Similarity analysis of silage, rumen and milk microbiota in dairy cows

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Abstract. Diseases that occur in such a multifactorial system as animal husbandry are determined not only by internal factors of the body, for example, the composition of the microflora of the digestive system, but also by external factors, such as feed. The aim of the study was to analyze the similarity of the microbiota of silage fed to cows with the composition of the microflora of their rumen and milk using the NGS sequencing method. The experiment was carried out on one of the commercial farms of the Leningrad region. The bacterial community of the contents of rumen, milk and silage from perennial cereals and legumes was evaluated by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) with primers for the V3-V4 region of the 16S rRNA gene. As a result of the study, 22 phylum of attributed microorganisms were found in the microflora of silage, 24 to 30 phylum of rumen, 18 phylum of milk. Similar taxa of microorganisms were identified in silage, rumen and milk, the main difference was noted in quantitative ratios ($P \leq 0.05$). For example, the amount of Firmicutes in silage was 52.9 ± 3.45%, in milk - 11.8 ± 0.78%. This suggests that there may be some relationship between the studied biotopes. Pathogenic microorganisms, including the causative agents of mastitis, were present in many samples. The genera Staphylococcus, Acinetobacter, Streptococcus and Fusobacterium were identified by us as the most represented ($P \leq 0.05$) in the composition of the milk microflora. Their content was 0.24±0.023, 1.8±1.23, 1.0±0.06 and 0.35±0.031%, respectively. Probably, the microflora of the rumen can influence the formation of the milk microbiota.

1 Introduction

One of the main tasks of animal husbandry is to maintain the health of animals at all stages of production activities. Animal health is of particular importance not only for the economic optimization of various stages of production, but also for the prevention of morbidity of consumers of livestock products (Klauke, 2012). Currently, it is of interest to introduce on-farm and inter-farm animal health management systems by regulating all links in the technological chain of livestock production (Petersen, et al., 2014).

For example, the rumen with the microflora inhabiting it, as one of the most important digestive organs in ruminants, plays one of the main roles in the feeding efficiency and

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productivity of cattle (Shabat, et al., 2016). Dysbiosis of the rumen microbiome can lead to such diseases of cows as digestive system dysfunctions, metabolic disorders, reproduction and inflammatory diseases (Matthews, et al., 2019). Despite extensive research in this area, significant progress has not yet been made in manipulating rumen microbiomes in dairy cattle to improve health and productivity.

Previously, some evidence was obtained for the presence of a biological binding pathway between the microbiota of the digestive system and the microflora of milk (Walker, Iyengar, 2015). Nevertheless, the question of the composition of the microflora of milk is poorly understood. For decades, it was believed that the milk of healthy cows is sterile. However, the results obtained by new generation sequencing methods showed that milk normally has its own resident microbial population, and the vast majority of these microorganisms are not associated with mastitis (Derakhshani, et al., 2018). It has been demonstrated that the composition of bacterial communities in milk samples may differ in cows kept on different bedding and in different geographical zones (Taponen, et al., 2019). It is interesting to establish whether the milk microbiome is formed under the influence of the rumen microbiome.

Diseases that occur in such a multifactorial system as animal husbandry are determined not only by internal factors of the body, but also by external factors, such as feed. Thus, properly prepared silage is the best food for ruminants. The main methods of obtaining high-quality and safe silage are the rapid achievement of a low pH level due to proper fermentation and maintenance of oxygen-free conditions. However, in case of violation of forage harvesting techniques (incorrect crop selection, non-compliance with the conditions of agricultural machinery, ramming, the use of ineffective preservatives, etc.), various undesirable, including pathogenic, microorganisms can develop in the silo (Driehuis et al., 2018).

Thus, factors contributing to the infection of animals and humans are constantly present in the interconnected various biotopes of the technological chain of livestock production. If a number of negative factors contributing to infection are summed up in several or all of these systems, this can trigger a chain of events with a possible unfavorable outcome.

