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SEQUENCING OF THE 16S rRNA GENE V3-V4 REGION TO DETERMINE THE COMPOSITION AND RELATIONSHIPS OF THE MICROBIOTA DURING INFLAMMATION OF THE UDDER AND REPRODUCTIVE TRACT IN COWS (*Bos taurus*)

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Abstract

Inflammatory diseases of the mammary gland and reproductive tract of cattle cause the greatest economic damage to dairy industry. 16S rRNA gene sequencing has significantly expanded the knowledge of microbiomes and uncultured in vitro bacteria that were not previously known to exist. This paper aims to expand the understanding of the composition of the microbiota of the reproductive tract and mammary gland of cattle with the identification of non-culturable microorganisms. New etiologically significant pathogens were identified for the first time. The role of microbiota of these animal loci in the development of inflammatory diseases was established. Our goal was to determine the common bacterial etiology of the inflammatory process in the mammary gland and reproductive tract of cattle. 16S rRNA gene variable regions sequencing was carried out in order to compare the composition of microbiota in mammary gland and the reproductive tract of cattle with inflammation. Four experimental groups were formed from each farm in five districts of the Sverdlovsk Province, animals without signs of inflammation of the mammary gland and reproductive tract (NP, group 1); animals with inflammation of the mammary gland, but without inflammation of the reproductive tract (M, group 2); animals with inflammation of the reproductive tract, but without inflammation of the mammary gland (E, group 3); animals with inflammation of the mammary gland and reproductive tract (EM, group 4). Samples of biological material (mammary gland secretion, cervical swabs) were obtained from each cow of all experimental groups; 16S rRNA gene sequencing was used for further studies. Sequence analysis of the 16S rRNA variable regions showed that the vast majority of identified OTU belong to the *Bacteria* domain, the rest belong to the *Euryarchaeota* type of the *Archaea* domain (0.38 % in mammary gland secretion samples, 0.44 % in cervical swabs samples). In mammary gland secretions, we revealed 19 bacterial types, including 43 classes, 85 orders, 165 families, and 484 genera. Half of the bacterial OTU are the *Firmicutes* phylum (680 OTU, 51.7 %), while *Actinobacteria* and *Bacteroidetes* are the second and third largest phyla (14.5 % and 11.3 %, respectively). Members of 22 bacterial phyla were found in cervical swabs, including 50 classes, 93 orders, 172 families and 365 genera. The predominant bacterial phylum is the *Firmicutes* phylum (876 OTU, 55.3 %), the second and third largest phyla are the *Bacteroidetes* (13.3 %) and the *Actinobacteria* (11.6 %), respectively. By the 16S rRNA gene sequencing, we revealed bacteria of the class *Clostridia* and the genus *Facklamia* not detectable by cultural methods. This confirms clinical significance of the 16S rRNA gene sequencing method for clarifying etiological agents in the case of unculturable or difficult-to-culture bacteria. We revealed the interrelation between the microbiota of the mammary gland and the reproductive tract

during inflammatory processes. The data contribute to a deeper understanding of the role of bacterial microbiota in the etiology and pathogenesis of inflammatory diseases of the mammary gland and the reproductive tract of animals.

Keywords: Holstein cattle, 16S rRNA, microbiota, mammary gland, reproductive tract

Microbiota can exhibit both synergistic and commensal properties, as well as have pathogenic effects, depending on the location in the host body. The presence of some bacteria does not lead to an inflammatory response in one organ system, but the same species can cause pathology in other organs [1]. Opportunistic bacteria normally found in the gastrointestinal tract of cattle, such as *Escherichia coli*, in the mammary gland or uterus can cause inflammation and an infectious process [1]. The healthy mammary gland is considered sterile, especially in animals during puberty before milk secretion begins [2]. During the first birth and after the start of milking, the mammary gland becomes a functionally open system that has a direct connection with the environment. It has been suggested that there is a natural community of microbes (microbiota) in the udder [3]. Inflammation of the mammary gland (mastitis) is most often caused by bacterial intramammary infection (IMI). Using classical culture methods, various species of bacteria that may be involved in the development of the inflammatory process have been described in milk samples taken from the affected lobes of the mammary gland of cows [2, 4]. Most often, staphylococci, enterobacteria and streptococci, which cause the vast majority of IMIs, are identified in mastitis milk samples [2, 5]. However, the ability of culture methods to study the full range of possible pathogens is limited.

