Original Research

Journal of Advanced Veterinary Research
(2026) Volume 16, Issue 2, 234-238

The microbiome of the digestive tract of ponies: Analysis based on 16S rRNA sequencing

Olena Kolchyk[1*], Anatoliy Paliy[1], Sergii Borovkov[2], Oleksandr Tarasov[2], Oksana Zlenko[1]

[1]National Scientific Center, Institute of Experimental and Clinical Veterinary Medicine, 83, Skovorodi Str., 61023, Kharkiv, Ukraine.
[2]Institute of Veterinary Medicine, the NAAS of Ukraine, 30, Donetska Str., 03154, Kyiv, Ukraine.

ARTICLE INFO

Received: 15 October 2025

Accepted: 23 December 2025

*Correspondence:

Corresponding author: Olena Kolchyk
E-mail address: kolchyk-elena@ukr.net

Keywords:

Ponies, Intestinal microbiome, Sequencing, Biodiversity, Bacterial communities

ABSTRACT

The study of the intestinal microbiome of ponies using 16S rRNA sequencing provided a deep insight into its structural organization, richness and ecological diversity. It was found that bacterial communities are characterized by high alpha diversity, a significant number of rare taxa, and the absence of dominance of individual species. The microbiome was dominated by four phyla; *Pseudomonadota*, *Bacillota*, *Bacteroidota*, and *Actinomycetota*, which accounted for over 90% of all reads. The presence of the genera *Escherichia* and *Bacteroides* may indicate transient changes or metabolic adaptation to environmental conditions. Analysis of β-diversity using the PCoA method revealed distinct individual differences caused by both internal (genetic) and external (ecological) factors. The similarity of microbiomes in animals with common housing conditions indicates a significant influence of diet, social structure, local microenvironment, and daily behavioural contacts. The predominance of rare taxa (according to Fisher's alpha and Rarity indices) emphasizes the ecological complexity, flexibility and stability of the microbial environment. Communities formed under natural grazing conditions proved to be more stable and adaptive. The results demonstrate the potential of microbiome monitoring for health diagnosis, feed optimization, probiotic strategy development, and prevention of intestinal dysfunction in ponies. Considering the microbiome as a complete ecosystem opens up new perspectives in veterinary medicine, particularly in the direction of personalised approaches to animal health maintenance. Further research considering the dynamics of the microbiota over time and the influence of seasonality may deepen our understanding of the relationship between the microbial profile, stress factors, and the physiological state of the animal.

Introduction

The digestive tract microbiome plays a key role in maintaining animal health by influencing digestion, immune response, metabolism, and resistance to pathogens. Compared to horses, the pony microbiome shows some differences in structure and functional potential, which may be related to differences in metabolism, anatomical features, feeding regimes, and genetic factors (Dougal *et al*., 2012; Blackmore *et al*., 2013; Stewart *et al*., 2018). For example, Welsh ponies, which are prone to obesity and metabolic syndrome, have been found to have a shift in the *Bacillota*/*Bacteroidota* ratio, which may be a biomarker for insulin resistance (Morrison *et al*., 2018).

Ponies, like other Perissodactyls, have an intestinal microbiome dominated by *Bacillota* and *Bacteroidota*, with significant contributions from Verrucomicrobiota and *Pseudomonadota*. The main genera commonly found include *Ruminococcus*, *Prevotella* and members of the *Lachnospiraceae* and *Ruminococcaceae* families, which are key fermenters and fibre degraders that support fermentation in the hindgut of horses (Willing *et al*., 2009; Antwis *et al*., 2018; Langner *et al*., 2020). The pony microbiome is dynamic and changes depending on age (bacterial diversity increases with the age of the animal), season (due to changes in diet), husbandry (stable/pasture), and medical interventions (use of antibiotics or antiparasitic agents) (Fernandes *et al*., 2014; Jovel *et al*., 2016; Videvall *et al*., 2019).

In addition, health conditions such as colic and laminitis correlate with reduced microbial diversity and dysbiosis, highlighting the role of the microbiome in managing pony health (Wylie *et al*., 2013; Luthersson *et al*., 2017). Diet is a major modulator: diets high in starch tend to enrich lactic acid bacteria such as streptococci and lactobacilli, increasing the risk of acidosis and colic, while diets high in fibre promote the development of cellulolytic taxa responsible for fibre degradation (Morrison *et al*., 2018; Adolph *et al*., 2019).

Despite growing interest in studying the microbiota of cattle, pigs and humans, research on the pony microbiome remains limited, even though these animals are an important model for studying intestinal microbial homeostasis in ungulates. Recently, thanks to the development of high-throughput sequencing technologies, in particular the 16S rRNA gene amplicon analysis method, it has become possible to study the taxonomic composition of microbiomes in more detail without the need to cultivate microorganisms. Applying this approach to the study of the pony microbiome provides a comprehensive picture of the bacterial environment of their gastrointestinal tract and identifies potential biomarkers of health or dysbiosis.

