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Comparison of the Microbiota of the Reproductive Tract and the Mammary Gland of Cows with and without Inflammation Using 16S rRNA Sequencing

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Abstract

Inflammatory diseases of the mammary gland and reproductive tract of cattle cause the greatest economic damage to dairy farming. Analysis of the relationship between the microbiota of these loci in animals without pathology and with mastitis and endometritis will provide new knowledge about the etiology of inflammatory diseases. This study performed sequencing of 16S rRNA variable regions, to determine and compare the composition of the mammary gland and the reproductive tract microbiota of cattle in normal conditions and inflammation. This research comprised 81 animals from five agricultural organizations of the Sverdlovsk oblast (Ural region, Russia), located in different areas. In each, 4 groups of experimental animals were formed: Group 1 animals with no pathology, i.e. without signs of inflammation of the mammary gland and reproductive tract (NP); Group 2 animals with signs of the mammary gland inflammation, but without signs of the reproductive tract inflammation (M); Group 3 animals with signs of the reproductive tract inflammation, but without signs of the mammary gland inflammation (E); Group 4 animals with signs of inflammation of the mammary gland and reproductive tract (EM). Samples of biological material (mammary gland secretion, cervical swabs) were obtained from each cow of all experimental groups; for further studies, the method of pooled sample for 16S rRNA metagenomic analysis was used. A comparison was made of the microbiota of the mammary gland and the reproductive tract of cattle with and without inflammatory processes.

Disciplinary: Microbiology & BioScience.

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1 Introduction

16S rRNA gene amplicon sequencing library preparation has significantly expanded the knowledge of bacterial communities (microbiomes) and revealed bacteria that were not previously known to exist because they could not be cultivated *in vitro*. In cattle, the study of the diversity of the main milk microbiota was carried out mainly by comparing milk samples from healthy and mastitis cows, since this disease is considered one of the most common diseases of dairy cattle, which cannot be fully controlled [1;2]. The mammary gland in cattle is the main reservoir of infectious pathogens, which include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Mycoplasma spp.* and *Corynebacterium bovis*. Opportunistic pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Streptococcus dysgalactiae* and *Streptococcus uberis* can also become a causative agent of infection [3]. The study of the mammary gland microbiota will allow us to assess its role in the occurrence of inflammatory diseases and to identify new strategies for the prevention of mastitis. [4]. A comparison of the reproductive tract microbiota of cattle was carried out between animals with no pathology and with postpartum inflammatory diseases. *Escherichia coli*, *Trueperella pyogenes*, *Prevotella spp.*, *Fusobacterium necrophorum*, *Bacteroidetes spp.* and *Firmicutes spp.* are predominant taxons associated with postpartum uterine diseases. However, the same bacterial species were found in the uterus of healthy cows [4]. The fact that affected and healthy cows carry similar pathogens indicates that uterine diseases are connected with the extensive growth of certain bacteria and the response of the immune system, but more research needs to be done on this issue [5].

Currently, the composition and characteristics of the microbiota of the mammary gland and the reproductive tract of cattle are widely described using the 16S rRNA sequencing method. However, this method has not been used to determine the general bacterial etiology of the inflammatory process in these loci of the animal organism. In addition, it is known that there are territorial differences in the microbiota structure of productive animals, which must be considered while planning treatment and preventive measures.

2 Materials and Methods

The study was conducted within the framework of the Russian Foundation for Basic Research (grant № 20-416-660004) “Molecular genetic and phenotypic characteristics of the reproductive tract microbiota of cattle”. The object of research was 81 animals from 5 agricultural organizations of the Sverdlovsk oblast (Ural region, Russia). In each of the agricultural organizations 4 groups of experimental animals were formed: Group 1 - animals with no pathology, i.e. without signs of inflammation of the mammary gland and reproductive tract (NP); Group 2 - animals with signs of the mammary gland inflammation, but without signs of the reproductive tract inflammation (M); Group 3 - animals with signs of the reproductive tract inflammation, but without signs of the mammary gland inflammation (E); Group 4 - animals with signs of inflammation of the mammary gland and reproductive tract (EM).

