Composition and functions of rumen and endometrial microorganisms associated with endometritis in dairy cows

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Abstract: Despite the attention given in the recent years to the microbiological causes of endometritis and the potential sources of uterine microbiota infestation, more questions than answers remain in this research field. This paper describes an experiment carried out at the livestock farm of the Leningrad region on 6 dairy cows of the Holsteinized Russian Black Pied breed of the second lactation in the period after calving. The animals were divided into two groups (n = 3): Group 1 included clinically healthy animals, Group 2 included animals diagnosed with purulent-catarrhal post-calving endometritis. Metagenomic sequencing was performed using the MiSeq genomic sequencer (Illumina, Inc., USA) with the MiSeq Reagent Kit v3 (Illumina, Inc., USA). Based on the next-generation sequencing of microbiota of endometrial scrapings, 7 phyla of microorganisms were detected in clinically healthy cows and only 4 phyla of microorganisms in cows diagnosed with endometritis. The increase in the proportion of Fusobacteriota taxon bacteria permanently present in endometrial scrapings and the decrease in Bacteroidota phylum bacteria in the group of animals with endometritis could be related to the occurrence of this disease. Bacteria Alloprevotella, Campylobacter, Caviibacter, Falsiporphyromonas, Veillonella present only in the endometrial tissue of sick cows may be the etiological origin of endometritis. In the rumen of animals with endometritis, there was an increase in Bacteroidota phylum microorganisms ($p \leq 0.05$) against a decrease in Firmicutes phylum bacteria ($p \leq 0.05$) compared to the clinically healthy group. Using the PICRUSt2 software package (v.2.3.0), it was shown that the microbiome of cows diagnosed with endometritis showed inhibition ($p \leq 0.05$) of the potential of 9 metabolic pathways compared to healthy animals. This could have negative consequences for the body of animals and be a consequence of metabolic disorders.

1 Introduction

The herd reproduction is one of the major issues hindering the development of dairy cattle breeding. The share of losses due to decreased reproductive capacity of cows exceeds 70%

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of all lost profits from production activity of dairy farms. The analysis of the causes of barrenness indicates the dominance of obstetric and gynecological pathology, among which post-calving endometritis occupies the leading position. According to statistics [1, 2], the incidence of clinical endometritis in cows in the post-calving period ranges from 7.5 to 61.6%. Endometritis is directly associated with increased service period, decreased percentage of fertilization, increased insemination index, decreased probability of pregnancy and increased embryonic mortality. This pathology often leads to the culling of animals and, consequently, to a decrease in the profitability of agricultural enterprises.

The occurrence of postpartum endometritis is promoted by high milk productivity, errors in feeding and keeping during the dry and transit periods. These circumstances can cause the failure of internal pathogenetic mechanisms that depress the functional activity of the reproductive system and to a total violation of the fragile balance of the reproductive system microbiota. According to the data, over 50% of cows of Holstein breed with productivity of over 9000 kg of milk in 305 days of lactation have clinical endometritis in the post-calving period.

Despite the attention given in the recent years to the microbiological causes of endometritis [3-5], more questions than answers remain in this research field. There is a steady emergence of new epidemic forms, masking of pathogens because of interspecific recombination, return of eradicated infections due to the entry of pathogens from epidemiologically unfavorable regions of the world, etc.

Appiah S.M. et al. [6] conducted a modern review of reproductive tract microbiota and concluded that the information about the potential sources of uterine microbiota colonization is rather scarce and contradictory. The available assumptions [7-9] certainly require clarification and additional evidence, so it is interesting to study the composition of rumen microbiome of cows with endometritis and without pathologies.

This study aimed to analyze the composition and functional features of the bacterial communities of the rumen and endometrium of cattle under normal and pathological conditions in the period after calving.

2 Materials and methods

The experiment was conducted in summer 2020. Examined were 6 cows (*Bos taurus*) of the dairy Holsteinized Russian Black Pied breed of the second lactation. The period after calving (average 23 days after calving) was studied. The average daily milk yield of cows was 27.0 L/head. The experiments met the requirements of the European Convention for the protection of vertebrate animals used for experiments or other scientific purposes (ETS №123, Strasburg, 1986). Animals were kept under the same conditions in tethered housing. The animals were divided into two groups (n = 3): Group 1 included clinically healthy animals; Group 2 included animals diagnosed with purulent-catarrhal post-calving endometritis (diagnosed on the 21st day after calving by standard methods of clinical diagnosis). Scrapings from endometrium and rumen were sampled under aseptic conditions as much as possible. The treatment of the cows was the same, none of the cows received antibiotics before sampling.

