Effects of transport stress on fecal microbiota in healthy donkeys

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SUBJECT AREAS
Animal Science

KEYWORDS
Transport stress, Donkeys, Fecal microbiota, 16S rRNA sequencing
Abstract
Background: With the development of large-scale donkey farming in China, long-distance transportation has become a common practice, and the incidence of intestinal diseases after transportation has increased. Intestinal microbiota is important for health and disease, and whether transportation disturbs donkey intestinal microbiota has not been investigated. This study aims to determine the effects of transportation on the fecal microbiota of healthy donkeys using 16S rDNA sequencing. Results: Fecal samples were collected from the rectum of 12 Dezhou donkeys before and after transportation. Results show that long-distance transportation can induce severe stress in donkeys and result in significantly lower level of bacterial richness index compared with that before transport (p=0.042) without distinct changes in diversity. This marked decrease in specific bacterial richness, such as for Eubacterium, Streptococcus, and Coriobacteriaceae, might be associated with the restricted synthesis of anti-inflammatory cytokines and metabolites, such as short chain fatty acids (SCFAs) that potentially contribute to disease development after the transport. Conclusions: Further studies are required to understand the potential effect of these microbiota changes on the development of donkey intestinal diseases. Preventative and therapeutic measures for donkeys before and after transportation should focus on providing diverse and rich bacterial microbiota and probiotic flora. Keywords: Transport stress, Donkeys, Fecal microbiota, 16S rDNA sequencing

Background
Domestic animals are transported due to various reasons, including breeding, slaughter, and biomedical purposes, which are also associated with animal welfare, food safety, marketing, and trade barriers [1, 2]. Transportation exposes animals to various potential stress factors, such as upload and offload, vehicle bumps, crowding, noise, temperature, and food and water deprivation, which can result in weight loss [3, 4], affect the quality of animal products [5], increase physical injury and tissue damage, attenuate animals’ immune function and susceptibility to disease [6, 7], and even lead to death [8]. Increasing evidence indicates that transport stress has caused substantial economic losses to the animal industry worldwide [5, 9].

Bacterial microbiota is complex and plays a key role in human and animal health. Imbalances of the
microbial communities can be associated with a wide range of diseases in equine gastrointestinal tract, including colitis [10], laminitis [11], equine grass sickness [12], and transient diarrhea in foals [13]. Administration of medications, transportation, fasting, and abrupt changes in diet could induce changes on horse intestinal microbiota [14–16]. However, the influence of these factors on donkeys has been poorly studied. Culture- and isolate-based conventional methods used to study microbiota have limited sensitivity for the assessment of complex microbial populations; for example, only less than 1% of microbes in any marine habitats can be cultured under standard laboratory conditions [17]. A recent next-generation sequencing and bioinformatics based technology allows for a detailed quantitative analysis of the microbiota and could be used to understand the influence of specific factors, such as antimicrobial treatment, diarrhea, and mild asthma, on horse intestinal or respiratory tract microbiota [18, 19].

Transporting donkeys from traditional donkey-concentrated areas for fattening and breeding has become a major breeding model in China and has been accompanied by the increase in long-duration transportation. Our previous statistics show that long distance transportation led to high morbidity and mortality among donkey during recovery period, and intestinal diseases are one of the main diseases that gradually became one of the key factors restricting the development of donkey breeding industry in China. Despite the high damage of donkey transportation, the effect of transportation on donkey gut microbiota is poorly understood. For the first time, we used high-throughput pyrosequencing to evaluate the effects of transport on donkey fecal microbiota, understand the pathophysiology of diseases related to gut microbiota, and develop effective preventive or treatment options.

Results
Transportation of donkey alters its hormonal levels
The concentration of serum Cortisol hormone (Cor), heat-shock protein 90 (HSP90), and adrenocorticotrophic hormone (ACTH) significantly differed between before and immediately after transportation. Serum ACTH, Cor, and HSP90 of the donkeys were significantly increased ($p<0.05$) on the day of arrival compared with those on the day before transportation (Fig. 1).