Samples of biological material (mammary gland secretion, cervical swabs) were obtained from each cow of all experimental groups in compliance with aseptic regulations. For further studies, the method of pooled sample was used, as a result of which 39 samples were obtained: mammary gland secretion (n=19), cervical swabs (n=20), from which total DNA was isolated and the 16S rRNA gene was sequenced. A comparison was made of samples of the mammary gland secretion between the groups: NP-M, NP-ME and samples of cervical canal swabs between the groups: NP-E, NP-ME, as well as a comparison of groups with an inflammatory process among themselves.

RNA extraction and 16S rRNA sequencing. The isolation of total DNA from biological samples was performed by the standard phenol method [7]. Based on the obtained DNA preparations, the library for sequencing was prepared using the commercial "Big DyeTM Terminator Cycle Sequencing Kit" (Applied Biosystems, USA) in strict accordance with the manufacturer's recommendations. Amplification of the 16S rRNA gene was performed using universal bacterial primers 16S-8-f-B (5'-AGRGTTTGATCCTGGCTCA-3') and 16S-1350-r-B (5'-GACGGGCGGTGTGTACAAG-3').

The sequencing of the prepared library was performed on the Illumina MiSeq outsourced platform at the Genomics Center for Collective Use of the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences (Novosibirsk). The search for similar sequences in nucleotide databases was performed using Blast programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and alignment was performed using the ClustalW program (http://www.ebi.ac. uk/Tools/msa/clustalw2).

2.1 Bioinformatics and Statistical Analysis

The resulting sequences were analyzed using the UPARSE pipeline in Usearch v.10.0 [8]. The UPARSE pipeline includes the ratio of paired read sequences, filtering sequences by quality, trimming them to the required length, merging identical sequences, excluding single sequences, excluding chimeras. OTU (operational taxonomic unit) clustering was carried out using the UPARSE-OTU algorithm, taxonomic assignment of sequence reads was carried out with the joint use of SINTAX [9], the 16S RDP v.16 simulator and BLAST + 2.12.0 [10].

OTU data sparseness was assessed individually for each sample using the Usearch program: the number of detected bacterial OTUs reaching a plateau with an increase in the number of sequences indicates a high quality of sampling, which is sufficient to compare the diversity of OTUs. The taxonomic structure of the microbiological composition of biomaterials was established by the ratio of the number of taxon-specific reads to the total number of sequences reads. Thus, the relative abundance of taxa (expressed as a percentage) was obtained. Comparison of the relative abundance of different bacterial taxa (phylum, family and at the level of OTUs) was carried out using a paired Mann-Whitney test for non-parametric data on unrelated measurements in the STATISTICA v.12 program (Statsoft, USA) between samples of milk and cervical swabs from

animals of all experimental groups; samples of milk and cervical swabs in the aggregate from each agricultural organization.

The obtained results were expressed in medians (M), minimum and maximum values. β-biodiversity indices were calculated using the Usearch program.

3 Results and Discussion

The results of high-throughput sequencing are available from the NCBI GenBank at the links: OK048814-OK050076 (bovine mammary gland secretion microbiota) and OK0037659-OK039186 (bovine cervical swabs microbiota).

Supplementary material in the form of tables is available on request addressed to the corresponding author.

Comparison of the results of the bacterial composition genotyping of mammary gland secretions samples from cows with no pathology (NP) and cows with mastitis (M) using the Mann-Whitney test (p-value < 0.05) indicates a number of statistically significant differences. Thus, a difference was found in the relative abundance of members of the *Neisseriaceae* family, which was predominant in animals with no pathology (Figure 1), but their abundance is generally extremely low (<1%). The species of other representatives of *Neisseriaceae*, such as *Kingella denitrificans*, *Neisseria perflava*, *Neisseria macacae*, *Snodgrassella alvi*, *Uruburuella suis* had also been established.