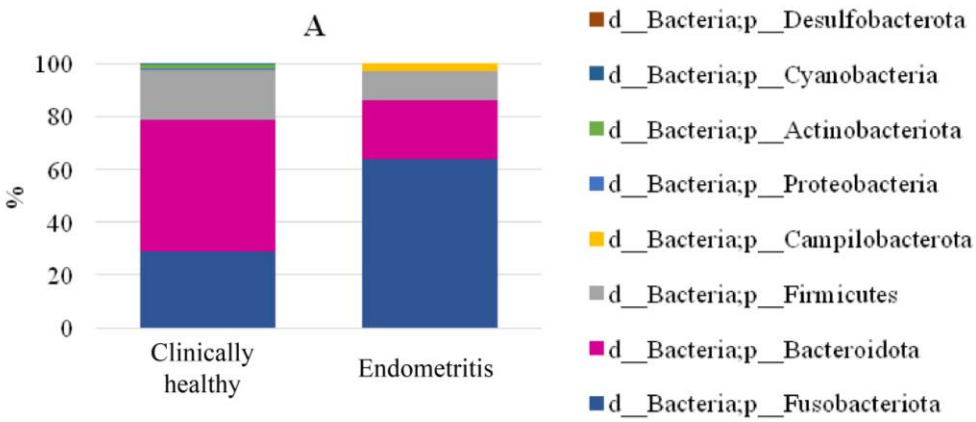
Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. Amplification for subsequent next-generation sequencing (NGS) was performed using the Verity DNA amplifier (Life Technologies, Inc., USA) using the eubacterial primers 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTACNVGGGGTWTCTAAT-3′), flanking the V1-V3 region of the 16S rRNA gene. Metagenomic sequencing was performed using the MiSeq genomic sequencer (Illumina, Inc., United States) with the MiSeq Reagent Kit v3 (Illumina, Inc., United States). The maximum length of the sequences obtained was

2x250 bp. Bioinformatic analysis of data was performed using Qiime2 software ver. 2020.8 (https://docs.qiime2.org/2020.8/). After the initial import of sequences into the Qiime2 format, paired-end reads were aligned. Noise sequences were filtered using the Deblur method, using a maximum trimming sequence length of 250 bp (https://msystems.asm.org/content/msys/2/2/e00191-16.full.pdf). The MAFFT software package was used to construct the de novo phylogeny, followed by masked sequence alignment. The Silva 138 reference database (https://www.arb-silva.de/documentation/release-138/) was used to analyze the taxonomy. Reconstruction and prediction of the functional content of the metagenome were performed using the PICRUSt2 software package (v.2.3.0)

Mathematical and statistical processing of results was performed by One-way ANalysis Of VAriance (ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). The results are presented as mean values (M) and standard errors of the mean (±SEM). The results were considered statistically significant at $p \leq 0.05$. Student's t-test was used to assess reliability.

3 Results and Discussion

The results of next-generation sequencing of microbiota of endometrial scrapings showed the presence of 7 phyla of microorganisms in clinically healthy cows and only 4 phyla of microorganisms in cows diagnosed with endometritis. Fusobacteriota, Bacteroidota, and Firmicutes were the dominant phyla in both groups of animals. At the same time, the ratio between these phyla was different. Thus, the number of Fusobacteriota was 34.6% higher and the number of Bacteroidota was 27.4% lower in cows diagnosed with endometritis compared to the group of clinically healthy animals ($p \leq 0.05$) (Fig. 1A). These data are consistent with previous studies. Most papers [10, 11] reported that reproductive organs of cows with endometritis and clinically healthy had the same dominant bacterial phylotypes, mainly Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria.



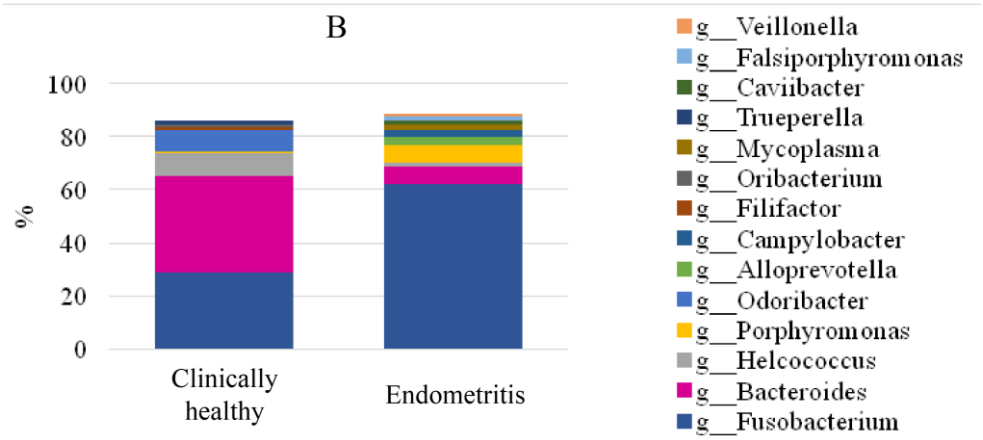


Fig. 1. Composition of the microbiota of scrapings from the endometrium of cows according to NGS of 16S rRNA amplicons: A - at the phylum level, B - at the genus level, significantly ($p \leq 0.05$) different between the groups. Clinically healthy group includes clinically healthy animals (n = 3), Endometritis group includes animals diagnosed with purulent-catarrhal post-calving endometritis (n = 3)

