Research Article

Diversity Characterization of the Gut Microbiome in Kazakh Horses using 16s rRNA Sequencing

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ABSTRACT

The Kazakh horse, an ancient breed native to Central Asia, holds cultural and historical significance in Kazakhstan. This study aimed to assess intestinal microbiota diversity in Kazakh horses, considering factors such as age, breed, and pasture type. The study was conducted in autumn-winter 2023, the research involved 24 horses aged 3-8 years from Pavlodar, Zhetysu, and Mangystau regions, grazing on natural grasslands. The study found that non-dominant bacteria, such as Tenericutes and Proteobacteria, were more abundant in horses from Pavlodar and Zhetysu. At the same time, the Mangystau region had higher levels of anaerobic cellulolytic and proteolytic bacteria. Horses on grazing had a more diverse microbiome than those on a high-protein diet. A link between lifestyle and microbiome adaptation was suggested, with 24-hour grazing promoting a richer microbiome. The microbiota in different regions revealed significant differences, with the Mangystau and Zhetysu regions showing highly diverse microbial communities. Horses from these areas even hosted unique bacteria associated with marine environments, likely due to the influence of seawater. In contrast, Pavlodar horses showed lower species diversity. The study highlights the importance of preserving the genetic traits of local horse breeds and suggests that gut microbiota plays a role in the growth and development of horses. Further research will focus on the resistome, metabolome, and the search for pharmabiotics to support the conservation and productivity of these breeds.

Keywords: Equine, 16S rRNA sequencing, Fecal microbiota, Intestinal microorganisms, Microbial community diversity

INTRODUCTION

The Kazakh horse, an ancient breed native to the steppes of Central Asia, holds significant cultural and historical value in Kazakhstan. This breed's physical characteristics exemplify its resilience and suitability for its challenging environment. The Kazakh horse's moderate size, sturdy conformation and well-developed musculature enable it to endure long journeys and navigate diverse terrains. Unique adaptations, such as efficient grazing habits, resistance to extreme temperatures, and an innate ability to detect water sources, allow the Kazakh horse to thrive in its natural habitat. Renowned for its exceptional endurance, adaptability, and hardiness, this breed has played a crucial role in the nomadic lifestyle of the Kazakh people for centuries (Kabylbekova et al. 2024).

The digestive system of a horse (Equus ferus caballus) has several distinctive features, including a small stomach volume and continuous secretion of gastric juice, which is

why wild horses graze around the clock. The large intestine of horses comprises three sections: the cecum, colon and rectum. The cecum in horses is considered analogous to the ruminant rumen, where up to 50% of all fiber and up to 40% of protein are digested with the aid of symbiotic microflora, including bacteria, archaea, micromycetes, protozoa, bacteriophages (Santos et al. 2011; Shepherd et al. 2012). The gut microbiota of herbivorous animals such as horses plays a crucial role in generating short-chain fatty acids, which serve as an essential source of energy for these animals (Flint et al. 2012; Di Pietro et al. 2021; Kinoshita et al. 2021).

The intestinal tract of horses harbors numerous beneficial microorganisms, including bacteria and fungi, collectively forming the gut microbiome (Costa and Weese 2012; Chaucheyras-Durand et al. 2022). These microorganisms play a pivotal role in equine health by providing enzymes that aid in fiber digestion and energy extraction and contribute to the development of immunity

(Julliand and Grimm 2016). Consequently, disruptions in the intestinal microbiome can precipitate diseases, compromise athletic performance and potentially influence equine behavior (Dougal et al. 2012; Morrison et al. 2018; Grimm et al. 2020).

The study by Li et al. (2023) stated that their catalog of bacterial genomes represents a valuable resource for the discovery of performance-enhancing microbes in horses and for further study of the gut microbiome. In their study, they analyzed 110 samples from the cecum and 132 samples from the rectum using metagenomic analysis, which assembles DNA fragments into probable bacterial genomes. Of the 4,142 genomes discovered, 4,015 correspond to new species. Another interesting discovery by scientists is a variety of antibiotic resistance genes, which confirms their widespread use in our time. Moreover, in English thoroughbred horses these genes have the greatest prevalence and quantity (Li et al. 2023).

The article authored by Kauter et al. (2019) on the gut microbiome of horses presents a comprehensive overview of the current state of research on equine enteral microbiota, shedding light on the intricate and vital role that gut microorganisms play in the overall health and performance of horses. This research has revealed that the equine gut harbors are a complex community of microorganisms, influencing digestion, nutrient absorption, and immunity. Moreover, the article explores the potential implications of the gut microbiome in equine diseases, such as colic and laminitis, as well as its impact on athletic performance, providing valuable insights for equine health and management. As a result, the article offers a promising glimpse into the future of equine microbiome research, suggesting that a deeper understanding of these microbial communities may lead to innovative strategies for enhancing the well-being and performance of horses (Kauter et al. 2019).

