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BIODIVERSITY AND PREDICTED METABOLIC FUNCTIONS OF THE RUMEN MICROBIOTA DEPENDING ON FEEDING HABITS AT DIFFERENT STAGES OF THE PHYSIOLOGICAL CYCLE OF DAIRY COWS

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Abstract

Under intensified cattle breeding, combined stress factors, in particular, extremely high milk productivity, inconsistency of neuro-humoral and hormonal regulation of feed intake and milk production, negative energy balance, feeds excessive in starch negatively impact the rumen microbiota and, consequently, a cow's physiology. This paper for the first time shows the phases of dairy cow lactation cycle as an important factor that determines the relative abundance of non-attributable bacteria from the candidate families vadinBE97 and WCHB1-41 which functions are practically not studied. The most pronounced changes in the metabolic potential of the microbiota, namely the inhibition of various metabolic pathways in the rumen chyme, e.g., energy (tricarboxylic acid cycle), protein, carbohydrate, lipid, including volatile fatty acid (VFA) synthesis, occurred in cows during stable and declining milk production phases as compared to dry, fresh and milked cows. The aim of this work is to study the composition and metabolic potential of the rumen microbiome in dairy cows during different physiological phases. The experiment (the JSC Agrofirma Dmitrova Gora, Tver Province, the summer 2020) was performed on 15 black-and-white Holsteinized dairy cows (*Bos taurus*) of the second and third lactations. The cows were assigned to five groups (5 cows each), including the dry cows (on average 30 days before calving, group I), the cows of 20 milking days (group II), of 90 milking days (group III), at day 208 of lactation (group IV), and in late lactation phase when the milk production is declining (day 310, group V). Dairy cows' diets were calculated using AMTS.Cattle.Professional software in accordance with the accepted requirements. Total DNA was extracted from rumen chyme samples (a Genomic DNA Purification Kit, Fermentas, Inc., Lithuania). The NGS procedure (a MiSeq platform, Illumina, Inc., USA) was performed using primers to the 16S rRNA V3-V4 region and reagents for NGS library preparation (Nextera® XT IndexKit, Illumina Inc., USA), PCR product purification (Agencourt AMPure XP, Beckman Coulter Inc., USA), and sequencing (MiSeq® ReagentKit v2, 500 cycle, Illumina Inc., USA). Bioinformatic analysis was performed with Qiime2 ver. 2020.8 software. Noise sequences were filtered by the Deblur method. The de novo phylogeny was constructed using the MAFFT software package. To analyze the taxonomy, the reference database Silva 138 (https://www.arb-silva.de/documentation/release-138/) was used. Reconstruction and prediction of the functional content of the metagenome was performed using PICRUSt2 software package v.2.3.0 with MetaCyc database for metabolic pathways and enzymes. Total RNA was isolated from the chyme samples (Aurum Total RNA kit, Bio-Rad, United States) followed by cDNA synthesis (iScript

RT Supermix kit, BioRad, USA). The relative expression of the bacterial L-lactate dehydrogenase gene *Ldh-L* and the *Ldb 0813* gene associated with D-lactate dehydrogenase synthesis was assessed using quantitative PCR (SsoAdvanced Universal SYBR Green Supermix kit, Bio-Rad, USA). The16S metagenomic sequencing revealed a decrease ($p \leq 0.05$) in the rumen bacteria α-diversity in group IV and group V. We have found twelve superphila and phyla of microorganisms. The superphylum *Bacteroidota* and the phylum *Firmicutes* we refer to the dominant rumen bacteria (up to 59.94±1.86 and 46.82±14.40 % of the population, respectively). The superphylum *Actinobacteriota* bacteria not found in lactating cows appeared only in dry cows. The bacteria of the superphylum *Armatimonadota* disappeared from the rumen of fresh cows and during stable lactation phase, and of the phylum *Chloroflexi* — during early and stable lactation phases. The cows differed significantly in eight bacterial families, the *Muribaculaceae*, *Prevotellaceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Saccharimonadaceae*, and candidate families WCHB1-41 and vadinBE97. The rumen genera *Asteroleplasma*, *Sharpea*, *Moryella*, *Oribacterium*, *Shuttleworthia* appeared after calving and persisted in the next phases of lactation. These bacteria were absent in dry cows. The predicted functional capability of 17 metabolic pathways of the microbiome varied ($p \leq 0.01$) in cows of different groups. The most pronounced changes, namely the suppression of various metabolic pathways in the rumen chyme, occurred in groups IV and V compared to group I, group II, and group III ($p \leq 0.01$). An increase in the expression of the *Ldh-L* ($p \leq 0.01$) and *Ldb 0813* ($p \leq 0.05$) genes associated with the synthesis of lactate dehydrogenases was characteristic of fresh cows compared to dry cows. There was a significant increase in the expression of the rumen bacteria genes *Ldh-L* (10.6-fold, $p \leq 0.001$) and *Ldb 0813* (2.8-fold, $p \leq 0.05$) when lactation declined as compared to group IV.

Keywords: rumen microbiome, ruminants, dairy cows, diet, starch, cellular tissue, NGS-sequencing, PICRUSt2, MetaCyc, metabolic pathway

Today's dairy farming strategy needs to maximize the utilization of nutrients in the feeds whilst minimizing the risks of rumen ecosystem dysbiosis, digestive and metabolic disorders. Microbial fermentation covers 70% of dairy cows' energy needs. This points to the critical role of rumen microbiota in animals' metabolism and to the need to investigate which microorganisms are present there and how they function [1].

Ruminants have one unique metabolic feature: due to the evolutionary symbiosis with the rumen microbiota, they are able to digest plant fiber that contains cellulose, hemicellulose, and xylans [2-4]. Microorganisms present in the digestive system, albeit of different phylogeny, are inextricably linked; their symbiotic relations and metabolic networks play a central role in the rumen functioning, especially in fiber digestion [5]. Thus, Akin et al. [6] and Janssen et al. [7] have shown the interdomain interdependence in the case of bacteria and archaea. Bacteria degrade lignocellulosic material and produce hydrogen [6] that methanogenic archaea need [7]. Similarly, most of the lactate produced by one category of microorganisms is further metabolized by the bacteria that need this substance [8]. This results in the production of volatile fatty acids (VFAs) that are absorbed through the stratified squamous epithelium of the rumen. On the one hand, VFAs directly replenish the energy substrates, mainly for gluconeogenesis [9], and thus make an important contribution to the formation of the animal's metabolic pool [10]. On the other hand, lactate-to-VFA conversion promotes the buffering of the rumen's contents, which is an important acidosis prevention mechanism [11].

