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THE INFLUENCE OF A DIETARY *Enterococcus faecium* STRAIN-BASED ADDITIVE ON THE TAXONOMIC AND FUNCTIONAL CHARACTERISTICS OF THE RUMEN MICROBIOTA OF LACTATING COWS

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Abstract

Today rations for dairy cows are designed to provide the highest growth rate and productivity in a short period of time. However, such intensive livestock farming affects, first of all, the health of animals, since metabolic pathways inherent in ruminants are disrupted. The use of 16S metagenomics approaches makes it possible to assess the genetic and metabolic diversity of the bovine microbiome, which allows identifying factors that can contribute to an increase in productivity and an improvement in the health of the host. In the feeding trial, dairy cows were fed with dietary probiotic Cellobacterin+ based on the *Enterococcus faecium* 1-35 strain (the winter-spring period of 2018, JSC PZ Plamya, Gatchinsky District, Leningrad Province). Two groups of ten Holsteinized black-and-white dairy cows (*Bos taurus taurus*) of the 2nd and 3rd lactation with an average annual milk yield of 7000-7500 kg were used. The basal diet was 10 kg compound feed, 2 kg yellow corn, 0.5 kg sunflower cake, 0.5 kg rapeseed cake, 1 kg hay, 25 kg grass silage, 1 kg beet molasses, and 0.2 kg MINVIT®-3 (Russia). In the morning, the test cows were fed with dietary Cellobacterin+ (OOO BIOTROF, St. Petersburg) at 40 g per cow. Cicatricial contents (10-50 g) were collected from three cows of each group at the end of the experiment. Fasting blood was taken for biochemical analysis from the tail vein with vacutainers. The blood was analyzed for total protein, total bilirubin, glucose, calcium, phosphorus, urea, reserve alkalinity, ketone bodies. The mass fraction of fat in milk was analyzed according to GOST 5867-90, protein according to GOST 23327-98, and the number of somatic cells according to GOST R 54761-2011. Total DNA from the studied samples was extracted using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. Amplification for subsequent NGS sequencing was run (a Veriti Thermal Cycler, Life Technologies, Inc., USA) using the eubacterial primers (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTANVGGGT-WTCTAAT-3′) flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing (a MiSeq system, Illumina, Inc., USA) was performed with a MiSeq Reagent Kit v3 (Illumina, Inc., USA). Chimeric sequences were excluded from analysis using the USEARCH 7.0 program (http://drive5.com/usearch/). The processing of the obtained reads using the bioinformatics platform CLC Bio GW 7.0 (Qiagen, the Netherlands) included overlapping, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to genus was determined using the RDP Classifier program (http://rdp.cme.msu.edu/). Mathematical and statistical processing of the results was carried out using the software packages Microsoft Office Excel 2003, R-Studio (Version 1.1.453)

(https://rstudio.com). The mean values (*M*) and standard errors of the means (±SEM) were calculated. The results were deemed significant at $p < 0.05$. Analysis of microbial β-diversity of the samples by the principal component method was performed according to the Weighted UniFrac PCoA Emperor method using the QIIME software package. Reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was performed using the PICRUSt2 software package (v.2.3.0). MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. Feeding the probiotic had a significant effect ($p = 0.049$) on an increase in milk yield, as well as on a decrease ($p = 0.003$) in the somatic cell number in milk by 38,000/ml per cow. The NGS sequencing provided a complete taxonomic and functional characterization of the cicatricial microbiota, including uncultivated representatives. Significant differences were found between the groups for 13 bacterial genera. In particular, in the rumen of cows treated with the probiotic Cellobacterin+, compared to the control group, a lower proportion of the order *Clostridia* were found, namely the bacteria of the genera *Anaerofilum* sp. (2.3 times lower, $p \leq 0.05$) and *Anaerostipes* sp. (1.8 times lower, $p \leq 0.05$) that produce lactate in the rumen as the end product of glucose metabolism. A decrease occurred in the abundance of the genera *Campylobacter*, *Gemella*, *Mycoplasma*, *Shewanella* ($p \leq 0.05$), and *Fusobacterium* (including *F. necrophorum*) ($p \leq 0.001$) among which pathogens are often found. Changes in the taxonomic structure of rumen microbiota as influenced by the probiotic were also associated with metabolic changes. The predicted functional potential of seven metabolic pathways was enhanced in cows fed Cellobacterin+ compared to the control animals. Thus, when fed Cellobacterin+, there was a 3.5-fold increase ($p \leq 0.05$) in the predicted level of microbiome metabolic capabilities associated with the synthesis of glyoxylate from allantoin, and 2.3-fold increase ($p \leq 0.05$) in the biosynthesis of propionate from L-glutamate. These findings allow us to suggest an important role of the biological product Cellobacterin+ for maintaining the homeostasis of metabolic processes.

