

## Microbiomes

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### RESULTS OF THE RESEARCH OF INTESTINAL MICROBIAL PROFILES OF *Equus ferus caballus* BY NGS SEQUENCING

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### Abstract

The symbiotic microbiome of the gastrointestinal tract of animals plays a vital role in the digestion and assimilation of feed nutrients, the development of immunity, disease resistance, and the breakdown of toxins. Significant amounts of starch are introduced into the diet of horses specialized for riding, in some cases (for example, before participating in exhibitions). This can lead to serious dysbiotic disorders of the microbiome. Disorders of the microbial community of the intestine can adversely affect animal health that become the cause of metabolic disorders, such as acidosis, a decrease in the digestibility of diet components, primarily fiber, hoof diseases, etc. The digestive system of *Equus ferus caballus* has a number of unique features compared to other mammals. In this work, for the first time in Russia, the diversity of the equine intestinal microbiome composition was demonstrated using the 16S metagenomics method. The study aimed to evaluate the microbiomes of the contents of the rectum of horses of different ages, physiological status, diets, sexes and breeds using NGS sequencing. The experiment was carried out in the summer 2017 at the Malanichevs' Farm (Grishkino settlement, Leningrad Province, Tosnensky District) with horses (*Equus ferus caballus*) specialized for riding and hippodrome trials. Samples of 10–50 g (in triplicate) were taken from the rectum of three stallions of the Hanoverian breed (3 years old), a mare (6 years old) and a stallion (7 years old) of the Trakehner breed. Five days before sampling, the mare was foaled. The diets of stallions and mares were different. The stallions' diet included grass (20 kg), hay (9 kg), carrots (1 kg), oats (3 kg), table salt (29 g). The mares' diet consisted of grass (26 kg), carrots (1 kg), rolled oats (2.5 kg), table salt (27 g). Total DNA from the samples was extracted using Genomic DNA Purification Kit (Fermentas Inc., Lithuania). Amplification for subsequent NGS sequencing was carried out on a Verity DNA amplifier (Life Technologies, Inc., USA) using eubacterial primers (IDT) 343F 343F (5'-CTCCTACGGRRSGCAG-CAG-3') and 806R (5'-GGACTACNVGGGTWCTAAT-3') flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing was performed on a MiSeq instrument (Illumina, Inc., USA). The taxonomic affiliation of microorganisms to genus was determined using the RDP Classifier program (<https://rdp.cme.msu.edu/classifier/classifier.jsp>). In five different studied individuals of *E. ferus caballus*, fairly similar microbiomes of intestinal profiles were revealed, regardless of the type of nutrition, physiological status, age, gender, and breed. High values of the Shannon and Simpson diversity indices testified to the species richness and biodiversity of the intestinal contents of horses. In the rectum, 25 phyla of microorganisms were found. The dominant phyla were *Firmicutes* (ranged from 32±1.9 to 40±3.8 %) and *Bacteroidetes* (from 34±2.1 to 40±4.7 %). It is important to emphasize that we revealed in the microflora a significant number of microorganisms associated with feed digestion, especially those decomposing cellulose. So, the content of bacteria synthesizing cellulases reached

significant values, up to  $23.8 \pm 1.30$  % for *Bacteroidales*, up to  $14.7 \pm 2.80$  % for *Lachnospiraceae*, up to  $10.2 \pm 3.30$  % for *Ruminococcaceae*, and up to  $6.6 \pm 0.6$  % for *Clostridiaceae*. A number of microorganisms were identified that can be associated with various diseases, e.g. horse with colic, acidosis, laminitis, etc. For example, in all samples of the rectum contents, we detected undesirable members of the order *Lactobacillales*, such as *Streptococcus equinus* and *Str. bovis*, which are associated with the occurrence of acidosis and laminitis in horses. The genus *Treponema* bacteria was revealed (from  $2.2 \pm 0.22$  to  $6.5 \pm 0.40$  %) which are associated with the occurrence of periodontitis in horses. The enterobacteria of the genera *Enterobacter*, *Serratia*, and *Escherichia* were detected, among which gastroenteritis pathogens can be often found. Further study of the intestinal microbiota profiles may contribute to the improvement of diagnosis and treatment of equine diseases.