In this regard, the aim of the study was to analyze the similarity of the microbiota of silage fed to cows with the composition of the microflora of their rumen and milk using the NGS sequencing method.

2 Materials and methods

The experiment was carried out on highly productive dairy cows of the black-and-white Holstein breed of the 2nd - 3rd lactation with an average weight of 500 kg on one of the commercial farms of the Leningrad region. The rations of cows were calculated automatically using the program "AMTS.Cattle.Professional" (https://agmodelsystems.com) in accordance with generally accepted requirements (Nekrasov et al., 2018). The animals were kept in the same conditions. The animal housing is tie-up.

Sampling of the contents of the rumen (in three repetitions from each cow) was carried out in 2 animals during the new body period (experimental group Rumen_1 – on the first day after calving, experimental group Rumen_2 – on the 20th day after calving), in 1 animal during the period of increasing the milk yield (100 days after calving, experimental group Rumen_3) and in 1 animal during lactation stabilization (117 days after calving, experimental group Rumen_4). Sampling of the rumen chyme – 30-50 g, was carried out from the upper part of the ventral rumen sac of cows with the maximum possible compliance with aseptic conditions manually using a sterile probe with this method.

Milk was aseptically taken from the mammary glands into sterile conical vials from cows from the groups Rumen_2, Rumen_3 and Rumen_4. The selection procedure was preceded

by the preparation of the mammary glands of cows before sampling milk, including sanitary treatment. When sampling milk for the study, the average sample was made up of proportional portions of all daily milk yields (morning, evening). 3-5 streams of milk were taken from each quarter of the udder. A combined sample was obtained from point samples. To create a combined sample, approximately 12 ml of milk was pumped from each quarter, i.e. only 48 ml from each cow.

Sampling for the analysis of silage from perennial cereals and legumes, which accounted for 19-24% of the cows' diet (depending on the feeding phase), was carried out using a sampler. 5 point samples were taken from the trenches in various places from a depth of 1.5-2.0 m. From the point samples, a combined sample was made, the mass of which was 3 kg. An average sample weighing 100 g was isolated from the combined silage sample by quartering.

The selected samples of the contents of the rumen, milk and silage were immediately placed in sterile plastic tubes. All samples were frozen at -20°C and transported in dry ice to the laboratory of BIOTROF+ LLC for subsequent DNA isolation.

Total DNA for microflora composition analysis was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA) according to the attached instructions. The bacterial community was evaluated by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) with primers for the V3-V4 region of the 16S rRNA gene. Direct primer: 5′-TCGTCGGCAG-CGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG-ACTACHVGGGTATCTAATCC-3′. PCR was performed under the following conditions: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (necessary for lengthening the sequence) (25 cycles); 5 min at 72 °C (final elongation). Sequencing was carried out with reagents for the preparation of libraries Nextera® XT IndexKit (Illumina, Inc.), for purification of PCR products - Agencourt AMPure XP (Beckman Coulter, Inc., USA) and for sequencing – MiSeq® ReagentKit v2 (500 cycle) (Illumina, Inc.). The maximum length of the obtained sequences was 2½250 bps.

Bioinformatic data analysis was performed using QIIME2 v. 2020.8 software (https://docs .qiime2.org/2020.8/). After importing sequences in the format .fastq from the sequencing device and the creation of matching files necessary for the work, containing metadata of the studied files, paired lines of readings were aligned. Further, the sequences were filtered by quality using the default settings. Noise sequences were filtered using the DADA2 method built into the QIIME2 package, which includes quality information in its error model, which makes the algorithm resistant to a sequence of lower quality, while using the maximum length of the trim sequence equal to 250 bps. (https://benjjneb .github.io/dada2/tutorial.html). To construct a de novo phylogeny, multiple sequence alignment was performed using the MAFFT software package (https://mafft.cbrc.jp/al-ignment/software/), then masked sequence alignment was performed to remove positions that differed significantly. The Silva 138.1 reference database was used to analyze the taxonomy (https://www.arb-silva.de/documentation/release-138.1/).