Under intensified cattle breeding, combined stress factors, in particular, extremely high milk productivity, inconsistency of neuro-humoral and hormonal regulation of feed intake and milk production, negative energy balance, feeds excessive in starch negatively impact the rumen microbiota and, consequently, a cow's physiology. As it is known, dry cows' diet should be designed to lower the risks of postpartum complications, which dictates the use of high-quality feed rich in fiber and moderate in concentrates. This stimulates chewing activity and rumen motility, raises pH, restores rumen microbiota, and thus helps recover the animal's general metabolic activity [12-14]. Some researchers [4, 15, 16] note the extreme diversity of rumen microbiota that in dry cows mainly consists of cellulo-

lytic bacteria.

According to Henderson et al. [17], unlike in monogastric animals, a ruminant's diet contributes much more to the formation of the rumen microbiome than the host's genotype and individual physiology. Physiological status and milk productivity are most responsive in newly calved cows [18]. Such cows have rapidly rising glucose demand; thus, energy output for milk production exceeds energy intake from feed, which results in a negative energy balance [14]. Low glucose and insulin concentrations in blood plasma trigger a physiological mechanism designed to overcome energy deficiency: the body actively mobilizes triglycerides from adipose tissue in order to cover the rising energy demands [19]. All this induces an imbalance of glucogenic and lipogenic compounds in plasma, which has dire consequences for metabolism [20].

Starch- and monosaccharide-based feeds, which are staple foods for lactating cows, are known to negatively affect the physiology of recently calved cows [21, 22]. Yet, starch and monosaccharides are the key sources of glycogenic precursors (e.g., propionate) and fermentable energy for the rumen microorganisms. Nevertheless, excessive production of short-chain fatty acids lowers rumen pH [23], as increased VFA production increases the population of acid-resistant bacteria of the phylum *Bacteroidetes* and culls the population of *Firmicutes* [24]. *Bacteroidetes* actively synthesize lactate as an intermediate of starch fermentation; coupled with the microbiota's inability to maintain an appropriate acid-base balance in the rumen, this process results in intraluminal accumulation of protons, with pH further falling below the physiological range [14, 25, 26]. The condition is commonly known as rumen acidosis. Acidosis multiplies the risks of fatty liver and ketosis, metritis, and abomasum displacement. Such disorders have far-reaching consequences for cattle health and productivity, resulting in lameness [27, 28], worsening reproduction, lower milk productivity, and shorter productive lifespan [29, 30]. Zebeli *et al.* argue [14] that the incidence and severity of metabolic disorders depend on feed intake and on the starch-richness of the diet. A gradual increase in concentrates by 0.25 kg of dry matter (DM) per day after calving enables better rumen microbiota adaptation than a 1 kg increase in the daily intake of concentrate (DM) [18]. This combination of a fast transition to a high-energy diet, postpartum and lactation stress, and negative energy balance is associated with a high risk of metabolic disorders in newly calved cows [31, 32].

During an increase in yield, stabilization and decline of lactation, cows are still at high risk of rumen microbiome disturbances and the resulting metabolic disorders [14]. Some researchers [26, 33, 34] have observed that high concentrations of soluble starch in cow rumen during early lactation were associated with a rising population of *Lactobacillus* sp. and of the amylolytic microorganism *Streptococcus bovis*, which produce lactate [34]. This induced lowering pH and suppression of cellulolytic and VFA-producing bacteria; it also disrupted the VFA synthesis processes.

Studying the rumen microbiome is one of the fundamental approaches to developing effective measures to prevent metabolic disorders at dairy farms. Of interest is a comprehensive analysis of change in the composition and metabolic potential of the rumen microbiome in various physiological periods in dairy cows. Both Russian researchers using classical methods of microbiology [35-37] and their international colleagues [13, 14, 23] have studied in detail the effects that physiological periods and diets have on the rumen microbiome taxonomy. However, the specific biological and metabolic functions of the rumen microbiome as exhibited in different physiological periods in dairy cows are yet to be fully discovered. Besides, microbiomes in animals raised in accordance with the Russian dairy practices have been little studied by means of molecular biology.

This paper for the first time shows the phases of the dairy cow lactation

cycle ~~as~~ an important factor that determines the relative abundance of non-attributable bacteria from the candidate families vadinBE97 and WCHB1-41 whose functions have been understudied thus far. The most pronounced changes in the metabolic potential of the microbiota, namely the inhibition of various metabolic pathways in the rumen chyme, e.g., energy (tricarboxylic acid cycle), protein, carbohydrate, lipid, including volatile fatty acid (VFA) synthesis, occurred in cows during stable and declining milk production phases as compared to dry, freshly calved, and milked cows.

The aim of this work is to study the composition and metabolic potential of the rumen microbiome in dairy cows during different physiological phases.

Materials and methods. The experiment was performed on 15 black-and-white Holsteinized dairy cows (*Bos taurus*) of the second and third lactations at JSC Agrofirma Dmitrova Gora, Tver Region, summer 2020. The choice of a commercial farm as a pilot site was based on the experiments described in earlier studies of microbiome in dairy cows [38, 39].

The animals were kept under the same conditions in tie-stall barns. They were split into five groups, 3 cows each: Group 1 of dry cows (30 days before calving on average), Group 2 of newly calved cows (20 days of milking on average), Group 3 of cows in early lactation (90 days in milk in average), Group 4 of cows in mid-lactation (208th day in milk), and Group 5 of cows in late lactation (310th day in milk). Dry cows were selected on the basis of the expected calving day. The number of animals per group was adjusted following the guidelines found in earlier studies [40, 41]. Group 1 had an average live weight of 703 kg. The daily average yield was 27.0 l/head, the fat content of milk was 3.7%, and the weight was 637 kg in Group 2. The daily average yield was 38.5 l/head, the fat content of milk was 3.7%, and the weight was 650 kg in Group 2. Group 4 and 5 animals had an average live weight of 667 and 681 kg, respectively.

Chyme (30-50 g per cow) were sampled manually from the upper ventral sac of the rumen using a sterile probe under as aseptic conditions as possible.

Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) per the kit manual [3]. Testing was based on DNA sedimentation from the substrate using solutions for cell wall lysis, DNA sedimentation, 1.2 M sodium chloride and chloroform.