Inflammatory diseases of the reproductive system are directly related to the development of the infectious process. The likelihood of bacterial infection and the development of inflammatory diseases of the reproductive system in cows increases during the postpartum period, since endometrial immune cells have a weak ability to neutralize pathogens, and tissues are susceptible to the influence of exogenous factors [6]. Previous metagenomic studies have found dramatic differences in microbiota composition between healthy cows and cows with reproductive diseases [7, 8]. Early detection of microbiota that causes inflammatory diseases of the reproductive tract is important for timely treatment and prevention of the infection [9-11].

Modern sequencing methods allow identification of microorganisms that inhabit various organs, systems or biological fluids, including those previously considered sterile [12], to study their physiological role, virulent and pathogenic properties in terms of significance in infections [13]. Thus, in the literature there is information that only 15% of the bacteria that make up the rumen microbiota can be cultured in laboratory conditions. Metagenomics projects have increased understanding of the rumen microbial ecosystem and its role in fiber digestion [14, 15]. Early studies of the uterine microbiota based on culture diagnostic methods identified a limited number of bacterial species involved in the development of inflammation. It was assumed that the main causative agents of postpartum endometrial pathology are *Escherichia coli*, *Fusobacterium* spp., *Prevotella* spp. and *Trueperella pyogenes*. However, in recent years, metagenomic approaches have uncovered previously underappreciated members of the microbial community that may be bacterial pathogens [16].

Sequencing of the 16S ribosomal subunit gene sequence is most commonly used to identify and compare the composition of bacterial communities [17]. Most literature sources provide separate results from studies of the microbiota of the mammary gland and reproductive tract of animals using 16S rRNA gene sequencing, concerning the study of the composition of normal flora, its

local and regional differences, changes in the development of mastitis and inflammatory diseases of the reproductive organs [18, 19]. However, there is no data on the metagenomic analysis for searching the relationship between the microbiota of the mammary gland and the reproductive tract of cows and to study the general bacterial etiology of diseases.

This study is the first to report that in the composition of the microbiota of the mammary gland and reproductive tract of cows during inflammation there are common bacteria not previously determined by culture methods, which suggests their connection in the etiology of inflammatory processes.

The purpose of the work is to compare bacterial communities in the secretions of the mammary gland and in the reproductive tract of cows to identify common agents of the inflammatory process.

Materials and methods. The research was carried out on five farms in the Sverdlovsk Province in 2021 on Holstein cows (*Bos taurus*) of the 2nd-3rd lactation. On each farm, 4 groups of 81 cows were formed. Group 1 was animals without inflammation (H, $n = 22$), group 2 was cows with the inflammation of mammary gland but not the reproductive tract (M, $n = 24$), group 3 had signs of the reproductive tract inflammation without the mammary gland inflammation (E, $n = 18$), and group 4 had inflammation of both the mammary gland and reproductive tract (EM, $n = 17$).

Biological material (mammary secretions, cervical washings) were aseptically sampled from each cow. On each farm from samples of each group bulk samples were formed, resulting in 39 bulk samples, including 19 samples of mammary gland secretion and 20 samples of cervical washings.

Total DNA was isolated from biological materials by the standard phenolic method [20]. The concentration of isolated DNA was measured a Qubit 4 fluorometer (ThermoFisher Scientific, USA).

The DNA library for sequencing was constructed using a commercial 16S V3-V4 Library Preparation Kit, Illumina, Inc., USA) according to the manufacturer's recommendations (<https://www.thermo-fisher.com/order/catalog/product/12358010>). Amplification (a CFX-96, Bio-Rad, USA) was carried out with universal bacterial primers 16S-8-f-B (5'-AGRGTGGATCCCTGGCTCA-3') and 16S-1350-r-B (5'-GACG-GGCGGTGTGTACAAG-3'). Amplification mode: initial denaturation at 98 °C for 30 s; 25 cycles: denaturation at 98 °C for 10 s, 55 °C for 15 s, 72 °C for 30 s; final elongation at 72 °C for 10 min.

Sequencing of the resulting library was performed with the Illumina MiSeq platform (Illumina, Inc., USA). Sequences were aligned using the ClustalW program (<http://www.ebi.ac.uk/Tools/msa/clustalw2>).

