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# 16S RRNA NGS SEQUENCING OF FECAL BACTERIAL MICROBIOTA IN SOME OBESE AND HEALTHY HORSES OF UKRAINIAN ORIGIN

*** S. B. Borovkov [1], O. V. Kolchyk [1], O. A. Tarasov [2], M. V. Bezymennyi [2]**

*[1] National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine", 83, Pushkinska Str., Kharkiv, Ukraine, 61023*

*[2] Institute of Veterinary Medicine the National Academy of Agrarian Sciences of Ukraine, 30, Donetska Str. Kyiv, Ukraine, 03154*

*E-mail: * Serg_b78@ukr.net, kolchyk-elena@ukr.net, ast97@ukr.net, nomax@ukr.net*

*ORCID: https://orcid.org/0000–0003–3021–2410; https://orcid.org/0000-0003-0497-2512, https://orcid.org/0000-0003-1481-5529, https://orcid.org/0000-0001-8042-0061*

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**Aim.** The aim of this study is to characterize and possibly differentiate the lower gut (fecal) bacteriota of healthy and obese horses using Next Generation Sequencing (NGS) of the 16S rRNA gene. **Methods.** The study involved 7 horses (4 stallions and 3 mares) of different breeds, aged 8–17 years: horses 1–4 of Ukrainian Saddle breed (horse 1 sports horse stallion Rebus, 10 y.o., horse 2 stallion Santes, 15 y.o., horse 3 stallion Sens, 14 y.o., horse 4, mare Siren, 17 y.o.), horse 5 of Heavy Draft breed (stallion Tsyhan, 8 y.o.), and non-thoroughbred horses 6 and 7 (mare Snezhynka, 10 y.o., mare Rumba 12 y.o.) Horses 2, 4, 5 and 7 were obese and horses 1, 3 and 6 were healthy. All horses were kept at the equestrian centre of the State Biotechnological University the Ministry of Education and Science of Ukraine (Kharkiv, Ukraine). Total DNA from rectal fecal samples were extracted using the PureLink Microbiome DNA purification kit (Invitrogen, USA), according to the manufacturer's instructions. To prepare libraries of the 16s rRNA of the bacteriota, we used the 16S rRNA barcoding kit 1–24 (Oxford Nanopore, USA). To purify the libraries obtained, magnetic particles NucleoMag NGS Clean-up and Size Select (Macherey-Nagel, Germany were used according to the recommended protocol of the rapid sequencing amplicons – 16S barcoding (SQK-16S024) (The manual to the sequencing kit). These conditions are based on standard protocols for 16S rRNA gene amplification, as described in Fujiyoshi et al (2020), and ensure robust amplification of bacterial DNA across a wide range of taxa. **Results.** Representatives of the bacterial phyla *Actinomycetota* (syn. *Actinobacteriota*), *Fibrobacterota, Lentisphaerota, Spirochaetota* (syn. *Spirochaetes*), *Bacteroidota*, *Firmicutes* (syn. *Bacillota*), *Planctomycetota*, *Verrucomicrobiota* (syn. *Verrucomicrobia*), *Candidatus* Melainabacteria*, Kiritimatiellota* and *Proteobacteria* (syn. *Pseudomonadota*) were detected. The dominating phylum was found to be *Firmicutes*, whose share was from 50 to 82 % of all the phyla detected. The number of *Firmicutes*, when compared to those of *Bacteroidota* varied considerably between healthy and obese horses. In the healthy horses 1,3 and 6 this was 2.5, 3.4 and 2.9 times higher for the *Firmicutes* and for the obese horses 2,4,5 and 7 it was 8.6, 8.2, 7.6 and 5.7 times higher. The number of *Firmicutes* compared to *Bacteroidota* varied significantly between healthy and obese horses. In healthy horses 1, 3, and 6, the number of *Firmicutes* was 2.5, 3.4, and 2.9 times higher, respectively, whereas in obese horses 2, 4, 5, and 7, the number of *Firmicutes* was 8.6, 8.2, 7.6, and 5.7 times higher, respectively. Increased numbers of *Proteobacteria* genera were observed in obese horses 2, 4, 5, and 7, ranging from 25 to 37 %, while in the healthy sport horses 1, 3 and 6 the level of *Proteobacteria* was between 1.07 and 3.43 %, which is typical for the microbiome of healthy animals. A low level of *Actinomycetota* (*Actinobacteriota*) was detected in the feces of the horses under study: 0.09 % in healthy sport horse 1, 0.09 % in healthy sport horse 3, and 0.15 % in healthy horse 6, respectively. In contrast, the level of this bacterial phylum varied in obese horses 2, 4, 5, and 7, ranging from 0.21 % to 0.48 %, respectively. It is important to note that the *Actinomycetota* phylum also includes the genus *Bifidobacterium*, which was not detected in any of the animals studied. **Conclusions.** For the first time in Ukraine, we sequenced the bacterial microbiota of the lower intestinal tract (fecal material) of seven horses of different ages, sexes, and breeds. In the feces of obese horses, there was a predominance of bacteria from the order *Eubacteriales* (phylum *Firmicutes,* class *Clostridia*), particularly from the families *Oscillospiraceae* and *Lachnospiraceae*, accompanied by a reduction in bacteria from the phylum *Bacteroidota* (FCB group clade) com-

pared to healthy horses. These alterations may be related to fat accumulation in the animals, possibly due to increased energy synthesis from feed. Cluster analysis revealed a high degree of similarity in bacteriota composition among the samples. Further studies, including larger sample sizes and exploration of physiological characteristics, are needed to obtain more comprehensive information.

