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THE RUMEN MICROBIOTA OF REINDEER (*Rangifer tarandus*) WITH CLINICAL MANIFESTATIONS OF NECROBACTERIOSIS

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Abstract

Necrobacteriosis is an infectious disease that affects many species of domestic and wild mammals, birds, and humans. The main clinical manifestations of the disease are associated with the development of purulent-necrotic lesions of the skin, mucous membranes, internal organs, and extremities as a result of infection of the host organism by strictly anaerobic pathogenic fuzobacteria, the *Fusobacterium necrophorum*. For reindeer herding, the problem of necrobacteriosis among other diseases of reindeer is one of the most significant, since it brings the most significant damage to the economic activity of the population of the Russian Arctic. This paper for the first time shows the obtained results on rumen microbiota composition differences between the clinically healthy *Rangifer tarandus* reindeer of the Russian Arctic and reindeer with necrobacteriosis. The purpose of the study was to characterize the distinctive features of rumen microbiota in the reindeer with the clinical manifestations of necrobacteriosis. The study was carried out on calves (4-6 months) and adults (3-6 years) animals, including clinically healthy individuals and those with necrobacteriosis. Samples of the rumen content were collected during the summer-autumn period in 2017 ($n = 3$ from each age group) in the Yamalo-Nenets Autonomous District. The total number of bacteria and fungi of the class *Neocallimastigales* was analyzed by quantitative PCR, the composition of the bacterial community was analyzed by T-RFLP (terminal restriction fragment length polymorphism) method. In individuals with clinical manifestations of necrobacteriosis, a significantly higher content of fusobacteria was detected, 1.79-fold in adults ($p < 0.05$), and 2.65-fold in calves ($p < 0.05$). In sick animals of both age groups, there was a significantly higher presence of bacteria of the genus *Staphylococus* ($p < 0.05$) and the family *Pseudomonadaceae* ($p < 0.05$), some species of which may cause purulent-necrotic lesions of animals. The 4-6 month old calves showed a significant increase ($p < 0.05$) in the content of family *Campylobacteriaceae* and family *Enterobacteriaceae* compared to clinically healthy animals. At the same time healthy individuals showed a greater number of cellulolytic and acid-utilizing bacteria. In general, it was noted that the rumen microbiome of calves with clinical signs of necrobacteriosis is characterized by large changes compared to the adult animals. In particular, in young reindeer with necrobacteriosis, there was a significant increase in the Shannon's diversity index of the rumen microbial community ($p < 0.05$), which indicates a greater heterogeneity of the bacterial community compared to healthy individuals. In addition, a significant ($p < 0.05$) decrease of cellulolytic chytridiomycetes of the class *Neocallimastigales* was detected in the rumen of this animal group. In this regard, the identified patterns may be due to the physiological features of this

stage of animal development in *Rangifer tarandus*. The obtained results can be a basis for recommendations to improve anti-necrobacteriosis measures in reindeer and to reduce mortality during summer and autumn period.

Keywords: *Rangifer tarandus*, reindeer, necrobacteriosis, laminitis, Fusobacteria, T-RFLP analysis, quantitative PCR, rumen microbiome, Russian Arctic

Infectious and invasive diseases are constantly recorded in the natural area of reindeer (*Rangifer tarandus*) [1]. They cause significant damage to reindeer husbandry which is strategically important for the population of the Far North. Parasitic diseases transmitted by blood-sucking insects, water infestations (edemagenosis, cephenomyiosis) and necrobacteriosis, an infectious bacterial disease, are of particular threat. The proportion of animals with necrobacteriosis averages from 7% to 33%. Necrobacteriosis is most pronounced in summer, when the number of sick animals can reach 65-70% [2].

Necrobacteriosis affects many species of domestic and wild animals, birds and humans. The main clinical manifestations of the disease are purulent necrosis of the skin, mucous membranes, and internal organs as a result of infection by strictly anaerobic pathogenic fusobacteria, mainly *Fusobacterium necrophorum* [3, 4]. The limbs are affected in ruminants. In reindeer, purulent lesions of hooves are noted only in the summer-autumn period [2, 5], mainly in July, August, with the onset of heat. In September, the number of diseases decreases sharply, and in October new cases are no longer recorded. This is due to the creation of optimal seasonal conditions for the development of infections, i.e. positive air temperatures, animal exhaustion, and insect activity. Outbreaks of necrobacteriosis occur both in the Russian Arctic [5] and in other areas of reindeer habitat [6].