In the studies conducted, the pony microbiome was analysed using 16S rRNA sequencing with the objective of determining the main bacterial taxa, their relative diversity, and potential changes depending on physiological or environmental factors. The objective of the study was to characterise the taxonomic composition and diversity of the intestinal microbiota of ponies by sequencing the 16S rRNA gene.

Materials and methods

Ethical approval

Animal experiments were approved by the Bioethics Committee of the Institute of Veterinary Medicine, NAAS (Protocol No. 01/25, dated 01 September, 2025). Work with experimental animals during the study was carried out in accordance with international standards specified in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123, 1986).

Animals and maintenance

We studied faecal samples from eight clinically healthy ponies from the same farm, of the same age, feeding and housing conditions. The animals were housed in individual stalls (2 × 2.5 m) with daily turnout for at least 6 hours. Feeding was standardized and consisted of high-quality meadow hay provided ad libitum (approximately 2.0–2.5 kg/100 kg body weight per day) and concentrate feed in the form of oats (0.5–0.7 kg/100

ISSN: 2090-6277/2090-6269/

kg body weight), divided into two meals (morning and evening). A mineral–vitamin supplement was included in the diet at the manufacturer's recommended dosage. Fresh water was available without restriction. Faecal samples were collected from eight clinically healthy ponies (mean body weight: 203.0±14.0 kg; mean age: 6 ± 1.5 years) maintained on the same farm under identical management conditions. One faecal sample was collected from the rectum of each animal using a rectal probe (Meidike, China) in an amount of 10 g, which was gently inserted into the rectum and placed in tubes with DNA/RNA Shield Sample Storage and Transport Medium (ZYMO Research, USA, Cat. No. R1100-50). The biological material samples were stored at -80°C for two hours after collection.

Evaluation

The PureLink Microbiome DNA purification kit (Invitrogen, USA) was used to extract DNA. This kit was chosen because faecal samples are difficult for DNA extraction due to their high inhibitor content and heterogeneous composition. Additionally, magnetic particles from the NucleoMag kit for cleanup and size selection of NGS library prep reactions (Macherey-Nagel) were used to further purify the isolated DNA.

The library was prepared for sequencing using the 16S Barcoding Kit 24 V14 (SQK_16S114.24, Oxford Nanopore, USA) according to the manufacturer's protocol (Jovel *et al*., 2016). The kit protocol involves amplification of the 16S RNA gene, barcoding of samples, and library purification on magnetic particles. DNA concentration was monitored on a Qubit v.3 fluorometer (Thermo Fisher Scientific, USA) using the Qubit 1xdsDNA HS assay kit (Invitrogen). DNA purity was determined on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Spectrophotometric evaluation with NanoDrop confirmed the acceptable quality of the DNA preparation: optical densities A260/A280 (1.8±0.1) and A260/A230 (1.9±0.1).

Library sequencing was performed on a MinION Mk1B (Oxford Nanopore) device with a R.10.4.1 flow cell (FLO-MIN114, Oxford Nanopore). Data accumulation in pod5 format was performed under the control of MinKNOW 24.11.10 software for 48 hours.

After the sequencing process was completed, raw database calling was performed using a super-high accuracy (sup) model with the dorado-0.9.1 software package. The obtained data were demultiplexed to distribute the read fragments among the corresponding samples.

Taxonomic classification of reads by the 16s genome was performed in the epi2me-labs/wf-16s v1.5.0 pipeline of the EPI2ME Desktop 24.02-01 software developed by Oxford Nanopore Technologies. Minimap2 was used as the classifier. The classification was performed using reference sequences from the ncbi_16s_18s database. The maximum level of taxonomic identification was set to the species level.

Further analysis of the microbiome was performed using the phyloseq (McMurdie and Holmes, 2013) and microbiome (Lahti and Shetty, 2017) packages in R (R Core Team 2024). Unclassified taxa were excluded from the analysis.

Microbial diversity was assessed at the level of individual specimens without division into groups. To assess intraspecimen microbial diversity (alpha diversity), a number of metrics listed below were calculated using the alpha function from the microbiome package.

The number of observed taxa (Observed richness) and the Chao1 estimator, which takes into account the number of rare taxa in the sample, were calculated as indicators of taxonomic richness. Taxonomic complexity and uneven distribution of abundant taxa were assessed using the Shannon index, Fisher's alpha, the inverse Simpson index, and the Gini–Simpson index.

The evenness of taxon distribution within samples was analysed using Pielou's evenness index, which normalises Shannon's value by the number of taxa detected. Coverage was assessed by calculating the number of taxa present in all counts (Coverage).

In addition, to characterize the structure of dominance, the relative dominance and Gini dominance indices were calculated. To describe the presence of low-frequency taxa, rarity metrics were used: Rarity (low abundance) and Rarity (rare taxa).