Sequencing quality data and alpha diversity analysis
The microbiota composition of the fecal samples was assessed by sequencing the bacterial 16S rRNA V3 + V4 region. A total of 1,233,776 pairs of reads were obtained from the 24 samples that were sequenced. Double-end read splicing and filtering resulted in 1,075,841 clean tags, and each sample produced 44,827 clean tags on average. With the use of QIIME (version 1.8.0) UCLUST software based on 97% sequence similarity, the tags were clustered into OTUs. The number of OTUs in fecal samples after transportation (AF1–12) decreased markedly relative to that before transportation (BF1–12) (Fig. 2A). The Venn diagram of OTUs was illustrated (Fig. 2B) and the Chao, Simpson, and Shannon indexes were calculated (Fig. 2C). A significant decrease in Chao index was found in the fecal samples after transportation (AF group) compared with that before transportation (BF group). No other differences in alpha diversity were identified.

Beta diversity analysis
Differential analysis, including PCoA (Fig. 3A) and UPGMA, between groups based on the weighted UniFrac algorithm were performed to further explore the relationship between different bacterial communities before and after transport (Fig. 3B). Fig. 3A shows that the percentage of variation explained by PC1 and PC2 were 43.01% and 17.01%, respectively. However, distinct clusters were not visually evident with PCoA, indicating no significant difference in the bacterial composition before and after transport. The phylogenetic tree based on the weighted UniFrac also revealed that the differences in bacterial community were difficult to discern visually, and this finding was in accordance with the PCoA result.

Phylogenetic analysis
The relative bacteria abundance of phylum in each sample (Fig. 4A) and group (Fig. 4B) were drawn using R language tools. As shown in Fig. 4B, 12 phyla had a mean relative abundance of more than 1%. The predominant phyla in each group were Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, Fusobacteria, Fibrobacteres, and Spirochaetes. The phylum-level analysis also revealed that the most prevalent phyla of the microbial community after transport were similar to those before transport. However, transport affected the relative abundance of phyla; for example, an increased relative abundance of Bacteroidetes (median: BF 32.6% to AF 40.9%) and Firmicutes
(median: BF 23.3% to AF 30.5%) and a decreased relative abundance of *Proteobacteria* (median: BF 18.1% to AF 10.3%) and *Verrucomicrobia* (median: BF 9.2% to AF 5.8%) were observed after transport.

The line discriminant analysis (LDA)-effect size (LEfSe) method was used for the quantitative analysis of biomarkers in the microbiota among each group. The LDA score was set at 2.0, and different genera with LDA threshold >2 were considered significant biomarkers. The cladogram is shown in Fig. 5A, and the LDA score distribution map is shown in Fig. 5B. Several genera were more abundant in BF samples than in AF samples according to LEfSe analysis. *Eubacterium* genus, *Coriobacteriaceae* family, *Streptococcus* species, *Atopostipes*, and *Pseudomonas* genera were enriched in BF samples compared with those in AF samples.

**Discussion**

The bacterial microbiota of foals plays an important role in various digestive tract diseases, and early colonization and development is a dynamic process. However, donkey bacterial microbiota is poorly studied. Initial culture-based studies only focus on a narrow spectrum of the fecal bacterial microbiota, and subsequent culture-independent studies based on 16S ribosomal RNA (rRNA) gene sequencing studies have provided insights into the rapid development of bacterial microbiota. In this study, the effect of transport on the phylogenetic composition of donkey fecal microbiota was analyzed. Differences in the relative abundances of phyla, classes, and orders and the loss of bacterial diversity and richness were observed.