Necrobacillosis is well-studied in cattle [6, 7], while studies on reindeer are much fewer. Recently, the possibility of interrelations between the causative agent of necrobacteriosis and the microflora of other ecotopes of the animal organism, in particular, the rumen, a being most actively discussed [8, 9].

Many authors believe that the health of ruminants is highly dependent on rumen digestion and is therefore ensured by the presence of certain microorganisms in the rumen [10]. According to modern concepts, *F. necroforum* belongs to the normal flora of the digestive tract of ruminants, especially the rumen, and can spread in the environment through excrement [6, 11]. It is known that fusobacteria are able to secrete a number of toxins that lead to tissue necrosis and the occurrence of secondary infections caused by actinobacteria. It has been reported that fusobacteria are able to penetrate only into damaged tissues, for example, with necrotic lesions of the extremities [12]. It has also been established that fusobacteria enter the animal through hemorrhagic lesions in the digestive tract [7].

The reasons of an increased reindeer susceptibility to infection with fusobacteria have not been studied much. Apparently, stress, high or low temperature, overpopulation, and poor nutrition can provoke it [12, 13]. Thus, Norwegian researchers, having studied the outbreak of necrobacteriosis in reindeer living in the north of Norway (2007-2008), confirmed that the *F. necroforum* was the causative agent of the infection, and concluded that the disease was provoked by the higher (compared to medium) values of temperature and humidity [6].

Biodiversity of pathogenic and conditionally pathogenic bacteria in the reindeer rumen under necrobacteriosis is practically not described.

This paper is the first report on the differences between the composition of rumen microbiota in clinically healthy reindeer in the Arctic zone of the Russian Federation and animals with manifestations of necrobacteriosis. Individuals with symptoms of necrobacteriosis have fewer cellulose-decomposing and acid-

reclaiming bacteria and more abundant fusobacteria and other pathogens (staphylococcus, pseudomonads, etc.), some of which may cause purulent necrotic lesions in animals. The most pronounced changes in the composition of the rumen microbiome as a result of necrobacteriosis were detected in 4-6-month-old calves in which the rumen bacterial community was more heterogeneous and characterized by increased counts of campylobacteria, enterobacteria, and decreased representation of *Chytridiomycetes* fungi.

The aim of the work was molecular analysis of the microbiota composition in the rumen of reindeer with clinical manifestations of necrobacteriosis.

Techniques. Samples of the rumen content were collected via a probe from 4-6-month-old calves and 3-6-year-old adult reindeer (*Rangifer tarandus*) of the Nenets breed (clinically healthy animals and individuals with manifestations of necrobacteriosis; $n = 3$ from each age group) in the summer-autumn period in 2017 in the Yamalo-Nenets Autonomous Area (Kharp urban settlement, forest-tundra natural climatic zone). Samples were frozen at –20 °C until analysis.

Total DNA from the samples was isolated using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) in accordance with the manufacturer's recommendations. The final concentration of the isolated total DNA in the solution was determined on a Qubit fluorimeter (Invitrogen, Inc., USA) with Qubit dsDNA BR Assay Kit (Invitrogen, Inc., USA) as per the manufacturer's instructions.

The total number of bacteria and *Chytridiomycetes* fungi of the *Neocallimastigales* class was assessed by quantitative PCR (DT Lite-4 amplifier; NPO DNA-Technology LLC, Russia) with a set of reagents for qPCR in the presence of EVA Green intercalating dye (Syntol CJSC, Russia). The following primers were used: F — 5′-ACTCCTAC-GGGAGGCAGCAG-3′, R — 5′-ATTACC-GCGGCTGCTGG-3′ (bacteria); F — 5′-GCACTTCATTGTGTGTACTG-3′, R — 5′-GGATGAAACTCGTTG-ACTTC-3′ (fungi). Amplification mode: 3 min at 95 °C (1 cycle); 13 s at 95 °C, 13 s at 57 °C, 30 s at 72 °C (40 cycles).