Keywords: biologicals, Cellobacterin+, lactating cows, rumen, 16S metagenomics, NGS sequencing, metabolism

Ruminants hold a specific place among other farm animals due to the unique features of the digestive system functioning. The rumen is inhabited by a large microbial community consisting of bacteria, archaea, and micromycetes, which allows the animal to use lignocellulosic material and convert non-protein nitrogen into microbial protein as a source of energy and amino acids [1, 2]. During polysaccharide fermentation, short-chain (so-called volatile) fatty acids are formed – acetate, butyrate, propionate, and others, which are absorbed through the rumen epithelium and are used by animals to maintain metabolism.

The rumen of ruminants is inhabited by a variety of traditionally non-culturable bacteria; therefore, it is often difficult to draw the right conclusions about their physiology and functions [3]. At the same time, these microorganisms can carry genes that determine a significant part of the microbiome metabolic diversity, play a decisive role in non-starchy polysaccharide and protein fermentation, synthesis of biologically active substances, engage in active intermicrobial interactions, and have a significant effect on the macroorganism. The advent of 16S metagenomics methods made it possible to establish DNA sequences for the whole population of microorganisms from natural sources and to obtain a complete taxonomic and functional characterization of cicatricial microbiota, regardless of the microorganism cultivation possibility [4]. High-throughput sequencing can assess the bovine microbiome genetic and metabolic diversity and identify factors that contribute to both ecological balance and host health [5].

Modern diets for dairy cows are designed to ensure the maximum growth rate and productivity in a short period [6, 7]. However, intensive technology affects primarily animal health since it disrupts the metabolic pathways inherent in ruminants. Because cicatricial microorganisms are practically the only enzyme source for plant feed digestion, as well as direct participants in metabolism, a violation of the rumen microbiocenosis composition can lead to many negative consequences. Conversely, a directed change in the rumen microbiota is accompanied by positive shifts in productivity, quality characteristics of milk, reproduction, and the duration of economic use, which can become one of the key factors in increasing dairy farming efficiency [8].

Due to the role of ruminants as producers of methane released into the atmosphere, researchers were focused mainly on methanogen microorganisms [9, 10]. However, at present, due to the physiology and nutrition problems of highly productive animals, there is a need to study the microbiome structure and metabolic pathways implemented by the cicatricial microbiota.

The regulating strategies for the microbiome composition include the nutritional intervention of feed additives (probiotics, prebiotics, phytobiotics, etc.) into the livestock diet [11-13]. The positive probiotic effects on the rumen microbiome are mainly associated with their positive effects on digestive processes, especially on cellulose digestion and microbial protein synthesis. *Saccharomyces cerevisiae* is the most popular yeast species used for rumen introduction [12]. As for probiotics based on bacteria, according to Fernández et al. [13], the use of genus *Lactobacillus* bacteria can become an alternative in the treatment and prevention of some diseases affecting ruminants. Thus, the calve nutritional intervention of *L. johnsonii* TP1.1, *L. reuteri* TP1.3B, *L. johnsonii* TP1.6, and *L. amylovorus* TP8.7 strains reduced the severity of diarrhea symptoms [13].

Although the positive effect of dietary supplements on the bovine rumen microbiome is well known, the need to study the properties of existing and new probiotics for animals remains high.

In this work, using PICRUSt2 and MetaCyc software, the fact was established of an increase in the predicted functional potential of some metabolic pathways in the cicatricial microbiota of cows fed dietary probiotic Cellobacterin+, containing *Enterococcus faecium* 1-35 strain.

The goal of the research was to assess the effect of the probiotic Cellobacterin+ on zootechnical indices, the cicatricial microbiome and its functional potential in dairy cows.

Methods. The experiment was carried out in the winter-spring period (JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018). Two groups (10 heads each) of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) of 2nd and 3rd lactation and 7000-7500 kg average annual milk yield were formed. The animals were kept in the same tie-stall barn.

The main diet included 10 kg of feed concentrate, 2 kg of yellow corn, 0.5 kg of sunflower cake, 0.5 kg of rapeseed cake, 1 kg of dry forage, 25 kg of grass silage, 1 kg of beet molasses, and 0.2 kg of MINVIT®-3 (AgroBalt Trade, Russia). The probiotic Cellobacterin+ (BIOTROF LLC, St. Petersburg), which included *Enterococcus faecium* 1-35 strain, was added at 40 g/head to the diet of the test group cows in the morning. Earlier, the dosage was tested on dairy cows [14]. The test duration was 60 days after a preparatory period of 15 days.

