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Postprint: Study on Gut Microbial Community Diversity in 3-6-Month-Old Yili Horses

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Abstract

This study aimed to investigate the diversity of gut microbial communities in 3- to 6-month-old Yili horses, reveal the succession patterns of gut microbiota in pre-weaning foals, and provide a theoretical basis for the growth, development, and intestinal health of foals at this stage from a microbiological perspective. Five 3-month-old Yili horses with an average body weight of (89.75$±$8.81) kg and the same birth date were selected for a 90-day feeding trial. Fecal samples were collected from the foals on day 0 (i.e., the day before the experiment began), day 30, day 60, and day 90 of the trial. Total microbial genomic DNA was extracted from each sample, and Illumina HiSeq sequencing technology was used to detect the microbial community diversity of the samples. The results showed: 1) Sequencing of 20 fecal samples from five foals yielded a total of 157,665 valid sequences and an average of 1,117 operational taxonomic units (OTUs). 2) The α-diversity indices (ACE, Chao1, Shannon, and Simpson indices) of the microbial communities in foal feces exhibited fluctuating changes with increasing foal age, but no significant differences were observed among time points ($P > 0.05$). 3) At the phylum level, the ten dominant bacterial phyla in foal feces were Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Spirochaetes, Fusobacteria, Tenericutes, Actinobacteria, TM7, and Euryarchaeota, among which Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia exhibited relatively high abundances; at the family level, the ten dominant bacterial families were Bacillaceae, Moraxellaceae, Planococcaceae, Carnobacteriaceae, BS11, RFP12, Lactobacillaceae, Ruminococcaceae, Lachnospiraceae, and Porphyromonadaceae; at the genus level, the ten dominant bacterial genera were Acinetobacter, Desemzia, Lactobacillus, Ureibacillus, Paludibacter, Bacillus, Escherichia, Carnobacterium, Treponema, and Mogibacterium. It can be concluded that Illumina HiSeq sequencing technology can accurately classify and study the gut microbial communities of 3- to 6-month-old Yili horses; Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia are the dominant phyla in

the gut of 3- to 6-month-old Yili horses.

Full Text

A Study on Intestinal Microbiota Diversity of 3- to 6-Month-Old Yili Horses

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Abstract

This study aimed to investigate the intestinal microbiota diversity of 3- to 6-month-old Yili horses, reveal the succession patterns of gut microbiota in pre-weaning foals, and provide a theoretical basis for the growth, development, and intestinal health of foals during this stage from a microbiological perspective. Five 3-month-old Yili foals with an average body weight of (89.75$±$8.81) kg and identical birth dates were selected for a 90-day feeding trial. Fecal samples were collected on day 0 (one day before the experiment began), day 30, day 60, and day 90 of the trial. Microbial genomic DNA was extracted from each sample, and Illumina HiSeq sequencing technology was employed to assess microbiota community diversity. The results showed that: 1) Sequencing of 20 fecal samples from the 5 foals yielded 157,665 effective sequences and an average of 1,117 operational taxonomic units (OTUs). 2) The alpha diversity indices (ACE, Chao1, Shannon, and Simpson) of the fecal microbiota exhibited fluctuating changes with increasing age, but no significant differences were observed among the time points (P>0.05). 3) At the phylum level, the ten dominant bacterial groups in foal feces were Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Spirochaetes, Fusobacteria, Tenericutes, Actinobacteria, TM7, and Euryarchaeota, with Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia showing the highest abundances. At the family level, the ten dominant groups were Bacillaceae, Moraxellaceae, Planococcaceae, Carnobacteriaceae, BS11, RFP12, Lactobacillaceae, Ruminococcaceae, Lachnospiraceae, and Porphyromonadaceae. At the genus level, the ten dominant genera were Acinetobacter, Desemzia, Lactobacillus, Ureibacillus, Paludibacter, Bacillus, Escherichia, Carnobacterium, Treponema, and Mogibacterium. These findings demonstrate that Illumina HiSeq sequencing technology can accurately classify and study the intestinal microbiota communities of 3- to 6-month-old Yili horses, with Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia being the dominant phyla in this age group.