Keywords: *Equus ferus caballus*, intestinal microbiome, *Bacteroidales*, *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Streptococcus equinus*, *Streptococcus bovis*, *Treponema*, *Enterobacter*, *Serratia*, *Escherichia*, NGS sequencing, BIOTROF, molecular methods

Gut microbiota (the gastrointestinal tract, GIT) plays a special role in herbivores, since the digestion of plant fibers with transformation to volatile fatty acids occurs with the participation of symbiont microorganisms [1]. The digestive system of the domestic horse (*Equus ferus caballus*) has a number of unique features as compared to other mammals. Thus, it is believed that cattle show a slightly greater resistance to the consumption of toxic feed [2] due to the active detoxification activity of the cicatricial normobiota before the feed is transferred to the small intestine. However, in horses that do not have a proventriculus, the intake of pathogens and toxins with food is one of the reasons for a dangerous disease, the symptom complex of colic leading to death [3, 4].

The large intestine of horses is well-formed, makes up approximately 64% of the gastrointestinal tract volume, and includes three sections, the cecum, the colon, and the rectum [5]. The cecum in horses (length about 1 m, volume of 30-35 l) is considered an analogue of the rumen of ruminants, since 40-50% of all fiber and up to 40% of protein are digested here [6, 7] with the participation of a variety of symbiotic microflora: bacteria, archaea, micromycetes, protozoa, and bacteriophages [1]. Microorganisms have a variety of functional activities necessary to facilitate feed digestion, e.g. hemicellulolytic and cellulolytic, amylolytic, proteolytic properties, and can utilize lactate [1]. The digestive system of *E. ferus caballus*, in comparison with that of wild relatives, is under severe anthropogenic pressure which has negative consequences for health. For example, an increased amount of dry matter in feed is an additional factor provoking colic symptoms, since, due to anatomical features, it significantly slows down chyme flow through the caecum [1]. Dysbiotic disorders of the microflora of the blind processes of the intestine, which takes an active part in digestion, together with unbalanced diets, increase the risk of colic symptom complex [1].

In special cases, e.g., before exhibitions, riding horses are fed high starch diets. By analogy with similar processes in cattle [8], this increases in the abundance of amylolytic bacteria that produce lactic acid in the gastrointestinal tract, primarily in the large intestine, which, in turn, causes a decrease in pH and triggers cascade mechanisms [1]. The abundance of cellulolytic forms which are pH-sensitive decreases, and the fiber assimilation processes in the cecum are disrupted, which becomes a provocative factor for the occurrence of colic [1].

This work is the Russia's first to disclose the diversity of the horse intestinal microbiome by 16S metagenomic sequencing method. Metagenomic analysis revealed a significant number of microorganisms associated with digestion of feed, primarily fiber, and a number of microorganisms related to occurrence of various diseases, e.g. colic, acidosis, and laminitis.

This research aimed to reveal profiles of rectal microbiomes in horses with regard to their age, sex, breed and diet, using high throughput sequencing technology.

**Material and methods.** The experiment was carried out in the summer 2017 at the Malanichevs' Farm (Grishkino settlement, Leningrad Province, Tosnensky District) on horses (*Equus ferus caballus*) specialized for riding and racetrack trials.

Samples of 10-50 g (in triplicate) from the rectum of three stallions of the Hanoverian breed (3 years old), a Trakehner mare (6 years old) and a Trakehner stallion (7 years old) were taken manually aseptically using sterile rubber gloves. Five days before sampling, the mare was foaled.

The stallions' and mare's diets differed. Stallions were fed rations with grass (20 kg), hay (9 kg), carrots (1 kg), oats (3 kg), and table salt (29 g); 9.89 energy feed units (EFU). The mare's ration consisted of grass (26 kg), carrots (1 kg), rolled oats (2.5 kg), and table salt (27 g); 9.87 EFU.

Total DNA was extracted with Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. Amplification for NGS sequencing was performed using a Verity DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers (IDT) 343F (5'-CTCCTACGGRRSGCAGCAG-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') flanking V1V3 region of the 16S rRNA gene. The following amplification mode was used: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (25 cycles); 5 min at 72 °C (1 cycle).

Metagenomic sequencing (MiSeq, Illumina, Inc., USA) was performed with the MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the obtained sequences was 2×300 bp. Chimeric sequences were excluded from analysis using the USEARCH 7.0 program (<http://drive5.com/usearch/>). The obtained 2×300 bp reads were processed using the bioinformatics platform CLC Bio GW 7.0 (Qiagen, the Netherlands). The processing included overlap, filtration by quality (quality value, QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to genus was determined using the RDP Classifier program (<https://rdp.cme.msu.edu/classifier/classifier.jsp>).