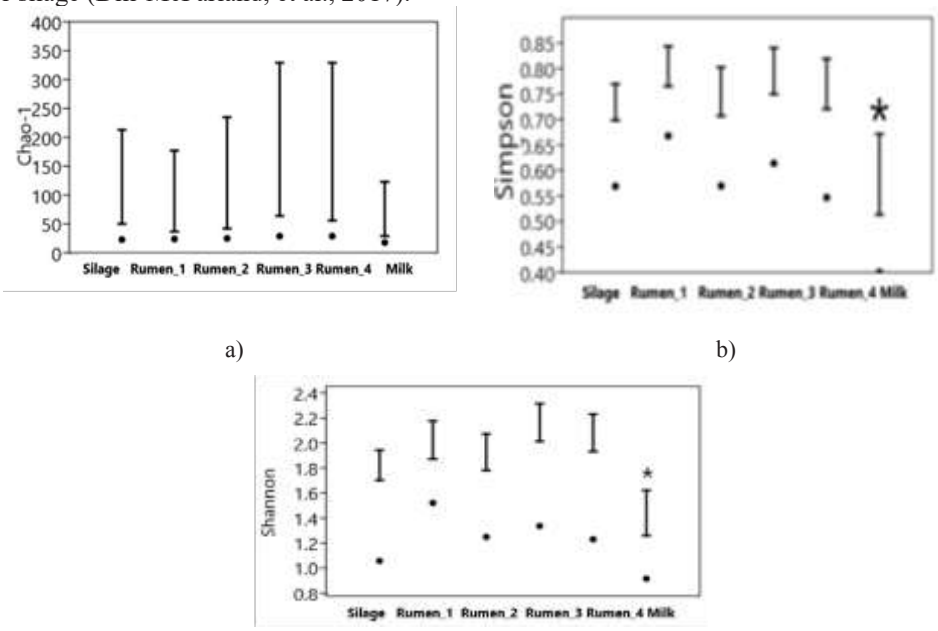
Based on the obtained table of operational taxonomic units (OTU – operational taxonomic unit), biodiversity indices were calculated using plug-ins of the QIIME2 software package. In the statistical analysis of the diversity indices, their additional transformation was not carried out.

Mathematical and statistical processing of the results was carried out by the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio v. 1.1.453 (https://rstudio.com). The results are presented as mean (M) and standard errors of mean (±SEM). The reliability of the differences was established by the Student's t-criterion, the differences were considered statistically significant at $P \leq 0.05$. The mean values were

compared using the significantly significant Tukey Difference (HSD) test and the TukeyHSD function in the R Stats Package. Cluster analysis was carried out to assess the difference in microbial profiles in the groups. The result of the cluster analysis was the constructed dendrogram. The Ward method (Bray-Curtis distance) was used to analyze the similarity (Ward, 1963).

3 Results of the study

As the results of the α-diversity analysis showed (Fig. 1), the values of the Simpson and Shannon indices were reduced in the milk microbiota compared with the microflora of the silage and the contents of the rumen of all the cows studied (P≤0.05). That is, it was demonstrated that microbial diversity was more complex in the rumen of cows and silage compared to milk. Of course, milk contains much less bacterial DNA than samples of rumen or silage (Dill-McFarland, et al., 2017).



a) b)

c)

Fig. 1. Absolute values of biodiversity indices Chao1(a), Simpson (b), Shannon (c) for the microflora of silage, rumen (experimental group Rumen_1 – on the first day after calving, group Rumen_2 – on the 20th day after calving, group Rumen_3 – on the 100th day after calving, group Rumen_4 – on 117-th days after calving) and cow's milk (2023). Calculated using plug-ins of the QIIME2 ver. 2020 software package.8. * - P≤0.05.

