foraged by sika deer and where sika deer rearing farms have

TABLE 3 Covariate coefficients and *p* values for a linear mixed model (sika deer ID as a random factor) of pathogenic bacteria in gut microbiota with training days in adult and sub-adult sika deer.

Age structure	Microbes	Covariates	Coefficients	Confidence interval		<i>p</i> Value
				(2.5%–97.5%)		
Adult	Spirochaetes	Intercept	1.329	0.939	1.703	<0.001
		Training day	−0.016	−0.023	−0.009	<0.001
	<i>Alistipes</i>	Intercept	2.195	1.784	2.596	<0.001
		Training day	−0.019	−0.028	−0.011	<0.001
	<i>Treponema</i>	Intercept	1.314	0.924	1.688	<0.001
		Training day	−0.016	−0.022	−0.009	<0.001
	<i>Oscillibacter</i>	Intercept	1.71	1.507	1.913	<0.001
		Training day	−0.015	−0.019	−0.01	<0.001
	<i>Clostridium_XIVa</i>	Intercept	1.441	1.17	1.714	<0.001
		Training day	−0.003	−0.009	0.003	0.356
Sub-adult	Spirochaetes	Intercept	0.604	0.204	0.995	0.009
		Training day	−0.006	−0.013	0.001	0.127
	<i>Alistipes</i>	Intercept	2.31	1.783	2.822	<0.001
		Training day	−0.021	−0.032	−0.011	<0.001
	<i>Treponema</i>	Intercept	0.592	0.191	0.982	0.01
		Training day	−0.006	−0.013	0.002	0.139
	<i>Oscillibacter</i>	Intercept	1.166	0.935	1.393	<0.001
		Training day	−0.01	−0.014	−0.005	<0.001
	<i>Clostridium_XIVa</i>	Intercept	2.848	2.247	3.448	<0.001
		Training day	−0.024	−0.038	−0.01	0.001

TABLE 4 Covariate coefficients and *p* values for a linear mixed model (sika deer ID as a random factor) for secondary metabolic pathways of gut microbiota in adult and sub-adult sika deer during natural training.

Age structure	Metabolism	Covariates	Coefficients	Confidence interval		<i>p</i> Value
				(2.5%–97.5%)		
Adult	Carbohydrate metabolism	Intercept	10.067	10.006	10.127	<0.001
		Training day	−0.001	−0.003	0.000	0.046
	Lipid metabolism	Intercept	2.838	2.800	2.876	<0.001
		Training day	0.000	0.000	0.001	0.337
Sub-adult	Carbohydrate metabolism	Intercept	10.170	10.088	10.254	<0.001
		Training day	−0.003	−0.005	−0.001	0.002
	Lipid metabolism	Intercept	2.754	2.690	2.819	<0.001
		Training day	0.003	0.002	0.005	<0.001

brick floors (limiting exposure to soil), gut microbial diversity increases as both adult and sub-adult forage and explore their new surroundings (Dallas & Warne, 2023; Diaz et al., 2023). Adult sika deer maintain a high level of dynamic gut microbial diversity to counterbalance the possible adverse effects of long-term captivity before nature training (Li, Wang, et al., 2018). In contrast, sub-adult sika deer initially displayed lower diversity, which rapidly increased to near adult levels as they adapted to

significant environmental and dietary changes during natural training.

4.2 | Gut microbiota in adult and sub-adult sika deer: Favorable/unfavorable for adaptation to the natural environment