The sequences were analyzed using the UPARSE pipeline (<https://drive5.com/uparse/>) in Usearch v.10.0 (<https://www.drive5.com/usearch/>) [21]. The UPARSE pipeline includes the ratio of paired read sequences, filtering sequences by quality, trimming to the required length, merging identical sequences, excluding single sequences and chimeras. Operational taxonomic units (OTUs) were clustered with the UPARSE-OTU algorithm, and taxa were assigned using SINTAX [22], the 16S RDP v.16 simulator, and BLAST+ 2.12.0 [23]. OTU data sparsity was assessed individually for each sample using the Usearch program.

The relative abundance of bacterial taxa at the level of phyla, families and OTUs was compared by the paired Mann-Whitney test for non-parametric data on unrelated measurements in the STATISTICA v.12 program (StatSoft, Inc., USA) for milk and cervical samples in two variants from animals in all groups and on each farm.

β -Biodiversity indices (comparisons of taxonomic diversity between studied samples) [24] were calculated using the KJ Jaccard coefficient (the Usearch program, <https://www.drive5.com/usearch/>). The results are presented as medians (*Me*), minimum and maximum values.

Results. High-throughput sequencing data are available in NCBI GenBank at links: OK048814-OK050076 (microbiota of bovine mammary secretions) and OK037659-OK039186 (microbiota of bovine cervical washings).

Taxonomic profiles of the microbiota of the reproductive tract and mammary gland. After quality testing and removal of chimeras, 1315 and 1584 OTUs were obtained for mammary gland secretions and cervical washings, respectively. Analysis of 16S rRNA gene sequences showed that the vast majority of OTUs belong to the *Bacteria* domain, the rest to the *Euryarchaeota* phylum of the *Archaea* domain, 0.38% in mammary gland secretions and 0.44% in cervical washings.

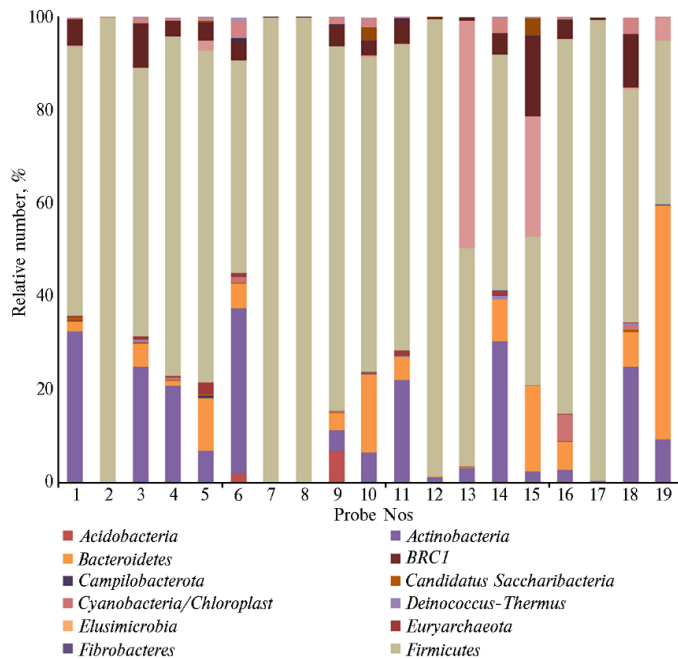


Fig. 1. Relative content of bacterial phyla OTUs in mammary gland secretion of Holstein cows (*Bos taurus*) (Sverdlovsk Province, 2021). Groups along the x-axis: animals without signs of the mammary gland and reproductive tract inflammations (H) (4-19), with inflammation of the mammary gland, but without inflammation of the reproductive tract (M) (1-16), with inflammation of the mammary gland and reproductive tract (ME) (3-18), with inflammation of the reproductive tract, but without of inflammation of the mammary gland (E) (2-17).