The bacterial community of the rumen was detected by NGS sequencing using MiSeq (Illumina, Inc., USA) with primers for the V3-V4 region of 16S rRNA (forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGATCTAATCC-3′).

Sequencing was performed using Nextera® XT IndexKit for library preparation (Illumina, Inc., USA), Agencourt AMPure XP kit for PCR product purification (Beckman Coulter, Inc., USA) and MiSeq® ReagentKit v2 (500 cycles) for sequencing (Illumina, Inc., USA). The obtained sequences had a maximum length of 2×250 bps.

Bioinformatics data analysis was performed by Qiime2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). Paired reads were aligned after the initial import of sequences into the Qiime2 format. Then the sequences were filtered by quality using the default settings. Noise sequences were filtered by the Deblur method using the maximum trimming length of 250 bps (https://msystems.asm.org/content/msys/2/2/e00191-16.full.pdf). To construct the de novo phylogeny, the MAFFT software was used with subsequent masked sequence alignment. For taxonomy analysis, we used the Silva 138 reference database (https://www.arb-silva.de/documentation/release-138/).

The resulting table of operational taxonomic units (OTUs) was used to

compute α-diversity indices in Qiime2 plugins and to plot curves of the OTU number as a function of the number of reads. No additional transformations were applied for the statistical analysis of diversity indices.

Reconstruction and prediction of the functional content of the metagenome were performed using the PICRUSt2 software package v.2.3.0 (https://picrust.github.io/picrust/). The software was used as recommended; all default settings were kept. Metabolic pathways and enzymes were analyzed using the MetaCyc database (https://metacyc.org/). The predicted MetaCyc metabolic pathway profiles were tested for the abundance of amplicon sequence variants [42].

Total RNA was isolated from chyme samples using the Aurum Total RNA kit (Bio-Rad, United States) per the manufacturer's manual. cDNA was synthesized on the RNA matrix using the iScript RT Supermix kit (Bio-Rad, USA) [43]. Quantitative PCR was used to analyze the relative expression of the *Ldh-L* and *Ldb 0813* genes of lactic acid fermentation-capable bacteria. Amplification with the primers of the *Ldb 0813*, the gene associated with the synthesis of D-lactate dehydrogenase (F: 5′-CTGGGATCCGTTGAGGGAGATGCTTAAG-3′, R: 5′-TCCGAAGCTTTTAGTTGACCCGGTTGAC-3′) and L-lactate dehydrogenase (gene *Ldh-L*) (F: 5′-CATCAAAAAGTTGTGTTAGTCGGCG-3′, R: 5′-TCAGCTAAACCGTCGTTAAGCACTT-3′) was run on a DT Lite-4 unit (a detection amplifier, NPO DNA-Technology LLC, Russia). Amplification conditions: 1 min at 95 °C (1 cycle); 15 s at 95 °C, 1 min at 50 °C (45 cycles). The reaction mixture for amplification (SsoAdvanced Universal SYBR Green Supermix kit, Bio-Rad, USA) was prepared in accordance with the manufacturer's protocol.

Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method [44] using the 16S-ribosomal subunit gene of prokaryotes (primer pair: F — 5′-AGGCC-TTCGGGTTGTAAAGT-3′, R — 5′-CGGGGATTCACATCTCACT-3′) for reference.

The Chao1, Shannon (H), and Simpson (D) biodiversity indices for the rumen microbiome were calculated as described in [45].

For mathematical and statistical processing of the results, a single-factor analysis of variance (ANOVA) was applied (Microsoft Excel XP/2003, R-Studio Version 1.1.453, https://rstudio.com). To correct Type I error, Tukey's HSD test was applied (https://www.rdocumentation.org/pack-ages/stats/versions/3.6.1/topics/TukeyHS). The results are shown as means (*M*) and standard errors of the mean (±SEM), with $p \leq 0.05$ as a threshold for the significance level verified by Student's *t*-test.

Results. Cows' diets for each group were designed in AMTS.Cattle.Professional (https://agmodelsystems.com) in accordance with the common standards [46-48], see Table. Premix recipes were adjusted for each group specifically. The premixes were designed to fully cover micronutrient needs for required productivity.

NGS sequencing revealed composition and structure of the bacterial community of bovine rumens. The Shannon and Chao1 indices were found not to differ between Groups 2 (newly calved) and 1 (dry cows), $p \geq 0.05$, see Fig. 1. This is an interesting finding, as both metrics estimate species diversity [49]. Newly calved cows were expected to show a more pronounced change in the diversity, whether an upward or downward change, from their dry counterparts, as they had been exposed to several negative factors on top of drastic change in the diet structure, calving stress, and the onset of lactation. Thus, Bach et al. [50] observed a decline in the H index of the digestive microbial community in freshly calved cows as compared to dry cows. In this study, the H index rose slightly ($p \leq 0.05$) in early lactation as compared to newly calved cows. A sharp decline ($p \leq 0.05$) in the H and Chao1 indices occurred as compared to the preceding physiological periods during mid-lactation in Group 4 and late lactation in Group 5. Digestive

microbiota is generally known to become less diverse in cases of dysbiosis, particularly in cases of treatment with antimicrobials [51] or gastroenteritis [52]. Nevertheless, the diversity of the microbial communities in the rumen is a fairly stable indicator, as in this study, Shannon and Chao1 indices did not decline in newly calved cows nor in early lactation. The Simpson index calculated for each tested group did not have significantly different values ($p \geq 0.05$), see Fig. 1, C.

Nutritional factors of diets for black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods

Parameter	Group				
	I	II	III	IV	V
Dry matter (DM) content, %	39.02	45.10	42.31	43.50	42.50
DM consumption, kg	11.82	18.94	25.00	25.60	27.60
Metabolic energy provided by the diet, % of the normal value	106.86	103.82	104.87	105.52	100.20
Metabolic protein provided by the diet, % of the normal value	103.47	97.08	104.57	105.33	105.43
Crude protein (CP), %	14.86	16.44	17.48	17.50	17.60
Net energy of lactation, MJ/kg	6.29	6.47	6.86	6.60	6.30
Neutral detergent fiber, % of DM	41.63	25.75	28.28	29.28	30.20
Acidic detergent fiber, % of DM	26.64	16.25	17.75	18.50	18.80
Rumen-degradable protein, % of CP	56.0	57.0	52.0	55.0	56.0
Rumen-nondegradable protein, % of CP	44.0	43.0	48.0	45.0	44.0
Starch, % of DM	16.20	23.54	26.64	26.45	24.50
Ca, % of DM	0.49	0.82	0.74	0.71	0.68
P, % of DM	0.51	0.39	0.40	0.40	0.41
Mg, % of DM	0.47	0.39	0.37	0.39	0.42

N o t e. For description of the groups, see the Material and methods section.