Keywords: Yili horses; Illumina HiSeq sequencing technology; intestinal microbiota; diversity

Horses are monogastric animals whose cecum functions similarly to the rumen of ruminants, capable of holding 28–36 L of digesta and digestive fluids. Cultivation-based detection of cecal contents or feces has revealed 400–500 microbial species with populations reaching 10^{10}–10^{12} cells/g [1-2]. The microorganisms colonizing the intestinal tract participate in the digestion, absorption, and synthesis of nutrients, representing a crucial component of the animal' s gut. Therefore, promoting the establishment of equine intestinal microbiota and maintaining the balance of normal gut flora are essential for digestion, intestinal health, and immune regulation in horses [3]. The equine gut is sterile at birth, with bacterial colonization occurring gradually through parturition, nursing, and environmental contact. As foals age and experience changes in living environment, dietary structure, and health status, the composition, structure, and abundance of intestinal microorganisms undergo corresponding changes [4]. Under stress conditions, alterations in these factors can disrupt the intestinal microbiota and cause diarrhea, which can be fatal in severe cases. Intestinal flora balance plays a vital role in equine growth, development, and health status [5]. Early supplementation feeding is crucial for foal development, and premature intake of supplemental feed can influence intestinal microbial structure. As 3-month-old foals experience reduced maternal milk, they gradually increase consumption of supplemental feed, transitioning from liquid to solid diets, which consequently alters the composition and abundance of intestinal microorganisms and affects gut health and growth. In view of this, the present study investigated 3- to 6-month-old Yili foals to examine the colonization and succession patterns of intestinal microbiota from 3 to 6 months of age by detecting fecal microbiota diversity, providing a reference for developing appropriate microbial preparations for foal health and preventing intestinal microbial diseases.

1 Materials and Methods

1.1 Experimental Period and Location

The experiment was conducted from July 2014 to October 2014 at Zhaosu Horse Farm in Ili Kazakh Autonomous Prefecture, Xinjiang.

1.2 Experimental Design

Five 3-month-old nursing Yili foals with an average body weight of (89.75$±$8.81) kg and identical birth dates were selected. The five foals and their dams were from the same grazing pasture, with dams of the same age (11 years) and parity (7th foaling). Under nursing conditions, each foal was supplemented with concentrate at 0.6% of body weight daily (adjusted every 30 days, see Table 1) and provided with equal amounts of high-quality alfalfa hay for a 90-day feeding trial. Fecal samples were collected at 12:00 on days 0 (one day before the experiment), 30, 60, and 90.

1.3 Management and Diet Composition

Experimental foals and dams were housed in outdoor stables (40 m wide × 75 m long). Foals were tethered from 08:00 to 18:00 and allowed free movement for the remaining time. The daily concentrate supplement was divided into three equal portions and fed at 08:00, 13:00, and 17:00 using specially designed feed bags (sized appropriately for foals to prevent respiratory interference and feed spillage). All foals received equal amounts [(1.00$±$0.25) kg] of high-quality alfalfa hay daily and had free access to water. On sampling days, foals were housed individually in indoor stalls with single troughs and partitions to prevent fecal cross-contamination. Stalls were cleaned after feeding in preparation for fecal collection at 12:00. The composition and nutrient levels of the concentrate supplement are shown in Table 2 .

1.4 Fecal Sample Collection

On days 0, 30, 60, and 90 of the experiment, foals were moved to indoor stalls at 08:00, tethered individually, and fitted with fecal collection bags. All feces expelled from entry until 12:00 were collected in buckets, mixed thoroughly, and sampled using a five-point sampling method (5 g per point). The 25 g composite sample was immediately placed in sealed bags and stored at -20°C until analysis.

1.5 Sample Grouping and Numbering

For subsequent analysis, samples from days 0, 30, 60, and 90 were designated as four groups, with samples from the five foals at each time point considered as one group.

1.6 Fecal High-Throughput Sequencing Analysis

Total DNA extraction, PCR amplification, Illumina HiSeq sequencing, and result analysis were completed with the assistance of Beijing Novogene Bioinformatics Technology Co., Ltd.

1.7 Data Processing

Metastats analysis was performed using R software to conduct permutation tests between groups at each taxonomic level to obtain P-values, which were then corrected using the Benjamini and Hochberg False Discovery Rate method. Significant inter-group differences were analyzed using t-tests in R software.