Comparison of the genotyping results of cervical swabs and mammary gland secretions from cows with no pathology and with inflammation of the reproductive tract and/or mammary gland using the Mann-Whitney test (p-value < 0.05) indicates a number of differences in the microbiota composition. Thus, a difference was found in the relative abundance of representatives of the *Peptoniphilaceae* family (40 OTUs) and belonging to this family species – *Peptoniphilus indolicus* in animals from groups NP and E, respectively (Figure 1).

Comparative analysis of obtained genotyping data of the bacterial composition of mammary gland secretion samples from cows with no pathology (NP) and cows with mastitis (M) using the Mann-Whitney test (p < 0.05) revealed a number of statistically significant differences. A difference was found in the relative abundance of members of the *Neisseriaceae* family and it appeared to a greater extent in animals with no pathology. Species investigation of this bacteria family revealed presence of *Neisseria oralis*, which according to previous data was found in humans' gingival plaque and its role in pathological or normal microflora in cattle has not been studied [11]. The species of *Neisseriaceae* representatives, such as *Kingella denitrificans*, *Neisseria perflava*, *Neisseria macacae*, *Snodgrassella alvi*, *Uruburuella suis* had also been established.

Our work revealed a significant difference (p=0.0449) in the relative content of *Ruminococcus bromii* in the mammary gland secretion of healthy animals. In the studies of Klieve A.V. et al. *Ruminococcus bromii* is defined as the dominant population of the bacterial community in the rumen of cattle, influencing the efficiency of starch utilization [12]. Asnicar Francesco and colleagues in their work showed that this type of bacteria belongs to specific microorganisms of the human microbiome involved in vertical transmission from mother to infant [13]. Thus,

Ruminococcus bromii is a part of a symbiotic microflora involved in the digestion process and probably plays an important role in the colonization of the gastrointestinal tract of newborn calves through colostrum consumption.