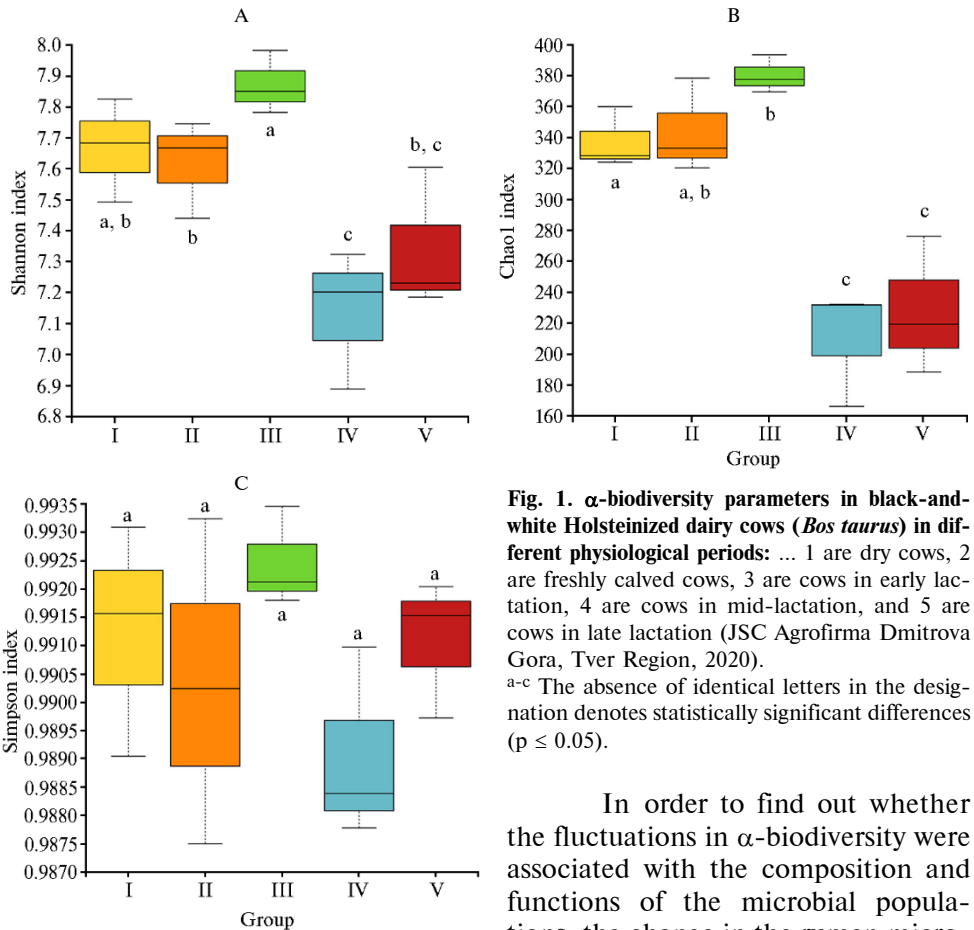

Fig. 1. α-biodiversity parameters in black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: ... 1 are dry cows, 2 are freshly calved cows, 3 are cows in early lactation, 4 are cows in mid-lactation, and 5 are cows in late lactation (JSC Agrofirma Dmitrova Gora, Tver Region, 2020).

[a-c] The absence of identical letters in the designation denotes statistically significant differences ($p \leq 0.05$).

In order to find out whether the fluctuations in α-biodiversity were associated with the composition and functions of the microbial populations, the change in the rumen micro-

biota taxonomy was estimated using NGS sequencing data.

Twelve superphyla and phyla of microorganisms were found in the rumen microbiota, see Fig. 2, where the superphylum *Bacteroidota* and the phylum *Firmicutes* could be considered the dominant bacteria in the rumen, as they represented up to 59.94±1.86 and 46.82±14.40% of the microbiota, respectively. These two taxonomic groups can be considered to be the core of the bacterial microbiome, as they have a significant presence in nearly all *Bos taurus* [12, 53, 54]. The dominant bacteria found in this study were likely responsible for most of the substance transformation in the rumen, in particular, that of cellulose, hemicellulose, starch, organic acids, and protein, as these substances are diet components and intermediate energy substrates [1].

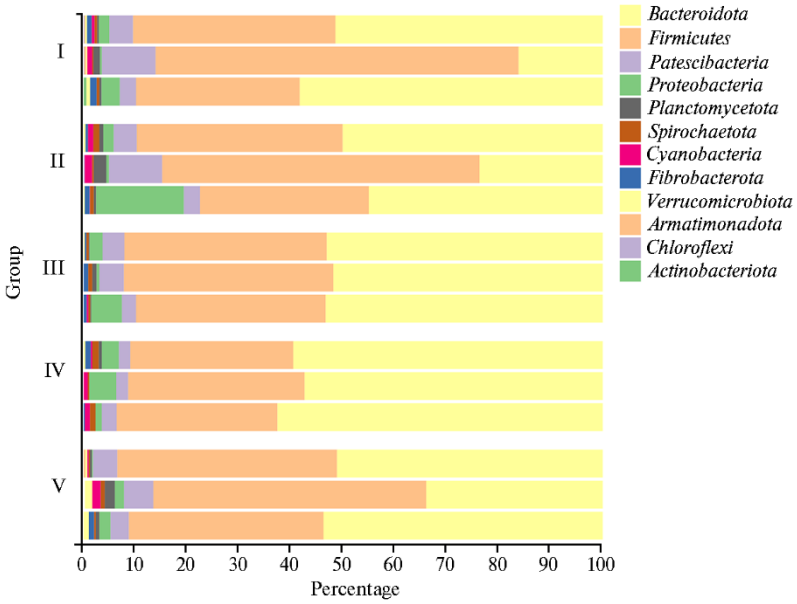

Fig. 2. Rumen microbiome taxonomy in black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

The decline in the α-diversity of the rumen microbiome was found to be related to the reduction in the relative population of some taxa, which was registered at the phylum level. Thus, bacteria of the superphylum *Actinobacteriota* were eliminated completely from the rumen in lactating cows but were found in dry cows; *Armatimonadota* were not found in newly calved cows or cows in mid-lactation; the phylum *Chloroflexi* was not found in cows in early or mid-lactation. The disappearance of these microorganisms might have contributed negatively to metabolism in lactating cows. Thus, *Actinobacteriota* are common symbionts in eukaryotes [55]; their cellulolytic enzymes enable more efficient fiber breakdown [56].