Mathematical and statistical processing was performed using the software packages Microsoft Office Excel 2003, PAST, R-Studio (Version 1.1.453) (PAST, 2011, <https://rstudio.com>) [9, 10]. The results were considered significant at  $p < 0.05$ . Numerical data are presented as means ( $M$ ) and standard errors of the mean ( $\pm$ SEM). The biodiversity indices of Chao1, Shannon (H), Simpson (D) were calculated as per [11].

**Results.** Analysis of  $\alpha$ -biodiversity parameters calculated from the results of profiling microbial community of the horse intestine by NGS-sequencing did not revealed differences between animals (Table 1). The values of the Shannon and Simpson indices of species diversity turned out to be rather high compared to those established for cattle. Thus, for fattening gobies fed various diets, the Shannon index of rumen microbiota varied from  $7.43 \pm 0.66$  to  $8.48 \pm 0.28$ , and the Simpson index from  $0.975 \pm 0.002$  to  $0.985 \pm 0.001$  [12]. It is known that the conditions in the rumen are optimal for the development of a variety of microflora, while the food stays here for 24-48 hours, which contributes to favorable conditions for microbiological processes and determines a high biodiversity level [13]. Nevertheless, the Chao1 index, which indicates the species richness of the microbiome, in the analysis of the horse intestinal microbiota was, on average, 5 times lower than in the study of cattle rumen [12].

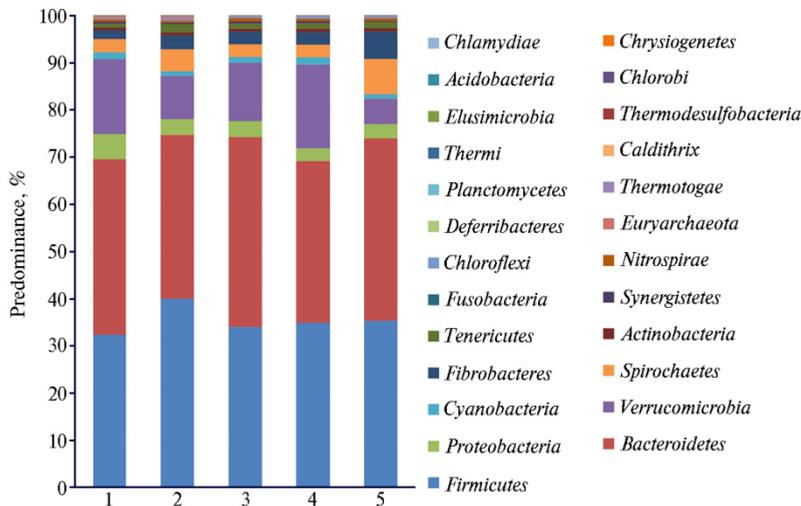
The rectal microbiome of the examined specimens contained 25 phyla of microorganisms (Fig. 1). *Firmicutes* (from  $32 \pm 1.9$  to  $40 \pm 3.8\%$ ) and *Bacteroidetes* (from  $34 \pm 2.1$  to  $40 \pm 4.7\%$ ) were dominant, *Verrucomicrobia*, *Proteobacteria*, *Spirochaetes*, *Fibrobacteres* were rather abundant, the rest of the phyla turned out to be minor. Earlier, other researchers noted the predominance of the phylum

*Firmicutes* in the feces of healthy horses together with a decrease in the number of the *Bacteroidetes* members [14]. At the same time, a decrease in the percentage of the phylum *Firmicutes* representatives with an increase in the percentage of the phylum *Verrucomicrobia* bacteria was associated with equine laminitis [15, 16]. A decrease in the proportion of phylum *Firmicutes* was also observed in horses with colitis and in those with symptoms of diarrhea. The work of P.K. Morrison et al. [17] demonstrated that in the feces of healthy horses, bacteria of the phylum *Bacteroidetes* predominated in percentage, while *Firmicutes* and *Fibrobacteres* were less represented.

**$\alpha$ -Diversity of the rectal microflora of horses (*Equus ferus caballus*) depending of the age, sex, breed and diet as calculated from NGS-sequencing data ( $M \pm SEM$ , Grishkino settlement, Leningrad Province, Tosnensky District, 2017)**

| Sample No. | Chao1 index  | Shannon index (H) | Simpson index (D) |
|------------|--------------|-------------------|-------------------|
| 1          | 255.6±18.235 | 7.5±0.68          | 0.99±0.063        |
| 2          | 259.5±12.442 | 7.6±0.44          | 0.99±0.073        |
| 3          | 341.8±16.329 | 7.9±0.42          | 0.99±0.047        |
| 4          | 324.1±15.321 | 7.9±0.39          | 0.99±0.054        |
| 5          | 224.3±12.637 | 7.3±0.53          | 0.99±0.068        |

Note. 1 — Trakehner stallion (7 years old), 2 — Trakehner mare (6 years old), 3, 4, 5 — Hanoverian stallions (3 years old). For animal diets, see *Material and methods*.