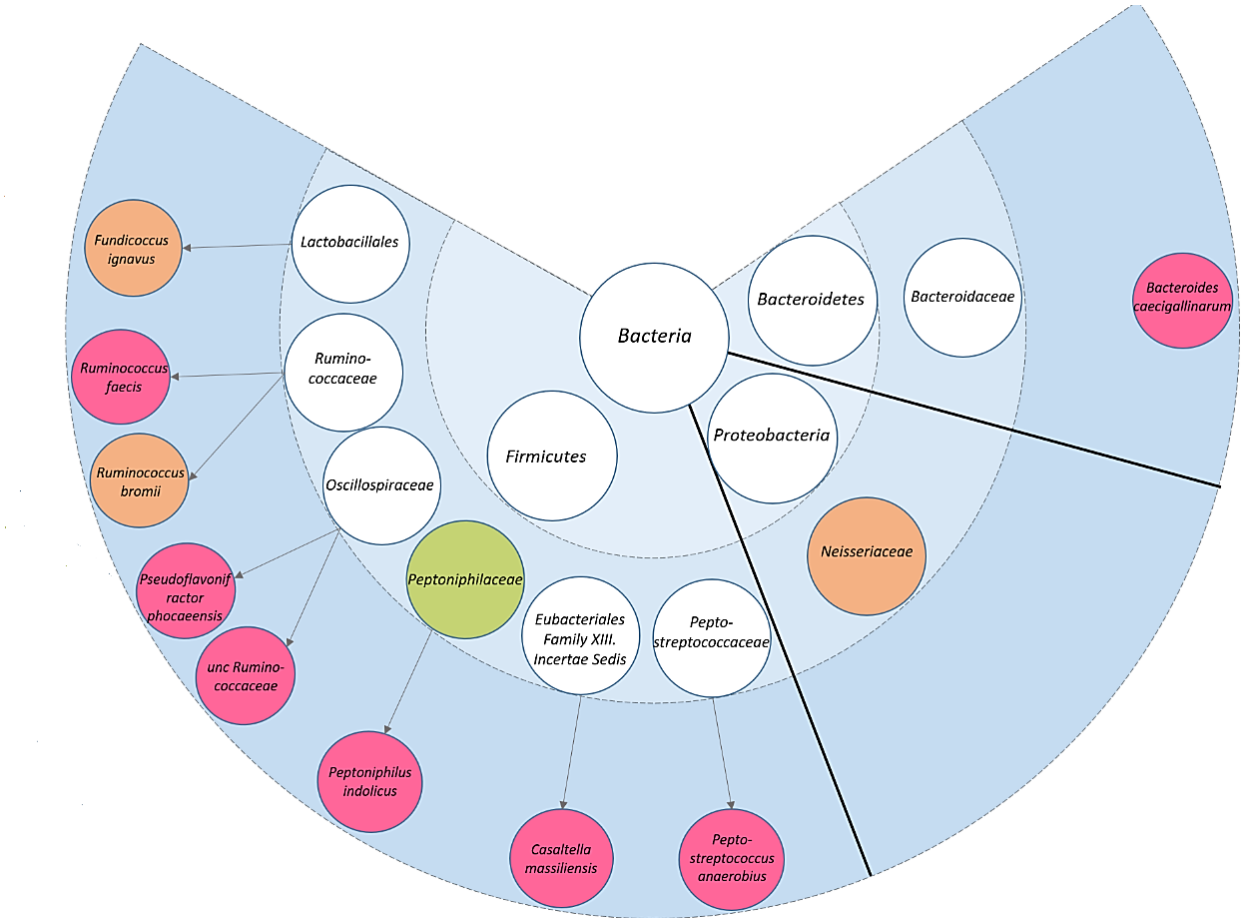

Figure 1:. Statistically significant differences in genotyping results of cervical swabs and mammary gland secretions from animal groups: NP – M (beige), NP – E (pink), NP – EM (green), non-significant (no color) (p-value<0,05).
NB: unc – unclassified.

After we raised the threshold of statistical significance to 0.05 < p-value < 0.1 [14], the number of other bacteria was determined with statistically significant differences in relative abundance among groups of samples. Following differences were observed by comparison of the phylum *Candidatus Saccharibacteria*, the families of *Aerococcaceae*, *Saccharinadaceae*, *Streptococcaceae*, *Peptoniphilaceae* and *Moraxellaceae* and OTUs belonging to certain bacterial species such as *Psychrobacter pasteurii*, *Facklamia miroungae*, *Coprococcus catus* that were determined in mammary gland secretion samples from animals with no pathology, however was not found in samples from animals with mastitis. Differences were in OTUs in samples from animals with no pathology (*Enterococcus faecalis*, *Coprococcus catus*), compared to animals with signs of endometritis and mastitis (*Peptoniphilus indolicus*, *Helcococcus ovis*). *Psychrobacter pasteurii*, *Helcococcus ovis* and *Peptoniphilus indolicus* bacteria were the most abundant in the EM group of OTUs, in contrast to bioassays of animals with mastitis (Figure 2).

Comparison of the genotyping results of cervical swabs and milk samples obtained from animals with no pathology and with reproductive tract and/or mammary gland inflammation using

