https://doi.org/10.31489/2025BMG1/44-52

UDC 57.083.18 Received: 29.05.2024 | Accepted: 22.11.2024

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Study of intestinal microbial profiles of Kazakh horsebreed using NGS-sequencing

The aim of the study was to evaluate the intestinal microbiome of horses (taking into account their maintenance, age, breed) by sequencing 16S rRNA amplicons. A total of 24 libraries were created from fecal samples of Kazakh breed horses from various regions of Kazakhstan. The alpha diversity (Chao 1 and ACE, Shannon and Simpson indices) of the intestines of Kazakh-bred horses showed that a rich microbial diversity was revealed in horses of the Mangystau, Pavlodar and Zhetysu regions, which were on natural pastures continuously. The species richness in horses of the Pavlodar and Zhetysu regions was 9.7, which was slightly higher than in horses of the Mangystau region (9.0 $p < 0.01$). Beta diversity was examined using Bray-Curtis distances, and the relationships between 24 horse fecal samples from three different regions of Kazakhstan formed distinct clusters based on their geographic origin. We identified the main intestinal microbiome of horses from different regions of Kazakhstan, consisting of Lactobacillus, Micrococcales, Bacillales, Bacteroidales, Clostridiales, Corynebacteriales, Burkholderiales. The study of the composition of the intestinal microbiota of local breeds, such as the Kazakh horse breed, is necessary to preserve biodiversity and choose ways to maintain and conduct productive horse breeding in agriculture.

Keywords: NGS-sequencing, 16S rRNA, Equus feruscaballus, Kazakh horses, Zhabe, Adai, gut microbiome, biodiversity.

Introduction

Herd horse breeding is one of the most important branches of animal husbandry in the Republic of Kazakhstan. A special feature of herd horse breeding is the year-round maintenance of horses in herds. Accordingly, the content of herds in different periods of the year is determined by geographical and soil-climatic conditions. In this regard, the organization of use of natural pastures in spring and summer, autumn and winter varies significantly. The botanical composition of the plots used in a particular season of the year includes various herbs that are eaten by horses at this time of year.

A valuable feature of herd horse breeding is that in such conditions, horses develop and consolidate signs of a strong constitution, high reproduction rates, and immunity to many diseases, the ability to withstand periods of poor feeding and maintain good fatness [1].

The horse's digestive system (Equus feruscaballus) has a number of features, a small stomach volume and regular secretion of gastric juice, so wild horses graze continuously. The large intestine of horses consists of three parts: the cecum, colon and rectum. The cecum in horses is considered an analog of ruminant rumen, where up to 50 % of all fiber and up to 40 % of protein are digested with the participation of symbiotic microflora: bacteria, archaea, micromycetes, protozoa, and bacteriophages [2].

Questions of the existence of a close relationship between the state of health and productivity of various living organisms (including farm animals) attract the attention of many researchers.

The intestinal microbiome of horses plays an important role in animal nutrition, allowing the horse to digest cellulose, which is the main component of grass consumed [3]. In contrast to ruminants, in which microbial digestion of cellulose occurs in the pancreas (rumen), in horses, the cellulolytic microbial community develops in the cecum and colon, which have a combined volume of ~100 liters with a food retention time of approximately 48–72 hours [4–6].

Representatives of resident bacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia are among the predominant types in the rectum of horses [7, 8].

A review of fecal microbiome microorganisms in horses older than 1 year showed a predominance of taxa from the groups Bacteroidales, Treponema, Bacteroidetes, Fibrobacter, and Lachnospiraceae; the study showed a significant correlation of microbial diversity in comparison with adults. As part of the contents of the rectum, 25 phyla of microorganisms were found. The dominant phylum was Firmicutes (content ranged from 32±1.9 to 40±3.8 %) and Bacteroidetes (from 34±2.1 to 40±4.7 %). Also, in a comparative aspect, scientists have found that the fecal microbiomes of Przewalski's horse, which is a representative of the wild

fauna of living horses, and domestic horses that were kept on natural pastures, contain the most diverse bacterial community compared to domestic horses [9].