The composition of the bacterial community of the reindeer rumen was analyzed by T-RFLP (terminal restriction fragment length polymorphism) [14]. PCR was performed on a Verity DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) labeled at the 5′-end (WellRed D4 fluorophore, Beckman Coulter, USA) and 1492R (5′-TACGGHTACCTTGTTACGACTT-3′), which allow amplification of a 16S rRNA gene fragment (from position 63 to 1492; numbering is indicated for *Escherichia coli* 16S rRNA gene), in the following mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 cycles); 5 min at 72 °C.

The fluorescently labeled amplicons of the 16S rRNA gene fragment were purified by the standard method [15]; 30-50 ng DNA was treated with restriction enzymes HaeIII, HhaI and MspI (Fermentas, Lithuania) for 2 h at 37 °C. Restriction products were precipitated with ethanol, then 0.2 μl of Size Standart-600 molecular weight marker (Beckman Coulter, USA) and 10 μl of Sample Loading Solution formamide (Beckman Coulter, USA) were added. The analysis was performed on CEQ 8000 (Beckman Coulter, USA) according to the manufacturer's recommendations (instrument error not more than 5%). The data obtained were processed with Fragment Analysis software (Beckman Coulter, USA). The height of the peaks and their area were calculated, based on which subtypes (phylotypes) were identified with an error of 1 nucleotide accepted in the study, and their proportion in the microbial community was estimated.

The taxonomic affiliation of bacteria was determined using the database (http://mica.ibest.uidaho.edu/trflp.php).

Statistical processing of the results was carried out by the dispersion analy-

sis method using the Microsoft Excel 2010 software. The mean (*M*) and the standard error of the mean (±SEM) were calculated, the significance of differences between the groups was evaluated by Student's *t*-test. The Past program calculated the Shannon and Simpson biodiversity indices (http://folk.uio.no/ohammer/past/).

Resultes. According to the assessment of the bacterial community by the T-RFLP method, most of the phylotypes belonged to the *Firmicutes* phylum (their total number reached 61% in adult reindeer). To a lesser extent, bacteria of the phyla *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* were found in the rumen microbiome of clinically healthy animals and individuals with symptoms of necrobacteriosis. Members of the *Fusobacteria*, *Acidobacteria*, and *Cyanobacteria* phyla comprised a minor amount. Also, there was significant proportion of phylotypes that could not be identified from the databases. The amount of unidentified taxa was the largest (up to 22.18%) in adults.

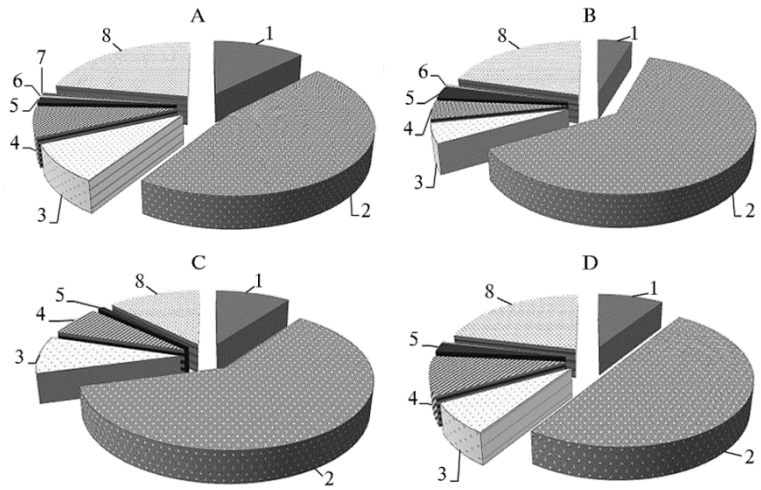

Fig. 1. Bacterial phyla in the reindeer (*Rangifer tarandus*) rumen community: A — adult individuals, B — adult individuals with clinical signs of necrobacteriosis, C — calves, D — calves with clinical signs of necrobacteriosis; 1 — phylum *Bacteroidetes*, 2 — phylum *Firmicutes*, 3 — phylum *Actinobacteria*, 4 — phylum *Proteobacteria*, 5 — phylum *Fusobacteria*, 6 — phylum *Cyanobacteria*, 7 — phylum *Acidobacteria*, 8 — unclassified nucleotide sequences (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