22 phylum of attributed microorganisms, as well as 38.5±2.34% of uncultivated bacteria were found in the microflora of silage, 24 to 30 phylum and 2.0±0.31–3.9±0.22% of uncultivated bacteria were found in the rumen microbiome, 18 phylum and 76.3±5.29% of uncultivated bacteria were found in milk.

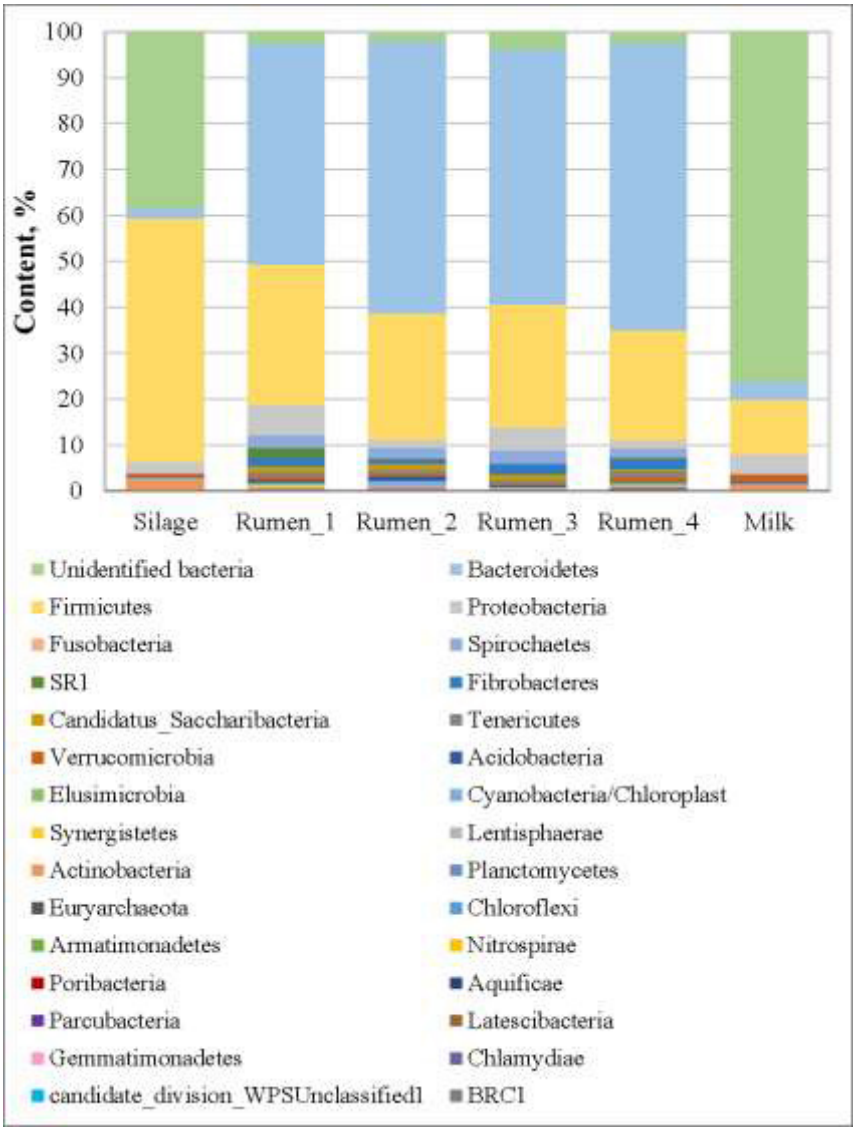


Fig. 2. The composition of the microflora of silage, rumen of cows (experimental group Rumen_1 – on the first day after calving, group Rumen_2 – on the 20th day after calving, group Rumen_3 - on the 100th day after calving, group Rumen_4 – on the 117th day after calving) and milk at the level of bacterial phylum (according to NGS-sequencing of amplicons of the 16S rRNA gene) (2023).