***Key words*:** 16S rRNA sequencing, microorganisms, metabolism, digestion, gut, colon.

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## INTRODUCTION

Horses are monogastric herbivores, which have a complex process of digestion of plant foods, mainly starch, and cellulose, different from ruminants, thanks to microbial populations (bacteria, archaea, fungi and viruses), also called microbiome, in the ileum, colon and cecum (Julliand et al, 2016, Ericsson et al, 2016) Two bacterial phyla, viz. *Firmicutes* (syn. *Bacillota*) and *Proteobacteria* (syn. *Pseudomonadota*), are prevalent in the horse intestines since their main task is the fermentation of sugars, protein breakdown, and cellulose hydrolysis (Dougal et al, 2017). Different sections of intestines have a specific microflora for feed digestion according to the degree of grinding, acidity of the feed masses, oxygenation of the intestinal lumen, and other factors (Zhu et al, 2021). For instance, it was determined that in the small intestine, there was a prevalence of *Firmicutes* and *Bacteroidota,* and in the large intestine, against the background of the decrease in *Bacteroidota,* a greater diversity of genera and species belonging to the phyla *Verrucomicrobiota* (syn. *Verrucomicrobia*), *Spirochaetota* (syn. *Spirochaetes*), *Fibrobacterota* and *Actinomycetota* (syn. *Actinobacteria)* (Dougal et al, 2012; Costa et al, 2015; Stewart et al, 2018). Despite the diversity of bacterial (including archaea) species it is possible to identify typical core bacteriota found in all parts of the gut, predominantly members of the *Bacteroidota* (53 to 58 %) and *Firmicutes* (31 to 35 %), together accounting for 90 % of the total bacterial population. Other species of this core include *Spirochaetota* (syn. *Spirochaetes*) (2 to 4 %), *Fibrobacterota* (syn. *Fibrobacter*) (1.5 to 4.2 %), *Proteobacteria* (syn. *Pseudomonadota*) (0.8 to 2.2 %), and *Cyanobacteriota* (syn. *Cyanobacteria*) (0.5 to 1.5 %) (Stewart et al, 2018). However, there proved to be considerable fluctuations in their ratios, among each other and among other phyla, depending on many factors, including the type of feeds, physiological state, age, disease and season (Morrison et al, 2018).

A non-invasive method of investigating the gut microbiome of horses is the one using feces, either taken from rectum or from the ground directly after deposition.

Recently the bacterial genus *Akkermansia*, which occurs widely in humans and many animals, including horses, was studied in more detail. This bacterium is of great interest to humans because it plays an apparent role in anti-inflammatory processes. Its numbers present in the microbiome are negatively correlated with the incidence of obesity, diabetes, or metabolic disorders (Everard et al, 2013; Xia et al, 2022).

Several studies of the horse microbiome have also identified the presence of *Phascolarctobacterium* spp. which use succinate to produce acetate and propionate (Su et al, 2020; Binnebose et al, 2023. This bacterial genus has been reported to be positively correlated with maintaining normal weight in children (Rampelli et al, 2018), as well as with reduced liver triglyceride levels after a high-fat diet in a rat model of non-alcoholic fatty liver disease (Panasevich et al, 2016). In horses, little is known about the exact role of this bacterium, but its relative abundance is reduced in the intestinal microbiota due to oligofructose-induced laminitis compared to that in healthy horses, accompanied by a lower relative abundance of the genera *Akkermansia* (and RFP12) and *Fibrobacter* (Tuniyazi et al, 2021; Clark et al, 2018). Furthermore, Edwards et al (2020) indicated that all of these OTUs are a part of the core hindgut microbiota of horses. The aim of the work was to determine and interpret qualitative and quantitative differences in the fecal bacterial microbiota of several obese and healthy horses using NGS sequencing of the 16S rRNA gene.

## MATERIALS AND METHODS

The clinical examination of the animals and sampling were conducted in 2023 in the equestrian complex of the State Biotechnological University, the village of Mala Danylivka, Kharkiv district, Kharkiv region, Ukraine. Feces samples were taken from 7 horses (4 stallions and 3 mares) of different breeds, aged 8–17 years: horses 1–4 of Ukrainian Saddle breed (horse 1 sports horse stallion Rebus, 10 y.o., horse 2 stallion Santes, 15 y.o., horse 3 stallion Sens, 14 y.o., horse 4, mare Siren, 17 y.o.), horse 5 of Heavy Draft breed (stallion Tsyhan, 8 y.o.), and non-thoroughbred horses 6 and 7 (mare Snezhynka, 10 y.o., mare Rumba 12 y.o.)

Horses 2, 4, 5 and 7 were obese and horses 1, 3 and 6 were healthy.

Fecal samples of 50 g were manually collected from the rectum using sterile gloves for each individual animal and placed into specialized medical plastic containers designed for human stool samples. After placing the stool in the containers, the samples were immediately snap-frozen in liquid nitrogen using a Dewar vessel for further transportation in thermal boxes filled with dry ice. In the laboratory, the samples were thawed at room temperature and subjected to further analysis according to the protocol.

The diets of clinically healthy animals (horses 1, 3, and 6) and obese horses (horses 2, 4, 5, and 7) did not differ in composition and included, in terms of dry matter: hay (7.65 kg), oats (2.64 kg), and salt (29 g). The feed was completely consumed by the animals. Feeding was carried out twice a day, and fecal samples were collected 3 hours after the second feeding. Access to water was ad libitum.

The total DNA from fecal samples (7 samples from 7 horses consisting of three breeds and two groups: healthy and obese) was extracted using the PureLink™ Microbiome DNA Purification Kit (Invitrogen™, USA), cat. No. A29789, following the manufacturer's instructions. This kit is specifically designed to ensure high-quality DNA isolation from complex microbiome samples such as feces, minimizing inhibitors that may affect downstream applications.

To prepare libraries for sequencing, we used the 16S Barcoding Kit 1-24 (cat. No. SQK16S024, Oxford Nanopore, USA) to amplify the (nearly) entire 16S rRNA gene region, using specific primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplification and barcoding of the samples were performed simultaneously, following the protocol provided by Oxford Nanopore Technologies.