The rumen microbiome of the animals with clinical manifestations of necrobacteriosis differed notably from that of healthy individuals. First, there should be noted a significantly higher presence of the *Fusobacteria* phylum in reindeer with symptoms of necrobacteriosis (Fig. 1), 1.79-fodl ($p < 0.05$) in adults and 2.65-fold ($p < 0.05$) in calves. The results obtained once again confirm the role of bacteria of the *Fusobacterium* genus (including *F. necrophorum*) in the etiology of necrobacteriosis.

Nevertheless, there are reports whose authors expressed doubts about this or provided evidence of the main role of other types of microorganisms in the etiology of necrobacteriosis in ruminants [16]. In this connection, the fact of joint presence and growth of a number of pathogens, including pathogens of purulent-necrotic infections, in the rumen of the individuals with symptoms of necrobacteriosis is of interest. Thus, in both age groups of reindeer with necrobacteriotic manifestations, the proportion of bacteria of the genus *Staphylococcus* ($p < 0.05$) and the family of *Pseudomonadaceae* ($p < 0.05$) significantly increased, some of which can cause purulent-necrotic infections of animals. Moreover, we revealed a 1.96-fold ($p < 0.05$) and 2.38-fold ($p < 0.05$) increase in campylobacteria (*Campylobacteriaceae* family) and enterobacteria (*Enterobacteriaceae*

family) populations, respectively, in 4-6-month-old calves as compared to clinically healthy animals.

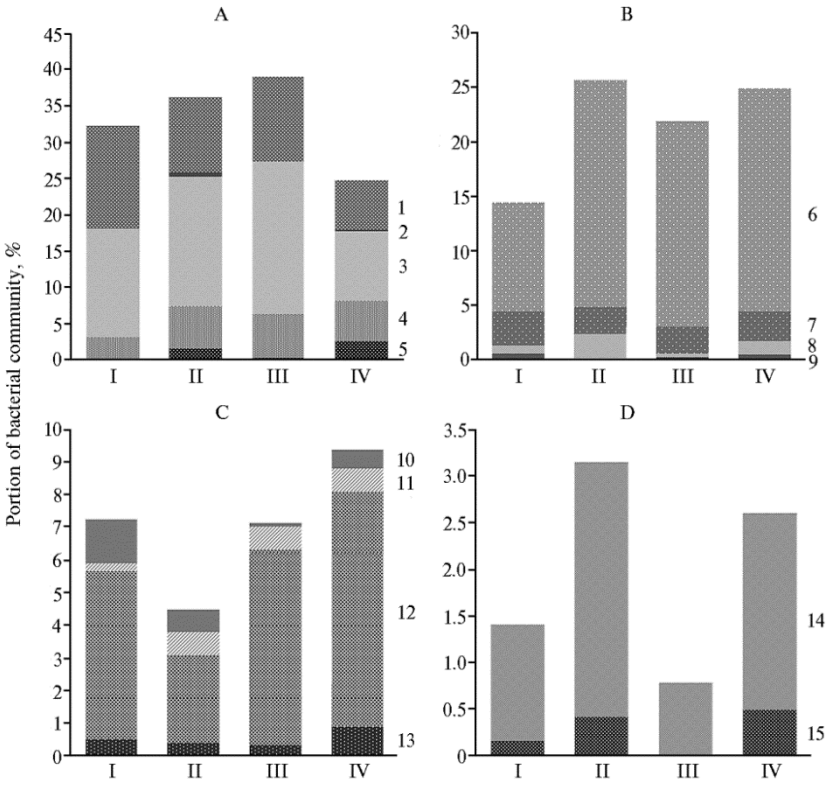

Fig. 2. Representation of bacterial taxa in rumen community of reindeer (*Rangifer tarandus*): I — adult individuals, II — adult individuals with clinical signs of necrobacteriosis, III — calves, IV — calves with clinical signs of necrobacteriosis (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

A — class *Clostridia* of phylum *Firmicutes*: 1 — family *Thermoanaerobacteraceae*, 2 — family *Lachnospiraceae*, 3 — family *Eubacteriaceae*, 4 — family *Ruminococcaceae*, 5 — family *Clostridiaceae*.