Similar attributed phylum were identified in silage, rumen and milk, the main difference was noted in quantitative ratios. For example, the largest number of Firmicutes was found in silage compared to other biotopes studied (52.9±3.45%, $P \leq 0.05$), which seems natural. Under anaerobic conditions, soluble carbohydrates in silage are fermented to organic acids by lactic acid bacteria of the phylum Firmicutes. As a result, lactic acid bacteria become dominant, and other undesirable microorganisms are partially suppressed (McDonald, et al., 1991). In the rumen, the number of Firmicutes was lower compared to silage ($P \leq 0.05$), but they still remained one of the dominant phylum ($P \leq 0.05$). Representatives of this taxon in the rumen play an important role in the decomposition of fiber and starch, and many of them produce

butyrate, which is associated with the health of the epithelium of the digestive system (Kim, et al., 2011). Firmicutes was the most predominant cultured phylum and in the composition of the milk microbiota (11.8±0.78%, P≤0.05). Although the specific role of these bacteria in milk has not yet been determined, gram-positive Firmicutes were previously considered as causative agents of infectious mastitis (Rodrigues, et al., 2017). On the other hand, some representatives of the phylum Firmicutes, such as Lactobacillus spp., are considered important microorganisms associated with fermentation for the production of fermented milk products (Widyastuti, et al., 2021). Earlier, researchers obtained similar data: it was shown that the most represented phylum in milk are Firmicutes, Proteobacteria, Bacteroidota and Actinobacteriota (Mary, et al., 2022).

In addition, we have shown that in the rumen of all the cows studied, Bacteroidota were found in a higher amount (P≤0.05) compared to other biotopes studied. The content of these bacteria was highest in cows of the Rumen_4 group – during the stabilization of lactation (P ≤ 0.05). The fact is that in the rumen, against the background of currently used diets rich in starch and monosaccharides, there is an increase in the number of bacteria of the Bacteroidota phylum, such as, for example, the Prevotellaceae family. It is known that these microorganisms use starch for the synthesis of volatile fatty acids (VFA) and lactate, however, excessive production of lactic acid and short-chain fatty acids is associated with a decrease in the pH of the rumen and can lead to lactate acidosis accompanied by dysbiosis (Meissner, et al., 2017). If at the beginning of lactation there is a danger of insufficient provision of cows with energy and other nutrients, then in the second phase (stabilization of lactation) there is a danger of excessive energy consumption due to advanced feeding. Violation of the microflora during this period leads to obesity of cows and metabolic diseases.

At the level of genera, it was confirmed that similar taxa were identified in the composition of the microbiota of the silage that was fed to cows, as well as in the microflora of the contents of their rumen and milk, among which conditionally pathogenic and pathogenic microorganisms are often found (Fig. 2). Thus, bacteria of the genera Mycoplasma, Mycobacterium, Porphyromonas, Treponema, Pseudomonas, Haemophilus, Staphylococcus, Acinetobacter, Streptococcus, Fusobacterium, Clostridium_sensu_stricto, Escherichia/Shigella were detected in silage, rumen and milk of cows.