PCR amplification was carried out in a total reaction volume of 25 μL, consisting of the following components:12.5 μL of 2X Platinum™ SuperFi II PCR Master Mix (Invitrogen™, USA),1 μL of forward primer (27F),1 μL of reverse primer (1492R),1 μL of template DNA (~10 ng), and nuclease-free water to adjust the final volume. The PCR cycling conditions were as follows: Initial denaturation: 98 °C for 30 seconds, 35 cycles of: denaturation at 98 °C for 10 seconds, annealing at 55 °C for 20 seconds, extension at 72 °C for 60 seconds. Final extension: 72 °C for 5 minutes. These conditions are based on standard protocols for 16S rRNA gene amplification, as described in Fujiyoshi et al (2020), and ensure robust amplification of bacterial DNA across a wide range of taxa.

To purify the libraries after PCR amplification, we used NucleoMag™ NGS Clean-up and Size Select magnetic particles (ref 744970.50, Macherey-Nagel™, Germany), in accordance with the manual of Oxford Nanopore Technologies (SQK-16S024). This step was crucial for removing PCR contaminants and short fragments, ensuring high-quality libraries for sequencing.

To purify the libraries, we used the magnetic particles NucleoMag™ NGS Clean-up and Size Select (ref 744970.50, Macherey-Nagel™, Germany) according to the manual of Oxford Nanopore Technologies (SQK-16S024).

NGS of the 16s rRNA was performed using the handheld ONT MinION Mk1B device (Oxford Nanopore, USA), Flow Cell Priming Kit (EXP-FLP002, Oxford Nanopore, USA) and Spot-on Flow Cell R9.4.1 (FLO-MIN106D, Oxford Nanopore, USA) according to the manufacturer's protocols. DNA concentration was determined using a fluorimeter (Qubit v.3, Thermo Fisher Scientific, USA) and the Qubit 1xdsDNA HS assay kit (Invitrogen, USA); DNA purity was measured using a spectrophotometer (Qubit v3.0, Thermo Fisher Scientific, USA) by the ratio of adsorption at wavelengths of 260/280 (at least 1.8) and 260/230 (at least 1.7). Libraries were normalized to equimolar concentrations and thereafter 70 fmol in 10 μl 10 mM Tris HCl pH 8.0 with 50 mM NaCl was used for sequencing. For 16S amplicons of ~1500 bp, 50–100 fmol corresponds to ~50–100 ng of DNA.

For basecalling we used Guppy 6.1.7 with GPU support, a super-accuracy model (dna_r9.4.1_450bps_sup.cfg) and all other default parameters.

The taxonomic classification based on the 16S rRNA gene was performed in the 16S workflow of EPI2ME Desktop 24.02-01 platform (Oxford Nanopore Technologies). For classification purposes, minimap2 mode was chosen against the ncbi_16s default reference database (Li, 2018).

In this study, we assessed alpha diversity using Simpson's index, Chao1 index and the Shannon diversity index, each capturing different aspects of microbial diversity in the samples. These diversity metrics were carefully selected to provide a comprehensive understanding of microbial richness, evenness, and dominance.

Beta diversity calculation was performed based on the Bray-Curtis distance metric prepared with vegan

2.6–8 library in R (Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, Weedon J (2024). _vegan: Community Ecology Package_. R package version 2.6-8, <https://CRAN.R-project.org/package=vegan>.). Principal coordinate analysis (PCoA) plots were built to visually interpret the results. Analysis of similarities (ANOSIM) was used to test the statistical significance of differences between sample groups.

## RESULTS

Thirty-four prevailing operative taxonomic units (OTU) were found in the gut microbiome of seven horses. The study determined a diverse range of microorganisms, including the phyla *Acidobacteriota, Actinomycetota* (syn. *Actinobacteriota*) (Oren and Garrity, 2021)*, Armatimonadota, Bacteroidota, Calditrichaeota, Candidatus Melainabacteria, Chlamydiota, Chloroflexota, Chrysiogenota, Cyanobacteriota* (syn. *Cyanobacteria*), *Deferribacterota, Deinococcota, Elusimicrobiota, Euryarchaeota, Fibrobacterota, Firmicutes* (syn. *Bacillota*), *Fusobacteriota, Gemmatimonadota, Ignavibacteriota, Kiritimatiellota, Lentisphaerota, Nitrospinae, Nitrospirota, Planctomycetota, Proteobacteria, Rhodothermota, Spirochaetota* (syn. *Spirochaetes*)*, Synergistota, Mycoplasmatota* (syn. *Tenericutes*), *Thermodesulfobacterota, Thermotogota, Verrucomicrobiota* (syn. *Verrucomicrobia*), and others, including unknown taxa belonging to the following phyla: *Actinomycetota, Fibrobacterota, Lentisphaerota, Spirochaetota, Bacteroidota, Firmicutes, Planctomycetota, Verrucomicrobiota*, *Candidatus* Melainabacteria (Oren, 2021)*, Kiritimatiellota* and *Proteobacteria* (syn. *Pseudomonadota*) were detected. *Firmicutes* were dominating, (50 to 82 % of all phyla, and *Bacteroidota* and *Proteobacteria* on second and third position, respectively, other phyla were minor (**Fig. 1**).

In the feces of obese horses (2, 4, 5, and 7), a significant increase in the abundance of *Firmicutes* was observed at the expense of *Bacteroidota* and/or *Proteobacteria*, namely by 8.6, 8.2, 7.6, and 5.7 times, respectively. In healthy horses (1, 3, and 6), the abundance of these bacteria was 2.5, 3.4, and 2.9 times higher, respectively.

An increase in the abundance of *Proteobacteria* was found in obese horses (4, 5, and 7), up to 19.64, 22.52, and 21.42 %, respectively, which may be associated with intestinal inflammation, endotoxin production, insufficient sugar fermentation, and protein breakdown. In healthy horses (1, 3, and 6), the percentage of *Proteobacteria* ranged from 1.07 to 3.43 %, which is characteristic of the microbiome of healthy animals.