Within the phylum Bacteroidota, bacteria *Odoribacter* spp. completely disappeared in the microbiota of cows diagnosed with endometritis, whereas in the group of healthy cows their content was 7.8±0.45%. This is explained by the fact that the main fermentation products of bacteria of this genus are acetic, propionic, succinic acids, butyric, isovaleric short-chain fatty acids [12], which have a wide range of health effects on epithelium, such as maintenance of barrier function and homeostasis, manifestation of anti-inflammatory and immunomodulatory activity in the mucosa, and suppression of pro-inflammatory cytokines [13]. Probably, dysbiotic disorders associated with the displacement of beneficial microorganisms *Odoribacter* spp. from the microbiota could be associated with the occurrence of endometritis.

At the same time, the microbiota of cows with endometritis contained new microbial genera that were absent in clinically healthy animals: *Alloprevotella* - 2.9% *Campylobacter* - 2.9%, *Caviibacter* - 1.8%, *Falsiporphyromonas* -1.4%, *Veillonella* - 1.4%. Previously, most researchers [14] did not find an exclusive association of a particular bacterial taxon with reproductive diseases of cows: no specific causative agents of endometritis could be identified. Most studies reported that reproductive organs of both diseased and clinically healthy cows had the same dominant bacterial taxa, and the difference was only in the ratios. However, the authors [15] recently discovered a completely new bacterium *Corynebacterium endometrii* sp. nov. associated with the occurrence of endometritis in cows.

Interestingly, we failed to identify lactic acid bacteria of the family Lactobacillaceae in the endometrial microbiota of cows. Indeed, the importance of lactobacilli in the reproductive tract of cows remains an open question [14]. Their unambiguous positive role in maintaining homeostasis, protecting the mucosa from pathogens and their dominance in the reproductive system in healthy individuals has been proved only in humans [16]. In contrast to humans, the presence of *Enterococcus* spp. and *Streptococcus* spp. species has been demonstrated for cows, with a minimal proportion of *Lactobacillus* spp. which is presumably associated with a higher pH than in humans [14]. However, it is still unclear whether this pH level is caused by the difference in genotype or it is a consequence of total dysbiosis of the reproductive system of cows.

As for the taxonomic diversity of the rumen microbiome, the same bacterial phyla were found in its composition as in the endometrial microbiota, excluding only representatives of the Fusobacteriota phylum (Fig. 2). Such phyla as Bacteroidota and Firmicutes also dominated in number in the rumen microbiome.

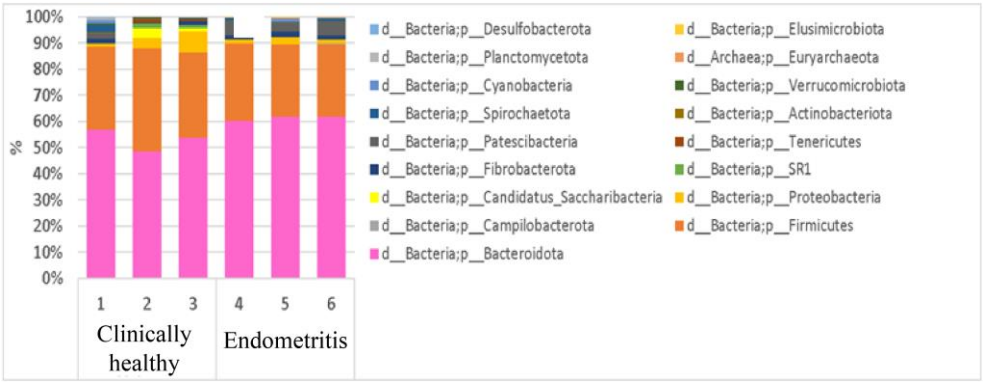


Fig. 2. Composition of the rumen microbiome of cows according to NGS of 16S rRNA amplicons at the phylum level. Clinically healthy group includes clinically healthy animals (n = 3), Endometritis group includes animals diagnosed with purulent-catarrhal post-calving endometritis (n = 3), 1-6 are numbers of experimental animals

Figure 2 shows that an increase in Bacteroidota phylum microorganisms ($p \leq 0.05$) against a decrease in Firmicutes phylum bacteria ($p \leq 0.05$) in the rumen of animals with endometritis was registered compared to the clinically healthy group. This may have had undesirable consequences for the animals and appeared to be related to metabolic diseases such as lactate acidosis. This is because a unique ability of many representatives of Firmicutes is the ability to degrade plant fiber containing cellulose, hemicellulose, and xylans [17]. Against the background of highly concentrated feeding, the number of Bacteroidota increases in the rumen. These bacteria form a powerful organic acid - lactate [18]. The accumulation of lactic acid causes rumen acidosis, because the rumen can no longer cope with buffering and absorption of acids, so its content become acidified. The number of Firmicutes, in turn, decreases against the background of pH drop due to lactate acidosis.