To visualize differences in the composition of microbiomes (beta diversity), principal coordinate analysis (PCoA) was performed based on the Bray–Curtis distance metric, which is sensitive to the quantitative composition of the detected taxa. For the objective of confirming the ordination results, hierarchical clustering was performed using the same distance (Bray–Curtis, 'average' method) using the *hclus*t() function from the base R package. This approach was used to identify natural groupings of samples without prior assumptions about animal groups.

Since the number of reads between samples varied significantly and given the sensitivity of the alpha and beta diversity metrics used to the number of reads, data rarefaction was performed to calculate these metrics. The rarefaction was performed using the rarefy_even_depth function from the phyloseq package in R to the minimum number of reads among all samples, which was 32,148.

The taxonomic composition of each sample was determined by visualization of the most abundant taxa at the genus and type (phylum) levels. To this end, non-rarefied data were used to preserve the full variability of taxonomic composition between samples. Mean values, standard deviations, and 95% confidence intervals were calculated for each of the most common taxa in the studied samples.

Statistical analysis

The study was descriptive in nature. Animals were not divided into groups for statistical comparison, and therefore no hypothesis-driven statistical tests were performed. Descriptive statistical methods were applied to characterize the microbial communities. Alpha diversity was assessed using a set of indices (Observed richness, Chao1, Shannon, Simpson, Fisher's alpha, Inverse Simpson, Gini–Simpson, Pielou's evenness, Coverage, and Rarity metrics) calculated with the microbiome package in R. Beta diversity was evaluated based on Bray–Curtis distance metric with principal coordinate analysis (PCoA). To confirm the ordination results, hierarchical clustering was also performed on Bray–Curtis distances using the 'average' linkage method implemented in the base R function *hclus*.

Taxonomic composition was summarized as relative abundances at the phylum and genus levels. For the most common taxa, mean values, standard deviations, and 95% confidence intervals were calculated.

Results

Analysis of rare taxa at the species level showed that the proportion of singletons is less than 0.1%, indicating a low level of random or false detections in rarefied data. At the same time, 20.9% of taxa (n=1821) had a total read count of less than 5, indicating the presence of a significant proportion of rare or low-abundance taxa. It cannot be ruled out that some of them may be artefacts associated with the technical limitations of long-read sequencing on the Oxford Nanopore platform (Table 1).

The assessment of alpha diversity at the species level revealed consistently high taxonomic richness and complexity of the microbiota in all samples studied. The number of taxa observed in individual samples ranged from 5,244 to 5,846, reflecting a partial overlap in taxonomic composition between samples and significant individual specificity of microbial communities.

Chao1 estimator values (7149–7715) indicate a potentially higher level of diversity, which is associated with the presence of low-abundance taxa and the possible underestimation of rare representatives. The Shannon index (6.10–6.73) and Inverse Simpson index (19.6–31.7) values indicate high taxonomic complexity, considering both the richness and evenness of taxon distribution. Fisher's alpha values (over 2000 in most samples) confirm the presence of numerous rare taxa.

The evenness of abundance distribution in the microbial communities of the samples remained moderate. Pielou's evenness values ranged

Table 1. Metrics of alpha diversity of the stomach microbiota in pony samples, calculated based on rarefied 16S rRNA gene sequencing data.

Metric	s13	s14	s15	s16	s17	s18	s19	s20
Observed richness	5796	5790	5556	5244	5747	5846	5846	5726
Chao1 estimator	7341.8	7714.8	7195.1	7148.6	7297	7596.7	7615.6	7337.5
Inverse Simpson index	31.75	19.6	25.84	24.3	30.56	26.51	26.14	29.24
Gini–Simpson index	0.97	0.95	0.96	0.96	0.97	0.96	0.96	0.97
Shannon index	6.73	6.51	6.6	6.1	6.57	6.5	6.49	6.56
Fisher's alpha	2064.2	2061	1937.6	1778.6	2038.1	2091.1	2091.1	2026.9
Coverage (shared taxa)	205	170	171	72	154	144	139	156
Pielou's evenness	0.78	0.75	0.77	0.71	0.76	0.75	0.75	0.76
Relative dominance	0.17	0.22	0.19	0.15	0.17	0.18	0.18	0.17
Gini dominance	0.78	0.8	0.8	0.84	0.79	0.79	0.8	0.79
Rarity (low abundance)	0.67	0.65	0.65	0.56	0.64	0.63	0.63	0.64
Rarity (rare taxa)	0.67	0.65	0.65	0.55	0.62	0.61	0.6	0.62

Values given include estimates of taxonomic richness (Observed richness, Chao1 estimator, Fisher's alpha), diversity indices (Shannon, Inverse Simpson, Gini–Simpson), evenness (Pielou's evenness), dominance (Relative dominance, Gini dominance), rarity (Rarity) and coverage (Coverage of shared taxa).

from 0.71 to 0.78, indicating the presence of dominant taxa without excessive dominance. The Relative dominance (14.6–22.3%) and Gini dominance (0.78–0.84) indices further confirm the moderate unevenness of the community structure.