Garber et al. (2020) stated a comprehensive and up-to-date exploration of the various factors that influence the composition and dynamics of the gut microbiota in horses. It synthesizes the existing knowledge on this subject, shedding light on the critical role of factors such as diet, environment, age, and health status in shaping the equine gut microbiome. The article provides valuable insights into the complex interactions between these factors and the microbial communities residing within the horse's digestive system, ultimately contributing to our understanding of how to optimize equine health, nutrition, and performance through microbiota management. This resource is indispensable for researchers, veterinarians, and equine professionals seeking a deeper understanding of the equine gut microbiome and its implications for horse management and care (Garber et al. 2020).

Ang et al. (2022) provided a valuable comparative analysis of the gut microbiome in feral and domesticated horses across various geographic regions. Through their research, the authors offer insights into how the gut microbiota composition differs between wild and domesticated equine populations, shedding light on the impact of domestication and geographic location on the horses' microbial communities. This study not only contributes to our understanding of the horse's evolving gut microbiome but also has broader implications for equine

health, nutrition, and management practices, highlighting the significance of environmental and lifestyle factors in shaping the gut microbiota of these animals. It serves as a vital resource for researchers and horse caretakers interested in optimizing equine well-being and performance through microbiome-related insights (Ang et al. 2022).

Summarizing the studies discussed above, the microbiota in the gastrointestinal tract of horses plays a significant role in the digestion of nutrients and supports the health and wellbeing of animals. Various factors can influence the microbial balance, often leading to conditions such as colic and laminitis (Venable et al. 2016). Research on the horse's microbiome aims to understand the functions of microbial communities in both health and disease states, as well as identify changes in microbial numbers depending on different factors in order to develop strategies for maintaining optimal intestinal health and preventing disease in horses.

In this study, we employed 16S rRNA gene amplicon sequencing to analyze the resident microbiome of 23 clinically healthy Kazakh horses from three ecologically distinct regions of Kazakhstan (Pavlodar, Zhetysu, Mangystau). Our study aimed to assess the diversity in the community structure and species composition of the intestinal microbiota of Kazakh horses, considering factors such as age, breed, and natural pastures.

MATERIALS AND METHODS

Ethical approval
The research protocol was discussed and approved at a meeting of the local ethical committee of the Kazakh National Agrarian University of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan dated March 11, 2024.

Animals and sample collection
The research was carried out in the autumn-winter period of 2023 in three different regions of Kazakhstan (Pavlodar, Zhetysu, Mangystau). The Kazakh-bred horses involved in this study were 3-8 years old and were located on natural grasslands of autumn grass, respectively, in the northern, western and southern regions of Kazakhstan. Rectum (fecal) samples were taken from 24 horses. All samples were frozen in liquid nitrogen, delivered to the laboratory and stored in a freezer at -20°C until DNA extraction. The study included 24 healthy horses. There were including Kazakh horses Zhabe type (n=8) from Pavlodar region, Kazakh horses Zhabe type (*n*=9) from Zhetysu region and Kazakh horses Adai type (*n*=6) from Mangystau region.

The adult animals were selected based on the following criteria: no drugs affecting gastrointestinal microbes were used within 4 months, no reported illness within the past 4 months of the study and, no gut-related disorders recorded until the beginning of the study. All the samples were in the same growing stage. Furthermore, the animals were clinically healthy based on their parasite profiles. 24 fecal samples were collected from the rectum of horses using long arm gloves. All samples were frozen in liquid nitrogen, delivered to the laboratory and stored in a freezer at -20°C until DNA extraction.

DNA extraction, library construction and sequencing

Microbiome DNA extraction was performed using the PureLink Microbiome DNA Purification Kit (ThermoFisher, USA) according to the manufacturer's protocol. The DNA concentration and purity were quantified with a Nanodrop 2000 ® (ThermoFisher, USA) and Qubit 3.0 (ThermoFisher, USA), respectively. 1% agarose gel electrophoresis was used to examine DNA quality.

The amplification of hypervariable regions for NGS sequencing was conducted using the Ion 16S Metagenomics Kit (ThermoFisher, USA) targeting the V2-4-8, V3-6, 7-9 region of the 16S rRNA gene, following the manufacturer's protocol. The Ion 530 chip was prepared for sequencing the obtained libraries with the Ion 510™&Ion 520™&Ion 530™ Kit – Chef. Metagenomic sequencing was performed on the IonTorrent S5 platform (ThermoFisher, USA) at the Kazakh National Agrarian Research University (Almaty, Kazakhstan).