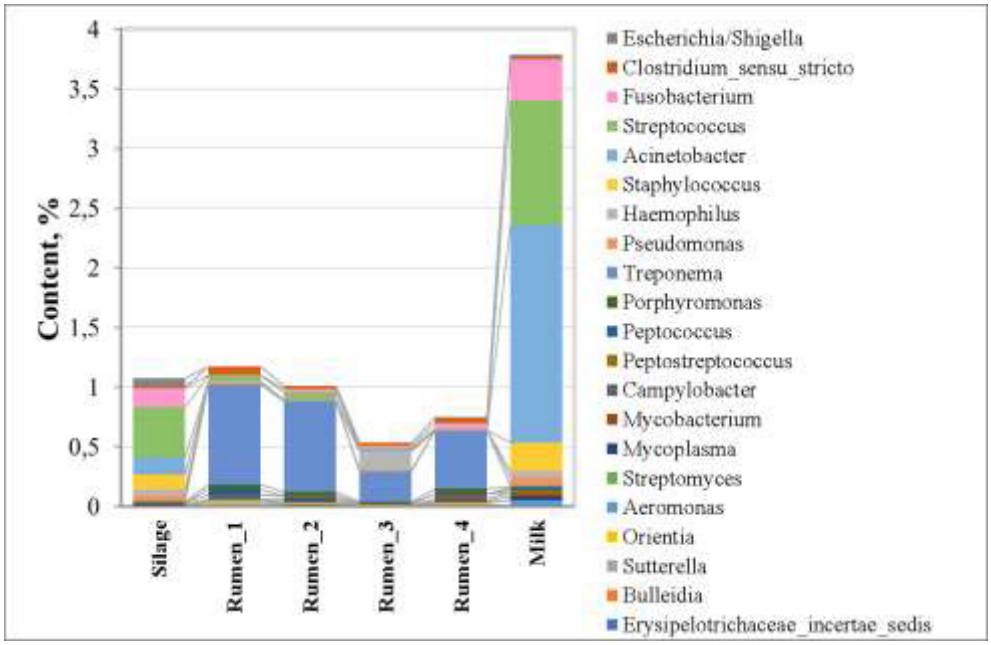


Fig. 3. The composition of conditionally pathogenic and pathogenic microflora of silage, rumen (experimental group Rumen_1 - on the first day after calving, group Rumen_2 – on the 20th day after calving, group Rumen_3 – on the 100th day after calving, group Rumen_4 – on the 117th day after calving) and cow milk at the level of bacterial genera (according to NGS sequencing of amplicons of the 16S rRNA gene) (2023).

At the same time, the genera *Staphylococcus, Acinetobacter, Streptococcus* and *Fusobacterium* were identified by us as the most represented ($P \leq 0.05$) in the composition of the milk microflora. Their content was 0.24±0.023, 1.8±1.23, 1.0±0.06 and 0.35±0.031%, respectively.

Streptococcus spp. previously was detected both in milk samples from healthy cows and in animals with mastitis (Quigley, et al., 2013).

Acinetobacter spp. is an important microorganism that causes diseases in humans, which can adapt to various environmental conditions. Representatives of *Acinetobacter* are often found in the milk of healthy cows and may be involved in the development of mastitis in cattle, if favorable conditions arise for the development of the pathogen (Bonsaglia, et al., 2017). From the point of view of consumer health protection, it is important to prevent the ingress of zoonotic pathogens of animal origin into livestock products (Hörügel, 2001).

Fusobacterium spp. is found in the rumen under acidosis conditions. This microorganism provokes necrobacteriosis of the rumen, liver, hooves, and also participates in the occurrence of mastitis (Nocek, 1997). Earlier, we detected the presence of *Fusobacterium* spp. in silage samples of a 30-day fermentation period (Yildirim, 2019). The survival of these microorganisms in silage is probably due to their resistance to low pH values and the use of lactic acid synthesized by lactobacilli as a nutrient substrate.

It is interesting to note the presence of *Clostridium* spp. in all the samples examined. Clostridium growth requires relatively high pH values (> 4.5), high feed moisture (>70%) and high water activity (from 0.952 to 0.971); therefore, they are suppressed in silos if the pH level drops to 4 or lower within 3 days (Muck et al., 2003). Consequently, factors that predispose to high humidity of the plant mass during harvesting or silage, or that restrain the pH decrease during silage, may contribute to the growth of clostridium in the silage.

Botulism, which can be caused by clostridia, is a serious disease in both humans and animals, the average mortality rate reaches 5-10% in humans and 10% in cattle (Payne et al., 2011). Earlier, during an outbreak of cattle botulism that occurred on several farms in the Netherlands, the content of *C. botulinum* type B and botulinum toxin type B was detected in siloed beer pellets and in grass silage on one of these farms (Notermans et al., 1981). Dry infant formula containing *C. botulinum* spores turned out to be the source of a case of childhood botulism in 2001 (Brett et al., 2005). Based on the results of our research and the data previously provided, it should be noted that dairy products, especially those used for the production of infant formulas, require special attention regarding the appearance of clostridium due to the potential risk of the disease.