A low level of *Actinomycetota* was detected in the feces of the horses studied, ranging from 0.09 to 0.15 % in healthy horses (1, 3 and 6) and from 0.21 to 0.48 % in obese horses (2, 4, 5 and 7). The phylum *Actinomycetota* includes the genus *Bifidobacterium*, which was not detected in any of the animals studied. Additionally, some lactic acid-producing bacteria (such as *Bifidobacterium lactis* or other lactic acid bacteria) were also absent, this could increase the permeability of the intestinal epithelial barrier to macromolecules from feed and could lead to a deficiency in secretory immunity. This, in turn, may cause the development of intestinal diseases (Artis, 2008; Patel et al, 2015). It is known that approximately 80 % of immunocompetent cells are located in the intestinal mucosa, and 25 % of tissues are immunologically active. The microflora contributes to both local immunity (activation of IgA production, phagocytic activity) and systemic immunity by providing continuous antigenic stimulation (Fagarasan et al, 2010; Bunker et al, 2015).

A low abundance of the *Fibrobacterota* was observed in obese horses (2, 4, 5, and 7), ranging from 0.13 to 0.2 %. In healthy horses (1, 3, and 6), the levels were higher, namely 0.72, 0.58, and 0.63 %, respectively. Very low levels of *Lentisphaerota* (0.01 %) and *Spirochaetota* (0.07 and 0.13 %) were detected in obese horses, particularly in horses 4 and 5. However, in contrast, higher levels of *Spirochaetota* were observed in healthy horses (1 and 3) and in obese horse 2, namely 0.72, 0.68, and 0.70 %, respectively. This is likely associated with the low levels of *Actinomycetota* in these animals (0.09, 0.13, and 0.48 %), which did not inhibit their growth.

In obese horses (4, 5, and 7), the *Verrucomicrobiota* phylum was barely represented, 0.004, 0.05, and 0.07 %, respectively. In healthy horses (1, 3, and 6), the levels were higher, namely 0.9, 0.23, and 0.2 %, respectively, and unexpectedly high in obese horse 2 (0.46 %).

The phylum *Planctomycetota* was completely absent in healthy horse 1; in the remaining horses, it was detected only at low levels (ranging from 0.02 to 0.05 %). These bacteria are considered transitional microbiota, typically found in water and soil, entering animals through the digestive tract (Godinho et al, 2024). The

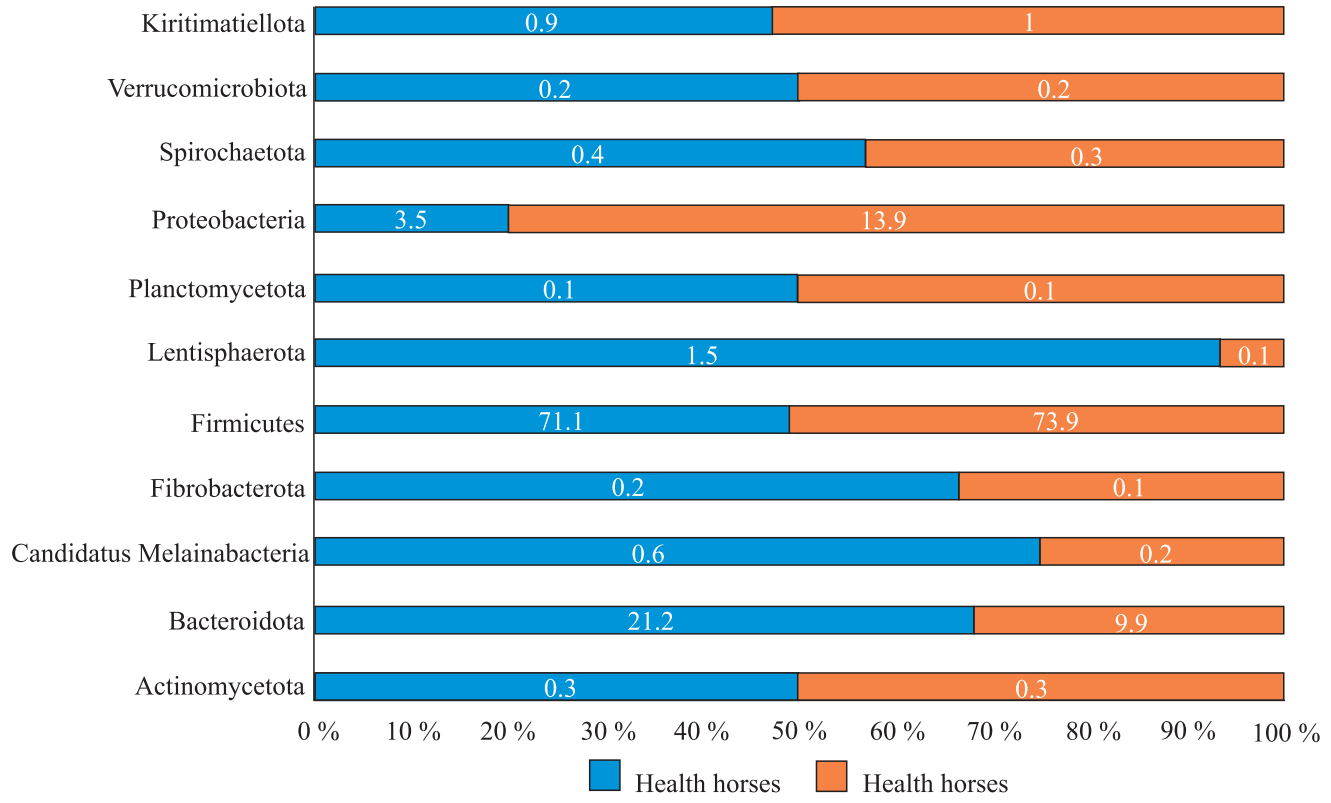


**Fig. 1**. Mean percentage of the different phyla of bacteriota present in the feces studied

"*Candidatus* Melainabacteria" phylum was present in the feces of obese horses at levels twice as low (from 0.22 to 0.31 %) compared to healthy horses (from 0.45 to 0.59 %).