ACTH and Cor levels increase under stress to deal with changes in the external environment. These hormones are important indexes in the stress reaction of animals, including beef cattle, piglets, chicken, and horses. During stressful situations, such as transportation, the ACTH and cortisol content in plasma increases variably [20–22]. HSP90 is an important stress protein in organisms because it is rapidly activated and synthesized during stress reaction [23]. In this study, the ACTH, Cor, and HSP90 levels significantly increased (p<0.05) after transport, and this finding is in agreement with other studies worldwide. Therefore, cold weather, crowded environment, bumpy transportation, and changes in environment and feeding patterns after arrival cause severe stress to the donkeys.
Transport stress rapidly affects the composition of gut microbiota and host physiology through the generation of bioactive metabolites [14, 24]. Our study revealed that donkey fecal microbiota after transport had significant decreases in specific bacterial richness compared with the controls, and this finding may be related to gastrointestinal diseases. LEfSe analysis revealed that after transport, significant decreases were found for the short chain fatty acids (SCFAs)-producing bacteria, including *Eubacterium* genus and *Coriobacteriaceae* family [25]. SCFAs are not included the diet but synthesized by colonic commensal bacteria from dietary carbohydrate; these substances plays a key role in the energy metabolism of the colonic epithelial cells and is important in the maintenance of colon health in humans [26]. SCFAs also act on immune cells, such as mononuclear phagocytes and lymphocytes, and participate in intestinal immune regulation by influencing the release of inflammatory factors and chemotaxis, thereby playing an important role in intestinal defense against pathogenic bacteria [27].

Interfering with the SCFAs synthesis in the colon may result in diarrhea; an increased production of SCFAs enhances the colonic fluid production and corrects the dehydration associated with acute diarrhea [28]. Moreover, the levels of butyrate produced by *Eubacterium* were significantly lower during and immediately after diarrhea than during a diarrhea-free period of normal health [29]. The number of *Coriobacteriaceae* family, which is found abundant in healthy gut, was significantly decreased in microscopic colitis cases [30]. The marked decrease in the relative abundance of these SCFAs-producing bacteria might therefore reflect that transport stress can interfere with SCFAs synthesis and may be important in the pathophysiology. Hence, donkeys are at a high risk of diarrhea after transport.

In addition to the marked decrease in SCFAs-producing bacteria, significantly low rate of *Streptococcus, Atopostipes*, and *Pseudomonas* was observed after transport. *Atopostipes* and *Pseudomonas* are bacteria producing branched chain fatty acids (BCFAs). *Atopostipes* is a bacterial genus of *Lactobacillales* order under the *Bacilli* class, a gram-positive bacterium. As a lactic acid bacteria, *Atopostipes* has a strong positive correlation with BCFAs by metabolizing valine and tryptophan to BCFAs [31]. *Pseudomonas* produce BCFAs through the deamination of branched chain...
amino acids such as leucine, isoleucine, and valine [31]. BCFAs, which are primarily saturated fatty acids (FA), are normal constituents in the gut throughout the human life cycle. Similar to SCFAs, BCFAs also have an important influence on intestinal health and are related to various health conditions. These compounds are metabolized by enterocytes and have a beneficial role against inflammation in the premature intestine, alter the microbiota, and increase the expression of anti-inflammatory cytokines [32]. *Streptococcus* is an enriched taxon according to LEfSe in the healthy horses [33]. *Streptococcus* and *Lactococcus* are negatively correlated with inflammatory parameters (TNF-α, LPS, and H₂O₂ yield) [34]. A probiotic combination containing *Streptococcus thermophilus* protected the bowel and improved colon inflammation in experimentally induced inflammatory bowel disease in rats [35]. These results indicate that transport stress significantly reduces the number of probiotics such as lactic acid bacteria and might disturb the synthesis of BCFAs, thereby increasing the rate of inflammatory bowel disease.

Previous studies have found that potential confounding factors, such as age [33], gender [36], diets [37] and environmental change [38] could impact the bacterial microbiota of different body sites. Thus, we have taken a series of measures to minimize the effects of these factors on the experiments, for example selecting donkeys with the same sex and age, consistency in fodder and feeding management. Environmental change is most likely to impact the intestinal microbial composition in this study because of the changing location where the donkeys were housed. We can’t avoid environmental bacteria being present in the intestinal microbiota, however, environmental bacteria have been shown to be only a minimal component of the intestinal microbiota in cattle [39].