Studies of scientists have identified significant changes in the composition of the microbiome, with weight loss or gain, as well as changes in the diet of horses, while indicators of the diversity of the microbial community were significantly higher in obese horses. The number of representatives of some taxa reached significant values: Bacteroidales — up to 23.8±1.30 %, Lachnospiraceae — up to 14.7±2.80 %, Ruminococcaceae — up to 10.2±3.30 %, Clostridiaceae — up to 6.6±0.60 %. This is an important observation, since the digestion of non-starchy feed polysaccharides in the gut is an exclusively microbiological process [10, 11].

In this study, for the first time in Kazakhstan, the diversity of the composition of the equine gut microbiome is shown using the 16S-metagenomics method. The composition of the microflora revealed a significant species diversity of microorganisms associated with the processes of feed digestion, as well as a number of microorganisms that contribute to the adaptation of horses to the pasture conditions of the corresponding region.

The aim of the study was to evaluate the gut microbiome of horses (taking into account their maintenance, age, and breed) using high-throughput sequencing.

Experimental

The study was conducted during the autumn-winter period of 2023 on Kazakh horses of the Zhabe and Adai breeds, located in three different regions of Kazakhstan: the peasant farms “Agro-Dam” in the Pavlodar region, “Kozhyr-Ata” in the Mangystau region, and “Akimbekov” in Zhetysu. The horses in this study were between 5 and 10 years old and were kept on natural pastures with autumn grass in each of these regions. They were clinically healthy and had not received antimicrobial treatment (antibiotics, anthelmintic, or non-steroidal anti-inflammatory drugs) for the previous four months. Rectal (fecal) samples were collected from a total of 32 horses, including Adai horses. All samples were immediately frozen in liquid nitrogen and then transported to a laboratory where they were stored in a deep freezer at -20 degrees Celsius until DNA extraction could be performed.

Samples with a volume of 10–20 grams were taken manually from the rectums of adult Kazakh horses in compliance with aseptic conditions. The samples were collected using sterile rubber gloves and transferred into 5 cubic centimeter sterile containers.

Microbiome DNA was extracted using the PureLink Microbiome DNA Purification Kit (stool samples) according to the manufacturer’s instructions (Invitrogen, Thermo Fisher). The DNA concentration and purity were quantified with a Nanodrop 2000 ® (ThermoFisher Scientific, USA) and Qubit3.0 (Life Invitrogen, USA), respectively. 2 % agarose gel electrophoresis was used to examined DNA quality.

Amplification hypervariable regions for NGS sequencing carried out on a DNA amplifier AmpliSense (Thermo Fisher Scientific) using two 16S primer sets Ion 16S Metagenomics Kit flanking region V2-4-8, V3-6, 7-9 of the 16S rRNA gene. For each sample, two reaction mixtures were prepared, one for 1 primer set and 2 for the second primer set, including a positive control with DNA E. coli and negative control. The PCR mixture contained 15 µl of 2X Environmental Master Mix, 3 µl. 16 S Primer Set (10X), 5 µl DNA and 7 µl ddH2O. The following amplification mode was used: 10 min at 95 °C (1 cycle); 30 sec at 95 °C, 30 sec at 58 °C, 20 sec at 72 °C (25 cycles); 7 min at 72 °C (1 cycle). The resulting amplicons were transferred into 1.5 ml Eppendorf tubes LoBind and cleared AgencourtAMpure XP beads on a magnetic tripod DynaMag -96 Bottom Magnet, before use, brought to room temperature and resuspended according to the manufacturer’s instructions. Then measured on Qubit (Invitrogen, USA) and amplicons from each sample were pooled equivalently. Subsequently, after each stage of preparation for creating libraries, Agencourt was cleaned AMpure XP beads on a magnetic stand.

To create fragment libraries, we used the Ion kit Plus Fragment Library Kit and corresponding barcoding of libraries using the IonCode TM set Barcode Adapters 1-96 Kit. Following the manufacturer’s instructions to obtain the recommended concentration of the resulting libraries of 10 pM, qPCR was carried out using the Ion kit Library TaqMan QuatitationAssay. The Ion ™ 530 chip was prepared using the Ion 510™ & Ion 520™ & Ion 530™ Kit — Chef. Metagenomic sequencing was performed on the IonTorrent platform S5, Thermo Fisher Scientific in the NAO Kazakh National Agrarian Research University (Almaty, Kazakhstan).

