High-Throughput Sequencing Analysis of Gut Microbiota Diversity in Blue Foxes (Postprint)

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Date: 2018-12-25T00:00:00+00:00

Abstract

This study aimed to investigate the composition and diversity of the gut microbiota in blue foxes. Eight healthy male farmed blue foxes in the growing period (5–6 months of age) were selected, and fresh fecal samples were collected for analysis of gut microbiota composition and diversity using high-throughput sequencing technology. The results showed that a total of 569,930 valid sequences were obtained from the feces of the eight healthy blue foxes, with operational taxonomic units (OTUs) ranging from 468 to 574, distributed across 209 genera within 16 phyla. At the phylum level, Firmicutes, Baeteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria were dominant, with Firmicutes having the highest proportion at 62.97%, followed by 22.05%, 8.89%, 5.15%, and 0.88%, respectively. At the genus level, Streptococcus had the highest proportion at 11.75%, followed by Lactobacillus (9.86%), Prevotella (9.28%), Megasphaera (8.21%), Collinsella (7.27%), Blautia (7.08%), and Bacteroides (5.64%). High-throughput sequencing revealed that blue foxes possess a complex gut microbiota structure with substantial inter-individual variation.

Full Text

Analysis of Intestinal Microbiota Diversity in Blue Foxes Using High-Throughput Sequencing Technology

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Abstract

This study aimed to investigate the composition and diversity of intestinal microbiota in blue foxes. Eight healthy growing male blue foxes (5–6 months

of age) under stall-feeding conditions were selected, and fresh fecal samples were collected for analysis of intestinal microbiota composition and diversity using high-throughput sequencing technology. The results showed that a total of 569,930 valid sequences were obtained from the fecal samples of eight healthy blue foxes, with operational taxonomic units (OTUs) ranging from 468 to 574, distributed across 209 genera within 16 phyla. At the phylum level, the dominant taxa were Firmicutes (62.97%), Bacteroidetes (22.05%), Actinobacteria (8.89%), Proteobacteria (5.15%), and Fusobacteria (0.88%). At the genus level, Streptococcus exhibited the highest relative abundance (11.75%), followed by Lactobacillus (9.86%), Prevotella (9.28%), Megasphaera (8.21%), Collinsella (7.27%), Blautia (7.08%), and Bacteroides (5.64%). These findings reveal that blue foxes possess a complex intestinal microbiota structure with substantial inter-individual variation.

Keywords: blue foxes; intestinal microbiota; high-throughput sequencing; diversity

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The blue fox (*Alopex lagopus*), also known as the Arctic fox, belongs to the phylum Chordata, class Mammalia, order Carnivora, family Canidae, and genus *Alopex* [**?**]. As a fur-bearing animal, blue foxes are raised in large numbers worldwide, with major farming regions in China concentrated in the Jiaodong Peninsula, Hebei Province, and the three northeastern provinces. Adapted to cold natural climates, blue foxes store substantial body fat and exhibit high utilization efficiency of high-protein, high-fat animal-based feeds, with a broad capacity to utilize various animal feed ingredients including different fish species, livestock and poultry by-products (boneless beef, mutton, pork, rabbit meat, chicken, etc.), and animal livers (such as rabbit, beef, sheep, chicken, duck, and goose liver). Currently, most research on blue foxes has focused on breeding, nutritional requirements, and pelt development [**?**], with few studies investigating intestinal microbiota composition and diversity.

The intestinal microbial system represents the most complex microecosystem within an animal, with numerous microorganisms participating in nutrient absorption, distribution, metabolism, and immune physiology. Dysbiosis of intestinal microbiota often leads to functional disorders in animals [**?**]. Therefore, understanding the normal distribution of intestinal microbiota in healthy animals is crucial for maintaining microbial balance and ensuring animal health. As carnivorous monogastric animals, foxes harbor gastrointestinal microorganisms primarily in the large intestine [**?**], yet the specific microbial composition and their roles in digestive physiology remain unclear. This study employed high-throughput sequencing technology to analyze the intestinal microbiota composition of blue foxes and investigate its diversity, providing a microbiological foundation for further elucidating the role of intestinal microbiota in nutrient metabolism and utilization in blue foxes.