B — other *Clostridia* members of phylum *Firmicutes*: 6 — order *Negativicutes*, 7 — genus *Bacillus*, 8 — genus *Lactobacillus,* 9 — genus *Peptococcus*.

C — phylum *Proteobacteria*: 10 — family *Burkholderiaceae*; 11 — family *Pseudomonadaceae*, 12 — family *Campylobacteriacea*e, 13 — family *Enterobacteriaceae*.

D — others: 14 — phylum *Fusobacteria*, 15 — genus *Staphylococcus*.

The obtained results confirm reports on the polyetiological nature of necrobacteriosis. Smith et al. [17, 18] showed the presence of microorganisms concomitant to fusobacteria, including enterobacteria and actinobacteria, which complicate the course of necrobacteriosis. The same opinion was held by Petrov and Tashev (quoted in [3]), who during the examination of 134 reindeer with purulent limb lesions revealed a blue pus bacillus (*Pseudomonas aeruginosa*), streptococcus (*Streptococcus* genus), staphylococcus (*Staphylococcus* genus) and their associations. Laishev et al. [5] report the isolation of micrococci, *E. coli*, staphylococcus, Proteus together with fusobacteria in experimental infection of reindeer with a pure culture of necrobacteriosis pathogen. According to Nocek [7], proliferation of pathogens living on the extremities of cattle (fusobacteria, staphylococcus, streptococcus, enterobacteria), along with an increase in blood levels of endotoxins and histamine (products of scarlet microbial lysis) and damage to blood vessels causes inflammation and necrosis of the dermal layers of hooves. Ostrovsky et al. [19] explained the mass spread of limb diseases in reindeer by the violation of the management technology, leading to injuries and

maceration of the skin, weakening its protective properties, followed by the introduction of microflora in the tissue.

In our work, the bacterial community of the rumen of clinically healthy animals differed from that of individuals with symptoms of necrobacteriosis by a high percentage of microorganisms involved in the fermentation of carbohydrates in plant feeds. For example, in calves with symptoms of necrobacteriosis, the proportion of *Clostridia* class bacteria (including those of the *Lachnospiraceae*, *Eubacteriaceae* and *Clostridiaceae* families), potentially capable of fermentation of plant feed polysaccharides with the formation of volatile fatty acids, was 1.32 times lower ($p < 0.05$). In addition, the number of *Bacteroidetes* phylum bacteria (including *Bacteroides*, *Prevotella*), which ferment starch, fiber, a number of other carbohydrates, proteins, and deaminate acids, was significantly lower ($p < 0.05$) in adults and young animals compared to healthy ones. Less calves had bacteria of the *Negativicutes* order (*Megasphaera*, *Selenomonas* genera), which prevent the pH reduction and lactate acidosis due to the utilization of organic acids (propionic, acetic, oil, lactic, etc.) formed during fermentation of plant tissue [7, 20, 21].

The calculation of environmental indices also demonstrated a general decrease in biodiversity in the rumen of animals with clinical manifestations of necrobacteriosis (Fig. 3). In particular, the Shannon index increases ($p < 0.05$) in young animals with necrobacteriosis, which indicates a greater heterogeneity of the bacterial community of their rumens compared to healthy individuals.

It should be noted that the total number of bacteria in healthy and diseased individuals did not differ significantly (Fig. 4). However, a significant decrease ($p < 0.05$) in the number of chytridiomycetes, which are known to produce a wide range of multifunctional polysaccharide enzymes, was revealed in the rumen of the young reindeer [22].