The superphylum *Verrucomicrobiota* had a lesser presence in cows in early or mid-lactation compared to dry cows ($p \leq 0.05$). Despite the ubiquity of these microorganisms, pure cultures only contain a few isolates [57], making their ecological significance ambiguous still. *Verrucomicrobia* are known to have a significant presence in termite intestines where they metabolized plant polysaccharides into acetate [57]. Some members of the superphylum *Verrucomicrobia* have recently been discovered to be able to oxidize methane (an ability that had earlier been observed in proteobacteria only) in an acidic environment (pH 0.8-2.0) [58]. Thus, a decrease of *Verrucomicrobiota* in this experiment, which was observed in early and mid-lactation, could be associated with the suppression of fiber

digestion and methanogenesis in cases of high-concentrate feeding.

Of special interest is that the superphylum *Patescibacteria* had a lesser presence in mid-lactation than in dry cows ($p \leq 0.05$). The fact is that *Patescibacteria* have significantly reduced genomes compared to most other microorganisms; they use simple intermediates of the host's digestive system in order to obtain energy, e.g., glucose and pyruvic acid; they have lost the ability to digest complex polysaccharides [59]. Loss of the *Patescibacteria* population in mid-lactation could be due to the disruption in the synthesis of these nutrient substrates in the rumen.

Fig. 3. Rumen microbiome taxonomy (family level) in black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

Detailed analysis of the microbiota composition (Fig. 3) showed some of the families to have a relatively even presence in the animals of different groups. However, eight families showed significant differences as well: *Muribaculaceae*, *Prevotellaceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Saccharimonadaceae*, and candidate families WCHB1-41, vadinBE97, see Fig. 3.

The family *Prevotellaceae* of the superphylum *Bacteroidota*, mainly of the genus *Prevotella*, has a greater presence in Group 4 than in Group 1 ($p \leq 0.05$). Such greater presence of *Prevotellaceae* in cows fed with starch- and monosaccharide-rich diets is only logical. These microorganisms are known [60, 61] to use starch to synthesize VFAs; however, excess production of short-chain fatty acids is associated with a decrease in ruminal pH and can lead to lactate acidosis accompanied by dysbiosis [62]. Besides, cows in Group 4 had smaller populations of the families *Oscillospiraceae* (the phylum *Firmicutes*) and *Saccharimonadaceae* (the superphylum *Patescibacteria*) than those in Group 1 ($p \leq 0.05$). This could be a sign

of dysbiosis due to high-concentrate feeding as *Oscillospiraceae* have a substantial set of glycoside hydrolases. The latter have a variety of functions and are involved in the degradation of cellulose and hemicellulose in feeds; thus, they are fundamental to the biological apparatus behind the destruction of glycosidic bonds [63].

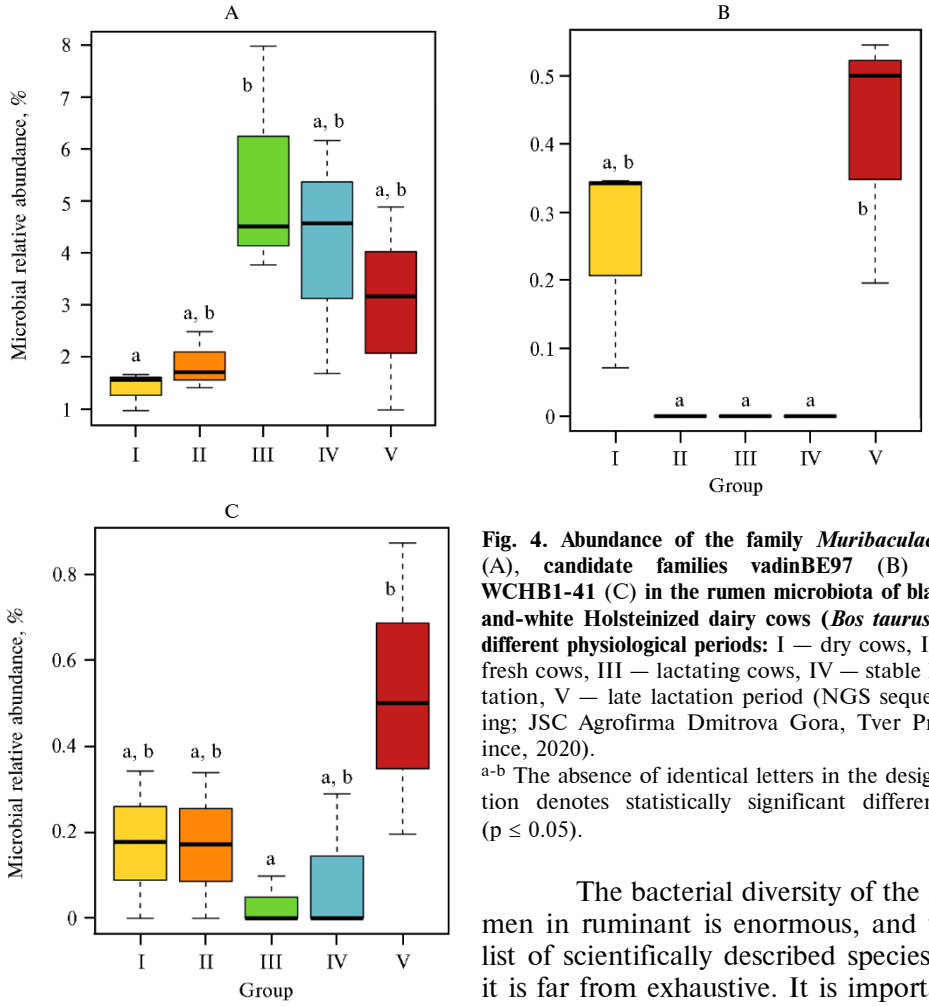

Fig. 4. Abundance of the family *Muribaculaceae* (A), **candidate families vadinBE97** (B) **and WCHB1-41** (C) **in the rumen microbiota of black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods:** I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020).
[a-b] The absence of identical letters in the designation denotes statistically significant differences ($p \leq 0.05$).