Sequence processing and statistical analysis

Data analysis was performed using RStudio version 2024.04.1+748 and R-based EasyAmplicon pipeline (Liu et al. 2023). Raw sequences were quality-filtered and dereplicated using VSEARCH v.2.21.1 subcommand -fastx_filter and -derep_fulllenght, respectively (Rognes et al. 2016). Then, the non-redundancy sequences were denoised into amplicon sequence variants (ASVs) with USEARCH v11.0.667 (Edgar 2010) (via -unoise3). Chimeras were removed by VSEARCH –uchime_ref against the SILVA database (Quast et al. 2013). Feature tables were created by vsearch –usearch_global. The taxonomy of the features was classified by the USEARCH sintax algorithm in SILVA v123.

Diversity analysis was carried out using the vegan v2.6–6.1 package (https://cran.r-project.org/web/packages/vegan/) and visualized by using the ggplot2 v3.5.1 (https://cran.r-project.org/web/packages/qqman/) package in R v4.4.0. LEfSe was conducted with the online platform ImageGP (http://www.ehbio.com/ImageGP-/index.php/ Home/Index/LEFSe.Html). Functional profile prediction of microbial communities was conducted by PICRUSt (Langille et al. 2013) with the Greengenes as the reference database.

RESULTS

The taxonomy rarefaction curve indicated that the sequencing depth used in this study was sufficient to saturate species richness in all samples. To analyze the sequence of 16S rRNA amplicons, 34,953. 557 readings were obtained at the paired ends from 39 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.

Analysis of microbial diversity in horse intestines

The data showed that at the phylum level, the sample group of the Mangystau region on average consisted mainly of Firmicutes (>70%), followed by Bacteroidota (9%) and Actinobacteria (18%), the sample groups of the Pavlodar and Zhetysu regions showed similar data, where Firmicutes (>50%) and then from Bacteroidota (>35%) as shown in Fig. 1-2.
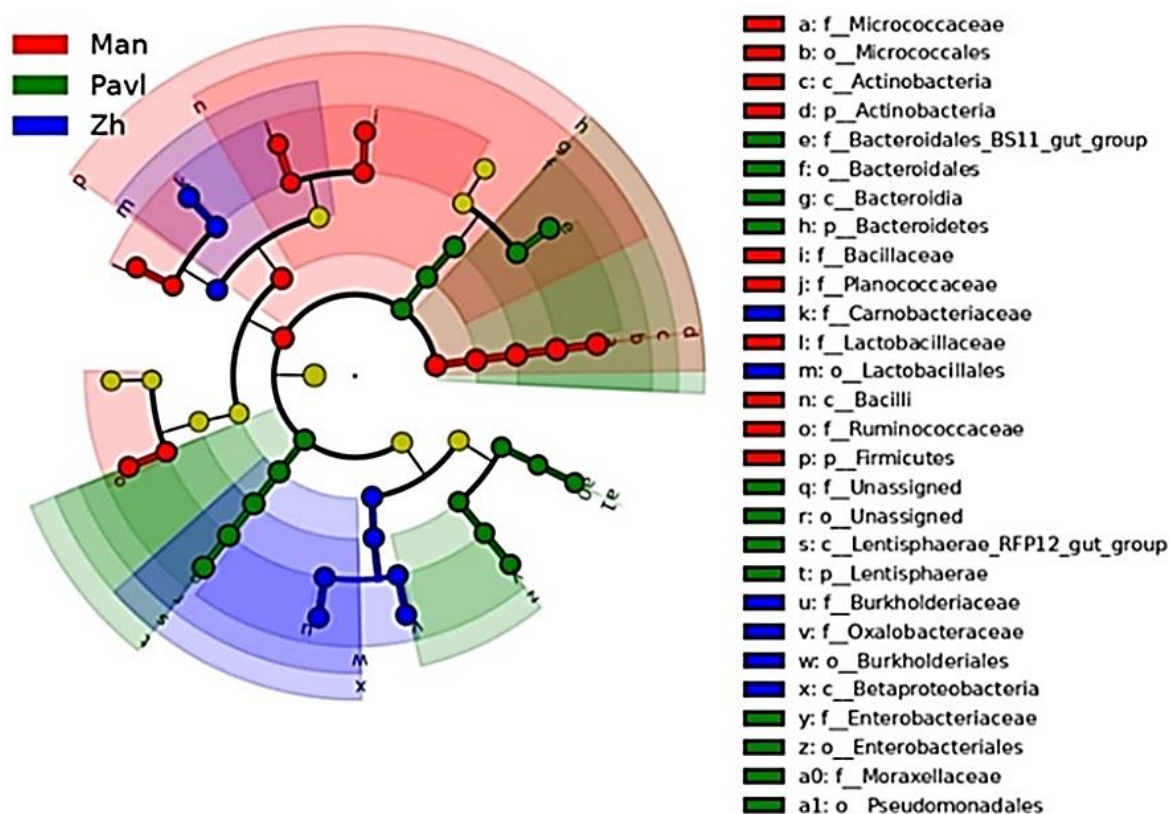