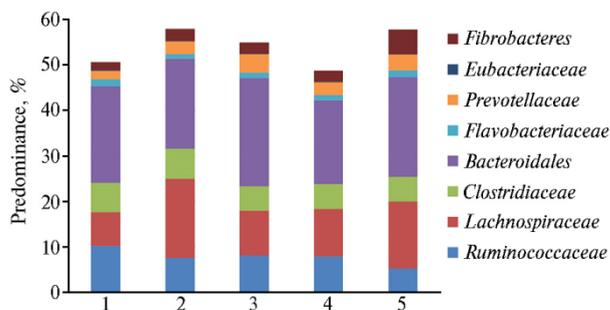


**Fig. 1. Predominance of bacterial phyla in the rectal contents of horses (*Equus ferus caballus*) depending of the age, sex, breed and diet: 1 — Trakehner stallion (7 years old), 2 — Trakehner mare (6 years old), 3, 4, 5 — Hanoverian stallions (3 years old) (Grishkino settlement, Leningrad Province, Tosnensky District, 2017). For animal diets, see *Material and methods*.**

In general, the microbial profiles we obtained for higher taxonomic rank found in the rectum of horses were characteristic of quite similar microbiomes, regardless of the type of nutrition, physiological status, age, sex and breed. An exception was the mare that had foaled 5 days before the examination, with the highest ( $40 \pm 3.8\%$ ,  $p \leq 0.05$ ) abundance of bacterial phylum *Firmicutes* compared to other individuals. It is interesting that the work of A. Schoster et al. [18] also revealed an increase in the abundance of the *Firmicutes* phylum representatives, in particular *Streptococcaceae*, in the intestine of mares after foaling, with a decrease in the relative abundance of the phylum *Proteobacteria* when compared to fecal samples before the foal.

In the rectum microbiome of the horses, a high diversity of taxa is notable (Fig. 2), i.e. the families *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Flavobacteriaceae*, *Prevotellaceae*, *Eubacteriaceae*, the order *Bacteroidales* and the

phylum *Fibrobacteres*, among which there are cellulase-producing bacteria. The abundance of some taxa was significant, up to  $23.8 \pm 1.30\%$  for *Bacteroidales*, up to  $14.7 \pm 2.80\%$  for *Lachnospiraceae*, up to  $10.2 \pm 3.30\%$  for *Ruminococcaceae*, and up to  $6.6 \pm 0.60\%$  for *Clostridiaceae*. This is an important observation, since the digestion of non-starchy polysaccharides in the gut is an exclusively microbiological process [19]. Representatives of the only genus *Fibrobacter* of the phylum *Fibrobacteres*, previously classified as the genus *Bacteroides*, the *F. succinogenes* and *F. intestinalis* [20] are known for their high efficiency in the hydrolysis of plant cellulose [21]. S.E. Salem et al. [22] observed a significant increase in the relative abundance of the phylum *Fibrobacters* upon using haylage in the diet of horses.



**Fig. 2. Predominance of taxa which can comprise cellulase-producing bacteria in the rectal contents of horses (*Equus ferus caballus*) depending of the age, sex, breed and diet:** 1 — Trakehner stallion (7 years old), 2 — Trakehner mare (6 years old), 3, 4, 5 — Hanoverian stallions (3 years old) (Grishkino settlement, Leningrad Province, Tosnensky District, 2017). For animal diets, see *Material and methods*.

to those in horses of 3 years of age (from  $0.03 \pm 0.001$  to  $0.05 \pm 0.002\%$ ) (at  $p \leq 0.05$ ).