At the end of the experiment, samples of ruminal digesta (10-50 g) were aseptically taken manually from three cows of each group with a sterile probe. Simultaneously, fasting blood was taken for biochemical analysis from the tail vein using vacutainers. In the blood serum, total protein, total bilirubin, glucose, calcium, phosphorus, urea, reserve alkalinity, and ketone bodies were determined by standard techniques [15]. The mass fraction of fat in milk was analyzed according to GOST 5867-90, protein according to GOST 23327-98, and the counts of somatic cells according to GOST R 54761-2011.

Total DNA was isolated using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. The method is based on selective detergent-mediated precipitation of DNA from a substrate using solutions for cell wall lysis and DNA precipitation, 1.2 M sodium chloride, and chloroform. Amplification (Veriti Thermal Cycler, Life Technologies, Inc., USA) for subsequent next-generation sequencing (NGS) was performed with eubacterial

primers (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTACNVGGGGTWTC-3′) flanking the V1V3 site of the 16S rRNA gene. Amplification mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (25 cycles); 5 min at 72 °C (1 cycle).

Metagenomic sequencing (MiSeq® system, Illumina, Inc., USA) was performed using MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the obtained sequences was 2×300 bp. Chimeric sequences were excluded from analysis using the USEARCH 7.0 program (http://drive5.com/usearch/). The processing of the obtained reads using the CLC Bio GW 7.0 bioinformatics platform (Qiagen, the Netherlands) included overlap testing, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to the genus was determined using the RDP Classifier program (http://rdp.cme.msu.edu/).

The α-biodiversity Chao1 index of the rumen microbiome was calculated [16]. Analysis of the microbial β-diversity by the method of principal components was carried out according to the Weighted UniFrac PCoA Emperor method using the QIIME software package [17]. Reconstruction and prediction of the metagenome functional content, gene families, and enzymes was carried out using the PICRUSt2 software package (v.2.3.0) [18]. The MetaCyc database (https://metacyc.org/) [19] was used to analyze metabolic pathways and enzymes. MetaCyc metabolic pathway profiles were assessed after normalization of the abundance of amplicon sequence variants using binary logarithm ($\log_2$) [18].

Mathematical and statistical processing of the results was carried out using the Microsoft Office Excel 2003 and R-Studio (Version 1.1.453) (https://rstudio.com) software packages. The mean values (M) and standard errors of the means (±SEM) were determined. Statistical analysis results were considered significant at $p < 0.05$.

Results. Dietary probiotic Cellobacterin+ did not have a statistically significant effect on fat and protein levels in milk, but there was a definite tendency of its positive effect on these indices (Table 1). However, feeding with Cellobacterin+ significantly influenced ($p = 0.049$) an increase in milk yield, as well as a decrease ($p = 0.003$) in the number of somatic cells in milk (by 38 thousand $\cdot$ ml^{-1} $\cdot$ head^{-1}). Previously, similar results were obtained on dairy cows by Spaniol et al. [20] who reported that the nutritional intervention of probiotics did not affect the milk biochemistry, but led to a decrease in somatic cells on day 15 of the experiment. In the work of Australian authors [21], the average daily milk yield of cows that consumed grass on pastures treated with probiotic bacterial strains was 1.21 l higher than that of control animals.

According to several studies based on the 16S rRNA gene characteristic, it was assumed that bovine mastitis with an increase in somatic cells in milk was the result of an imbalance between the normal biota of the mammary gland and pathogens [22]. Perhaps the decrease in milk somatic cells in the authors' test was associated with the modulation of the animal's immune system under the influence of a bacterium probiotic strain. Previously, it was demonstrated that through interaction with monocytes, macrophages, and dendritic cells, probiotics could modulate the balance of helper T-cells and thus influence the adaptive immune response [23, 24]. Other researchers [20] have shown that probiotic administration is associated with an increase in circulating cytokines (tumor necrosis factor, interleukin-4, and interferon) in the bovine blood.

Almost all of the studied biochemical parameters of the bovine blood were within the normal range or did not significantly exceed their limits (Table 2). The biochemical blood profiles of cows in the control and experimental groups did not

differ significantly.

1. Milk productivity of Holsteinized black-and-white dairy cows (*Bos taurus taurus*) fed dietary probiotic Cellobacterin+ (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018)

Parameter	Control group (n = 10)	Treatment group (n = 10)	p values between groups
Average daily milk yield of natural milk, kg	31.7±1.50	33.3±1.60	0.049
The fat content of milk, %	3.68±0.150	3.97±0.200	0.260
The protein content of milk, %	2.88±0.170	3.14±0.140	0.250
Average daily milk yield of 4% fat, kg	29.2±1.20	33.0±1.40	0.048
Somatic cells, thousand · ml^{-1} · head^{-1}	163±8.5	125±6.9	0.003

N o t e. See the group description in the Methods section.