Members of 19 bacterial phyla, including 43 classes, 85 orders, 165 families and 484 genera, were identified in mammary gland secretions. Half of the bacterial operational taxonomic units were the phylum *Firmicutes* (680 OTUs, 51.7%), the second and third phyla in terms of the number of OTUs were *Actinobacteria* and *Bacteroidetes* (14.5% and 11.3%, respectively). In some samples of mammary gland secretion, OTUs of phyla were found whose contribution is also significant. In samples No. 11 and No. 18, we detected OTUs of the phylum *Fusobacteria* (49% and 17%, respectively) (Fig. 1). The most numerous (481 OTUs, 36.6%) was the class *Clostridia*, which was dominated by the family *Ruminococcaceae* (192 OTUs, 14.6% of the total number of OTUs), the families *Lachnospiraceae* (113 OTUs, 8.6%) and unc_ *Clostridiales* (89 OTUs, 6.8%) were the second and the third in abundance (Fig. 2).

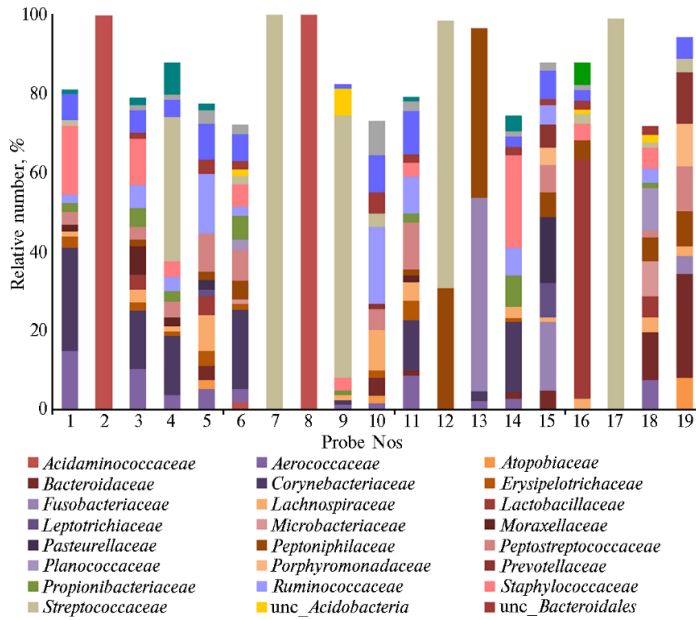


Fig. 2. Relative content of predominant bacterial family OTUs in mammary gland secretion of Holstein cows (*Bos taurus*) (Sverdlovsk Province, 2021). Groups along the x-axis: animals without signs of the mammary gland and reproductive tract inflammations (H) (4-19), with inflammation of the mammary gland, but without inflammation of the reproductive tract (M) (1-16), with inflammation of the mammary gland and reproductive tract (ME) (3-18), with inflammation of the reproductive tract, but without of inflammation of the mammary gland (E) (2-17). The total OTU content for some samples does not reach 100%, since data were used only for OTUs with contribution of $\geq 5\%$; unc — unclassified.

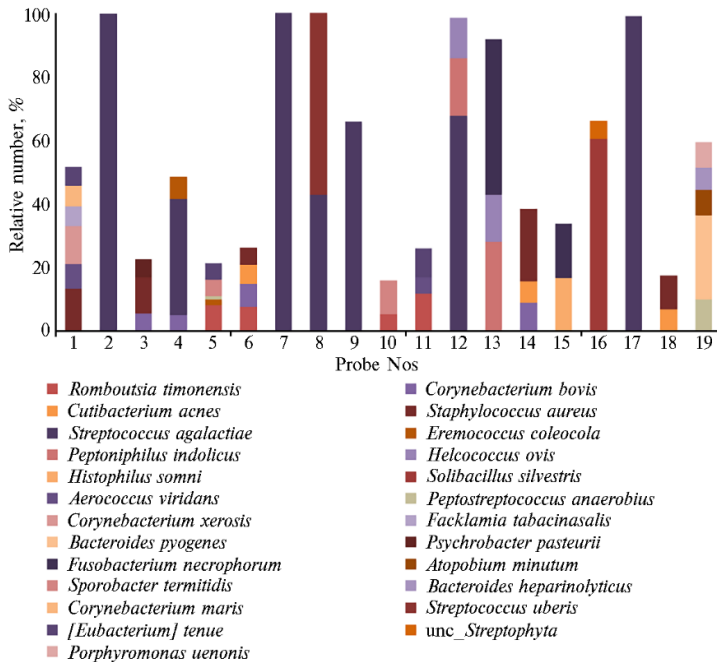


Fig. 3. Relative content of predominant bacterial OTUs in mammary gland secretion of Holstein cows (*Bos taurus*) (Sverdlovsk Province, 2021). Groups along the x-axis: animals without signs of the mammary gland and reproductive tract inflammations (H) (4-19), with inflammation of the mammary gland, but without inflammation of the reproductive tract (M) (1-16), with inflammation of the mammary gland and reproductive tract (ME) (3-18), with inflammation of the reproductive tract, but without of inflammation of the mammary gland (E) (2-17). The total OTU content for some samples does not reach 100%, since data were used only for OTUs with contribution of $\geq 5\%$; unc — unclassified.