Materials and Methods

On September 17, 2017, eight healthy growing male blue foxes (F1–F8) aged 5–6 months were selected from Changli County, Hebei Province (39.72°E, 119.15°N). All animals exhibited good mental condition, normal feeding behavior, and clean living environments. Fresh fecal samples were collected immediately after defecation using sterile collection bags to prevent contamination from ground contact. Approximately 15 g of fresh feces were aliquoted into 5 mL cryovials (5 g per tube, three tubes per animal) and transported to the laboratory in liquid nitrogen for subsequent analysis. The foxes were fed a standard farm diet, with composition and nutrient levels shown in .

DNA Extraction

Microbial genomic DNA was extracted from blue fox fecal samples using the Fast DNA R SPIN Kit for Feces according to the manufacturer's instructions. Briefly, 0.5 g of fecal sample was placed in a Lysing Matrix E tube with 825 L phosphate buffer and 275 L PLS solution, then mixed thoroughly. After centrifugation at 14,000×g for 5 min, the supernatant was discarded. The pellet was resuspended in 978 L phosphate buffer and 122 L MT buffer, mixed, and homogenized in a FastPrep R24 device at 6.0 m/s for 40 s. Following another centrifugation at 14,000×g for 5 min, the supernatant was transferred to a 2 mL centrifuge tube. Then 250 L PPS solution was added, mixed, and incubated at 4 °C for 10 min before centrifugation at 14,000×g for 2 min. The supernatant was transferred to a new 2 mL tube, mixed with 1 mL Binding Matrix Solution, and oscillated for 3 min. After centrifugation at 14,000×g for 2 min, the supernatant was discarded, and the pellet was washed twice with wash solutions according to the kit protocol. Finally, DNA was eluted in 100 L TES solution and stored at -20 °C.

Extracted genomic DNA was evaluated by 1% agarose gel electrophoresis (120 V for 23 min, using III DNA Marker). DNA products appeared at approximately 14,000 bp with high band intensity, showing no obvious degradation or contamination, confirming suitability for subsequent experiments [**?**]. The target DNA was sent to Beijing Novogene Bioinformatics Technology Co., Ltd. for sequencing on the Illumina MiSeq platform (HiSeq PE250) with a read length of 250 bp.

PCR Amplification and Sequencing

Bacterial 16S rRNA V3/V4 region-specific primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5' -GGACTACHVGGGTWTCTAAT-3') were used for PCR amplification with extracted total DNA as template. The 50 L PCR reaction mixture contained 1 L each of forward and reverse primers, 1 L DNA template, 25 L Taq polymerase, and 22 L ddH O. Amplification conditions were: initial denaturation at 95 °C for 5 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; final extension at 72 °C for 10 min. PCR products were stored

at 4 °C and verified by 2% agarose gel electrophoresis.

Bioinformatics Analysis

Raw data were assembled and filtered to obtain high-quality sequences for accurate and reliable results. Effective sequences were clustered and taxonomically classified, with sequences sharing 97% similarity defined as one operational taxonomic unit (OTU). Microbial composition was statistically analyzed at each taxonomic level for each sample. Based on OTU clustering results, microbial richness and alpha diversity were calculated, with Ace and Chao1 indices representing richness and Simpson and Shannon indices representing diversity. Species abundance and diversity were statistically analyzed at both phylum and genus levels.

Results

Diversity Indices As shown in , a total of 654,716 sequences were obtained from eight samples, with individual sample sequence numbers ranging from 57,002 to 95,638. Among these, 569,930 valid sequences were retained (ranging from 52,758 to 84,112 per sample). Clustering analysis at 97% similarity yielded 4,071 total OTUs (ranging from 468 to 574 per sample). Richness indices averaged 559 for Ace (range: 480–699) and 595 for Chao1 (range: 465–1036). Diversity indices averaged 5.27 for Shannon (range: 4.05–5.80) and 0.936 for Simpson (range: 0.847–0.957). Detailed parameters for each sample are presented in .