The coverage coefficient (shared taxa) was 72–205 taxa found in each count, ensuring an adequate level of reliability of the results. The rarity metrics (low abundance) and rarity (rare taxa) indicate that more than 60% of taxa in each sample were classified as rare or low frequency.

Taken together, the alpha diversity indices obtained reflect a high level of biodiversity, a complex taxonomic structure of the microbiota, and pronounced individual variability between animals.

PCoA analysis based on Bray–Curtis distances revealed pronounced interindividual variability in the composition of the pony microbiome. The most closely grouped samples (s17, s18, s19, s20) indicate a similar composition of bacterial communities, possibly due to common housing or feeding conditions. Samples s13 and s14 show the greatest distance from the previous ones, indicating differences in the microbiota profile. Hierarchical clustering based on Bray–Curtis distances revealed the presence of several groups of samples with similar taxonomic composition. The closest cluster was formed by samples s18, s19 and s17, indicating a high similarity in the microbiota profile of these animals. Sample s20 joins this group at the next level of clustering, indicating a partial similarity to the previous three. Other samples (s13, s15, s16, and s14) show greater inter-sample variability. Among them, s13 and s15 have the highest degree of similarity, which is consistent with their proximity on the PCoA graph.

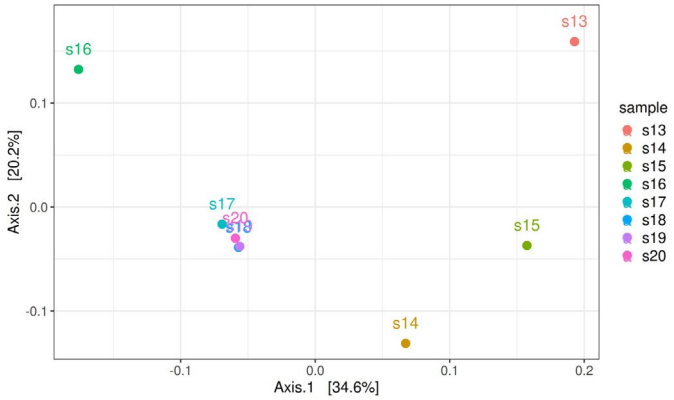

Figure 1. Results of principal coordinate analysis (PCoA) based on the beta diversity matrix (Bray–Curtis distance) for eight samples. Each point corresponds to a separate microbiota sample, and the distance between points reflects the level of taxonomic similarity.

Overall, the dendrogram confirms the presence of two main groups of samples, one of which is characterized by high internal taxonomic consistency.

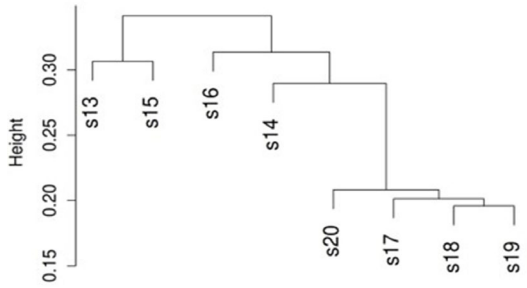

Figure 2. Hierarchical clustering of pony faecal microbiome samples using the Bray–Curtis metric. The y-axis shows the height of the cluster, which reflects the distance between clusters at the time of their aggregation. Lower height values indicate greater similarity between bacterial profiles.

Analysis of the taxonomic composition of the microbiota at the phylum level showed that the dominant share was represented by members of the phyla *Pseudomonadota*, *Bacillota*, *Bacteroidota*, and *Actinomycetota*, which together accounted for over 90% of all classified reads (Table 2, Figure 3). The most represented phylum was *Pseudomonadota* with an average relative abundance of 37.8% (95% CI: 35.3–40.2%), indicating its leading role in the formation of the bacterial community. The second most abundant phylum was *Bacillota* – 25.5% (95% CI: 23.8–27.1%), which together with *Pseudomonadota* forms the basis of the taxonomic structure. A significant proportion was also accounted for by *Bacteroidota* (17.4%, 95% CI: 15.2–19.5%) and *Actinomycetota* (10.7%, 95% CI: 9.8–11.5%). Less numerous but consistently present phyla included *Fibrobacterota*, *Cyanobacteriota*, *Spirochaetota*, *Euryarchaeota*, *Mycoplasmatota*, and *Planctomycetota*, each with an average abundance below 2%.