2 Results

2.1 Sequencing Results and Alpha Diversity Analysis

Sequencing results and alpha diversity data are presented in Table 3 . Sequencing of 20 fecal samples from the 5 foals yielded 157,665 effective sequences,

which were clustered into 4,471 operational taxonomic units (OTUs) at 97% sequence identity. The sequencing coverage for all four time points exceeded 96%, indicating that the obtained sequences represented over 96% of bacterial phylotypes in the microbial community, with good sequencing coverage and abundant species observed at each time point. The alpha diversity indices (ACE, Chao1, Shannon, and Simpson) of the fecal microbiota showed fluctuating changes with increasing age, but no significant differences were detected among time points ($P>0.05$).

2.2 Beta Diversity Analysis

The principal component analysis (PCA) results based on OTU levels are shown in Figure 1 [Figure 1: see original paper]. Principal component 1 (PC1) explained 17.28% of the total microbial variation, while principal component 2 (PC2) explained 9.68%. The 20 samples from the four groups could be clearly distinguished, with days 0 and 30 separating distinctly from days 60 and 90, and day 60 separating from day 90, indicating similar microbial community composition within the same time points.

Figure 2 [Figure 2: see original paper] displays the unweighted pair group method with arithmetic mean (UPGMA) clustering tree on the left and relative abundance distribution at the phylum level for each group on the right. Days 0 and 30 clustered together, while days 60 and 90 formed separate clusters, suggesting that microbial communities became more complex and diverse as foals aged.

2.3 Composition and Abundance Distribution of Foal Intestinal Microbiota

Based on comprehensive analysis of sequence numbers, this study examined the microbial community structure in foal feces at four time points with inter-time-point comparisons. According to taxonomic annotation results, the top 10 most abundant species at each taxonomic level for each sample were selected to generate stacked bar charts of relative abundance for intuitive visualization of dominant species and their proportions at different taxonomic levels.

2.3.1 Microbial Abundance at Phylum Level in 3- to 6-Month-Old Foals Microbial abundance data at the phylum level are presented in Table 4 . The ten dominant phyla in foal feces were Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Spirochaetes, Fusobacteria, Tenericutes, Actinobacteria, TM7, and Euryarchaeota, accounting for over 98% of classified bacteria, with approximately 1% representing other unclassified phyla. The stacked bar chart of phylum-level abundance is shown in Figure 3 [Figure 3: see original paper]. Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia were the dominant phyla. Microbial abundances changed to varying degrees with increasing age. Firmicutes abundance was 33.19%, 32.90%, 73.72%, and 67.89% on days 0, 30, 60, and 90, respectively, being significantly higher on day 60 ($P<0.01$)

and day 90 (P<0.05) compared to days 0 and 30. Actinobacteria abundance reached 1.34% on day 60, significantly higher than on days 0 and 30 (P<0.05). Tenericutes abundance increased with age, reaching 3.05% on day 90, significantly higher than on days 0 and 30 (P<0.05). Bacteroidetes abundance was 40.97%, 43.44%, 0.49%, and 2.75% on days 0, 30, 60, and 90, respectively, being significantly lower on days 60 and 90 (P<0.01) compared to days 0 and 30. Spirochaetes abundance decreased initially and then stabilized, with 3.13% on day 0 being significantly higher than on days 30, 60, and 90 (P<0.05). No significant changes were observed for Proteobacteria, Verrucomicrobia, Fusobacteria, TM7, or Euryarchaeota throughout the trial (P>0.05), though Proteobacteria abundance decreased on day 30 and increased on days 60 and 90 to levels similar to day 0.