Rarefaction curves generated using mothur for OTUs at 97% similarity ([Figure 1: see original paper]-A) showed that curves for all samples (except F8) gradually plateaued when sequencing depth exceeded 40,000 reads. Similarly, Shannon curves ([Figure 1: see original paper]-B) also approached saturation, indicating that the sequencing depth was sufficient to capture the majority of microbial diversity.

Phylum-Level Microbial Structure At the phylum level, microbial communities were taxonomically assigned to 17 phyla. Firmicutes was the most abundant phylum in blue fox intestinal microbiota, accounting for 62.97% of sequences, followed by Bacteroidetes (22.05%), Actinobacteria (8.89%), Proteobacteria (5.15%), and Fusobacteria (0.88%). These five dominant phyla collectively represented 99.94% of the total microbiota, with detailed distribution shown in [Figure 2: see original paper]. Notably, sample F4 exhibited lower similarity to other foxes, with Bacteroidetes as the most abundant phylum in its gut, whereas Firmicutes dominated in all other individuals.

Genus-Level Microbial Structure At the genus level, microbial communities were classified into 210 genera, with 30 genera accounting for 85.95% of total relative abundance. Streptococcus was the most abundant genus (11.75%),

followed by Lactobacillus (9.86%), Prevotella (9.28%), Megasphaera (8.21%), Collinsella (7.27%), Blautia (7.08%), Bacteroides (5.64%), Alloprevotella (5.50%), Peptoclostridium (5.07%), and Megamonas (3.55%). Detailed relative abundance at the genus level is presented in [Figure 3: see original paper].

The proportions of the top 10 genera in each individual blue fox are shown in . The dominant genus in F1 was Collinsella (17.45%), while Prevotella dominated in F2, F3, and F4 (14.52%, 13.06%, and 22.16%, respectively). Lactobacillus was most abundant in F5 and F7 (17.56% and 21.55%, respectively), and Streptococcus predominated in F6 and F8 (15.80% and 25.72%, respectively). Notably, F8 showed particularly high proportions of Streptococcus, Lactobacillus, and Megasphaera, which collectively accounted for 71.00% of its gut microbiota.

Discussion

Analysis of Diversity Indices Alpha diversity analysis typically reflects sample richness and diversity. In this study, sequencing coverage for all samples exceeded 0.997, indicating high bacterial coverage and adequate sequencing depth for intestinal microbiota analysis [**?**]. Rarefaction curves plateaued for all samples except F8 when sequencing depth surpassed 40,000 reads, demonstrating that the dataset was sufficient and that additional sequencing would yield minimal new OTUs [**?**]. Similarly, flattening Shannon curves indicated adequate sequencing depth to represent the majority of microbial communities [**?**]. Higher Shannon indices denote greater microbial diversity, and the data in reveal that F8 exhibited lower gut microbiota diversity. The rarefaction curve for F8 showed a steeper slope compared to other individuals, suggesting insufficient sampling depth may have contributed to its lower diversity. Overall, blue fox gut microbiota showed an average of 509 OTUs, Chao1 index of 595, and Shannon index of 5.27. In comparison, Fan et al. [**?**] reported that farmed mink gut microbiota contained 294 OTUs, Chao1 index of 350, and Shannon index of 3.74. Thus, blue foxes appear to harbor higher richness and diversity than mink, another fur-bearing animal.