Table 2. Relative abundance of dominant phyla in the study group of samples (n=8)

Phylum	Mean	SD	CI lower	CI upper
Pseudomonadota	37.77	3.52	35.34	40.2
Bacillota	25.49	2.37	23.84	27.14
Bacteroidota	17.38	3.14	15.2	19.56
Actinomycetota	10.65	1.22	9.81	11.49
Fibrobacterota	1.52	0.97	0.85	2.19
Cyanobacteriota	1.23	0.36	0.98	1.48
Spirochaetota	1.13	0.13	1.05	1.21
Euryarchaeota	0.88	0.1	0.82	0.94
Mycoplasmatota	0.73	0.18	0.61	0.85
Planctomycetota	0.7	0.08	0.64	0.76

Data are presented as mean ± standard deviation (SD) and 95% confidence interval (CI), calculated based on the relative abundance of taxa in percent.

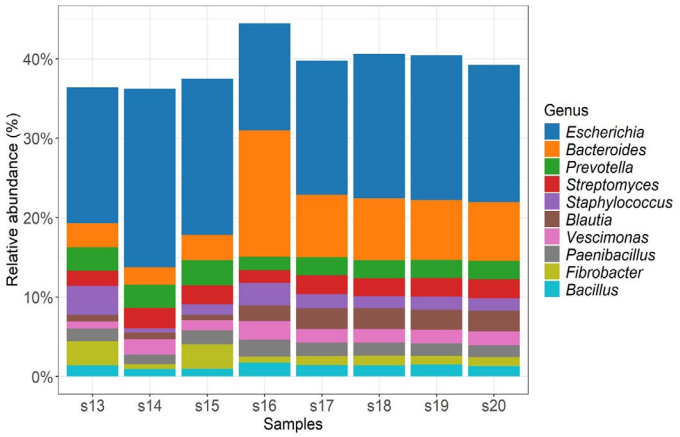

Figure 3. Taxonomic composition of microbiota in pony samples at the genus level.

The most dominant taxa in terms of average relative abundance included representatives of the genera *Escherichia* – 17.92%, *Bacteroides* – 6.87%, *Prevotella* – 2.49% and other members of the intestinal microbiota ranging from 0.89 to 2.23%. These genera play a key role in fibre digestion and maintaining metabolic homeostasis in horses. Interindividual variability in the relative abundance of individual taxa indicates individual differences in microbial composition.

Discussion

Sequencing of 16S rRNA genes provided a valuable snapshot of the bacterial composition of the pony's intestine and its changes depending on age and diet. Analysis of alpha diversity showed that the pony's intestinal microbiome was abundant and diverse, with a similar composition of microorganisms in all 8 study animals. The Chao1 index ranging from 7149 to 7715 indicates the presence of a significant number of rare, small taxa that are not fully recorded in the selected samples. This approach to assessing richness is explained by the theoretical essence of Chao1 — the assessment of undetected taxa based on single and duplicate observations. The Shannon index (6.10–6.73) and Inverse Simpson index at the level (19.6–31.7) emphasise that the communities are not dominated by one or two species, but contain a significant number of representative taxa (without excessive dominance).

High Fisher's alpha values (> 2000) indicate the presence of a large number of rare microorganisms, each of which is present in very low numbers, but together they constitute a significant part of biodiversity (Imdad *et al*., 2024; Cassol *et al*., 2025). The coverage coefficient (shared taxa) with the presence of (72–205) taxa common to all counts indicates a stable microbiome base, which minimizes the risk of randomness in interpretation and confirms the reliability of the results, especially in the context of Chao estimates and richness. The Rarity index, with over 60% of taxa, reflects the ecological complexity of the microbiota. This is typical for natural habitats, where a large number of rare bacteria are observed in alpha diversity (Willis, 2019; Kers and Saccenti, 2022). The total high presence of rare lines (Fisher's alpha, Rarity) indicates a rather complex and stable community, characteristic of natural conditions.

Despite the stability of the main characteristics, significant variations between pony samples in richness and evenness indices reflect the strong influence of individual ecological or genetic factors.

PCoA based on Bray–Curtis clearly demonstrated significant interindividual variability of microbiomes in 8 study ponies. These results are confirmed by many studies on horses, which have shown that animal personality is one of the main factors explaining β diversity ($R^2 \approx 0.24–0.33$) (Weinert-Nelson *et al*. 2022). The clustering of samples s17, s18, s19, and s20 on the PCoA reflects their similar bacterial composition and is an indicator of the structural conservatism of microbiomes characteristic of animals with similar environments (Salem *et al*. 2019). A similar pattern has been described in horses — common conditions (diet, housing) unite microbiomes (Aleman *et al*. 2022).

In ponies, the microbiome is formed by common husbandry in a herd and geographical location (environment), which once again confirms the idea of the influence of individual animals on the composition of each other's microbiomes and, ultimately, the group (Antwis *et al*., 2018).