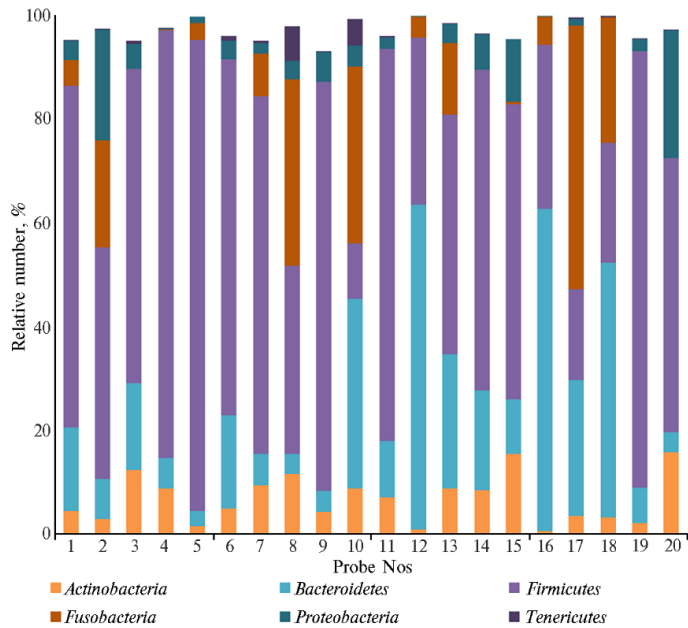


Fig. 4. Relative content of bacterial phyla OTUs in cervical flushes of Holstein cows (*Bos taurus*) (Sverdlovsk Province, 2021). Groups along the x-axis: animals without signs of mammary gland and reproductive tract inflammation of the (H) (4-20), with inflammation of the mammary gland, but without inflammation of the reproductive tract (M) (1-17), with inflammation of the mammary gland and reproductive tract (ME) (3-19), with inflammation of the reproductive tract, but without signs of inflammation of the mammary gland (E) (6-18). Inflammation of the reproductive tract, but without inflammation of the mammary gland (E) (2-17). The total OTU content for some samples does not reach 100%, since data were used only for OTUs with contribution of $\geq 5\%$.

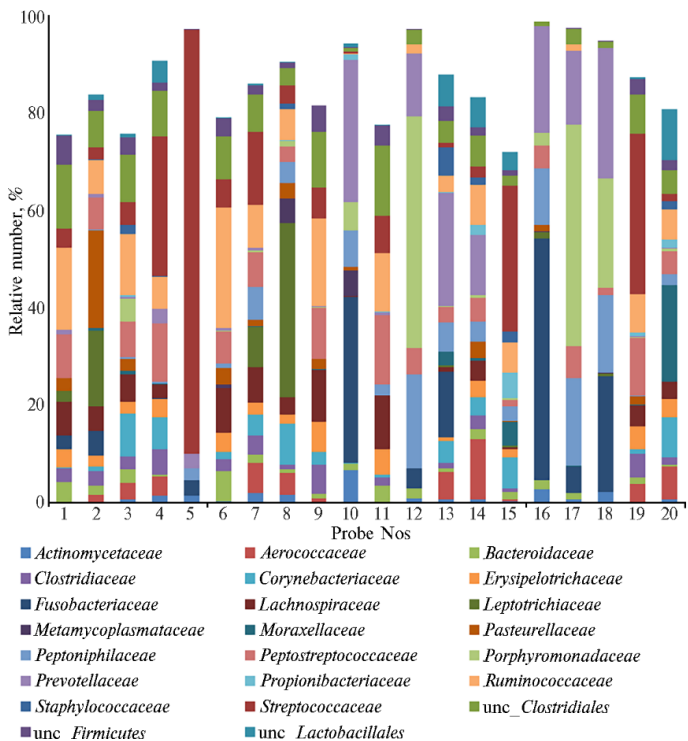


Fig. 5. Relative content of predominant bacterial family OTUs in cervical flushes of Holstein cows (*Bos taurus*) (Sverdlovsk Province, 2021). Groups along the x-axis: animals without signs of mammary gland and reproductive tract inflammation of the (H) (4-20), with inflammation of the mammary gland, but