The bacterial diversity of the rumen in ruminant is enormous, and the list of scientifically described species in it is far from exhaustive. It is important to focus on the microorganisms that are yet uncultured in order to properly analyze such important processes as nutrient metabolism cycles and modulation in the body's functions associated with the host's health. In this regard, it was interesting to identify the family *Muribaculaceae* of the superphylum *Bacteroidota* in the rumen microbiome, see Fig. 4. Until 2019 [64], this taxon was classified as an uncultured family S24-7 despite being ubiquitous in the intestinal microbiota in many vertebrates. Taxonomic ambiguity made agent-host interaction analysis difficult.

In this experiment, *Muribaculaceae* had a greater presence ($p \leq 0.05$) in lactating than in dry cows, see Fig. 4. Earlier, Ormerod et al. [65] demonstrated the presence of a substantial and versatile set of carbohydrate breakdown-associated enzymes in the analyzed genomes of S24-7 species. The ability to degrade carbohydrates might explain the presence of these microorganisms in cow rumen during high-concentrate feeding. The fact that *Muribaculaceae* have mechanisms to protect themselves from some organic acids [64] might explain their colonization behavior.

The facts that the non-attributable bacteria from the candidate family vadinBE97 disappeared from the rumen microbiota in lactating cows in Groups 2, 3, and 4, and that the presence of the candidate family WCHB1-41 was reduced in Groups 3 and 4 (see Fig. 4) confirms that feeding is an important factor of the relative abundance of these microorganisms in the rumen. This finding is of fundamental interest, as the functions of the bacteria of these families in the superphylum *Verrucomicrobiota* remain virtually unstudied due to the impossibility of laboratory culturing.

Attention-worthy is the fact that microbiota of the genera *Asteroleplasma*, *Sharpea* (fam. *Erysipelatoclos-tridiaceae*), and *Moryella*, *Oribacterium*, *Shuttleworthia* (fam. *Lachnospiraceae*), which were not found in dry cows' microbiota, appeared in the rumen shortly after calving and persisted through the subsequent lactation periods. These genera could be referred to as marker taxa that appeared in response to calving-related stressors, negative energy balance, and dietary changes.

Interestingly, *Asteroleplasma anaerobium*, being the only *Asteroleplasma* in the microbiome of the tested cows, has lactate dehydrogenases that are activated by fructose-1,6-biphosphate [66]. Lactate dehydrogenases are key enzymes of lactic acid fermentation that produces lactate [67, 68]. Lactate is also one of the key metabolic end products of *Moryella* [69], *Oribacterium* [70], and *Shuttleworthia* [71]. The fact that these taxa appeared in the microbiome during lactation could be due to metabolic disorders that are often observed in high-concentrate feeding [34], as lactate induces a lower pH and causes cellulolytic and VFA-synthesizing microorganisms to die [34].

Let us focus on *Sharpea azabuensis*, the only *Sharpea* species found in newly calved cows' microbiome as well as during early and mid-lactation. In the rumen, this bacterium is able to synthesize intermediate trans-11-isomers of linoleic and linolenic acid [72]. Concentrate-based diets of ruminants mainly contain C18 unsaturated fatty acids (α-linolenic, linoleic, oleic, etc.) [73]. A diet rich in starch and low in fiber is known to cause the rumen metabolism to shift towards producing trans-10 isomers of fatty acids via trans-11 intermediates [73]. The presence of trans-10 intermediates in the rumen is often associated with a decrease in milk fat [74].

Nevertheless, there were no typical changes [34, 75] indicative of possible metabolic disorders in the cows' microbiome. In particular, there was no increase in the population of *Lactobacillaceae* that in most cases [34] induce a decrease in pH and trigger metabolic disorders on top of a high-concentrate diet. *Fusobacterium necrophorum*, a highly virulent ruminant pathogen capable of producing hemagglutinin, endotoxin, and leukotoxin [76], often gaining a competitive advantage from high-concentrate feeding [76], was totally absent from the microbiome.

To find out whether the physiological period-related compositional changes in the rumen microbiome were associated with functional changes, the research team reconstructed and predicted the functional content of the metagenomic community of the rumen using PICRUSt2 and the Kyoto Encyclopedia of Genes and Genomes (KEGG: Kyoto Encyclopedia of Genes and Genomes) (https://www.genome.jp/kegg/). This reconstruction made it possible to annotate 282 various metabolic pathways. For 17, the predicted functional potential changed ($p \leq 0.01$) in cows of different groups, see Fig. 5. The most pronounced ($p \leq 0.01$) changes in the metabolic potential of the microbiota, namely the inhibition of various metabolic pathways in the rumen chyme, e.g., energy (tricarboxylic acid cycle), protein, carbohydrate, lipid, including volatile fatty acid (VFA) synthesis, occurred in cows of Groups 4 and 5 as compared to Groups 1, 2, and 3, see Fig. 5.