The results of the analysis of the microbiota of the large intestine of ponies showed that bacterial communities in ponies have a clearly expressed dominant structure, represented mainly by four phyla: *Pseudomonadota*, *Bacillota*, *Bacteroidota* and *Actinomycetota*. These types accounted for over 90% of all reads, which corresponds to the general structure of the microbiota in horses described in scientific papers (Costa *et al*., 2015; Arnold *et al*., 2020).

The increased number of *Pseudomonadota* (37.8% on average) can be considered a potential indicator of dysbiosis, although in herbivores it can be variable within the normal range and includes a variety of Gram-negative bacteria, some of which are opportunistic pathogens (*Escherichia*, *Klebsiella*, *Enterobacter*) (Steelman *et al*., 2012; Warzecha *et al*., 2017). In horses with a high-fibre diet, *Pseudomonadota* may dominate without pathological signs (Costa *et al*., 2015).

The phylum *Bacillota* (25.5%) includes the following key genera: Clostridium, *Ruminococcus*, *Lactobacillus*, which are involved in the breakdown of fibre, fermentation of sugars and production of light fatty acids. A decrease in the proportion of *Bacillota* has been observed in animals with intestinal disorders, making them an indicator of microbiota stability and depending on housing conditions, feeding, physical activity, and the use of medications (Whitfield-Cargile *et al*. 2018; Kauter *et al*. 2019; Zakia *et al*. 2023).

The *Bacteroidota* type (17.4%) is also widely represented in the microbiomes of herbivores. Its representatives are responsible for the digestion of complex carbohydrates and the synthesis of vitamins. The balance between *Bacillota* and *Bacteroidota* is often considered an indicator of the metabolic status of the intestine. In horses, the stable presence of *Bacteroidota* is observed under normal metabolic conditions (Ericsson *et al*. 2016; Costa and Weese, 2018). The average proportion of *Actinomycetota* (10.7%) is characteristic of healthy horses. This phylum includes the genera *Bifidobacterium* and *Corynebacterium*, which have prebiotic or protective effects, modulate immunity, and participate in maintaining the intestinal barrier (Boucher *et al*., 2024).

The taxa *Fibrobacterota*, *Cyanobacteriota*, *Spirochaetota*, *Euryarchaeota*, *Mycoplasmatota*, and *Planctomycetota* were less abundant in the overall microbiome of the pony intestine, as they are necessary for maintaining intestinal homeostasis, fermenting fibre, and producing short-chain fatty acids, which have anti-inflammatory and immunomodulatory properties (Daly *et al*., 2012).

The microbiome showed a predominance of representatives of the genera *Escherichia* (17.92%) and *Bacteroides* (6.87%). However, the excessive presence of *Escherichia* is considered a marker of disturbed homeostasis or a transitional microbiotic state (Pickard *et al*., 2017).

The genus *Prevotella*, important for the complex breakdown of polysaccharides, accounted for 2.49%, which probably reflects altered food substrates and destabilisation of the microbial ecosystem (Liu *et al*., 2021). Eleven other genera, which are important symbionts that promote the utilisation of cellulose and hemicellulose and participate in the production of butyric acid (butyrate), which is critical for the energy supply of colonocytes and the preservation of the integrity of the intestinal mucosal barrier in ponies (Antharam *et al*., 2013).

Summarizing the results obtained, it should be noted that understanding the composition of the pony intestinal microbiome can aid in diagnosis and treatment, including diet development, prebiotic/probiotic interventions, and treatment strategies to alleviate metabolic disorders. Validated microbial biomarkers identified by 16S sequencing may form the basis for future microbiome-based health monitoring.

Conclusion

Based on the results of the studies, the profile of the intestinal microbiota of clinically healthy ponies was presented, obtained on the basis of amplicon sequencing of the 16S rRNA gene using the Oxford Nanopore platform. The results indicate high taxonomic richness and structural complexity of bacterial communities: a total of 8,704 unique taxa were identified at the species level; the number of species observed in the samples ranged from 5,244 to 5,846.

Alpha diversity metrics (Chao1, Shannon, Simpson's inverse index, Fisher's index) indicate significant richness and evenness, typical of the microbiota of healthy animals. Beta diversity analysis (PCoA and hierarchical clustering) did not reveal clear clustering of samples, indicating the individual specificity of microbial communities.

In the taxonomic structure of the microbiota at the phylum level, the dominant representatives are *Pseudomonadota* (38%), *Bacillota* (25%), *Bacteroidota* (17%) and *Actinomycetota* (11%) — this ratio is consistent with the microbiota of other ungulates. About 21% of taxa has extremely low abundance (<5 reads), confirming the presence of a significant proportion of rare or transient taxa. Prospects for further research. The results of the studies complement existing knowledge about pony microbiota and create a basis for further comparative or functional studies in veterinary microbiomics.