the Mann-Whitney test (p-value <0.05) revealed a number of differences in the microbiota composition. A difference was found in the relative abundance of bacteria of the family *Peptoniphilaceae* and the species *Peptoniphilus indolicus* in animals from groups NP and E, respectively. A statistically significant difference was established in the relative abundance of bacteria of the *Aerococcaceae* family in the normal flora compared with animals with signs of mastitis. In studies by Wu H. et al., the bacterium *Aerococcaceae* is classified as the most common taxon of the microbiota of milk and airborne dust on farms [15]. Differences were observed when comparing the microbiota composition of mammary gland secretion samples by phylum of bacteria, such as *Candidatus Saccharibacteria*. Representatives of these taxonomic groups are symbiotic microorganisms involved in the fermentation of cellulose in the rumen [15]. The microbiota of the mammary secretion of animals without organ pathology is represented by bacteria: *Facklamia miroungae*, *Coprococcus catus*, *Enterococcus faecalis*. These bacteria may originate from environmental components that contaminate the teat's skin (such as bedding, feces) [16]. It is known that the bacterium *Coprococcus catus* is a representative of the intestinal microflora of animals and humans. The presence of *Coprococcus catus* in bioassays indicates non-compliance with sanitary and hygiene standards [17]. *Peptoniphilus indolicus* (M = 2.67), *Helcococcus ovis* (M = 2.31) were present in the microbiota of milk samples during inflammation, and the latter was found only in the bacterial composition of samples from cows with simultaneous inflammation of the mammary gland and reproductive tract. Both microorganisms identified by us are associated with the occurrence of mastitis in cows. *Peptoniphilus indolicus* is etiologically associated with the development of the so-called "summer" acute mastitis in Europe and belongs to the group of pyogenic bacteria [18, 19]. *Helcococcus ovis* was first identified in sheep with subclinical mastitis in Spain and the United Kingdom in 1999, in later reports the causative role of this pathogen in clinical mastitis in cows was revealed [20, 21]. Our studies confirm the significance of *Helcococcus ovis* and *Peptoniphilus indolicus* in the pathogenesis of mastitis in cows in the Sverdlovsk region, which coincides with the data of other authors.

Bacteroides plebeius is part of the gut microbiota and has been most commonly found in human feces [22], however, there is evidence of *Bacteroides* as a genital microbiota biomarker for predicting the pathogenesis of reproductive disorders in cows. [23].

Ruminococcus faecis (*Mediterraneibacter faecis*) is a representative of the *Clostridia* class and was found by foreign scientists in the microbiota of the bird's intestines [24], therefore, the question of its role in the reproductive tract microbiota of cows remains open. *Pseudoflavonifractor phocaeensis* refers to representatives of the bacterial community of the digestive tract of cattle. The data obtained indicate a significant relationship between the microbiota of the gastrointestinal tract and the colonization of the reproductive tract of animals, which can be explained by the presence of a hematogenous route of microorganism transmission [25].