There were some limitations to this study, including the limited sample size and inevitable environmental factors. Besides, faecal microbial composition does not fully reflect the microbial composition of different regions in the gastrointestinal tract. Further studies about effects of transport stress on donkey various intestinal segments microbiota changes are required.

**Conclusions**

This study provided a comprehensive analysis of the effects of transport stress on the fecal microbiota in healthy donkeys for the first time. Transport causes severe stress to the donkeys and
markedly decreases the bacterial richness. This decrease might be associated with the restricted synthesis of anti-inflammatory cytokines and metabolites (SCFAs and BCFAs), thus contributing to disease development such as colitis or diarrhea after transport. Preventative and therapeutic measures for donkeys before and after transportation should focus on providing diverse and rich bacterial microbiota and probiotic flora, especially those producing SCFAs or BCFAs such as members of the *Eubacterium* and Coriobacteriaceae mentioned in this study.

**Declarations**

**Abbreviations**

OTUs: Operational Taxonomic Units; LefSe: line discriminant analysis (LDA)-effect size; PCoA: Principal coordinate analysis; SCFAs: short chain fatty acids; BCFAs: branched chain fatty acids; Cor: cortisol hormone; HSP90: heat-shock protein 90; ACTH: adrenocorticotrophic hormone.

**Ethics approval and consent to participate**

This study was performed by strictly following Animal management regulations of the People’s Republic of China. The study was approved by National Engineering Research Center for Gelatin-based TCM.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

This study was supported by National Key Research and Development Project, China (2018YFD0502205) and Agriculture Improved Varieties Project of Shandong Province, China (2017LZGC020).

**Authors’ contributions**

FWZ designed research, participated in all experiments and bioinformatics analysis. GMJ, XHZ, WPG, PX, TW, YLF performed research. GMJ wrote the first draft of the manuscript. FWZ, LL, XSZ, CLJ, ZPZ
contributed to modify the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Methods

This study was performed by strictly following Animal management regulations of the People’s Republic of China and approved by National Engineering Research Center for Gelatin-based TCM.

Animals and transport

Healthy male Dezhou donkeys were selected from Inner Mongolia Dong-E Black Donkey Animal Husbandry Co., Ltd in Chifeng City, Inner Mongolia Province, China. Dezhou donkeys are a large somatotype ass and unique indigenous breed in China. These selected donkeys were then transported to Dong-E E-Jiao Co., Ltd in Shandong Province for breeding.

The experiment was conducted on 12 male Dezhou donkeys aged 10–12 months weighing (140.8±5.2 kg, mean±SD). The donkeys were clinically healthy and provided free access to water and feed composed of straw and commercial concentrates daily. All donkeys had no previous experience of road transport. During transportation, the average environmental temperature and humidity were −10 °C and 28%, respectively. The surrounding walls of the truck (13.4 m long and 5.6 m wide) were equipped with iron guardrails, and the floor was iron with extremely thin bedding materials. The truck
did not have roof coverings, and the donkeys were therefore exposed to different weather conditions. The transport started from Chifeng City in Inner Mongolia Province at 17:00 p.m on January 7, 2018, and arrived at Dong-E City in Shandong Province at 14:00 p.m on January 8, 2018, which represents a travel time of about 21 hours and a distance of 950 km. The routes were secondary roads and expressways. The donkeys were not fed or watered during transportation. Before and after transportation, diet and water were not changed, and all donkeys were stabled with daily access to hay and water. The donkeys were housed in different areas of the same barn, without any contact with other animals. The same feeding methods and times were used between before and after transportation. The fodder was transported from the original location, thereby minimizing the effects of environment and food on the experiments.

Sample Collection
In brief, 15 ml of blood sample was collected from the jugular vein of each donkey and placed in separate vials. Each vial contained 5 ml of the blood sample. All the samples were collected pre- and post-transport. The blood samples were placed on ice, immediately transferred to the laboratory for analysis, and centrifuged at 3000 g for 20 min at 4 °C. The supernatants were stored in microtubes and stored at −80 °C until analysis. All laboratory analyses were performed within 24 h. Fecal samples were collected from the rectum (n = 12), stored in the microfuge tube, and frozen at −80 °C pending DNA extraction. After sampling, all donkeys were backed into the barn to continue to be raised.