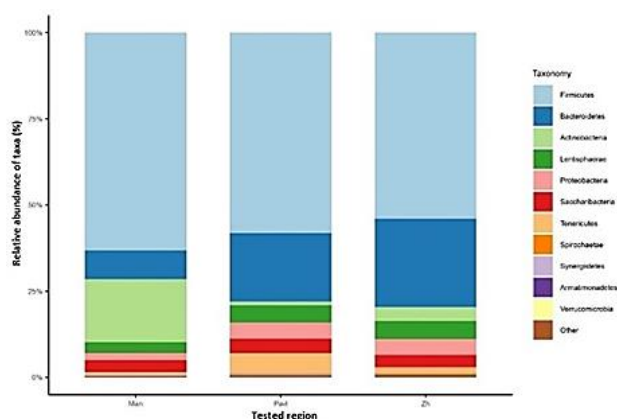**Fig. 1:** Phylogenetic tree representing taxa obtained from fecal samples of Kazakh breed horses

Fig. 2: Relative abundance of bacterial phylum divided into three tested regions: Mangystau (Man), Pavlodar (Pavl) and Zhetysu (Zh).

Fig. 3: Relative abundance of microbiota family divided into samples of each group based on tested region: Mangystau (Man), Pavlodar (Pavl) and Zhetysu (Zh).

Firmicutes (~55%) was the most abundant phylum, followed by Bacteroidetes (20%), Actinobacteria (4-5%) for samples from the Mangystau region (20%), Lentisphaerae (5%) and Proteobacteria (5%), Saccharibacteria (4%). Firmicutes, Bacteroidetes, Actinobacteria, Lentisphaerae and Proteobacteria were observed in all samples. Tenericutes were observed only in samples from the Pavlodar region. Firmicutes were the most abundant phylum (54%) in samples from the Zhetysu region, while Actinobacteria (20%) and Firmicutes (60%) were the two most abundant phyla in the Mangistau 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*.

Bacterial taxa differed in equine fecal samples from the respective regions. At the phylum level, Firmicutes and Bacteroidetes both showed a remarkable difference in the three groups (Fig. 4c-f). In addition, the ratio of F and B (Firmicutes and Bacteroidetes) in horses from the Mangistau region (1.403±1.013) was significantly higher than in horses from the Pavlodar (0.904±0.55) and Zhetysu regions (0.936±0.41). The number of Tenericutes and Verrucomicrobia was significantly higher in horses of the Pavlodar region. However, an abundance of Bacillus and. Cyanobacteria was observed in horses from the Mangistau region (Fig 4, a-h).

Alpha diversity

Chao1, ACE, Shannon and Simpson indices were used to calculate species richness and uniformity within each tested region. Comparison of each alpha diversity indicator showed that the intestinal microbiomes of horses from three different regions differed significantly (Fig. 5). 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. Species richness in horses of the Pavlodar and Zhetysu regions was 9.7, which was slightly higher than in horses of the Mangistau region (P<0.01). 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.

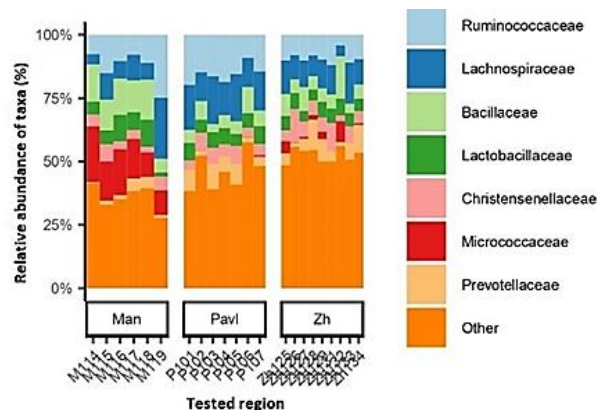The coverage of horses of Zhetysu, horses of Pavlodar

and horses of Mangystau regions was 0.9527, 0.9426 and 0.9586, respectively, indicating that the proportion of undetected species in the sample is relatively small. The observed species index in horses of the southern and western regions was significantly higher than in horses of the northern region. Statistical analysis showed that horses from three regions had a rich and diverse gut microbiota. It is interesting that the horses of the Mangistau region also had a 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.

Beta diversity

We examined the relationship between 24 horses' 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. 6). Horses from the Pavlodar and Zhetysu regions formed close, overlapping, but separate clusters, and samples from the Mangistau region group were grouped clearly from each other. In the PCoA plot, 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<0.001), confirming the difference in the structure of the intestinal microbial community of horses from the Pavlodar, Zhetysu and Mangistau regions.