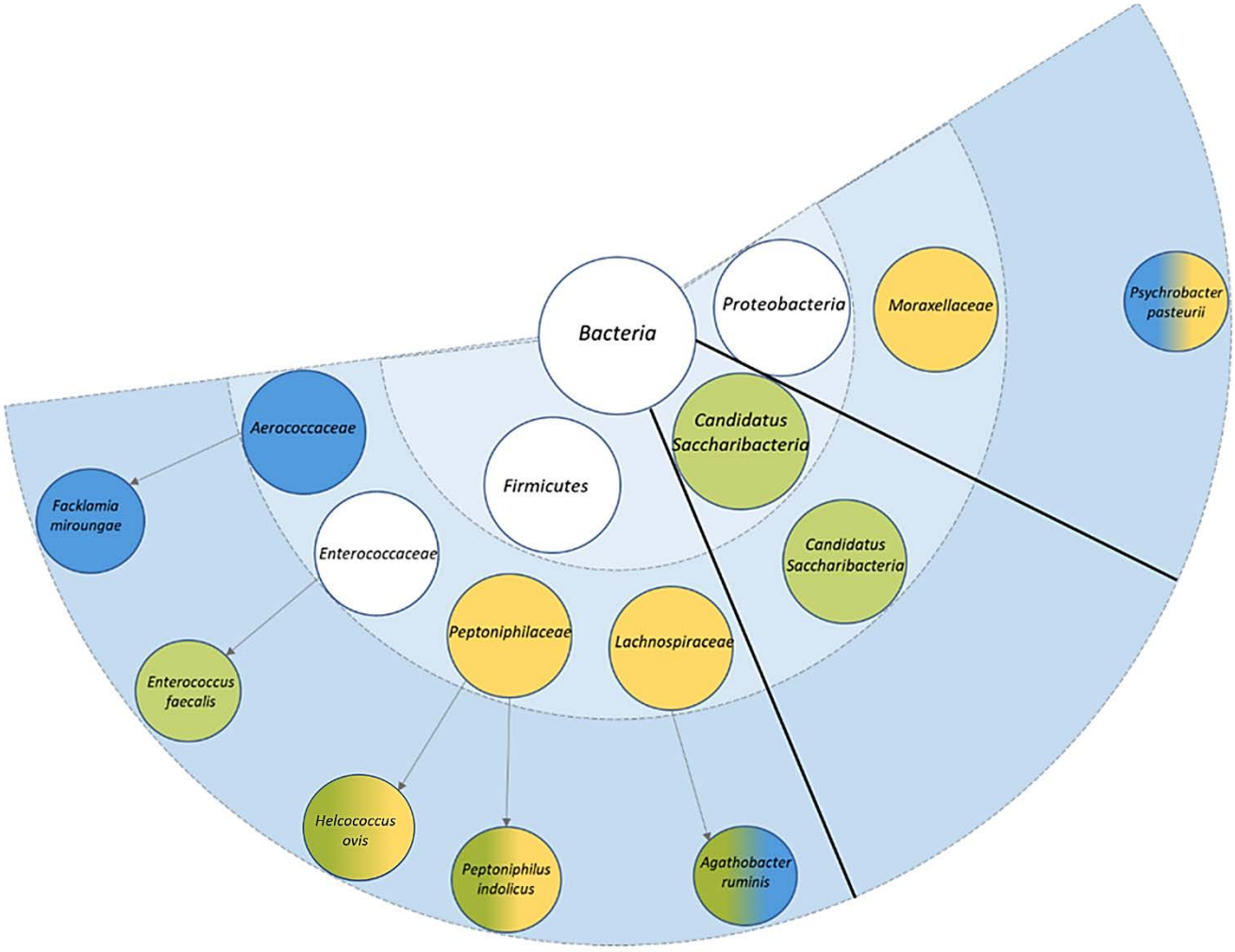

Figure 2:. Statistically significant differences in genotyping results of cervical swabs and mammary gland secretions from animal groups: NP – M (blue), NP – EM (green), M – EM (yellow), non-significant (no color) (0,05<p-value<0,1).
NB: unc – unclassified.

Differences are also observed when comparing types, families and OTUs.

An increase in the relative abundance of *Bacteroidetes* (M=19.26), *Veillonellaceae* (M=0.53) in cervical swabs of cows from the EM group and *Porphyromonadaceae* (M=2.47), *Peptoniphilaceae* (M=11.58) in cervical swabs of cows from the group M was established by comparison with animals with no pathology. The relative abundance of *unc_Bacteroidales*, *unc_Bacteroidetes*, *unc_Bacteria*, *Muribaculaceae*, *unc_Deltaproteobacteria* was significantly higher in the microbiota of cervical swabs of cows without pathology of the reproductive tract. The relative abundance of *Bacteroidaceae* (M = 2.17) was significantly higher in the microbiota of cervical swabs of cows with simultaneous inflammation of the reproductive tract and mammary gland, in comparison with animals with endometritis (group E).

Analysis of the relative abundance showed that the main predominant operating taxonomic units in the group of cows with inflammation of the reproductive tract were *Porphyromonas levii, Parvimonas micra, Peptostreptococcus anaerobius, Casaltella massiliensis, Moraxella osloensis*, in comparison with the group of animals without pathology. On the contrary, there was a significantly higher abundance of OTUs belonging to *Butyrivibrio proteoclasticus, unc_Bacteroidales, Flintibacter butyricus (Sporobacter termitidis), Alistipes finegoldii, Kineothrix alysoides, Sporobacter termitidis,*

Bacteroides caecigallinarum, Agathobacter ruminis plautii, Anaerotignum faecicola, Emergencia timonensis, unc_Firmicutes, Lachnotalea glycerini in the microbiota of cervical swabs of animals without signs of inflammatory reproductive pathology. A comparative analysis of the E group and the EM group revealed a significant increase in the relative abundance of *Kineothrix alysoides, unc_Bacteroidales, Bacteroides plebeius* in the microbiota of cervical swabs in case of simultaneous inflammation of the reproductive tract and mammary gland in cows (Figure 3).