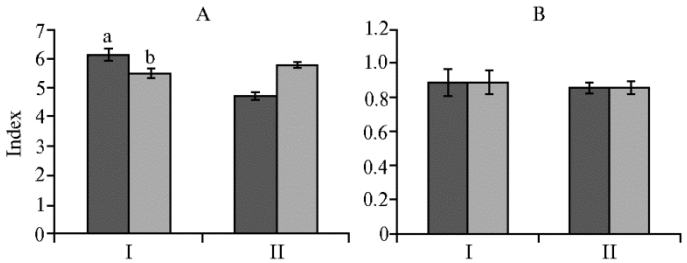

Fig. 3. Biodiversity of the rumen bacterial community in adult reindeers (*Rangifer tarandus*) (I) and calves (II): A — Shannon index, B — Simpson index; a — clinically healthy animals, b — animals with clinical signs of necrobacteriosis (*M*±SEM, (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

Previously, a microbiotic imbalance in the digestive tract under necrobacteriosis infection was reported to lead to a faster proliferation of the pathogenic strain of fusobacteria [23, 24]. In addition, infection of cattle with fusobacteria may occur as a result of the lactate acidosis syndrome, which is characterized by abnormal rumen microbiota composition [4, 7]. At the same time, the number of cellulose-decomposing and acid-recycling bacteria decreases in the cattle rumen; the number of *Streptococcus* and *Lactobacillus* genera synthesizing lactates increases, which reduces the rumen pH [25]. Lactate acidosis leads to damage to the rumen epithelium and penetration of pathogenic fusobacteria through the mucous membrane into the bloodstream. In turn, this leads to further infection of the body, in which, depending on the biotype of the pathogen (A, B or AB), a corresponding clinical picture is observed (lesions of hooves, mucous membranes or skin, liver abscesses or other internal organs). Simultaneous isolation of fusobacteria from other organs (liver, kidneys) and the rumen

has been repeatedly shown for the cattle [7, 8]. Interestingly, there was no increase in the representation of lactobacillus in the rumen of the reindeer we surveyed, which indicates the need to continue studying the relationship between the rumen microbiome and the development of necrobacteriosis. For example, the causes of necrobacteriosis in reindeer may be contaminated feed or the penetration of microorganisms through the respiratory tract. The latter was shown in the United States during outbreak of necrobacteriosis caused by lesions of the respiratory tract of white-tailed deer (*Odocoileus virginianus*) by fusobacteria *F. necrophorum* and *F. varium* [8, 26].

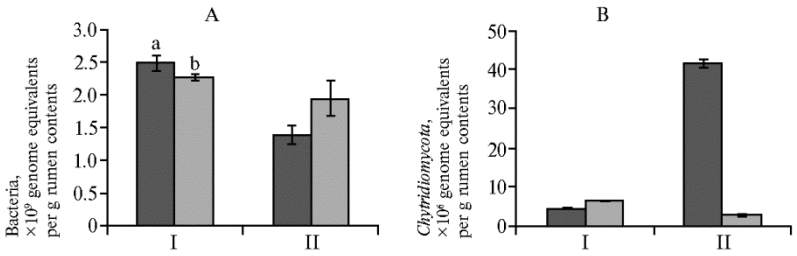

Fig. 4. The counts of microorganisms in rumen of adult reindeers (*Rangifer tarandus*) (I) and calves (II): A — bacteria, B — chytridiomycetes; a — clinically healthy animals, b — animals with clinical signs of necrobacteriosis (*M*±SEM, (Yamal-Nenets Autonomous Area, urban settlement Kharp, 2017).

Thus, in reindeer with manifestations of necrobacteriosis the composition of rumen microbiome is significantly disturbed compared to clinically healthy animals. The latter had more cellulose-decomposing and acid-utilizing bacteria and less fusobacteria and other pathogens (*Staphylococcus*, *Pseudomonas*, etc.). The rumen microbiome in calves with clinical signs of necrobacteriosis is characterized by more significant changes than in sick adults. These are expressed in greater heterogeneity of the bacterial community, increased representation of campylobacteria, enterobacteria, as well as a decrease in the number of chytridiomycetes. Therefore, the revealed regularities could be conditioned by physiological peculiarities of this stage of development in reindeer. The obtained results can be a base for recommendations on effective protection against reindeer necrobacteriosis and reducing mortality.

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