Sequence analysis

Removal of primers, quality control, denoising, splicing of double-terminal sequences, removal of chimera and identification of amplicon sequence variants (ASVs) were performed using DADA. For taxonomic classification, we selected the Greengenes database (version 13.8). ASVs that were identified in only a single sample or classified as non-bacterial were discarded. The sequence of each horse was randomly selected to achieve a uniform sequencing depth for fair comparison the maximum length of the obtained sequences was 300 bp. The obtained 300 bp. reads were processed using the bioinformatics platform Metagenomics 16S w1.1 Detects population diversity from a metagenomics sample from Ion semiconductor reads from the Ion 16S Metagenomics Kit. Released with: Ion Reporter Software 5.2. Workflow Version: 1.1., Version: 5.20. The taxonomic affiliation of microorganisms to the genus was determined using the program Sample Group: Multi Research Category: 16S rRNA Profiling Reference: Curated MicroSEQ (R) 16S Reference Library v2013.1. The results of the statistical analysis were considered significant at $p < 0.05$.

Results and Discussion

To analyze the sequence of 16S rRNA amplicons, 34,953. 557 readings were obtained at the paired ends from 24 samples, ranging from 27,417 to 203,026. After removing dimers of adapters, low-quality and polyclonal reads, 13,009. 688 sequence reads were saved for subsequent analysis.

The taxonomy rarefaction curve indicated that the sequencing depth used in this study was sufficient to saturate species richness in all samples (Fig. 1).

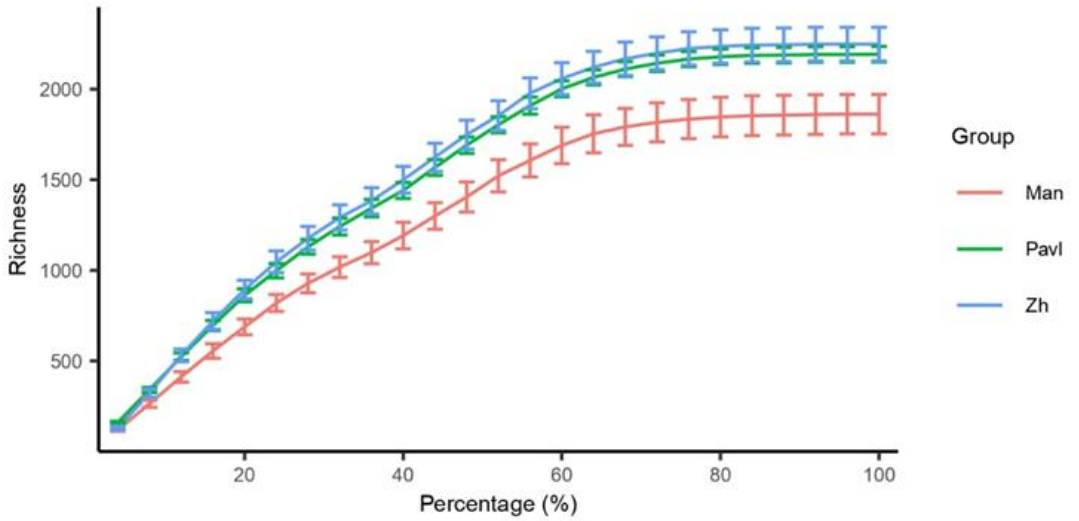

Figure 1. Rarefaction curves for all samples used in this study.
Each curve is color coded depending on the group it belongs to; Mangystau region (n = 6),
Pavlodar region (n = 7), Zhetysu region (n = 9).

Analysis of microbial diversity in horse intestines

The data showed that at the phylum level, the Mangystau region sample group on average consisted mainly of *Firmicutes* (more than 70 %), followed by *Bacteroidota* (9 %) and *Actinobacteria* (18 %), the Pavlodar and Zhetysu region sample groups showed similar data, where Firmicutes (more than 50 %) and then from *Bacteroidota* (more than 35 %) as shown in Figure 2.