It is also important to note the presence of *Mycobacterium* spp. in silage, rumen of some cows and milk. One of the representatives of the genus - *Mycobacterium bovis* - is the causative agent of bovine tuberculosis, which is a zoonotic disease of global concern due to the growing number of infected animals in herds, especially in developing countries, and its adverse effects on human health.

When clustering the results of NGS sequencing of samples (Fig. 4), two clusters were formed: the first consisted entirely of samples taken from the rumen of cows (it can also be divided into two subclusters), and the second cluster included samples of milk and silage. Based on the analysis of the dendrogram, it is possible to conclude about a certain level of similarity of the bacterial profiles of the microbiota of silage and milk.

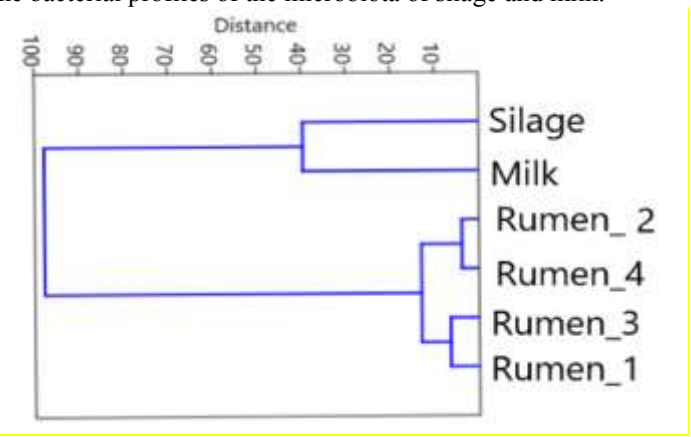


Fig. 4. Hierarchical clustering by the spectrum of microorganisms of silage, rumen (experimental group Rumen_1 – on the first day after calving, group Rumen_2 – on the 20th day after calving, group Rumen_3 – on the 100th day after calving, group Rumen_4 – on the 117th day after calving) and milk (according to NGS sequencing of amplicons 16S rRNA gene), the Ward method (Bray-Curtis distance), where each endpoint of the dendrogram branch represents the composition of the microflora of the sample, the length of each branch characterizes the measure of similarity between the samples under consideration: the more homogeneous the groups, the shorter the branch length (2023).

4 Conclusion

Similar taxa of microorganisms were identified in silage, rumen and milk, the main difference was noted in quantitative ratios. This suggests that there may be some relationship between these compartments. Interestingly, pathogenic microorganisms, including the causative agents of mastitis, were present in many samples. Probably, the microflora of the rumen can influence the formation of the milk microbiota, which partially confirms the previously stated

enteromammary theory (Rodríguez, et al., 2014; Hu, et al., 2022). Previously, Ma et al. (Ma et al., 2018) successfully induced inflammation in the mammary gland by transplanting feces from mastitis cows to mice without bacteria. This showed that the microbiota of the digestive system is an important cause of mastitis. The presence of *Fusobacterium* spp. in the rumen and in milk, which are companions of lactate acidosis, may indicate both fecal and hematogenic transmission of bacterial infection, since lactate acidosis is associated with damage to the rumen epithelium, as well as liver abscesses (Kleen et al., 2003). The transfer of microorganisms to milk can also occur during milking.

Thus, in conditions of intensive animal husbandry and a decrease in adaptive potential, cows may have pronounced stress reactions, in which animals become more susceptible to infections caused by potentially infectious bacteria contained in feed and in the body. The obtained results provide an experimental basis for the prevention of animal and human diseases by regulating the rumen microbiota in dairy cows and feed microbiota.

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