The LEfSe effect size was used to identify differentially abundant biomarkers based on the geographic origin of equine fecal samples. LEfSe identified 140 (adjusted FDR <0.05, LDA >2.0) significant features. A list of 41 essential features has also been compiled (Fig. 7) based on overlapping characters, *Arthrobacter, Lactobacillus, Bacillus* were obtained. And *Planococcus,* which included in the four biomarkers with an LDA index >5.0., for the group of the Mangistau region, for the Pavlodar region only *Klebsiella* and *Acinetobacter, Carnobacterium, Noviherbaspirillum* were identified as bacterial biomarkers.
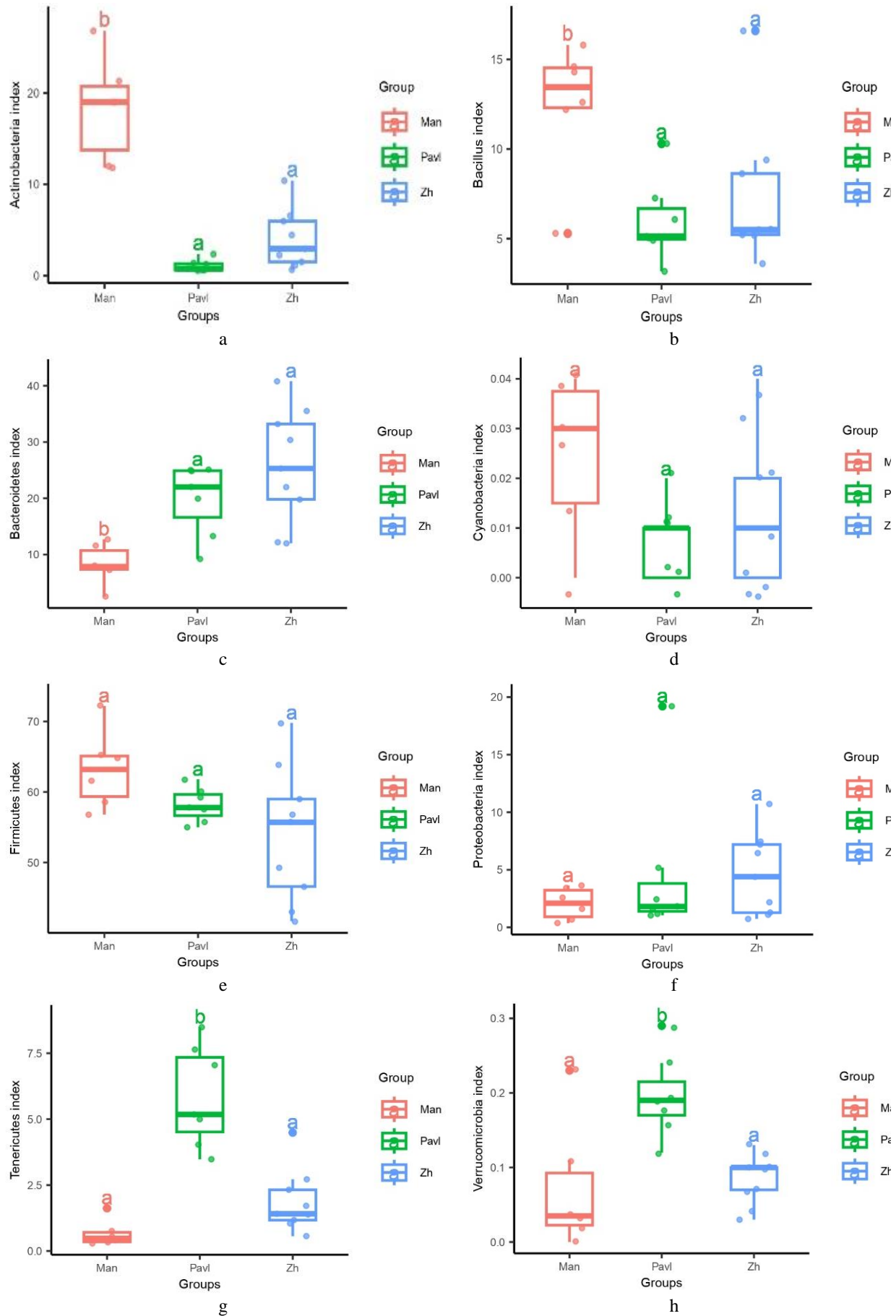