Hormonal analysis
Cortisol hormone (Cor), heat-shock protein 90 (HSP90), and adrenocorticotrophic hormone (ACTH) were determined through an ELISA-based technique using the commercial kits of Enzyme-linked Biotechnology (Shanghai Enzyme-linked Biotechnology Co., Ltd. China).

DNA extraction and pyrosequencing
Total bacteria DNA was extracted from the fecal sample stored at −80 °C using the genomic DNA extraction kit (TIANGEN Biotech, China). The V3 and V4 regions of the 16S rRNA gene were amplified by PCR using specific bacterial primers (F: 5′-ACTCCTACGGGAGGCAGCA–3′, R: 5′-GGACTACHVGGGTWTCTAAT–3′). High-throughput pyrosequencing of the PCR products was performed
on an Illumina MiSeq platform at Biomarker Technologies Co., Ltd. (China).

**Bioinformatics and data analysis**

The raw paired-end reads from the original DNA fragments were merged using FLASH32 and assigned to each sample according to the unique barcodes. QIIME (version 1.8.0) UCLUST software was used based on 97% sequence similarity, and the tags were clustered into operational taxonomic units (OTUs). Alpha diversity index was evaluated using Mothur software (version, v.1.30). The number of sequences contained in each sample was standardized to compare the diversity index among the samples. Analysis treasure included Shannon, Chao1, and Simpson indexes. For beta diversity analysis, principal coordinate analysis (PCoA) and unweighted pair-group method with arithmetic mean (UPGMA) were obtained using QIIME. The line discriminant analysis (LDA)-effect size (LEfSe) method was used for the quantitative analysis of biomarkers in each group. LEfSe analysis, an LDA threshold \(>2\), the non-parametric factorial Kruskal–Wallis sum-rank test, and the unpaired Wilcoxon rank-sum test were performed to identify the most differently abundant taxa. \(P\) values were calculated by the two-tailed Student’s t-test using GraphPad Prism software, and a \(p\) value of \(<0.05\) was considered significant for all comparisons.

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**Figures**

![Figure 1](image)

Changes in the levels of serum Cor, HSP90 and ACTH before and after transport. Data are shown as means ± standard deviations (SD) (n = 12). * denotes p <0.05; ** denotes p <0.01.
OTU analysis and alpha diversity indices of the healthy donkey fecal bacterial microbiota before (BF) and after transport (AF). a The vertical axis (OTU Number) represents the final OTU number after taxonomic analysis. b Venn diagram of OTUs. The overlap section represented the shared OTUs between BF and AF group. c Shannon index, simpson index and chao index of the fecal bacterial microbiota between BF and AF group. Horizontal line represents the median. * denotes p <0.05.
Figure 3

Beta analysis between BF and AF group based on the weighted UniFrac algorithm. a Principal coordinate analysis (PCoA) of the fecal bacterial microbiota of healthy donkeys (n = 12) before (BF) and after transport (AF). PCoA scores plot based on weighted UniFrac distance and each symbol represented each sample from BF and AF group. b UPGMA clustering tree and histogram combination drawing based on the weighted UniFrac. The left part is the phylogenetic tree based on the similarity between samples; the right part is the heatmap of relative abundance in phylum level.
Composition and relative abundance of dominant phyla in the bacterial communities of different samples (a) and groups (b) before and after transport. Other: Bacterial taxa with ≤1% abundance, Unknown: Sequences which could not be classified.
Figure 5

Linear discriminant analysis identifying differences between BF and AF group. a Phylogenetic profile of specific bacterial taxa and predominant bacteria among the two different groups. b LDA score of 2.0 were used as thresholds for significance in LEfSe analyses.

Supplementary Files

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