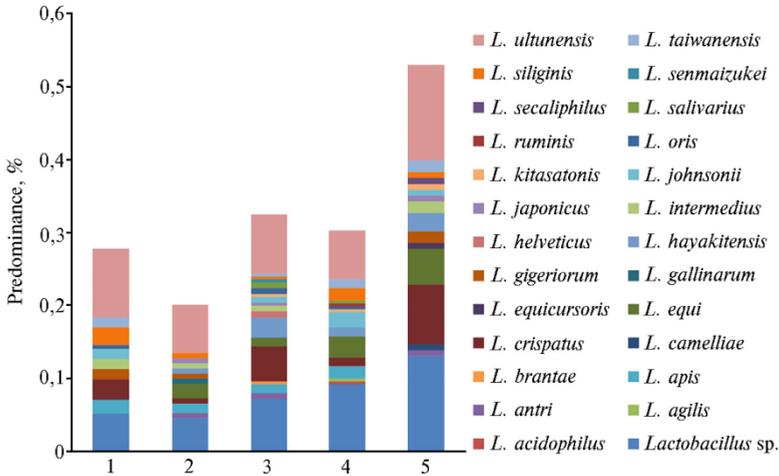
Among the representatives of *Firmicutes*, the counts of bacteria from the order *Lactobacillales* were rather low in all samples, not exceeding  $1.35 \pm 0.070\%$  of the total abundance of microorganisms. At the same time, typical intestinal microorganisms of the *Bifidobacteriaceae* family [26] were almost completely absent in the rectal contents.

On the one hand, the presence of lacto- and bifidobacteria in the intestine is an important marker of animal health. It is widely known that lactobacteria perform a number of significant functions. They protect the host organism from pathogens, exhibit immunomodulatory activity, and participate in the synthesis of vitamins and essential amino acids. Lacto- and bifidobacteria are also probiotics [18, 27, 28]. In our study, despite the low percentage of the genus *Lactobacillus* members (from  $0.2 \pm 0.01$  to  $0.5 \pm 0.03\%$ ), their species diversity was quite wide, up to 25 species (Fig. 3).

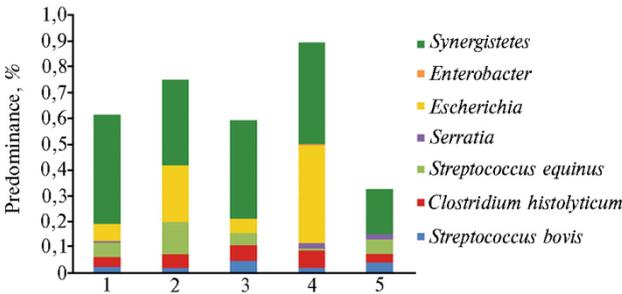
On the other hand, we revealed unwanted members of the order *Lactobacillales*, such as *Streptococcus equinus* and *Str. bovis*, in all samples of the rectal contents (Fig. 4). The presence of *Str. bovis* in the horse digestive tract is associated with laminitis [29]. In horses [30], as in cattle [8], the counts of bacteria producing lactic acid increases in response to a significant proportion of starch in the diet, which often leads to lactic acidosis. This is accompanied by a decrease in the pH of the large intestine, a violation of the processes of fiber digestion and the improper quantitative ratio of volatile fatty acids. In many cases, as a result of acidosis in animals, hoof lesions, the laminitis can occur. As noted by C. Bergsten [31], in experimental models on horses and bulls, laminitis was easily provoked by an excessive amount of carbohydrates in the diet. It is assumed that high amounts

It is interesting to find in the intestines of horses the members of phylum *Euryarchaeota*, including methanogens [23, 24] that produce methane, halophilic bacteria that remain viable at extreme salt concentrations, and thermoanaerophiles that survive at temperatures of  $41-122\text{ }^\circ\text{C}$  [25]. We found a significantly higher abundance of the phylum *Euryarchaeota* in horses aged 6-7 years of (from  $0.31 \pm 0.020$  to  $0.44 \pm 0.020\%$ ) as compared

of endotoxins and histamine secreted by pathogenic forms, primarily *F. necrophorum*, which increase in abundance under acidic pH conditions, lead to damage to the mucous membrane of the digestive tract [31]. Endotoxins are an extremely powerful trigger for the prostaglandin cascade. This causes blood clots which clog up the small blood vessels (capillaries) of the lamina propria resulting in impaired circulation. Decreased oxygen and nutrient supply damages cells that form the keratinized laminae of the corium.



**Fig. 3. Predominance of *Lactobacillus* bacteria in the rectal contents of horses (*Equus ferus caballus*) depending of the age, sex, breed and diet:** 1 — Trakehner stallion (7 years old), 2 — Trakehner mare (6 years old), 3, 4, 5 — Hanoverian stallions (3 years old) (Grishkino settlement, Leningrad Province, Tosnensky District, 2017). For animal diets, see *Material and methods*.