Fig. 4: Statistical comparisons of relative abundance for each of dominant phylum (a. Actinobacteria, b. Bacillus, c. Bacterioideles, d. Cyanobacteria, e. Firmicutes, f. Proteobacteria, g. Tenericutes, h. Verrucomicrobia) grouped by tested region: Mangystau (Man), Pavlodar (Pavl) and Zhetysu (Zh) (P<0.05).
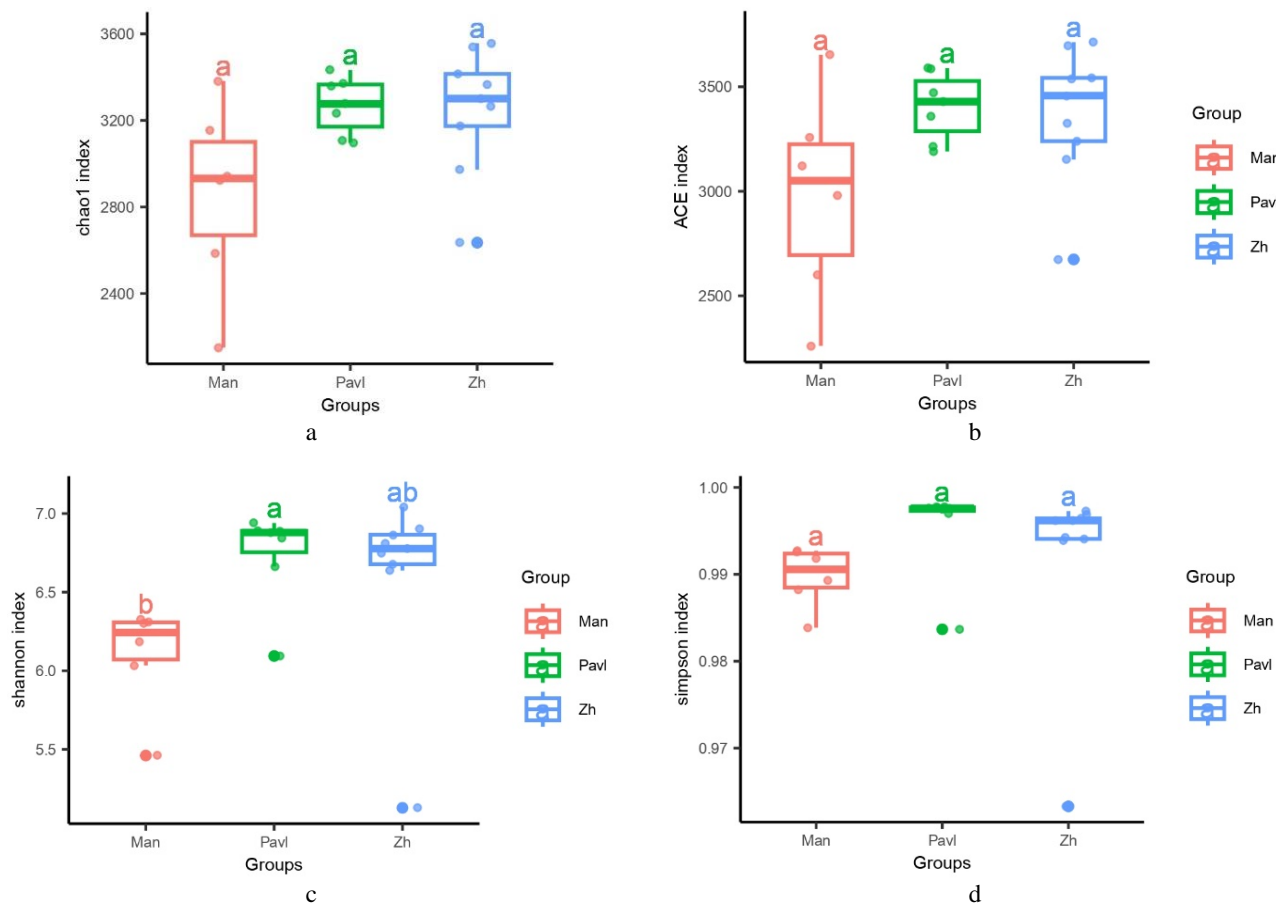
Fig. 5: Comparison of alpha diversity indices between microbial communities obtained from intestinal samples from Kazakh horses. Samples are grouped by tested region and represent by a different color: Mangistau (red), Pavlodar (green) and Zhetysu (blue). ANOVA tests (Chao1 and ACE) and Kruskal-Wallis tests (Shannon and Simpson) were used for between-group comparisons.
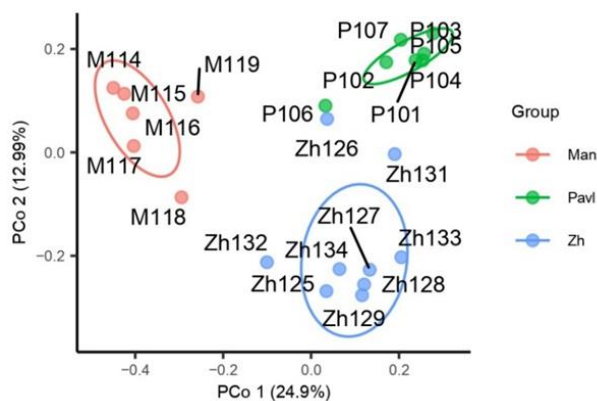