Acknowledgements

The authors express their gratitude to the horse farm (Kharkiv region, village of Lisne) for the opportunity to conduct experiments on ponies.

Conflict of interest

The authors have no conflict of interest to declare.

References

Antharam, V.C., Li E.C., Ishmael, A., Sharma, A., Mai, V., Rand, K.H., Wang, G.P., 2013. Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea. J. Clin. Microbiol. 51, 2884–92. doi:10.1128/JCM.00845-13.

Adolph, S., Schedlbauer, C., Blaue, D., Schöniger, A., Gittel, C., Brehm, W., Fuhrmann, H., Vervuert, I., 2019. Lipid classes in adipose tissues and liver differ between Shetland ponies and Warmblood horses. PLoS One 14:e0207568.doi: 10.1371/journal.pone.0207568.

Aleman, M., Sheldon, S., Jospin, G., Coil, D., Stratton-Phelps, M., Eisen, J., 2022. Caecal microbiota in horses with trigeminal-mediated headshaking. Vet Med Sci. 8, 1049-1055. doi: 10.1002/vms3.735.

Arnold, C.E., Isaiah, A., Pilla, R., Lidbury, J., Coverdale, J.S., Callaway, T.R., Lawhon, S.D., Steiner, J., Suchodolski, J.S., 2020. The caecal and fecal microbiomes and metabolomes of horses before and after metronidazole administration. PLoS One. 15, e0232905. doi: 10.1371/journal.pone.0232905.

Antwis, R.E., Lea, J.M.D., Unwin, B., Shultz, S., 2018. Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. Microbiome 6, 207. doi: 10.1186/s40168-018-0593-2.

Blackmore, T.M, Dugdale, A., Argo, C.M., Curtis, G., Pinloche, E., Harris, P.A., 2013. Strong Stability and Host Specific Bacterial Community in Faeces of Ponies. Plos ONE 8, e75079. doi:10.1371/journal.pone.0075079.

Boucher, L., Leduc, L., Leclère, M., Costa, M.C., 2024. Current Understanding of Equine Gut Dysbiosis and Microbiota Manipulation Techniques: Comparison with Current Knowledge in Other Species. Animals (Basel) 14, 758. doi: 10.3390/ani14050758.

Cassol, I., Ibañez, M., Bustamante, J.P., 2025. Key features and guidelines for the application of microbial alpha diversity metrics. Scientific Reports 15, 622. doi:10.1038/s41598-024-77864-y.

Costa, M.C., Silva, G., Ramos, R.V., Staempfli, H.R., Arroyo, L.G., Kim, P., Weese, J.S., 2015. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet J. 205, 74-80. doi:10.1016/j.tvjl.2015.03.018.

Costa, M.C., Weese, J.S., 2018. Understanding the intestinal microbiome in health and disease. Vet. Clin. North Am. Equine Pract. 34, 1–12. doi: 10.1016/j.cveq.2017.11.005.

Daly, K., Proudman, C.J., Duncan, S.H., Flint, H.J., Dyer, J., Shirazi-Beechey, S.P., 2012. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. Br. J. Nutr. 107, 989–995. doi: 10.1017/S0007114511003825.

Dougal, K., Harris, P.A., Edwards, A., Pachebat, J.A., Blackmore, T.M. *et al.*, 2012. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiol Ecol. 82, 642-52. doi:10.1111/j.1574-6941.2012.01441.x.

Ericsson, A.C., Johnson, P.J., Lopes, M.A., Perry, S.C., Lanter, H.R., 2016. A Microbiological Map of the Healthy Equine Gastrointestinal Tract. PLoS One 11, e0166523. doi: 10.1371/journal.pone.0166523.

Fernandes, K.A., Kittelmann, S., Rogers, C.W., Gee, E.K., Bolwell, C.F., Bermingham, E.N., Thomas, D.G., 2014. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PLoS One 9, e112846. doi:10.1371/journal.pone.0112846.

Imdad, S., So, B., Jang, J., Park, J., Lee, S.J., Kim, J.H., Kang, C., 2024. Temporal variations in the gut microbial diversity in response to high-fat diet and exercise. Sci Rep. 14, 3282. doi: 10.1038/s41598-024-52852-4.

Jovel, J., Patterson, J., Wang, W., Hotte, N., O'keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A., Madsen, K., Wong, G., 2016. Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. Frontiers in Microbiology. 7, 459. doi: 10.3389/fmicb.2016.00459.

Kauter, A., Epping, L., Semmler, T., Antao, E., Kannapin, D., Stoeckle, S.D., Gehlen, H., Lübke-Becker, A., Günther, S., Wieler, L.H., Walther, B., 2019. The gut microbiome of horses: current research on equine enteral microbiota and future perspectives. Anim. Microbiome. 1, 14. doi: 10.1186/s42523-019-0013-3.