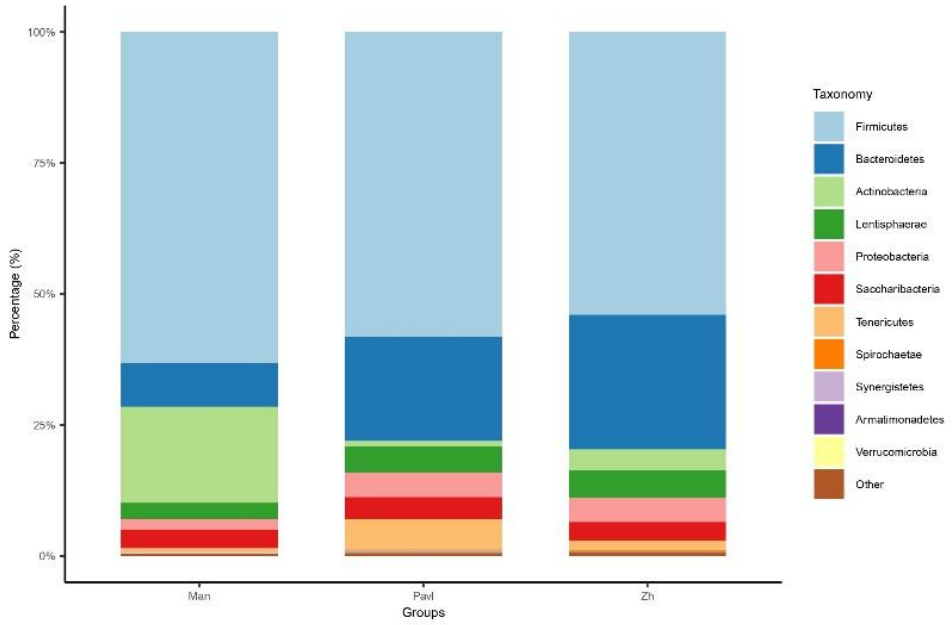

Figure 2. Relative abundance of microbiota phyla, divided into three groups by region, color coded. Group 1 consisted of animals from the Mangystau region, group 2 — Pavlodar region, group 3 — Zhetysu region.

We identified a core microbiome consisting of the following 7 genera in the intestines of horses from different regions of Kazakhstan. These were *Lactobacillus, Micrococcales, Bacillales, Bacteroidales, Clostridiales, Corynebacteriales, Burkholderiales* (Fig. 3).

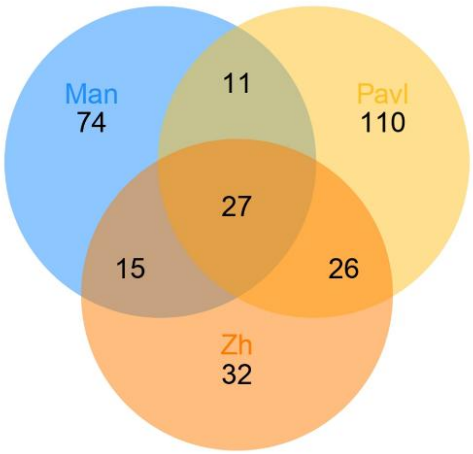

Figure 3. Venn diagram of three groups illustrating the most common bacterial genera identified in fecal intestinal samples of Kazakh horses from the Mangystau, Pavlodar and Zhetysu regions and scattered animals (with relative abundances by group > 0.1 %). Seven genera of bacteria (Lactobacillus, Micrococcales, Bacillales, Bacteroidales, Clostridiales, Corynebacteriales, Burkholderiales) at the intersection of all three groups have been identified as the core microbiome