without inflammation of the reproductive tract (M) (1-17), with inflammation of the mammary gland and reproductive tract (ME) (3-19), with inflammation of the reproductive tract, but without signs of inflammation of the mammary gland (E) (2-18). The total OTU content for some samples does not reach 100%, since data were used only for OTUs with contribution of $\geq 5\%$; unc — unclassified.

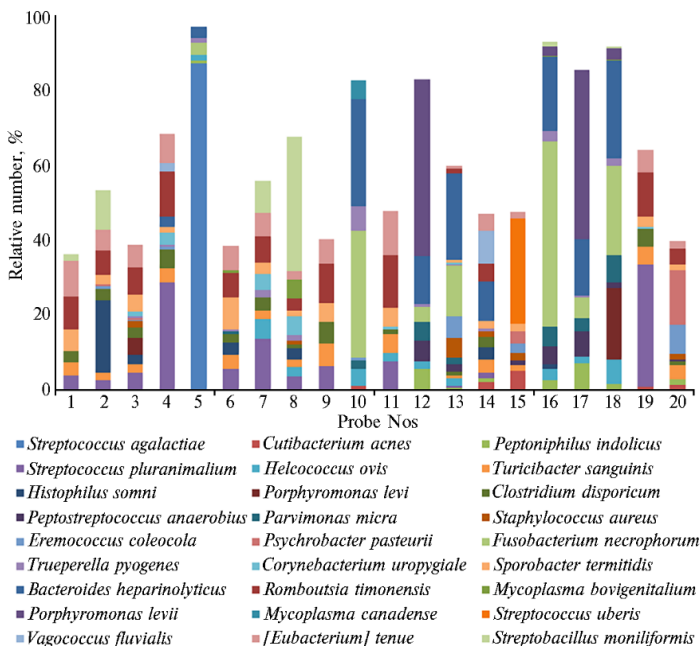


Рис. 6. Relative content of predominant bacterial OTUs in cervical flushes of Holstein cows (*Bos taurus*) (Sverdlovsk Province, 2021). Groups along the x-axis: animals without signs of mammary gland and reproductive tract inflammation of the (H) (4-20), with inflammation of the mammary gland, but without inflammation of the reproductive tract (M) (1-17), with inflammation of the mammary gland and reproductive tract (EM) (3-19), with inflammation of the reproductive tract, but without signs of inflammation of the mammary gland (E) (2-18). The total OTU content for some samples does not reach 100%, since data were used only for OTUs with contribution of $\geq 5\%$; unc — unclassified.

For all mammary gland secretions, only 25 OTUs (or 1.9% of all OTUs) had $\geq 5\%$ contribution to the relative abundance. These OTUs correspond to members of 6 phyla, 8 classes, 11 orders, 16 families and 22 genera, which is numerically much smaller in taxonomic diversity than the entire list of OTUs (Fig. 3).

In cervical washings, representatives of 22 bacterial phyla were found, including 50 classes, 93 orders, 172 families and 365 genera. The predominant bacterial phylum was *Firmicutes* (876 OTUs, 55.3%), the second and third most abundant taxa were *Bacteroidetes* (13.3%) and *Actinobacteria* (11.6%) (Fig. 4). In the *Clostridia* class which was the largest group by the number of OTUs (637 OTUs, 40.2%) the dominant family was *Peptoniphilaceae* (256 OTUs, 16.2%), the families *Lachnospiraceae* (149 OTUs, 9.4%) and unc_ *Clostridiales* (130 OTUs, 8.2%) were in second and third place (Fig. 5).

For the bacterial community across all cervical lavage samples, only 27 OTUs (1.7% of the total number of OTUs) had a contribution to the microbiota composition of $\geq 5\%$. These OTUs represented 6 phyla, 8 classes, 12 orders, 18 families, and 24 genera of bacteria, but taxonomic diversity was much higher for all OTUs (Fig. 6).