At the beginning of natural training, the sika deer were fed a diet rich in carbohydrates, proteins, fats,

and amino acids (Supporting Information S1: Figure 1). As the training progressed, the amount of concentrated feed was gradually reduced, requiring the sika deer to consume large amounts of fiber-rich plants to obtain the nutrients needed for survival in the wild. The gastrointestinal tract of ruminants can ferment starch and sugars from fibrous plant material, but it cannot produce the necessary fiber-degrading enzymes on its own; these enzymes must be produced by the bacteria colonizing their intestines (Hu et al., 2017). Firmicutes and *Roseburia* are important fiber-digesting bacteria that increase with the intake of fiber-rich foods (Mirande et al., 2010; Leth et al., 2018). They encode three major cellulose-digesting enzymes—endoglucanase, β -glucosidase, and cellulose 1,4-beta-cellobiosidase (Wang et al., 2019)—which break down fiber into volatile fatty acids that are available to the host. *Roseburia*, in particular, is an important butyrate-producing bacterium in Firmicutes. Butyrate has wide-ranging anti-inflammatory and metabolic regulatory roles in various disease models (Canfora et al., 2015; McNabney and Henagan, 2017; Tamanai-Shacoori et al., 2017).

During natural training, we also observed an increase in the relative abundance of Verrucomicrobia and Actinobacteria, both of which are bacteria beneficial to the organism. Verrucomicrobia is found in humans mainly as *Akkermansia*, a common inhabitant of the human gut, accounting for about 1–3% of the total gut microbiota (Almeida et al., 2020). *Akkermansia* is considered a next-generation beneficial microbe (Cani, 2017; Cani et al., 2022; Zhai et al., 2019) with important roles in maintaining gut health, regulating host metabolism and immune response (Macchione et al., 2019; Naito et al., 2018), and protecting against cardiovascular diseases (Li et al., 2016). Its products can provide energy for ruminants, and *Akkermansia* may act as an energy sensor, being abundant during caloric deficits and scarce during energy surpluses, reflecting a co-evolutionary mechanism for energy absorption when available (Chevalier et al., 2015). *Akkermansia* levels have been shown to be elevated in malnourished mice (Preidis et al., 2015). The relative abundance of *Akkermansia* tends to increase as the amount of feed is gradually reduced during natural food training.

Actinobacteria are mainly distributed in organic matter-rich and slightly alkaline soils and in the gastrointestinal tract of herbivorous animals, aiding digestion. The lactic and acetic acid produced by Actinobacteria can regulate gut pH, inhibit the proliferation of harmful bacteria, maintain intestinal health, and slow down gut aging (Di Gioia et al., 2014; Yang et al., 2020). In a study of goats transitioning from a forage-based diet to a high-grain diet, the abundance of Actinobacteria tended to decrease as dietary starch content increased (Grilli et al., 2016). This finding aligns with our study, which showed that the abundance of Actinobacteria

increased as the amount of forage in the sika deer diet increased and the amount of edible starch decreased.

Long-term captive environments can increase the risk of disease in animals, leading to problems such as excessive obesity (Ley, 2010; Vallianou et al., 2019) or gut inflammation (Mosca et al., 2016), which can threaten survival and reduce reintroduction success (Dallas and Warne, 2023). During natural training, we found a tendency for a decrease in *Spirochaetes*, *Alistipes*, *Treponema*, *Oscillibacter*, and *Clostridium_XIVa* in the gut microbiota of adult and sub-adult sika deer. These are pathogenic bacteria mostly associated with inflammation (Abdel-Moein et al., 2015; Feng et al., 2015; Frosth et al., 2023; Kim et al., 2020; Zhou et al., 2018), and they have been linked to the development of osteoporosis (Wei et al., 2021), various types of cancers (Parker et al., 2020; Yin et al., 2023; Zhang et al., 2019), chronic kidney disease (Kim et al., 2020), and certain psychiatric disorders (Jolivet-Gougeon & Bonnaure-Mallet, 2018; Naseribafrouei et al., 2014; Yang et al., 2021).

Overall, our results indicate that the beneficial/fiber-digesting bacteria in the gut microbiota of sika deer showed an increasing trend during natural training, while pathogenic bacteria showed a decreasing trend. This suggests that natural training can help sika deer adapt to a high-fiber diet in the natural environment by enhancing the presence of beneficial bacteria and reducing pathogenic bacteria, thereby promoting better health and adaptability to the natural environment.