Fig. 6: Beta-diversity visualized with PCoA (Principal Coordinates Analysis) of 22 microbial samples.

DISCUSSION

Productive horse breeding in Kazakhstan is a traditional branch of animal husbandry, whose task is the production of horse meat and koumiss. The Kazakh horse breed is distinguished by higher meat and dairy qualities, unpretentiousness to the conditions of year-round pasture-breeding. Its development is facilitated by the huge scale of natural pastures (Kargaeyeva et al. 2023).

It is essential to investigate the influence of the microbiome on nutrient absorption, digestion and the formation of normal immunity. However, the classification of taxons is also a fundamental aspect of the research. The findings of this study provide a fundamental understanding of the microbiome of Kazakh horses, which could possibly be utilized for the development of immunomodulatory correction of the equine microbiome with the goal of reducing the risk of disease.

In natural spring pastures, freshly extracted feces are often eaten by monthly foals for colonization with intestinal microbiome; such a technique is characteristic of wild horses and explains the mechanism of transmission of specific resident microbiome. The predominant microbiome phylum in Kazakh-bred horses was identified as Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, which aligns with the findings of other scientific studies in this field (Kauter et al. 2019; Morrison et al. 2020; Di Pietro et al. 2021).

The relative abundance of all dominant types was the same in free-fed horses, while the abundance of non-dominant types Tenericutes and Proteobacteria was significantly higher in horses from the Pavlodar and Zhetysu regions. The results of beta diversity showed that the bacterial communities in horses of the Mangystau region identified target groups of microorganisms - anaerobic cellulolytics of the phyla Bacteroidota and Chloroflexi, as well as proteolytic bacteria of the phylum Firmicutes.

The gut microbial communities of horses kept on grazing may retain specific non-dominant bacteria and contain a more diverse microbiome than that of captive horses. The results suggest that host lifestyle may be a
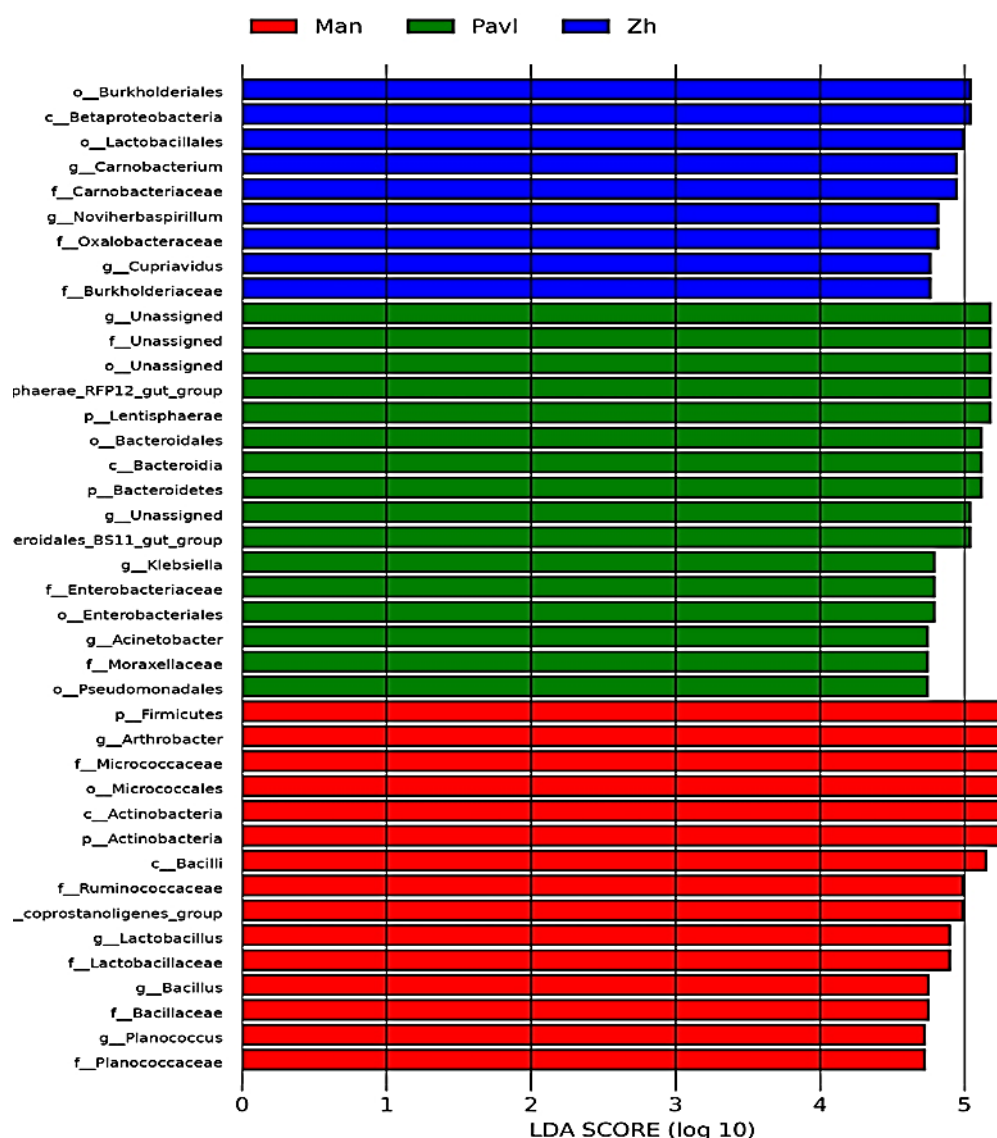
Fig. 7: Important biomarkers that are differentially present in intestinal fecal samples grouped according to their geographic origin. Using LEfSe algorithms, 11 consensus differentially abundant genera were identified and are shown in the figure in ascending order of their LDA score. Columns representing individual genera are color-coded to indicate the specimen group (geographical origin) in which they are most abundant