Alpha diversity

The assessment of the alpha diversity of the species index and uniformity were calculated for samples from 3 different regions. Chao1 and ACE indices were used to calculate species richness, and Shannon and Simpson indices were used to calculate uniformity. All four indicators of alpha diversity showed that the intestinal microbiomes of horses from three different regions differed significantly in species richness and alignment ($p < 0.05$) (Fig. 4). Despite the autumn herbage of the natural grasslands of various regions, a sample of horse samples from the Zhetysu and Pavlodar regions showed a high index of species richness. The species richness of horses of the Pavlodar and Zhetysu regions was 9.7, which was slightly higher than

that of horses of the Mangystau region (9.0 p<0.01) (additional file table). Samples from the Mangystau region were high in species richness, given the harsh natural conditions, and samples from all three regions were leaders in species evenness. Statistical analysis showed that horses from three regions had a rich and diverse gut microbiota. It is interesting that the horses of the Mangystau region also had the rich species diversity, despite the sparse grass stand, in comparison with the other two regions, where the natural conditions are more favorable and are characterized by fairly good pastures with high grass stand. This phenomenon emphasizes the connection with the place of origin of horses.

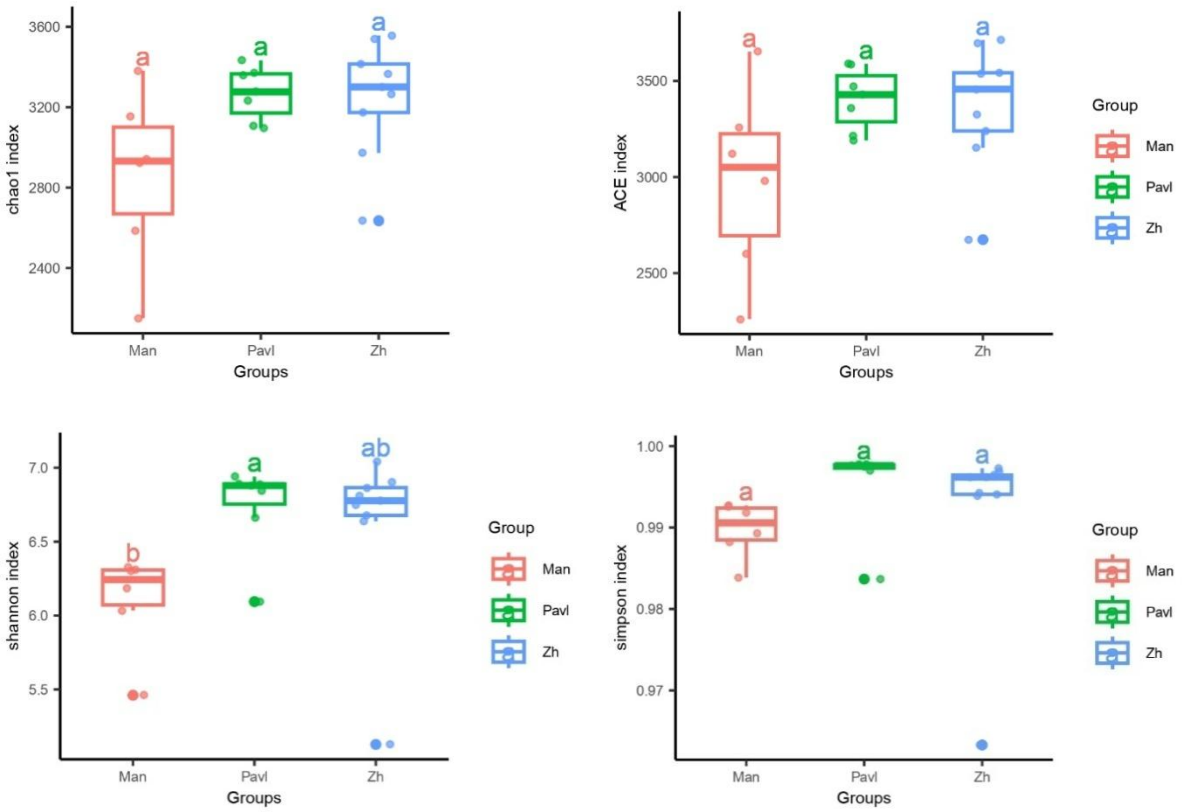

Figure 4. Graphs representing alpha diversity values of microbial communities obtained from intestinal samples from healthy horses. The samples are grouped and color-coded according to their geographic origin (i.e. Mangystau, Pavlodar and Zhetysu regions). The Chao 1 and ACE indices measure species richness and the Shannon and Simpson indices measure evenness. ANOVA tests (Chao 1 and observed ASV) and Kruskal-Wallis tests (Shannon and Simpson) were used for between-group comparisons