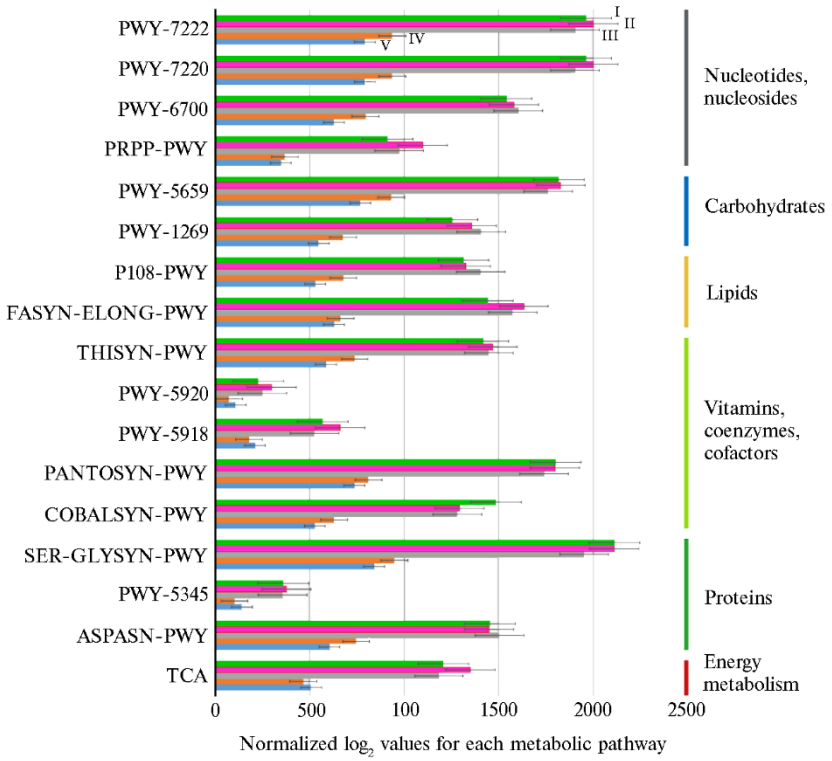

Fig. 5. Data ($p \leq 0.01$) of the functional annotation of metabolic pathways of metagenomics community of the rumen microbiota of black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods: I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (NGS sequencing; JSC Agrofirma Dmitrova Gora, Tver Province, 2020). TCA stands for the Krebs cycle, ASPASN-PWY is for the biosynthesis of L-aspartate and L-asparagine, PWY-5345 is for the biosynthesis of L-methionine via sulfhydrylation, SER-GLYSYN-PWY is for the biosynthesis of L-serine and glycine, COBALSYN-PWY is for the biosynthesis of adenosylcobalamin from cobinamide I, PANTOSYN-PWY is for the biosynthesis of pantothenate and coenzyme A I, PWY-5918 is for the biosynthesis of heme from glutamate, PWY-5920 is for the biosynthesis of heme from glycine, THISYN-PWY is for the biosynthesis of thiamine diphosphate I, FASYN-ELONG-PWY is for the chain lengthening of saturated fatty acids, P108-PWY is for pyruvate fermentation into propanoate I, PWY-1269 is for the biosynthesis of CMP-3-desoxy-D-mannooctulosonate I, PWY-5659 is for the biosynthesis of GDP-mannose, PRPP-PWY is for the biosynthesis of histidine, purine, and pyrimidine, PWY-6700 is for the biosynthesis of queuosine , PWY-7220 is for adenosine deoxyribonucleotide biosynthesis, PWY-7222 is for guanosine deoxyribonucleotide synthesis.

Groups 4 and 5 had lower ($p \leq 0.01$) Krebs cycle-related predicted metabolic capacities of the microbiome than Groups 1, 2, and 3, which could disrupt energy metabolism in the rumen as the Krebs cycle is the central regulator in macroorganisms and microorganisms alike. It involves a complex multistep sequence of reactions that supply energy and plastic substrates, reduced and phosphorylated cofactors for the major biosynthetic pathways [77] that largely determine the subsequent intensity and direction of the major metabolic flows [78].

Consequently, it seems natural that the microbiome had a lower capacity to synthesize pantothenate and coenzyme A (CoA) in Groups 4 and 5 than in Groups 1, 2, and 3 ($p \leq 0.01$). Acetyl coenzyme A (acetyl-CoA) is a key link in the regulation of the activity of pyruvate and alpha-ketoglutarate dehydrogenases in the Krebs cycle [79]. CoA biosynthesis precursor 3-methyl-2-oxobutanoate functions as an intermediate in the biosynthesis of L-valine. First, this compound converts into (R)-pantoate, then into (R)-4′-phosphopantothenate. Further L-cysteine and (R)-4′-phosphopantothenate produces (R)-4′-phosphopantetheinyl-

L-cysteine, which is then decarboxylated into 4′-phosphopantetheine. The final reaction is catalyzed by dephospho-CoA kinase (EC 2.7.1.24) that converts 4′-phosphopantetheine into CoA [80, 81].

Groups 4 and 5 had a lower energy balance potential in the rumen than Groups 1, 2, or 3 ($p \leq 0.01$), possibly due to the disrupted synthesis of microbial amino acids (L-aspartate, L-asparagine, L-methionine, L-serine, and glycine), see Fig. 5. Indeed, energy and nitrogen are the key determinants of how much microbial protein is synthesized in the rumen [82]. Microbial protein is the primary source of amino acids for ruminants [83]. The aforementioned changes in the potential could be due to feeding. Microbial protein synthesis rates had been shown [84] to drop in cows on a high-concentrate diet.

Cows in Groups 4 and 5 could have health issues due to less genes being involved in pyruvate-propanoate conversion, as propanoate is an important gluconeogenesis precursor in ruminants and can only be produced by the rumen microbiota [9]. Phase I: succinyl-CoA is converted into (R)-methylmalonyl-CoA that breaks down and transfers a C1 unit onto pyruvate. This reaction is catalyzed by methylmalonyl-CoA-carboxyl transferase and produces propanoyl-CoA and oxaloacetate. Newly produced propanoyl-CoA donates coenzyme A for another cycle phase where it is transferred onto the succinate. The subsequent succinate CoA transferase-catalyzed reaction produces the final product: propanoate [85]. High-concentrate feeding-induced lower pH in the rumen inhibits the reproduction of acidification-sensitive VFA producers, in particular propanoate producers [86]. Thus might have lowered the rates of propanoate production in Groups 4 and 5, resulting in inhibited gluconeogenesis.

Besides, Group 4 and 5 cows had lower ($p \leq 0.01$) metabolic potential for the synthesis of adenosylcobalamin from cobinamide in the rumen. Adenosylcobalamin is rumen microbe-produced vitamin B_{12} [87]. Many bacteria lack a complete set of genes required to synthesize adenosylcobalamin de novo; however, they are able to convert cobinamide into cobalamin [88]. To that end, the upper ligand of 5′-deoxyadenosine connects to adenosylcobinamide, which is further phosphorylated to produce adenosylcobinamide phosphate, an intermediate of de novo biosynthesis.

These modifications of the functional potential in Group 4 and 5 cows could be due to metabolic disorders [89]. Previously, Lima et al. [90] showed that the change in the number of B_{12} biosynthesis-associated genes in the rumen microbiota was due to the amount of feed intake. Ogunade et al. [91] studied the functional annotation of the rumen microbiome in young Holstein bulls and found the livestock with symptoms of subacute acidosis to have altered potential of 10 metabolic pathways including carbohydrate, amino acid, energy, vitamin, and cofactor metabolism; the change also affected the formation of bacterial biofilms. In the present research, changes in metabolic potential corresponded to the decrease in the rumen α-biodiversity, as well as to the disappearance of some taxa (the superphylum *Armatimonadota*, the phylum *Chloroflexi*) and a decrease in the population of other taxa (the superphylum *Verrucomicrobiota*, the family *Oscillospiraceae* of the phylum *Firmicutes*, the family *Saccharimonadaceae* of the superphylum *Patescibacteria*, etc.) in the rumen in Groups 4 and 5.