The relationship between the microbiota of the mammary gland and the reproductive tract during inflammation. To compare the β -biodiversity of the microbiota according to metagenomic analysis of the 16S rRNA gene in inflammatory pathologies, we used the Jaccard Similarity Index (JSI). The average JSI value determined by comparing five farms with

each other, was 0.885. The JSI value ($0 \leq \text{JSI} \leq 1$) directly reflects the degree of difference, the higher the value of the coefficient, the greater the differences between samples [24]. However, this index includes all OTUs with those which contribution is extremely insignificant. It is advisable to consider OTUs that have a significant contribution ($\geq 1\%$) to the taxonomic composition of the bacterial community. Therefore, we assessed differences in the composition of the microbiota of mammary gland secretions and cervical washings based on the relative content of OTUs that were dominant in number (Table).

Microorganisms common to the microbiota in inflammation of the mammary gland and reproductive tract in Holstein cows (*Bos taurus*) from five farms surveyed (Sverdlovsk Province, 2021)

No. 1	No. 2	No. 3	No. 4	No. 5
		В и д		
<i>Turicibacter sanguinis</i> (A)	<i>Helcococcus ovis</i> (D)	<i>Helcococcus ovis</i> (D)	<i>Turicibacter sanguinis</i> (A)	<i>Peptostreptococcus anaerobius</i>
<i>Sporobacter termitidis</i> (B)	<i>Peptoniphilus indolicus</i>	<i>Facklamia tabacinasalis</i>	<i>Cutibacterium acnes</i>	<i>Sporobacter termitidis</i> (B)
<i>Bacteroides tenuis</i> (C)		<i>Fusobacterium necrophorum</i>	<i>Staphylococcus aureus/simiae</i>	<i>Bacteroides tenuis</i> (C)
<i>Clostridium saudiense/disporicum</i>				
<i>Romboutsia timonensis</i>				
<i>Butyrivibrio proteoclasticus</i>				

Note. Letters (A-D) indicate matching types of microorganisms.

Comparing biological samples by OUT revealed that during inflammation of both the mammary gland and the reproductive tract, *Turicibacter sanguinis*, *Staphylococcus aureus*, *Sporobacter termitidis*, *Clostridium saudiense*, *Peptostreptococcus anaerobius*, *Romboutsia timonensis*, *Bacteroides tenuis*, *Butyrivibrio proteoclasticus*, *Peptoniphilus indolicus*, *Helcococcus ovis*, *Facklamia tabacinasalis*, *Fusobacterium necrophorum*, and *Cutibacterium acnes* were present in the pathological biomaterial.

Of the bacteria for which a relationship has been established in the relative content in the secretion of the mammary gland and cervical washings during inflammation, *Turicibacter sanguinis*, *Staphylococcus aureus*, *Peptostreptococcus anaerobius*, *Peptoniphilus indolicus* and *Helcococcus ovis* are described as infectious agents that provoke inflammations [25–28]. *Fusobacterium necrophorum* is a gram-negative, non-spore-forming anaerobic bacillus that lives in the gastrointestinal tract, but when introduced into other tissues it can become a pathogenic microorganism causing necrotic diseases [29]. Its role in the rumen is the fermentation of lactic acid and the degradation of feed and epithelial proteins [30]. Recent studies have shown that *F. necrophorum* is involved in the occurrence of mastitis in dairy cows, endometritis, interdigital dermatitis, and esophageal ulcers [29, 31–33]. *F. necrophorum* is one of the predominant bacteria causing intrauterine infection in cows and is found in greater numbers in cows with metritis than in healthy cows. *F. necrophorum* isolated from cattle liver abscesses has been shown to possess several virulence factors such as adhesins, leukotoxins, endotoxins, hemolysins, hemagglutinins, and proteases. However, the mechanisms by which colonization of the reproductive tract occurs remain unknown [29].

Sporobacter termitidis was previously identified in samples of mammary gland secretions from both healthy and mastitic patients [34], and this microorganism is also known as a representative of the normal flora of the rumen of cattle [35]. *Romboutsia timonensis*, *Facklamia tabacinasalis*, and *Bacteroides tenuis*, also known as *Eubacterium tenue*, have been detected in mammary gland secretions, udder skin of cows, and collected milk [36, 37].