Kers, J.G., Saccenti, E., 2022. The Power of Microbiome Studies: Some Considerations on Which Alpha and Beta Metrics to Use and How to Report Results. Front. Microbiol. 12, 796025. doi: 10.3389/fmicb.2021.796025.

Lahti, L., Shetty, S., 2017. Tools for microbiome analysis in R. Microbiome package version 1.1.10013.

Langner, K., Blaue, D., Schedlbauer, C., Starzonek, J., Julliand, V., Vervuert, I., 2020. Changes in the faecal microbiota of horses and ponies during a two-year body weight gain programme. PLoS One 15, e0230015. doi: 10.1371/journal.pone.0230015.

Liu, B., Liu, X., Liang, Z., Wang, J., 2021. Gut microbiota in obesity. World Journal of Gastroenterology. 27, 3837-3850. doi:10.3748/wjg.v27.i25.3837.

Luthersson, N., Mannfalk, Parkin M., Tim, D.H., Harris, P., 2017. Laminitis: Risk Factors and Outcome in a Group of Danish Horses. Journal of Equine Veterinary Science 53, 68–73. doi: 10.1016/j.jevs.2016.03.006.

Morrison, P.K., Newbold, C.J., Jones, E., Worgan. H.J., Grove-White, D.H., Dugdale, A.H., *et al.*, 2018. The Equine Gastrointestinal Microbiome: Impacts of Age and Obesity. Front Microbiol. 9, 3017. doi: 10.3389/fmicb.2018.03017.

McMurdie, P.J., Holmes S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." Edited by Michael Watson. PLoS One. 8, e61217. doi:10.1371/journal.pone.0061217.

Pickard, J.M., Zeng, M.Y., Caruso, R., Núñez, G., 2017. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease Immunological Reviews. 279, 70-89. doi:10.1111/imr.12567.

R Core Team. 2024. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Salem, S.E.,Hough, R., Probert, C., Maddox, T.W., Antczak P., 2019. A longitudinal study of the faecal microbiome and metabolome of periparturient mares PeerJ. 7, e6687. doi: 10.7717/peerj.6687.

Steelman, S.M., Chowdhary, B.P., Dowd, S., Suchodolski, J., Janečka, J.E., 2012. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Vet Res. 8, 231. doi: 10.1186/1746-6148-8-231.

Stewart, H.L., Pitta, D., Indugu, N., Vecchiarelli, B., Engiles, J.B., Southwood, L.L., 2018. Characterization of the fecal microbiota of healthy horses. Am J Vet Res. 79, 811-819. doi: 10.2460/ajvr.79.8.811.

Videvall, E., Song, S.J., Bensch, H.M., Strandh, M., Engelbrecht, A., Serfontein, N., Cornwallis, C.K., 2019. Major shifts in gut microbiota during development and its relationship to growth in ostriches. Molecular Ecology, 28(10), 2653-2667. doi.org/10.1111/mec.15087.

Warzecha, C.M., Coverdale, J.A., Janecka, J.E., Leatherwood, J.L., Pinchak, W.E., Wickersham, T.A., Mccann, J.C. 2017. Influence of short-term dietary starch inclusion on the equine caecal microbiome. J Anim Sci. 95, 5077-5090. doi:10.2527/jas2017.1754.

Willis, A.D., 2019. Rarefaction, alpha diversity, and statistics. Front. Microbiol. 10, 2407. doi: 10.3389/fmicb.2019.02407.

Willing, B., Vörös, A., Roos, S., Jones, C., Jansson, A., Lindberg, J.E., 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training.Equine veterinary journal, 41(9), 908-914.

Wylie, C.E., Collins, S.N., Verheyen, K.L., Newton, J.R., 2013. Risk factors for equine laminitis: a case-control study of horses and ponies registered with veterinary practices in the United Kingdom between 2009 and 2011. Vet J. 198, 57–69. doi:10.1016/j.tvjl.2013.08.028.

Whitfield-Cargile, C.M., Chamoun-Emanuelli, A.M., Cohen, N.D., Richardson, L.M., Ajami, N.J., Dockery, H.J., 2018. Differential effects of selective and non-selective cyclooxygenase inhibitors on fecal microbiota in adult horses. PLoS One 13, e0202527. doi:10.1371/journal.pone.0202527.

Weinert-Nelson, J.R., Biddle, A.S., Williams, C.A., 2022. Fecal microbiome of horses transitioning between warm-season and coolseason grass pasture within integrated rotational grazing systems. Anim. Microbiome 4, 41. doi: 10.1186/s42523-022-00192-x.

Zakia, L.S.; Gomez, D.E.; Caddey, B.B.; Boerlin, P.; Surette, M.G.; Arroyo, L.G., 2023. Direct and culture-enriched 16S rRNA sequencing of cecal content of healthy horses and horses with typhlocolitis. PLoS One 18, e0284193. doi: 10.1371/journal.pone.0284193.