Beta diversity

We examined the relationship between 24 horse fecal samples from three different regions of Kazakhstan using Bray-Curtis distances. We used PCoA (principal coordinate analysis) to study the community structure of the intestinal microbiota of Kazakh horses. The samples formed clear clusters based on their geographic origin (Fig. 5). Horses from the Pavlodar and Zhetysu regions formed close, overlapping, but separate clusters, and samples from the Mangystau region group were clearly grouped from each other. In the PCoAplot, the bacterial communities were grouped and separated from each other along the principal coordinate axis 1 (PC1), and the cluster analysis was similar, explaining the greatest amount of variation (24.9 %). Analysis of variance of beta diversity values showed that clear clustering was statistically significant (p value <0.001), confirming the difference in the structure of the intestinal microbial community of horses from the Pavlodar, Zhetysu and Mangystau regions. Comparisons of the results of studies of the intestinal microbiota of horses with studies of scientists [12–14] around the world show that the resident microbial profile of horses is similar, but tends to be dependent on the type of maintenance, age and place of origin.

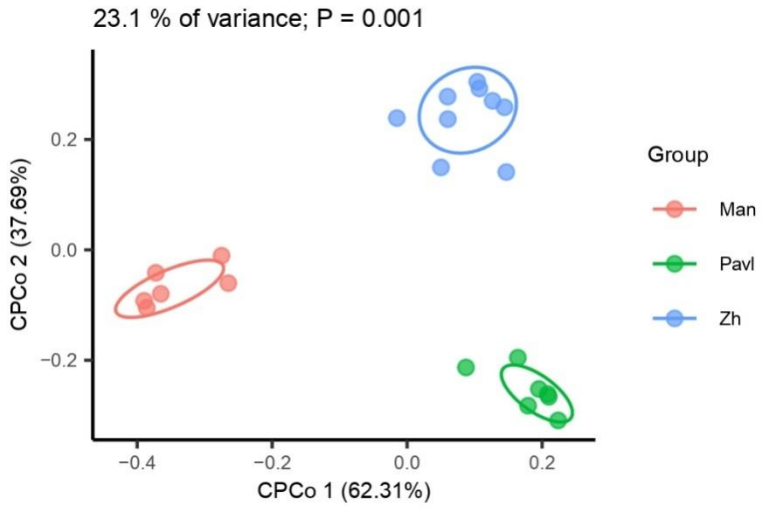

Figure 5. Inter relationships of the intestinal microbiota of horses from three samples. Maps representing beta diversity based on Bray-Curtis analysis. Graphs are generated based on the Bray-Curtis distance. The samples are grouped and color-coded according to their geographic origin, with blue dots representing the Zhetysu region group (Zh), red dots representing the Mangystau region group (Man), and green dots representing the Pavlodar region group (Pavl)

Conclusion

Based on our research, the ecosystems of different regions in Kazakhstan influence the composition of the microbial communities in horses. This study is the first to characterize the gut microbiota of the Kazakh horse breed through the sequencing of 16S rRNA amplicons. We compared the microbial diversity in the intestines of Kazakh horses and found that horses from the Mangystau, Pavlodar, and Zhetysu regions, which are located on natural pastures, had a richer microbial diversity. Now, when considering the species at the taxonomic level, the diversity of the Kazakh horse breed in different regions has been determined. This indicates the unique characteristics of the breed. Studying the composition of the microbiota in local breeds, such as the Kazakh horse, is essential for preserving biodiversity and promoting safe and sustainable horse breeding practices in agriculture. The analysis of the gut microbiota from healthy Kazakh horses will help create an information bulletin and contribute to further research on developing strategies to ensure the survival and well-being of this local breed.

Funding

This work was carried out within the framework of grant funding of the Ministry Science and Higher Education of the Republic of Kazakhstan: AP14869181 “The study of microbiome ecogenomics of Kazakh horse breed by NGS sequencing” (2022–2024).