Butyrivibrio proteoclasticus and *Cutibacterium acnes* are members of the

rumen microbiota, but the latter has also been isolated from dairy products [38, 39]. *Clostridium saudiense* has been detected in the gastrointestinal tract of pigs by 16S rRNA gene sequencing, but its role as an infectious agent requires further investigation [40].

The important role of ruminal and intestinal microbiota in the reproductive health of cows has been noted in a number of studies [41-43]. It is assumed that colonization of the genital tract and mammary gland by the microbiota of the gastrointestinal tract influences the pathogenesis of mastitis and endometritis, and it is possible to correct the microbiocenosis of the genital tract by influencing the rumen microbiota [44].

Both in the structure of microbial communities of mammary gland secretions and in the structure of microbiocenosis of cervical washings in cattle, the predominant phyla were *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. These data are consistent with general ideas about the composition of the microbiota of the reproductive tract of cows [45]. In some samples of mammary secretions and cervical washings collected from animals with concurrent mastitis and endometritis (ME), we noted a high relative abundance of the phylum *Fusobacteria*. In most animals, this phylum dominated in cervical lavages and only in some individuals in both the secretion of the mammary gland and cervical lavages simultaneously. Moreover, the phylum *Proteobacteria* was present in all cervical washings, as well as in most samples of mammary gland secretion. In mammary gland secretions, the relative content of this group of bacteria in terms of the number of OTUs was 17%. Here, proteobacteria were represented by the species *Histophilus somni*. *Histophilus somni* is a representative of the *Pasteurellaceae* family, involved in the development of inflammatory processes in various diseases of cattle (thrombotic meningoencephalitis, respiratory diseases, myocarditis, and mastitis) [46]. In cervical washings, the abundance of proteobacteria (12% in the microbiota composition with a relative content of 7.8% based on the number of OTUs for which the contribution to the microbiota composition exceeds 1%) was represented by *Psychrobacter pasteurii* (3.3%), *Enterobacteriaceae* (2.1%), *Mailhella massiliensis* (1.3%), *Oligella ureolytica* (1.2%). According to British scientists, *Psychrobacter* species rarely cause disease in animals [47]. *Mailhella massiliensis* has sulfate-reducing properties that inhibit rumen methane production in cattle [48, 49], and *Oligella ureolytica* is rarely detected in microbiological studies using both culture methods and 16S rRNA gene sequencing (50). It is worth noting that in the studies known to us, the reproductive diseases in cows were associated with a high relative abundance of bacteria of the *Fusobacteria* phylum [16, 51], but we obtained such data on *Proteobacteria* for the first time. We also note that with the dominance of the main phyla *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, the composition of the microbiota of each sample still differs in the presence and relative abundance of other significant taxa, the determination of the species composition of which requires a more detailed analysis.

Thus, based on the results of microbiota genotyping using 16S rRNA, we determined taxonomic profiles of mammary gland secretions and cervical washings from healthy cows and animals with mastitis and endometritis. Representatives of 19 bacterial phyla, including 43 classes, 85 orders, 165 families and 484 genera, were identified in mammary gland secretions. Half of the bacterial operational taxonomic units were the phylum *Firmicutes* (680 OTUs, 51.7%), the second and third phyla in terms of the number of OTUs were *Actinobacteria* and *Bacteroidetes* (14.5% and 11.3%, respectively). In cervical lavage samples, the bacterial phylum *Firmicutes* predominated (876 OTUs, 55.3%), the second and third were *Bacteroidetes* (13.3%) and *Actinobacteria* (11.6%), respectively. 16S rRNA sequencing to identify differences in the microbiota profile of the

reproductive tract and mammary gland during the inflammatory process was used in this work for the first time. This made it possible to detect bacteria that can be considered as common etiological agents of mastitis and endometritis. Our data on infectious agents that provoke the inflammatory process coincide with the results of previous studies. Thus, during inflammation, common pathogens were found both in the secretion of the mammary gland and in cervical washings. These are *Turicibacter sanguinis*, *Staphylococcus aureus*, *Peptostreptococcus anaerobius*, *Peptoniphilus indolicus* and *Helcococcus ovis*. The results we present can help understanding the role of bacterial microbiota in the pathogenesis of inflammatory diseases of the mammary gland and reproductive tract and for developing an ecosystem approach to therapeutic and preventive measures.

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