Phylum-Level Microbial Structure Analysis At the phylum level, Firmicutes and Bacteroidetes were the dominant taxa in blue fox gut microbiota. While Firmicutes predominated in most samples, Bacteroidetes was most abundant in sample F4. Research indicates that Firmicutes primarily hydrolyze carbohydrates and proteins, whereas Bacteroidetes metabolize steroids, bile acids, and polysaccharides, thereby facilitating polysaccharide absorption and protein synthesis [**?**]. The Firmicutes/Bacteroidetes ratio influences obesity, with obese mice showing increased Firmicutes and decreased Bacteroidetes abundance, associated with enhanced energy harvest from diet [**?**]. Dietary shifts from low-fat, high-fiber to high-fat, high-sugar diets significantly reduce Bacteroidetes proportions [**?**], promoting obesity. Therefore, the microbiota in F4 may exhibit stronger metabolic capacity for high-fat, high-sugar diets, though body weight was not monitored in this study.

Intestinal microbiota composition is influenced by multiple factors including age, sex, environment, diet composition, physiological status, and genetics [?]. Despite identical living conditions and diet, F4 and F8 showed distinct microbiota compositions compared to other individuals, likely related to individual physiological and genetic differences. This demonstrates that inter-individual variation exists even under identical environmental conditions.

Comparative analysis reveals substantial differences in gut microbiota among canid species. In wild wolves (*Canis lupus*), Firmicutes (60.0%) dominates, followed by Bacteroidetes (16.9%), Proteobacteria (9.2%), Fusobacteria (9.2%), and Actinobacteria (4.6%) [?]. In dholes (*Cuon alpinus*), the five most abundant phyla are Firmicutes (20.97%–44.01%), Bacteroidetes (21.63%–38.97%), Proteobacteria (9.33%–17.60%), Fusobacteria (9.11%–17.90%), and Actinobacteria (1.22%–2.87%) [?]. Domestic dogs show Firmicutes (47.7%), Proteobacteria (23.3%), Fusobacteria (16.6%), and Bacteroidetes (12.4%) [?], while pet dogs exhibit Firmicutes (64.17%), Bacteroidetes (19.89%), Fusobacteria (13.58%), Actinobacteria (1.5%), and Proteobacteria (0.86%) [?].

Notably, blue foxes showed higher Actinobacteria proportions compared to other canids. This phylum was dominated by Coriobacteriaceae and Bifidobacteriaceae (99.99% of Actinobacteria), whereas Bifidobacteriaceae were scarce in wolves and dholes [?]. Blue fox Bifidobacteriaceae consisted solely of bifidobacteria, which are known to produce lactic acid and acetic acid that regulate intestinal pH and inhibit pathogen proliferation [?]. Bifidobacteria also play important roles in maintaining intestinal health and slowing gut aging [?]. The relatively high abundance in these 5–6 month old growing foxes may reflect their younger age. Compared to other animals, blue foxes exhibited notably higher Actinobacteria proportions.

Coriobacteriaceae bacteria increase significantly under stress conditions and may contribute to anxiety and tension [?]. This family is also associated with hypercholesterolemia [?], obesity, and type 2 diabetes [?]. Compared to wolves and blue foxes, domestic dogs show higher Fusobacteria and lower Bacteroidetes proportions [?, ?]. According to Bergey' s Manual of Systematic Bacteriology, Bacteroidetes comprises four classes: Bacteroidia, Flavobacteria, Sphingobacteria, and Cytophagia [?], with Bacteroidia accounting for 99.77% of Bacteroidetes in blue foxes. The lower Bacteroidetes proportion in domestic dogs likely reflects human intervention in their diet, which increases carbohydrate content and reduces fat compared to other canids. Overall, blue fox gut microbiota diversity patterns are generally similar to other canids, with major differences limited to Fusobacteria and Actinobacteria abundances. However, whether these differences influence host ecology requires further investigation.

Mink, another major fur-bearing animal in China, show gut microbiota dominated by Firmicutes (60.0%), Bacteroidetes (16.2%), Fusobacteria (11.5%), Actinobacteria (5.9%), and Proteobacteria (5.3%) [?]. Except for higher Fusobacteria in mink, other phyla were similar to blue foxes. Increased dietary fiber reduces Fusobacteria abundance [?], and the lower fiber content in mink

diets compared to blue fox diets may explain the higher Fusobacteria in mink. Notably, herbivorous horses lack Fusobacteria entirely [**?**], and human gut microbiota contains less than 1% Fusobacteria [**?**], suggesting this phylum may be influenced by plant-based dietary components.