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Ш. Касымбекова, В. Строчков, Д. Кабылбекова, А. Махмутов, Ж. Бименова

NGS-секвенирлеу арқылы қазақ тұқымды жылқыларының ішек микробтық профильдерін зерттеу

Зерттеудің мақсаты 16S рРНҚ ампликондарын секвенирлеу арқылы жылқылардың ішек микробиомасын (жануарларды ұстау түрі, жасын, тұқымын ескере отырып) бағалау. Қазақстанның түрлі аймақтарынан қазақ тұқымды жылқылардың ішек нәжісінің үлгілерінен барлығы 24 секвенирлеуге арналған кітапханалар құрылды. Қазақ жылқыларының ішек микробиомасының Альфа әртүрлілігі (CHAO 1 және ACE, Шеннон және Симпсон индекстері) тәулік бойы табиғи жайылымдарда болған Маңғыстау, Павлодар және Жетісу облыстарының жылқыларында жоғары микробтық әртүрлілік анықталғанын көрсетті. Павлодар және Жетісу өңіріндегі жылқылардың микробтық түрлік көрсеткіші 9,7 құрады, бұл Маңғыстау өңіріндегі жылқыларға қарағанда (9,0 $p < 0,01$) шамалы жоғары болды. Бета әртүрлілігі Брей-Кертис арақашықтықтарын, Қазақстанның үш түрлі аймағындағы жылқылардың ішек нәжісінің 24 үлгісі арасындағы байланысты пайдалана отырып зерттелді, олардың географиялық орналасуына байланысты нақты кластерлер қалыптастырылды. *Lactobacillus, Micrococcales, Bacillales, Bacteroidales, Clostridiales, Corynebacteriales, Burkholderiales*-тен тұратын Қазақстанның түрлі өңірлеріндегі жылқылардың негізгі ішек микробиомасы анықталды. Қазақтың жылқы тұқымы сияқты жергілікті тұқымдардың ішек микробиотасының құрамын зерттеу биоәртүрлілікті сақтау және ауыл шаруашылығында өнімді жылқы шаруашылығын ұстау және жүргізу тәсілдерін таңдау үшін қажет.

Кілт сөздер: NGS-секвенирлеу, 16S рРНҚ, *Equus ferus caballus*, қазақ жылқылары, Жабы, Адай, ішек микробиомы, биоәртүрлілік.

Ш. Касымбекова, В. Строчков, Д. Кабылбекова, А. Махмутов, Ж. Бименова

Изучение кишечных микробных профилей лошадей Казахской породы методом NGS-секвенирования

Целью исследования являлась оценка микробиома кишечника лошадей (с учетом их содержания, возраста, породы) путем секвенирования ампликонов 16S рРНК. Всего было создано 24 библиотеки из образцов фекалий лошадей казахской породы из различных регионов Казахстана. Альфа-разнообразие (Chao 1 и ACE, индексы Шеннона и Симпсона) кишечника лошадей казахской породы показало, что богатое микробное разнообразие было выявлено у лошадей Мангистауской, Павлодарской и Жетысу-

ской областей, которые круглосуточно находились на естественных пастбищах. Видовое богатство у лошадей Павлодарского и Жетысуского региона составило 9,7, что было незначительно выше, чем у лошадей Мангистауского региона (9,0 P <0,01). Бета-разнообразие исследовали, используя расстояния Брея-Кертиса; взаимосвязь между 24 образцами фекалий лошадей из трех разных регионов Казахстана сформировала четкие кластеры в зависимости от их географического происхождения. Мы идентифицировали основной микробиом кишечника лошадей различных регионов Казахстана, состоящий из *Lactobacillus, Micrococcales, Bacillales, Bacteroidales, Clostridiales, Corynebacteriales, Burkholderiales*. Изучение состава микробиоты кишечника местных пород, таких как казахская порода лошадей, необходимо для сохранения биоразнообразия и выбора способов содержания и ведения продуктивного коневодства в сельском хозяйстве.

Ключевые слова: NGS-секвенирование, 16SrRNA, *Equusferuscaballus*, казахские лошади, Жабе, Адай, кишечный микробиом, биоразнообразие.

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