**Fig. 4. Predominance of bacterial taxa related to animal pathology in the rectal contents of horses (*Equus ferus caballus*) depending of the age, sex, breed and diet:** 1 — Trakehner stallion (7 years old), 2 — Trakehner mare (6 years old), 3, 4, 5 — Hanoverian stallions (3 years old) (Grishkino settlement, Leningrad Province, Tosnensky District, 2017). For animal diets, see *Material and methods*.

The abundance of genus *Bacillus* bacteria, the microorganisms with broad antagonistic properties, in the rectal contents of the horses was also quite low and did not exceed  $0.32 \pm 0.020\%$ , despite the fact that these microorganisms, due to spore formation are more resistant to aggressive environment of the gastrointestinal tract than many other forms [32]. Their functional role is associated with the ability to colonize the digestive tract, which en-

sure interaction with the intestinal epithelium of the host organism [33]. Genes associated with the synthesis of antimicrobial compounds account for about at least 4-5% of the total genome of *Bacillus* sp. strains [34]. On average, 87% of antimicrobial bacterial metabolites of *Bacillus* sp. are organic acids, alcohols, ketones, alkanes, aldehydes, alkenes and 13% of the total pool of antimicrobial compounds are other substances, e.g. ribosomal peptides (bacteriocins and enzymes), polyketides, nonribosomal peptides [35]. Most strains of bacilli also exhibit broad enzymatic activity and are involved in the metabolism of various nutrient substrates [32].

The presence of *Treponema* bacteria in the rectal contents of the examined animals (from  $2.2 \pm 0.22$  to  $6.5 \pm 0.40\%$ ) deserves attention. We identified 11 *Treponema* species of which *Treponema bryantii* predominated. *Treponema* bacteria are associated with periodontitis in horses [36]. However, recent studies have reported the presence of 2–3% *Treponema* bacteria in healthy horses [14].

Identification of relatively new phylum *Synergistetes* in the intestinal microflora of horses (see Fig. 3) is of special interest. Bacteria of this taxon are often detected in cysts and abscesses in humans [37, 38], and therefore they are classified as opportunistic pathogens [38, 39]. It is believed that some representatives of the phylum *Synergistetes* are also involved in occurrence of gastrointestinal infections (37) and, accordingly, may be associated with the colic symptom complex. Identification of enterobacteria from genera *Enterobacter*, *Serratia* and *Escherichia* in the gastrointestinal microbiomes of the studied animals also worth attention, since among these bacteria causative agents of gastroenteritis often occur. In addition, we detected bacteria of the genus *Clostridium*. A significantly higher level of the genus *Clostridium* was found in horses aged 6–7 years (from  $4.1 \pm 0.25$  to  $4.3 \pm 0.29\%$ ) compared to animals aged 3 years (from  $3.1 \pm 0.18$  to  $3.3 \pm 0.13\%$ ) (at  $p \leq 0.05$ ). In particular, we revealed *Clostridium histolyticum*, the causative agent of necrotic infections [40].

It should be emphasized that we have found a number of microorganisms in the horse gut microbiome that could accompany the onset of the colic symptom complex [3, 4], the main cause of horse mortality. Horses, especially those specialized for horse riding and racetrack competitions, have a very high risk of laminitis due to the significant load on the hooves [15, 16]. Maintaining a healthy gut microbiome and preventing dysbiosis and acidosis as the main causes of laminitis are effective in prevention of laminitis. When developing preventive measures to regulate the intestinal microbiome, one should bear in mind that the modern concept of pathogen control implies optimization of microecological niches based on the principles of self-regulation and ecologization [41]. Natural feed additives, such as bacterial strains with probiotic properties [42–44] and vegetable essential oils [45–47], can effectively maintain the balance of the normobiota of a horse digestive system.

Our findings on the equine microbiomes can be helpful to develop biomarkers as predictors of response to treatment with drugs, probiotics, and to changed diet composition.

Thus, high-throughput sequencing of rectal contents revealed rather similar intestinal microbiomes in five *Equus ferus caballus* individuals of different ages, sexes, and breeds, and fed different diets. Both normal microbiota and pathogenic microorganisms detected in the rectal contents show high biodiversity. It is worth to indicate a significant abundance of microorganisms associated with digestion of feed, primarily non-starchy polysaccharides, in the microbiomes. This is of interest and will be further investigated. In addition, it can be assumed that the gut microbiome of horses is associated with their overall health status and wellbeing.

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