2.3.2 Microbial Abundance at Family Level in 3- to 6-Month-Old Foals
Microbial abundance data at the family level are presented in Table 5 . The ten dominant families in foal feces were Bacillaceae, Moraxellaceae, Planococcaceae, Carnobacteriaceae, BS11, RFP12, Lactobacillaceae, Ruminococcaceae, Lachnospiraceae, and Porphyromonadaceae. The stacked bar chart of family-level abundance is shown in Figure 4 [Figure 4: see original paper]. Bacillaceae abundance was 22.31% on day 60, significantly higher than on days 0 and 30 (P<0.01) and day 90 (P<0.05). Lactobacillaceae abundance was 5.77% on day 90, significantly higher than on days 0 and 30 (P<0.01). Ruminococcaceae abundance was 11.93%, 10.19%, 3.44%, and 7.66% on days 0, 30, 60, and 90, respectively, being significantly lower on day 60 (P<0.01) compared to days 0 and 30, and significantly lower than day 90 (P<0.05). Lachnospiraceae abundance was 2.58%, 0.40%, 0.02%, and 0.14% on days 0, 30, 60, and 90, respectively, with day 60 being significantly lower than days 0 and 30 (P<0.01). BS11 abundance was 8.95%, 15.98%, 0.06%, and 0.53% on days 0, 30, 60, and 90, respectively, with days 60 and 90 being significantly lower than days 0 and 30 (P<0.05). No significant changes were observed for Moraxellaceae, Planococcaceae, Carnobacteriaceae, or RFP12 throughout the trial (P>0.05).

2.3.3 Microbial Abundance at Genus Level in 3- to 6-Month-Old Foals
Microbial abundance data at the genus level are presented in Table 6 . The ten dominant genera in foal feces were Acinetobacter, Desemzia, Lactobacillus, Ureibacillus, Paludibacter, Bacillus, Escherichia, Carnobacterium, Treponema, and Mogibacterium. The stacked bar chart of genus-level abundance is shown in Figure 5 [Figure 5: see original paper]. Lactobacillus abundance was 5.77% on day 90, significantly higher than on days 0, 30, and 60 (P<0.01). Bacillus abundance was 4.11% on day 60, significantly higher than on days 0 and 30 (P<0.01).

3 Discussion

3.1 Intestinal Microbiota Diversity in 3- to 6-Month-Old Foals

Illumina high-throughput sequencing technology has been widely used in microbiology research in recent years. This technology enables deeper, more intuitive, and accurate reflection of animal gastrointestinal health and growth status through assessment of gut microbiota stability and diversity [3,6-8]. Proughbred et al. [9] sequenced 14 fecal samples from 8 castrated Thoroughbred horses, obtaining 488,213 effective sequences with OTU ranges of 1,200–3,000. Yatsunenk et al. [10] reported that high-throughput sequencing detected microbial bacteria in human feces with over 97% coverage and more than 2,000 OTUs. Costa et al. [11] obtained 6,536,523 effective sequences from 11 horse fecal samples. In the present study, Illumina HiSeq sequencing of 20 fecal samples from 5 foals at four stages yielded 157,665 effective 16S rDNA sequences, with the number increasing from 23,549 on day 0 to 53,617 on day 90 as foals aged. An average of 1,117 OTUs were obtained, increasing from 1,005 at the beginning to 1,347 at the end of the trial. Alpha diversity analysis revealed that ACE, Chao1, and Shannon indices gradually increased with age, while the Simpson index decreased, indicating increasingly rich intestinal microbiota diversity with age and dietary changes, consistent with findings reported by Sakaitani et al. [4]. As foals aged, solid forage became the primary nutrient source, and dietary type change was the main factor influencing intestinal microbiota structure [12]. In this trial, as dam milk production decreased, foals gradually replaced maternal milk with concentrate supplement and alfalfa hay. As dam milk production decreased, foals consumed more roughage (alfalfa hay) while receiving supplemental concentrate, resulting in gradual dietary transition. Consequently, the microbial species detected in foal feces became more abundant, diverse, and complex. These results align with Combes et al. [13] in rabbits, who found that age significantly affected intestinal microbiota structure, diversity, and complexity, directly related to dietary changes.

3.2 Changes in Intestinal Microbiota Community Structure in 3- to 6-Month-Old Foals

Research on equine intestinal microbiota colonization is limited compared to ruminants. Foal meconium is sterile, and the newborn gut becomes colonized through bacterial infection during parturition, nursing, and environmental contact. By 12 weeks of age, foals exhibit levels of fiber-hydrolyzing bacteria and lactic acid bacteria similar to adult horses [14]. At 42 days of age, foals harbor more abundant microbial populations than mares, with 60% similarity. In other studies, foals aged 42–80 days showed 50% similarity to mare intestinal microbiota. Therefore, studying early establishment of foal intestinal microbiota is crucial for foal intestinal health and growth.