Genus-Level Microbial Structure Analysis At the genus level, Streptococcus was the most abundant taxon in blue fox gut microbiota, consistent with findings in farmed mink [**?**]. Streptococcus ferments simple sugars and degrades proteins, playing a crucial role in host energy metabolism [**?**]. Lactobacillus, the second most abundant genus, has attracted increasing attention as a probiotic. Lactobacillus interacts with other probiotics to treat irritable bowel syndrome (IBS) [**?**], ferments carbohydrates to produce lactic acid and butyric acid that lower intestinal pH and inhibit pathogen growth, generates hydrogen peroxide to suppress fungal pathogens such as *Candida* [**?**], and activates immune cells to eliminate pathogenic microorganisms, thereby enhancing immunity [**?**].

Both Streptococcus and Lactobacillus belong to Lactobacillales within Firmicutes, which accounted for 22.5% of blue fox gut microbiota. Prevotella, belonging to Bacteroidales within Bacteroidetes, promotes protein degradation and synergistically degrades cellulose with other microbes in the rumen [**?**]. Diet strongly influences Prevotella abundance, as evidenced by 53% prevalence in Burkina Faso children compared to minimal levels in European children of the same age [**?**]. Western diets rich in protein and animal fat favor Bacteroides, while carbohydrate-based diets favor Prevotella [**?**].

Collinsella, belonging to Coriobacteriales within Actinobacteria, ferments carbohydrates to produce formic acid, lactate, and ethanol, and correlates negatively with body weight and BMI [**?**]. Blautia, a Firmicutes genus, converts intestinal gases into acetic acid, thereby reducing gas accumulation [**?**]. As a member of Lachnospiraceae, Blautia degrades hemicellulose in ruminants [**?**] and is abundant in blue foxes, though its specific function in fox gut requires further investigation.

Thus, blue fox gut microbiota comprises diverse functional groups including Bacteroidetes (metabolizing steroids, bile acids, and polysaccharides), Streptococcus (protein degradation), Lactobacillus and Collinsella (carbohydrate fermentation and pathogen inhibition), and Prevotella and Blautia (fiber degradation), forming a rich and diverse community.

Blue foxes are carnivorous, but farming practices reduce animal protein and increase plant protein proportions to lower costs. No studies have reported intestinal microbiota of wild blue foxes or those fed animal protein-based diets. The current study used a plant protein-based diet, so these results may not represent the microbiota of carnivorous (wild or animal protein-fed) blue foxes. Notably, cellulose-degrading Prevotella was absent in wild canids (wolves, corsac foxes, red foxes, raccoon dogs, and dholes) [**?**] but was a dominant genus in our plant protein-fed blue foxes, suggesting dietary composition shapes microbiota

structure. Future studies should compare intestinal microbiota between wild blue foxes and those fed animal protein-based diets to clarify these differences.

Individual variation analysis at the genus level revealed that F8 showed high proportions of Streptococcus, Lactobacillus, and Megasphaera but low proportions of other genera, possibly due to insufficient sampling depth. Additionally, host genetics influences intestinal microbiota composition and diversity [**?**], explaining inter-individual variation even under identical environmental conditions. These dominant genera are closely associated with nutrient metabolism, primarily functioning in protein degradation and fiber degradation enhancement, though the underlying mechanisms require further investigation.

Conclusion

Under the dietary conditions of this study, the intestinal microbiota of eight blue foxes was distributed across 16 phyla, dominated by Firmicutes (62.97%), Bacteroidetes (22.05%), Actinobacteria (8.89%), Proteobacteria (5.15%), and Fusobacteria (0.88%), which collectively accounted for 99.94% of total phyla. These phyla comprised 209 genera, forming a rich and diverse intestinal microbiota community. At the genus level, Streptococcus showed the highest relative abundance (11.75%), followed by Lactobacillus (9.86%).

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Note: Figure translations are in progress. See original paper for figures.