Determination of the alpha biodiversity parameters (Shannon, Simpson and Chao1) showed that there were no significant differences in the composition of all seven horses studied (**Table 1**).

Bacterial families important for the breakdown of fibrous material in the intestines of healthy and obese horses were represented as follows: the family *Eubacteriaceae* (ranging from 11.29 to 12.48 %) and the family *Clostridiaceae* (ranging from 11.38 to 15.56 %). *Lachnospiraceae* were present at low levels of 10.81 and 13.71 % in healthy horses, but at higher levels in obese horses, particularly with *Eubacteriaceae* ranging from 45.7 to 48.9 %, *Clostridiaceae* from 68.5 to 75.6 %, and *Lachnospiraceae* from 25.1 to 40.9 %, respectively. Starch-degrading bacteria, such as those belonging to the *Ruminococcaceae* and *Flavobacteriaceae*, were not detected, while representatives of the *Prevotellaceae* were present only in healthy horses at 1.35, 1.51 and 1.73 %, respectively. An increase in starch levels, in the absence of bacteria capable of breaking it down, leads to the suppression of fibre-degrading bacteria, which in turn results in lactate accumulation and a decrease in pH. This can cause intestinal acidosis

**Table 1.** Diversity indices for fecal bacteriota, based on 16s rRNA sequencing for 4 obese and 3 healthy horses

| Horse | Shannon index (H) | Simpson index (D) | Chao1 index |
|---|---|---|---|
| 1 healthy | 4.29 | 1.05 | 2444.0 |
| 2 obese | 5.0 | 1.02 | 2745.0 |
| 3 healthy | 5.37 | 1.02 | 3505.0 |
| 4 obese | 4.45 | 1.04 | 2444.0 |
| 5 obese | 4.88 | 1.02 | 2675.0 |
| 6 healthy | 4.67 | 1.02 | 2567.0 |
| 7 obese | 4.65 | 1.03 | 2872.0 |

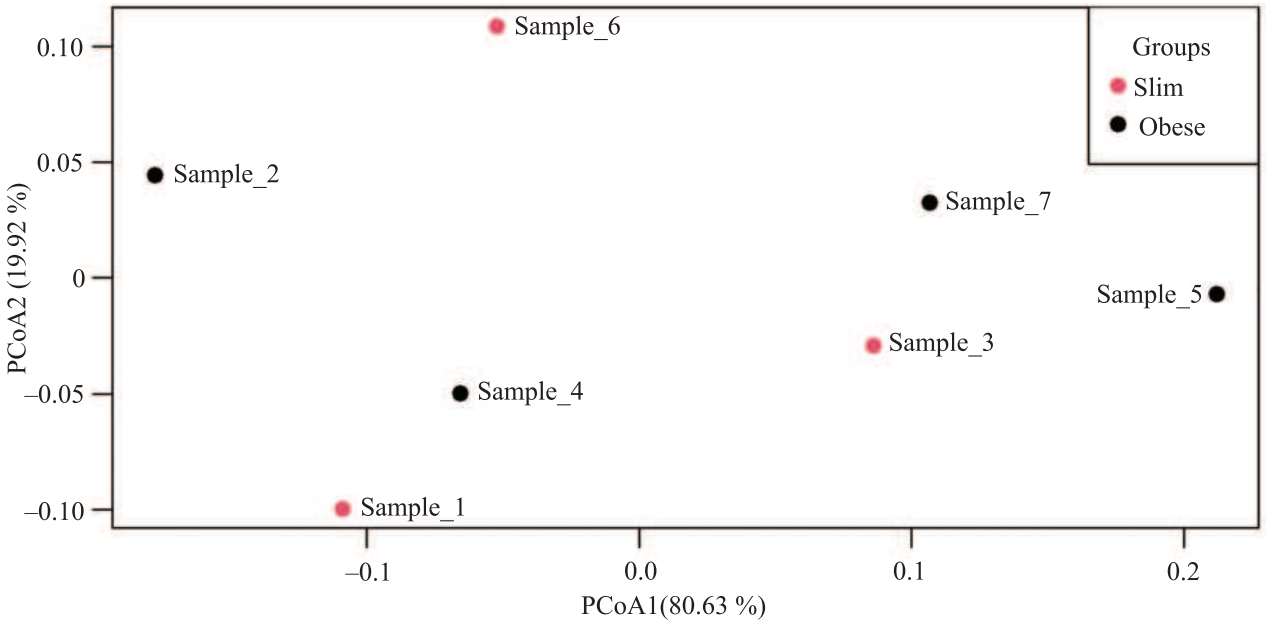


**Fig.2.** Results of cluster analysis (PCoA) based on Bray-Curtis dissimilarity distance of feces bacteriota based on 16s rRNA sequencing. Black dots indicate the samples from obese horses, red dots the ones from healthy horses. The graph indicates that analysis of these 7 samples did not separate bacteriota composition in feces of healthy and obese horses