Xu et al. [15] used Illumina high-throughput sequencing to study the cecal microbiota of weaned rabbits, finding that Firmicutes, Bacteroidetes,

Proteobacteria, and Verrucomicrobia dominated at the phylum level, while Ruminococcaceae, Lachnospiraceae, Bacteroidaceae, and Porphyromonadaceae dominated at the family level. Bai et al. [16] used PCR-DGGE to analyze weaned rabbits and found Firmicutes and Lachnospiraceae as dominant flora. Gu [17] used 454 high-throughput sequencing to show that Firmicutes and Bacteroidetes were dominant in mice under different dietary conditions. The present study detected nine phyla accounting for over 99% abundance: Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Actinobacteria, TM7, and Euryarchaeota, with Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes being dominant. These findings indicate that Firmicutes and Bacteroidetes are predominant in both large and small monogastric animals. The detection of more diverse flora at the phylum level in foals is related to interspecies differences. Dougal et al. [6] identified Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria, and Actinobacteria as dominant in the large intestinal contents of Thoroughbred and pony horses, suggesting that intestinal microbiota composition is more similar within the same species.

Furthermore, studies in mice, pigs, horses, and cattle have identified Firmicutes and Bacteroidetes as dominant gastrointestinal flora [18-20], with Firmicutes being the primary fiber-degrading group in fiber-fed animals [21]. Firmicutes can extract more energy from fibrous feed to meet animal growth requirements [22]. Gastrointestinal flora abundance is directly related to digestive capacity [23-24]. The predominance of Firmicutes in foals is associated with the cecum and colon being the primary digestive sites in equids. Although Bacteroidetes abundance decreased with age, it remained a dominant group. Bacteroidetes is the primary carbohydrate-utilizing group in herbivores [25], directly related to fiber intake. In pigs, dietary antibiotics reduce Bacteroidetes abundance [26]. The concentrate supplement in this trial contained no antibiotics, though dams were treated with anthelmintics (mainly closantel) before the experiment; whether this contributed to reduced Bacteroidetes abundance requires further investigation.

In this study, Bacillaceae, Lactobacillaceae, and Ruminococcaceae (belonging to Firmicutes), Porphyromonadaceae (Bacteroidetes), and Lachnospiraceae (Spirochaetes) showed significant changes with age. Research indicates that bacteria including Lachnospiraceae and Fibrobacter in herbivore (ruminant and non-ruminant) intestines are associated with herbivorous characteristics [27-28]. Twelve-week-old (approximately 3-month) foals have similar fiber-degrading bacterial levels to mares [4]. Changes in these bacterial abundances at the family level are directly related to increased dietary fiber content. Lachnospiraceae bacteria participate in cellulose degradation [29], primarily degrading pectin, fructose, and cellobiose from plant cell walls to produce volatile fatty acids (acetate, propionate, butyrate) that provide energy for animals and intestinal microbes. Studies show that dietary change is the primary cause of intestinal microbiota alteration [12]. In this trial, as dam milk production decreased, foals gradually increased solid feed intake, leading to higher fiber consumption,

which likely promoted increased abundance of fiber-degrading bacteria.

Additionally, Proteobacteria is a dominant phylum in animal digestive tracts with diverse clinical significance for gastrointestinal disease diagnosis. Many pathogenic bacteria including Escherichia coli, Salmonella, Vibrio cholerae, and Helicobacter pylori belong to the γ-proteobacterial class [30]. In this study, Proteobacteria abundance showed no significant changes throughout the trial, and no harmful Proteobacteria were identified among the top 10 dominant groups at the family and genus levels. This suggests that increased abundance of beneficial bacteria such as Firmicutes and Bacteroidetes with age may have suppressed or stabilized harmful Proteobacteria, thereby maintaining intestinal microbiota stability and ensuring gut health.

4 Conclusions

This study used Illumina HiSeq sequencing technology to obtain 157,665 effective sequences from 20 fecal samples collected at four time points from 5 foals, with an average of 1,117 OTUs, meeting sequencing requirements.

Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia were the dominant bacterial groups in the intestines of 3- to 6-month-old Yili horses.

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