significant factor in the differentiation of the microbiome during the process of adaptation. It can therefore be hypothesized that 24-hour grazing is an appropriate physiological adaptation for mono-ungulates in order to maintain a relatively high level of richness in the gut microbiome.

The abundance of Clostridiales was found to be significantly lower in horses that were fed a diet based on grazing conditions in comparison to horses that were fed a high-protein diet at home. The relationship of the diversity and function of the intestinal microbiota with the health and phenotypes of horses, as well as their habitat, is currently an urgent area of research (Li et al. 2023; Lv el al. 2023).

The correlation between the microbiota of horses and their geographical location was evaluated, specifically in the Mangystau (Caspian semi-desert territory) and Zhetysu (Lake Alakol) regions of Kazakhstan. It was concluded that the intestinal microbiomes of horses from these regions have highly diverse microbial communities. This includes a strain of the epiphytic marine bacterium *Plannomicrobium okeanokoites*, which is associated with the holobiont microbiome of the brown algae *Himantothallus grandifolius* (Armstrong et al. 2001; Croft et al. 2006; Saini et al. 2023). This study has confirmed several interesting facts regarding the adaptation of horses

to appropriate natural pastures and the utilization of seawater from the Caspian Sea and Lake Alakol. In comparison, the microbial community of the sample of horses in the Pavlodar region exhibited a paucity of species diversity.

It is important to preserve local horse breeds with their unique genetic characteristics, which have been passed down from generation to generation to adapt to certain geographical regions and natural conditions. The study of their characteristic microbial profiles allows us to obtain the defining characteristics of their successful growth and development of local horse populations.

Conclusion

Based on our findings, ecosystems of various regions of Kazakhstan affect the composition of microbial communities of horses. Our study was the first to characterize the gut microbiota of Kazakh-bred horses by sequencing 16S rRNA amplicons. A comparison of the microbial diversity of the intestines of Kazakh-bred horses showed that a rich microbial diversity was determined in horses of the Mangystau, Pavlodar and Zhetysu regions, which were located around the clock on natural pastures. At the taxonomic level of the phylum, the analyzed samples were similar to horses of other breeds and directions, in the

results of research by other scientists. Now, when considered at the species taxonomic level, the diversity in the regions of the Kazakh horse breed has been determined, which indicates the distinctive features of the breed. Five genera have been identified that reliably correlate with the growth of the body of horses. From this we can conclude about the effect of the microbial profile of the intestine on the growth of horses. Studying the microbiota composition of local breeds such as the Kazakh horse breed is necessary for preserving biodiversity and promoting safe methods of productive horse breeding in agriculture. Further research will focus on the resistome, metabolome, and the search for pharmabiotics. Analysis of the obtained gut microbiota of healthy Kazakh horses will serve to create a fact sheet and further research to develop strategies to ensure the conservation and well-being of local horse breeds.

Auhtor's Contribution: Sh. Kassymbekova: data interpretation, critical revisions, interpretation of results. V. Strochkov: conceptualization, research design, technical support. Y. Ussenbekov: literature review, manuscript writing, overall study supervision. A. Kalykova: data analysis, statistical work. D. Kabylbekova: funding acquisition, project administration, supervision, methodological development. Zh. Bimenova: data acquisition and data collection. S. Khizat: manuscript writing, data analysis.

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