The fact that unculturable bacteria of candidate phylum SR1 completely disappeared from the rumen of healthy animals may also indicate dysbiotic microbiome disorders in cows with endometritis. Bacteria of this phylum are found in the intestines of termites [19]. It is known that termites are in close symbiosis with their microbiome providing them with carbohydrate and energy supply.

As shown using the PICRUSt2 software package (v.2.3.0), 767 predicted metabolic pathways were detected in the cow microbiome. Differences ($p \leq 0.05$) between animals from different groups were observed for 13 predicted metabolic pathways. Cows diagnosed with endometritis showed inhibition ($p \leq 0.05$) of the potential of 9 metabolic pathways compared to healthy animals, including GALACTARDEG-PWY - D-galactarate I degradation, GLUCARGALACTSUPER-PWY - degradation of D-glucarate and D-galactarate, P125-PWY biosynthesis of (R,R)-butanediol, PWY-3781 - aerobic respiration I, PWY-5304 - sulfur oxidation, PWY-5676 - fermentation of acetyl-CoA to butanoate II, PWY-57472 - 2-methylcyrate cycle II. This could have negative consequences for the animal organism and be caused by metabolic disorders. The results obtained seem to be logical, since the relationship between the occurrence of metritis in cows and feeding disorders in the dry period and ketosis was shown earlier [20].

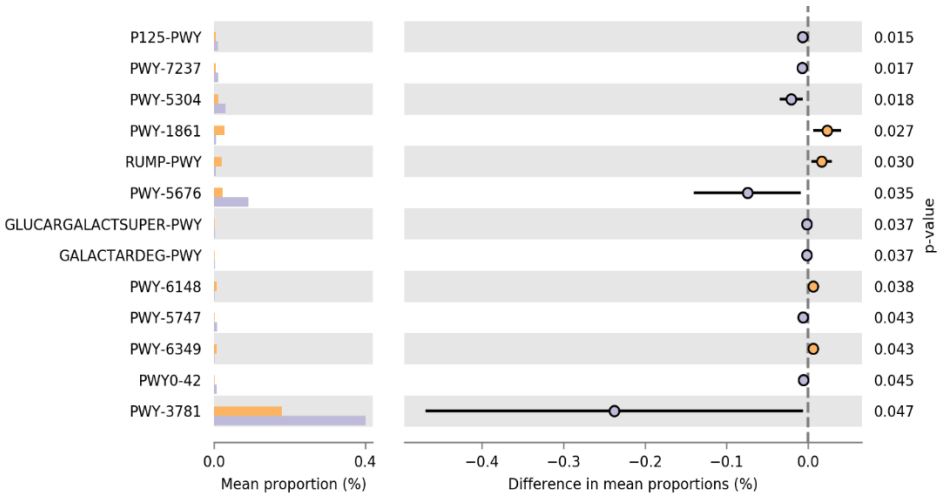


Fig. 3. Predicted metabolic pathways of the microbiome in the rumen of cows constructed using the software package PICRUSt2 (v.2.3.0), which differed between groups ($p \leq 0.05$): lilac color indicates the group of clinically healthy cows, orange color indicates the group of animals diagnosed with purulent-catarrhal post-endometritis

4 Conclusion

The increase in the proportion of Fusobacteriota taxon bacteria permanently present in endometrial scrapings and the decrease in Bacteroidota phylum bacteria in the group of animals with endometritis may be related to the occurrence of this disease. Bacteria *Alloprevotella*, *Campylobacter*, *Caviibacter*, *Falsiporphyromonas*, *Veillonella* present only in the endometrial tissue of sick cows may be the etiological origin of endometritis. It is likely that the change in the ratio of microorganisms in the rumen of cattle, which is expressed in the increase in the proportion of Bacteroidota phylum and decrease in the proportion of Firmicutes phylum, is an important bacteriological risk factor for the occurrence of clinical post-osseous endometritis. This is confirmed by the data that cows diagnosed with endometritis showed inhibition ($p \leq 0.05$) of the potential of 9 metabolic pathways compared to healthy animals. Similar changes in the rumen were observed in animals at risk for metabolic diseases such as lactate acidosis.

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