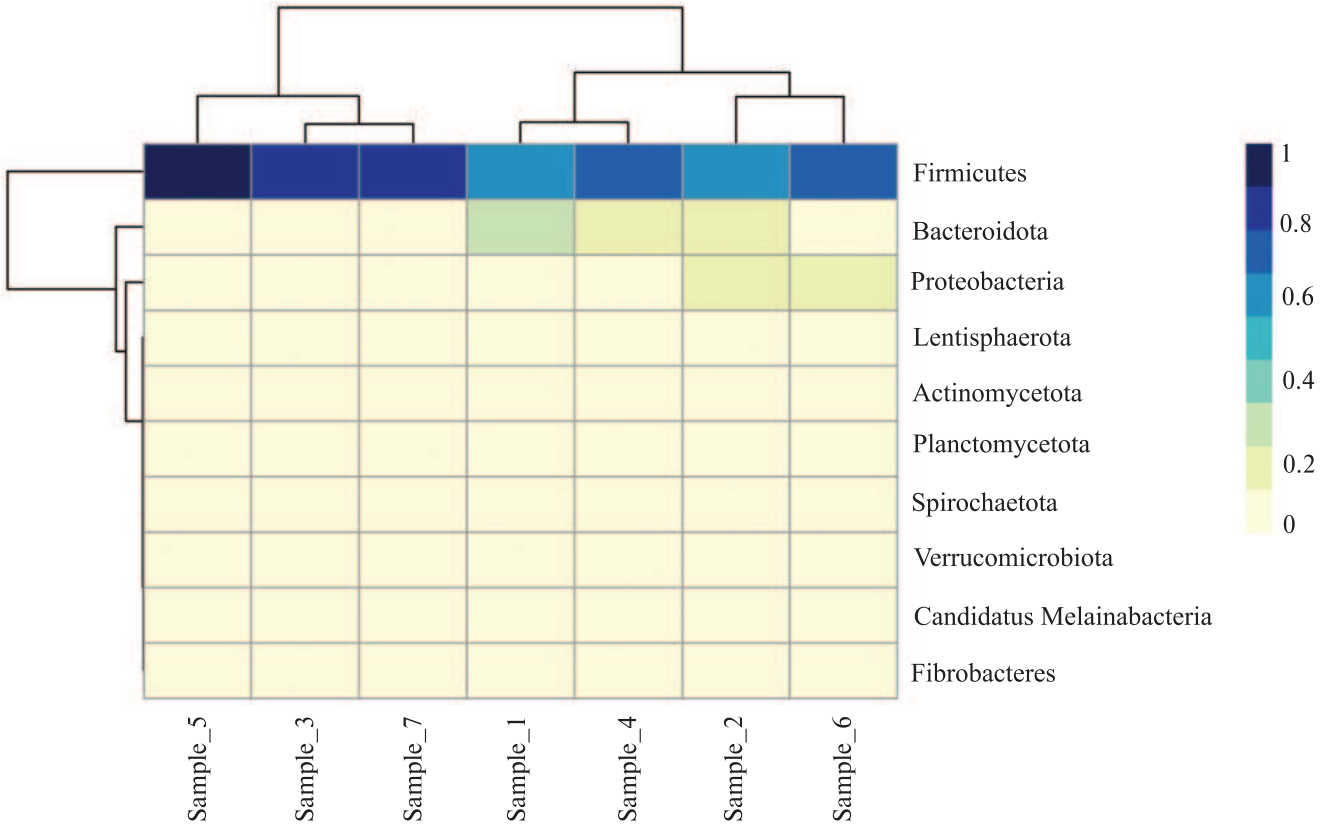


**Fig. 3.** Heatmap of taxonomic clustering of bacteriota in 7 samples of feces of obese (samples 2, 4, 5 and 7) and healthy horses (samples 1,3 and 6).

and increase the risk of developing colitis and laminitis (De Fombelle et al, 2001; Respondek et al, 2008).

When comparing the quantitative indicators of phylotypes in the large intestines of obese and healthy

horses, significant multidirectional differences were observed between the bacterial phyla.

Thus, the most diverse population of fecal bacteriota was observed in obese horse 2, where the *Firmicutes*, (mainly the order *Clostridia*), predominated (75.6 %). Among them, the families *Oscillospiraceae* (16.5 %) and *Lachnospiraceae* (40.9 %) were prominent. The phylum *Bacteroidota* was represented at 9.8 % only. In our opinion, the increase in the proportion of *Firmicutes* and the decrease in *Bacteroidota* may contribute to fat accumulation in animals by increasing energy synthesis from feed when compared to our results obtained with sport horses.

The representation of the *Oscillospiraceae* ranged from 19.1 to 28.3 %, while in obese horses, they were not detected at all. Meanwhile, the bacterial species diversity in the feces of healthy sport horse 1 was characteristic of a healthy microbiome and differed from the diversity of the obese animal in terms of ratios. In the feces of healthy sport horse 1, *Firmicutes* were dominate, up to 71.5 % of the total bacterial population, with a low level of Bacteroidota (8.6 %).

The class *Clostridia* with the family *Oscillospiraceae* was present at a level of 27.5 %, and *Lachnospiraceae* at 34.7 %, with the appearance of a new class, Bacilli, which was not observed in the obese horse, at a level of 33.2 %. This included representative of the family *Planococcaceae* (genus *Solibacillus*) and the family *Bacillaceae* (genus *Lysinibacillus*), which are characterized by high metabolic properties. However, lactobacilli and bifidobacteria were not detected in the gut microbiota of the sport horse, indicating reduced resistance, possibly due to intense physical activity.

The PCoA (**Fig. 2**) did not show statistically significant differences between the fecal bacteriota of obese and healthy horses ($R = 0.375$, $P = 0.053$). This absence of clustering is also clear from the heatmap constructed from our data (**Fig. 3**). The heatmap shows in addition that the phyla *Firmicutes, Bacteroidota* and *Proteobacteria* were most represented in the horse feces.

Both the samples from obese horses (2, 4, 5, 7) and those from healthy ones (1, 3 and 6) are grouped into only one cluster, which confirms the absence of considerable changes in the microbiota populations and diversity in the feces taken from the rectum.

## DISCUSSION

In this study, we analyzed the fecal microbiota of four obese and three healthy horses. Many studies have already been published investigating the effect of obesity phenotype on the intestinal tract and the intestinal environment in horses, using a heterogeneous population (Biddle et al, 2018; Coleman et al, 2019) or sampling at baseline and endpoints of a homogeneous diet-controlled population in homogeneous studies at a single location (Morrison et al, 2018; Morrison et al, 2020). However, studies on horses of the Ukrainian Riding breed and other breeds kept in the Kharkiv region of Ukraine were conducted for the first time.

Dougal et al (2014) recommend performing studies at several locations over time, using microbiological and metabolite analysis, to better understand physiological processes in horses. In our study, the animals had a stable, unchanged diet for four months, which, in our opinion, allowed for the stabilization of digestive processes and avoided changes in the intestinal bacterial microbiota during dietary shifts.