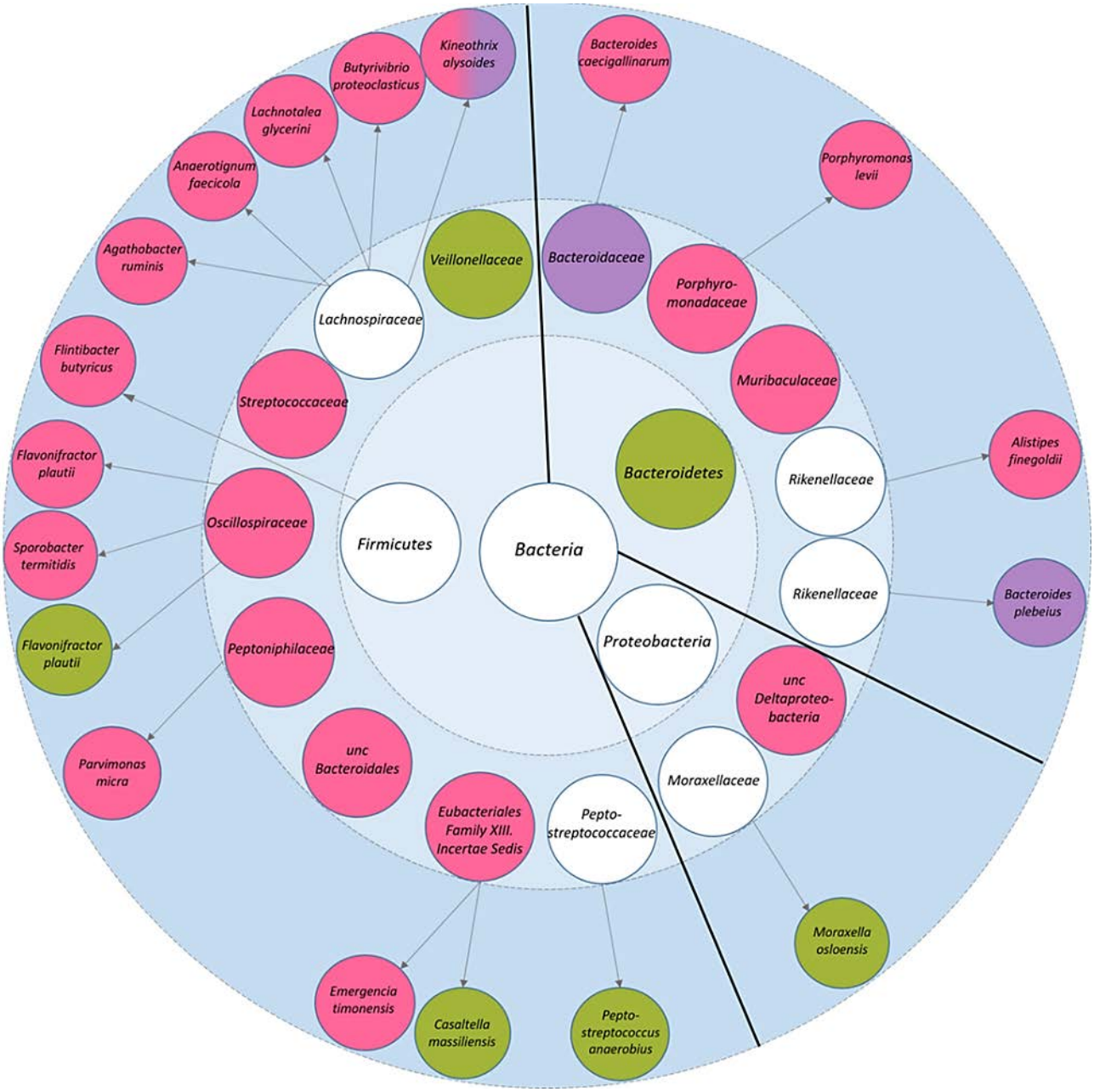

Figure 3:. Statistically significant differences in genotyping results of cervical swabs and mammary gland secretions from animal groups: NP – M (pink), NP – E (green), E – EM (violet), non-significant (no color) (0,05<p-value<0,1).
NB: unc – unclassified.