Thus, the most pronounced differences were observed in: the composition of the rumen microbiome between early/mid-lactation and dry cows; the metabolic potential between mid-/late lactation and dry cows. Data suggests that the biodiversity and functions of microorganisms depend on the condition and feeding of cows in different physiological periods. The findings also suggest that the rumen microbiome and its functional potential are more susceptive to negative effects in

early, mid-, and late lactation than shortly after calving, which probably means that common conceptions might need some reviewing [13, 14].

As noted, the production of lactic acid by the rumen microbiota is a key mechanism behind the metabolic disorders in ruminants [92]. The authors compared taxonomic research data and metabolic potential estimates for rumen microorganisms against the analysis of the expression of lactate dehydrogenase synthesis-associated bacterial genes. For analysis, quantitative PCR with reverse transcription was used, a bacterial gene expression assessment method whose reproducibility, high sensitivity, and specificity are commonly recognized [93]. Lactate is produced by lactic acid fermentation from precursors under the influence of two different forms of NAD-bound lactate dehydrogenases: one (EC 1.1.1.27) produces L(+)-lactate and is encoded primarily by the *Ldh-L* gene [51]; the other one (EC 1.1.1.28) produces D(–)-lactate and is encoded by *Ldb 0813* [94]. The authors of [95] believe there is a significant difference between the effects of the two enantiomers on ruminants, although stereoisomers have similar physical and chemical properties. The important difference between the isomers lies in their renal excretion capacity, which is lower for D-lactate and thus determines its primary role in provoking metabolic acidosis [95]. This is why the authors believe the *Ldh-L* and *Ldb 0813* genes are important candidate biomarkers that could indicate the activity of lactic acid synthesis in the bovine rumen.

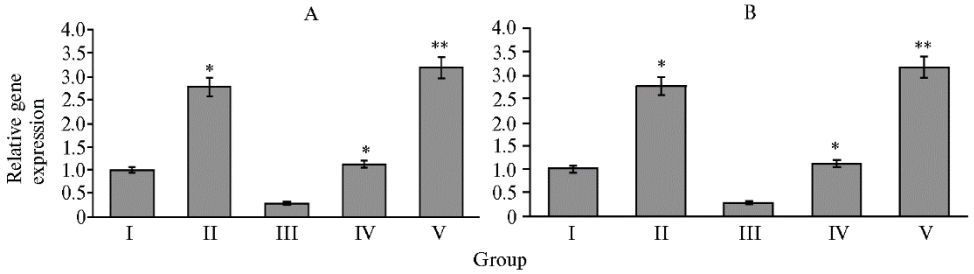

Fig. 6. Relative expression (compared to the gene of cows from Group 1) **of the genes *Ldh-L*** (A) **and *Ldb 0813*** (B) **in the microbial community of the rumen microbiota of black-and-white Holsteinized dairy cows (*Bos taurus*) in different physiological periods:** I — dry cows, II — fresh cows, III — lactating cows, IV — stable lactation, V — late lactation period (JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

*, ** Differences of Groups II, III, IV, and V from Group I are statistically significant at $p \leq 0.01$ and $p \leq 0.001$.

Gene expression was reduced 11.3-fold for Ldh-L and 9.9-fold for Ldb 0813 in Group 3 (early lactation) as compared to newly calved cows (Group 2) ($p \leq 0.001$). That being said, animals in early lactation were able to better resist the negative effects of starch enrichment than their newly calved counterparts. This could be due to the emergence of adaptive mechanisms in the rumen microbiota.

A notably increased expression (10.6-fold for *Ldh-L* at $p \leq 0.001$, 2.8-fold for *Ldb 0813* at $p \leq 0.05$) occurred in the late lactation period (Group 5 vs. Group 4). This could be due to the rising populations of *Asteroleplasma*, *Sharpea*, *Moryella*, *Oribacterium*, *Shuttleworthia*, which produce lactate as one of the major fermentation products.

These variations in gene expression corresponded to the reduction in the α-diversity of the rumen microbial community and to the inhibition of the carbohydrate, protein, energy, and lipid metabolism potential of the microbiota. Based on these findings, one can conclude that in late lactation, animals are, too, at risk of metabolic disorders, which should be borne in mind when devising a strategy for less metabolic diseases in dairy cows. Some changes in the composition and

functions of the rumen microorganisms could be due to the specifics of metabolism, feeding, stress (e.g. postpartum and early lactation stress), immune and hormonal status in different physiological periods.

Thus, we used bioinformatics methods to study in detail the rumen microbiome structure and to predict its functional potential in different physiological periods of dairy cows. 16S metagenomic sequencing showed a reduction in the α-diversity of the bacterial microbiota of the rumen in mid- and late lactation. Twelve superphyla and phyla of microorganisms were found; the superphylum *Bacteroidota* and the phylum *Firmicutes* could be considered the dominant bacteria in the rumen, as they represented up to 59.94±1.86 and 46.82±14.40% of the microbiome, respectively. A relation has been found between the presence of certain taxa of rumen microorganisms and physiological periods in dairy cows. The superphylum *Actinobacteriota* was only found in dry cows' rumens, fully eliminated from lactating cows. The superphylum *Armatimonadota* was absent from the rumen in newly calved cows and cows in mid-lactation; the phylum *Chloroflexi* was absent in early and mid-lactation. Detailed microbiome analysis showed the animals to differ significantly in terms of eight bacterial families (*Muribaculaceae*, *Prevotellaceae*, *Erysipelatoclostridiaceae*, *Oscillospiraceae*, *Ruminococcaceae*, *Saccharimonadaceae*, candidate families WCHB1-41, vadinBE97). The genera *Asteroleplasma*, *Sharpea*, *Moryella*, *Oribacterium*, *Shuttleworthia*, which were absent from dry cows, appeared after calving and persisted through subsequent periods of lactation. The emergence of these bacteria in the microbiome during lactation could be due to metabolic disbalance since lactate is one of their major products. The predicted functional potential of the rumen microbiota differed across 17 metabolic pathways. Changes, namely the inhibition of various types of chyme metabolism, were most pronounced ($p \leq 0.01$) during mid- and late lactation. Increased expression was detected in newly calved cows vs. dry cows for the *Ldh-L* ($p \leq 0.01$) and *Ldb 0813* ($p \leq 0.05$) that are connected to lactate dehydrogenase synthesis. The bacterial community of the rumen exhibited a significant increase in the expression of *Ldh-L* (10.6x at $p \leq 0.001$) and *Ldb 0813* (2.8x at ≤ 0.05) in late lactation versus mid-lactation.

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