The composition of the main taxa of bacteriota found in horse feces in this study is consistent with that of other studies, where also *Firmicutes* and *Bacteroidota* dominated, and *Firmicutes* prevailing over *Bacteroidetes* with much less representation of *Spirochaetota* and *Fibrobacterota* (Costa et al, 2015; Ang et al, 2022; Kawaida et al, 2024). We determined that that *Firmicutes* prevailed over *Bacteroidota* in the feces of obese horses as well, that was also found by Biddle et al, 2018 and Wester RJ et al, 2024), However, in a study of obese Welsh mountain horses the opposite was found (Morrison et al, 2020). This suggests that reports (including ours) on the possible importance of microbiome-level changes in relation to obesity should be interpreted with caution (Antwis et al, 2015).

The taxa present at the family and genus levels was generally similar among all horses and included the most common types of fecal bacteria (Fig. 1). Among the Firmicutes, the *Clostridia* class was more prevalent, reaching up to 75.6 in obese horses compared to 15.56 in healthy ones. *Lachnospiraceae* were present at 40.9 in obese horses and at a lower level of 13.71 in healthy animals. This increased presence of *Clostridia* has been associated with the development of intestinal diseases, particularly in relation to physical activity (Campbell et al, 2016; Petriz et al, 2014, Mach et al, 2020), while a reduction in their numbers has been linked to colitis in horses (Lara et al, 2022). Representatives of the *Oscillospiraceae* in healthy animals reached 28.3 %, whereas in obese horses, they were absent, as was the family *Prevotellaceae*, which was present only in healthy horses at levels of up to 1.73 %, respectively.

Additionally, families such as *Ruminococcaceae* and *Lachnospiraceae* were frequently observed in healthy animals, but rarely in horses with colitis or diarrhoea (Elzinga, 2016, McKinney CA et al, 2020). In all obese horses, the absence of bacteria from the *Ruminococcaceae*, *Flavobacteriaceae*, and *Prevotellaceae* families was noted, which is often observed in animals with metabolic disorders. These bacteria are involved in the fermentation of complex carbohydrates and the production of short-chain fatty acids, which are essential for maintaining energy balance and gut health. Their deficiency leads to metabolic disruption and the development of obesity (Rylova et al, 2019).

Diet in general has been found to influence the microbiome of the equine intestines (Kristoffersen et al, 2016; Gerstner et al, 2018; Massacci et al, 2020), and certain effects of dietary restrictions on equine gut microbiota have also been noted (Morrison et al, 2020; Coleman et al, 2019). Therefore, when studying horses, one of the significant advantages of horses is the strict regulation and homogeneity of their diet. In our study, this was reflected in the generally stable taxonomic composition of the fecal bacteriota in both obese and healthy horses, which is also consistent with findings from other studies (Ang et al, 2022; Garber et al, 2020).

The experimental horses were fed the same food, consisting of 1.5 to 4 kg of oats and 10–15 kg of hay, although this diet was not nutrient-balanced. It is advisable to adapt the diet of horses based on factors such as gender, age, breed, weight, physical condition, and type of work (Bulmer et al, 2019; Destrez et al, 2019).

Horse nutrition largely depends on the physical load given to these horses. During intense physical activity, horses expend a lot of energy and strength, which requires an increase in the dose of mineral and vitamin supplements, as well as adjustments to the proportions of rough, juicy, and concentrated feeds. In contrast, horses that do not engage in physical work should adhere to a more modest, balanced diet (De Fombelle et al, 2003, Fernandes et al, 2014).

In turn, obese horses 2, 4, 5, and 7 showed a tendency towards changes in their bacterial microbiota, specifically the absence of lacto- and bifidobacteria, *Ruminococcaceae, Flavobacteriaceae, Prevotellaceae*, further presence of only low numbers of *Actinomycetota*, *Fibrobacterota*, families *Eubacteriaceae, Clostridiaceae, Lachnospiraceae*, and an increase in numbers of *Proteobacteria* (Arroyo et al, 2020).

To increase the diversity of microorganisms in the gut of experimental horses and thereby improve the health and stability of their microbiome, it is necessary to provide feeds with a high fibre content (Cotillard et al, 2018). Ingredients containing easily digestible fibre, such as beet and apple pulp, oilcake, and dried hay, may work together as probiotics and promote the growth of beneficial microflora. The stable presence of such feeds in the horses› diet will help the intestinal microflora cope with sharp changes in hay batches and transitions from pasture to hay and back (Zhang et al, 2021; Plaizier et al, 2018).

In this study, the seven horses differed in gender, breed, weight, and origin, but they were kept in the same facility and had a common feeding type and similar surrounding microflora. Given the sample size of this study, investigations will be extended for a statistical assessment of the impact of the intestinal bacterial microbiota on the formation of obesity in horses.

## CONCLUSIONS

This study represents the first instance in Ukraine where we sequenced the 16S rRNA gene of the bacterial microbiota of the lower gut (fecal samples) from seven horses of varying ages, genders, and breeds. The feces of obese horses exhibited a dominance of bacteria belonging to the order *Eubacteriales,* phylum *Firmicutes* (syn. *Bacillota*), class *Clostridia*, particularly from the families *Oscillospiraceae* and *Lachnospiraceae*. This went together with a reduction in the proportion of *Bacteroidota* (FCB group clade) when compared to healthy horses. These alterations may be linked to fat accumulation in the animals, possibly due to an increase in energy synthesis from their diet. Cluster analysis indicated a significant similarity in the bacteriota composition across the samples. Additional research, involving larger sample sizes and investigations into physiological factors, is necessary to gain a more comprehensive understanding.

***Adherence to ethical principles.*** While conducting the experimental research, all the manipulations with horses involved in the study were conducted in compliance with the main principles of bioethics, according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and General Ethical Principles for Experiments on Animals, adopted by the First National Bioethics Congress (Kyiv, 2012), and approved by the Bioethics Committee of the National Scientific Center ‘Institute of Experimental and Clinical Veterinary Medicine,’ authorized on September 5, 2023, protocol No. 3–23 dated December 26, 2023.”