As a result of the research, a difference was found in the relative abundance of *Bacteroidetes* (M=19.26), *Veillonellaceae* (M=0.53) in the cervical swabs of cows from the EM group and *Porphyromonadaceae* (M=2.47), *Peptoniphilaceae* (M=11.58) in cervical swabs of cows from group M

changing upwards in comparison with healthy animals. In the microbiota of cervical swabs of cows without pathology of the reproductive tract, the abundance of *unc_Bacteroidales*, *unc_Bacteroidetes*, *unc_Bacteria*, *Muribaculaceae*, *unc_Deltaproteobacteria* was significantly higher. In the microbiota of cervical swabs of cows with simultaneous inflammation of the reproductive tract and mammary gland, there was a significantly higher relative abundance of *Bacteroidaceae* (M = 2.17) in comparison with animals with endometritis (group E).

Certain differences were found in the microbiota composition of the mammary gland secretion of cows with mastitis and with simultaneous inflammation of the mammary gland and reproductive tract. For instance, *Streptococcaceae* (M=66.43) was the predominant family in the taxonomic profile of cows with mastitis, and in cows of the second group, there were statistically significantly more representatives of the *Peptoniphilaceae* (M=8.62) and *Moraxellaceae* (M=0.44) families. It is noteworthy that members of the *Peptoniphilaceae* are anaerobic acidogenic bacteria, and members of the *Moraxellaceae* family are described as a component of the milk microbiota of Holstein cows and are also stably present in bedding and in airborne dust [26]. It is known that possible seasonal fluctuations in the occurrence of mastitis are mainly associated with a change in the microbiota composition of the housing facilities for animals, namely, bedding and air, which, when in contact with the teats of the mammary gland, affect the milk microbiota [26].

The microbiota of cervical swabs of animals with no pathology was characterized by the presence of *Bacteroides plebeius*, *Ruminococcus faecis* (*Mediterraneibacter faecis*) and a higher relative abundance of *Pseudoflavonifractor phocaeensis*, in contrast to cows with signs of inflammation of the reproductive tract.

Among the bacteria for which an interrelation in the relative abundance in milk samples and cervical swabs of cows during inflammation has been established – *Turicibacter sanguinis*, *Staphylococcus aureus*, *Peptostreptococcus anaerobius*, *Peptoniphilus indolicus*, *Helcococcus ovis* are described as infectious agents provoking the inflammatory process [18–21, 27, 28]. The rest of the bacteria, such as *Sporobacter termitidis*, *Clostridium saudiense*, *Romboutsia timonensis*, *Bacteroides tenuis*, *Butyrivibrio proteoclasticus*, *Facklamia tabacinasalis*, *Fusobacterium necrophorum*, *Cutibacterium acne* are representatives of the gastrointestinal microflora or saprophytic microorganisms living in the external environment.

4 Conclusion

16S rRNA-based analysis of microbiota allowed for the determination of taxonomic profiles of biosamples of mammary gland secretions and cervical swabs of cattle, including the detection of difficult-to-culture and uncultivatable bacterial species. In this study, a comparative analysis of the microbiota of animals with no pathology and with inflammatory processes was carried out. For the first time, the species diversity of the microbiota composition of animals with different physiological statuses was shown, however, the changes, that bacterial microbiota undergoes during disease and remission, require further research. Moreover, common bacteria were found in

inflammation of the mammary gland and reproductive tract of cows, which gives reason to assume a common etiology of the inflammatory processes of these organs.

5 Availability of Data and Material

Information can be made available by contacting the corresponding author.

6 Acknowledgments

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