4.3 | Metabolic pathways of gut microbial functions in adult and sub-adult sika deer

A previous study showed that carbohydrate metabolism, lipid metabolism, and other processes are the main functions of the gut microbiota in tigers (Jiang et al., 2020). During natural training, as the variety of available diets decreases, sika deer must consume more food rich in hard-to-digest fiber to maintain the nutrients and energy required for survival. This shift leads to a decrease in carbohydrate metabolism in adult and sub-adults as they adjust to digesting and breaking down their fibrous diets. Moreover, adult sika deer are more competitive for food than sub-adults, which was observed during the natural training; adults would drive away sub-adults when food was scarce to secure more nutrients for themselves. Lipid metabolism involves the biosynthesis and degradation of lipids such as fatty acids, triglycerides, and cholesterol (Schoeler & Caesar, 2019). Short-chain fatty acids, which are bacterial metabolites produced by gut microbiota through the anaerobic fermentation of fibers, serve as major energy sources. These include acetate, propionate, and butyrate, which play roles in immunity, inflammation, and the regulation of lipid metabolism (Dalile et al., 2019; Hu et al., 2018; Zhang et al., 2023). Natural training for sika deer takes

place between August and October, so sub-adult sika deer require a low-fat, low-sugar, high-fiber diet to maximize lipid metabolism, allowing them to store more energy in preparation for impending food shortages and the cold winter in the wild. This is consistent with previous studies that showed gut microbiota in mice increases intestinal absorption, energy homeostasis, and fat burning during cold periods (Ufarté et al., 2015). In addition, these changes improve digestive efficiency and SCFA production in ruminants living in cold environments and at high altitudes (Earle et al., 2015; Geva-Zatorsky et al., 2015). However, these metabolic changes may also correspond to a reduced abundance of microbes related to host immune function and an increased risk of disease (Cummings et al., 1987; Hooper et al., 2002; Rooks and Garrett, 2016).

5 | CONSERVATION IMPLICATIONS

To improve the reintroduction success rate of sika deer and other captive ungulates, we put forward the following recommendations based on the findings of this study: (1) Breeders often raise large numbers of sika deer in a limited space and feed them high-sugar and high-fat diets to maximize economic benefits. These practices can increase the potential pathogenicity of the animals, which is not conducive to successful reintroduction. This issue can be mitigated by rationally planning breeding numbers, ensuring a clean environment, and increasing the fiber content of the feed. Such measures will improve the overall health of the animals in captivity, creating a healthier and more viable breeding stock for reintroduction efforts. (2) Before releasing captive animals into the natural environment, it is recommended to implement a period of natural, localized training. This allows the animals to establish a stable gut microbiota that is better adapted to the natural environment and the new food composition. Such training is likely to improve the success of reintroduction by helping animals acclimate more effectively to their new surroundings. (3) Adult sika deer tend to have more stable gut microbiota diversity, composition, and function during natural training, which develops under long-term captive conditions to cope with possible adverse effects of such environments. In contrast, the gut microbiota of sub-adult sika deer, though less stable initially, can rapidly approach adult levels after natural training, demonstrating good plasticity. Therefore, we recommend prioritizing sub-adults when reintroducing ungulates, as they may adapt more effectively to the wild. These findings could be essential for the successful reintroduction of large ungulates in future conservation efforts.

AUTHOR CONTRIBUTIONS

Guangshun Jiang designed this research; Wentao Zhang, Feifei Yang, Jiale Sun, Yanhui Guan, Shixian Guo, and Jixu Sun contributed to the data

collection work; Data analysis was mainly carried out by Wentao Zhang, Feifei Yang, Heng Bao, Jiale Sun, Wannian Cheng, Shiyu Chen and Nathan J. Roberts; Wentao Zhang wrote the first draft of the manuscript; Feifei Yang, Heng Bao, and Nathan J. Roberts revised and improved it. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Nathan J. Roberts  <http://orcid.org/0000-0001-8299-4499>

Guangshun Jiang  <http://orcid.org/0000-0001-6321-9489>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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