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**Секвенування 16S рРНК з використанням NGS фекальної бактеріальної мікробіоти у здорових коней українського походження та коней з ожирінням**

* С. Б. Боровков [1], О. В. Кольчик [1], О. А. Тарасов [2], М. В. Безименний [2]

[1] Національний науковий центр "Інститут експериментальної та клінічної ветеринарної медицини", вул. Пушкінська, 83, м. Харків, Україна, 61023
[2] Інститут ветеринарної медицини Національної академії аграрних наук Україна, вул. Донецька, 30, м. Київ, Україна, 03154

E-mail: * Serg_b78@ukr.net, kolchyk-elena@ukr.net, ast97@ukr.net, nomax@ukr.net

orcid: https://orcid.org/0000–0003–3021–2410; https://orcid.org/0000-0003-0497-2512, https://orcid.org/0000-0003-1481-5529, https://orcid.org/0000-0001-8042-0061

**Мета.** Охарактеризувати та, можливо, диференціювати бактеріоту нижнього кишечника (фекальний) здорових та ожирілих коней за допомогою секвенування наступного покоління (NGS) гена 16S рРНК. **Методи.** У дослідженні взяли участь 7 коней (4 жеребці та 3 кобили) різних порід віком 8–17 років: коні 1–4 породи українська верхова (кінь 1 – спортивний жеребець Ребус, 10 років, кінь 2 – жеребець Сантес, 15 років, кінь 3 – жеребець Сенс, 14 років, кінь 4 – кобила Сирена, 17 років), кінь 5 – породи важкий драфт (жеребець Циган, 8 років) та не чистопородні коні 6 і 7 (кобила Сніжинка, 10 років, кобила Румба, 12 років). Коні 2, 4, 5 і 7 були ожирілими, а коні 1, 3 і 6 – здоровими. Коні утримувалися в кінному центрі Державного біотехнологічного університету Міністерства освіти і науки України (Харків, Україна). Загальна ДНК з ректальних фекальних зразків була екстрагована з використанням набору для очищення ДНК PureLink Microbiome (Invitrogen, США) відповідно до інструкцій виробника. Для підготовки бібліотек 16S рРНК бактеріальної мікробіоти ми використовували набір для баркодування 16S рРНК 1–24 (Oxford Nanopore, США). Для очищення отриманих бібліотек використовувалися магнітні частинки NucleoMag NGS Clean-up та Size Select (Macherey-Nagel, Німеччина). Ці умови засновані на стандартних протоколах для ампліфікації гена 16S рРНК, описаних у дослідженні Fujiyoshi et al (2020), і забезпечують надійну ампліфікацію бактеріальної ДНК на широкому діапазоні таксонів. Для очищення бібліотек ми використали магнітні частинки NucleoMag™ NGS Clean-up та Size Select (ref 744970.50, Macherey-Nagel™, Німеччина) відповідно до інструкцій Oxford Nanopore Technologies (SQK-16S024). **Результати**. Були виявлені представники бактеріальних филів *Actinomycetota* (syn. *Actinobacteriota*), *Fibrobacterota, Lentisphaerota, Spirochaetota* (syn. *Spirochaetes*), *Bacteroidota*, *Firmicutes* (syn. *Bacillota*), *Planctomycetota*, *Verrucomicrobiota* (syn. *Verrucomicrobia*), *Candidatus* Melainabacteria*, Kiritimatiellota* and *Proteobacteria* (syn. *Pseudomonadota*). Домінуючим філом виявився *Firmicutes*, частка якого становила від 50 до 82 % від усіх виявлених филів. Кількість *Firmicutes* у порівнянні з *Bacteroidota* значно варіювала між здоровими та ожирілими конями. У здорових коней 1, 3 і 6 це було в 2.5, 3.4 та 2.9 рази вище для *Firmicutes*, а у ожирілих коней 2, 4, 5 і 7 – в 8.6, 8.2, 7.6 і 5.7 рази вище відповідно. Збільшена кількість родів *Proteobacteria* спостерігалася у ожирілих коней 2, 4, 5 і 7, яка становила від 25 до 37 %, тоді як у здорових спортивних коней 1–3 рівень *Proteobacteria* знаходився в межах від 1.07 до 3.43 %, що є типовим для мікробіому здорових тварин. Низький рівень *Actinomycetota* (*Actinobacteriota*) спостерігався у фекаліях досліджуваних коней: 0.09 % у здорового спортивного коня 1, 0.09 % у здорового спортивного коня 3 та 0.15 % у іншого здорового коня. У той же час, рівень варіювався від 0.21 до 0.48 % у ожирілих коней 2, 4, 5 і 7. Цей тип також включає рід *Bifidobacterium*, який не був виявлений у жодної з вивчених тварин. **Висновки.** Вперше в Україні було проведено секвенування бактеріальної мікробіоти товстого кишечника (фекального матеріалу) семи коней різного віку, статі та порід. У фекаліях коней із ожирінням спостерігалося переважання бактерій *Eubacteriales* (філ *Firmicutes*, клас *Clostridia*), зокрема з родин *Oscillospiraceae* та *Lachnospiraceae*, що супроводжувалося зменшенням частки філуму *Bacteroidota* (кластер FCB) у порівнянні зі здоровими конями. Ці зміни можуть бути пов'язані з накопиченням жиру у тварин, потенційно через збільшений синтез енергії з кормів. Кластерний аналіз виявив високу ступінь схожості в складі мікробіома серед зразків. Необхідні подальші дослідження, включаючи збільшення обсягу вибірки та вивчення фізіологічних основ, щоб надати більш комплексну інформацію.

**Ключові слова:** 16S РНК секвенування, мікроорганізми, обмін речовин, травлення, кишківник, товста кишка.

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