2. Blood biochemical parameters of Holsteinized black-and-white dairy cows (*Bos taurus taurus*) fed dietary probiotic Cellobacterin+ (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018)

Parameter	Control group (n = 10)	Treatment group (n = 10)	p values between groups	Standard
Total protein, g/l	78.3±4.10	81.8±4.90	0.62	70-89
Albumin, % of total protein	50.7±2.90	40.9±2.00	0.07	38-50
Total bilirubin, mmol/l	2.2±0.20	2.33±0.110	0.6	0.17-5.13
Glucose, mmol/l	2.19±0.100	2.28±0.130	0.62	2.22-3.33
Calcium, mmol/l	2.28±0.140	2.37±0.190	0.73	2.6-3.5
Phosphorus, mmol/l	2.52±0.200	2.09±0.100	0.07	1.29-2.25
Alkali reserve, vol.% CO_2	57.8±2.30	55.0±2.30	0.45	46-56
Urea, mmol/l	4.4±0.30	3.77±0.150	0.16	3.3-6.7
Ketone bodies	–	–		–

N o t e. See the group description in the Methods section. Dashes indicate that no ketone bodies were detected.

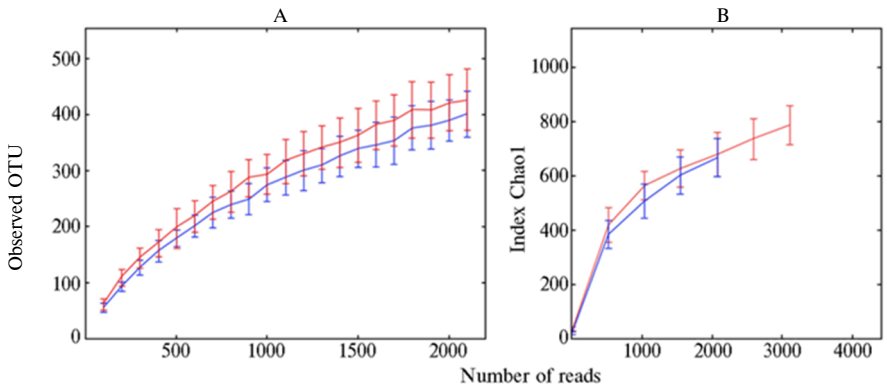

Fig. 1. α-Biodiversity of the rumen microbiome in Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) in the control (blue graph) **and when fed dietary probiotic Cellobacterin+** (red graph): A — variation of operational taxonomic units (OTU), B — Index Chao1 (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

Based on the NGS sequencing data, the parameters of the rumen microbiome α-biodiversity which was characterized by the abundance of operational taxonomic units (OTUs) within communities [25, 26] were calculated (Fig. 1). There were no significant differences in the number of OTUs and the Chao1 index between the test and control variants.

The results of the assessment of β-diversity, that is, a diversity between communities [25, 26], are presented as a three-dimensional graph of the PCoA Emperor (Fig. 2). The principal component PC1 described 67.97% of the data, PC2 described 15.96%, PC3 described 7.87%, i.e., in general, the method made it possible to characterize the changes in the microbiome, while retaining 91.8% of the information.

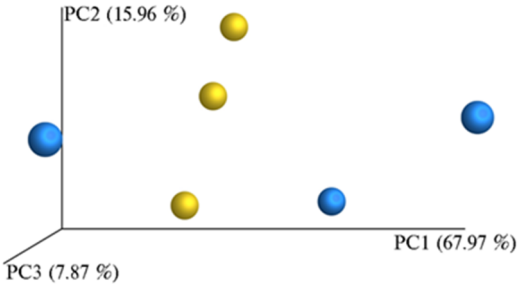

Fig. 2. Principal component analysis of β-diversity between rumen microbiomes in Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) (one point corresponds to one animal) **in the control** (yellow balls) **and when fed dietary probiotic Cellobacterin+** (blue balls) (JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

Comparison of the cicatricial microbiota of cows from different groups using the method of principal components showed that the microbiomes of three cows from the control group were combined into one cluster, and the microbiomes of cows from the test group partially formed their cluster, which may indicate the probiotic effect on the microbiome structure. Nevertheless, clustering was more pronounced in the control than in the probiotic group, i.e., the shift along the axis of the PC1 component was less.

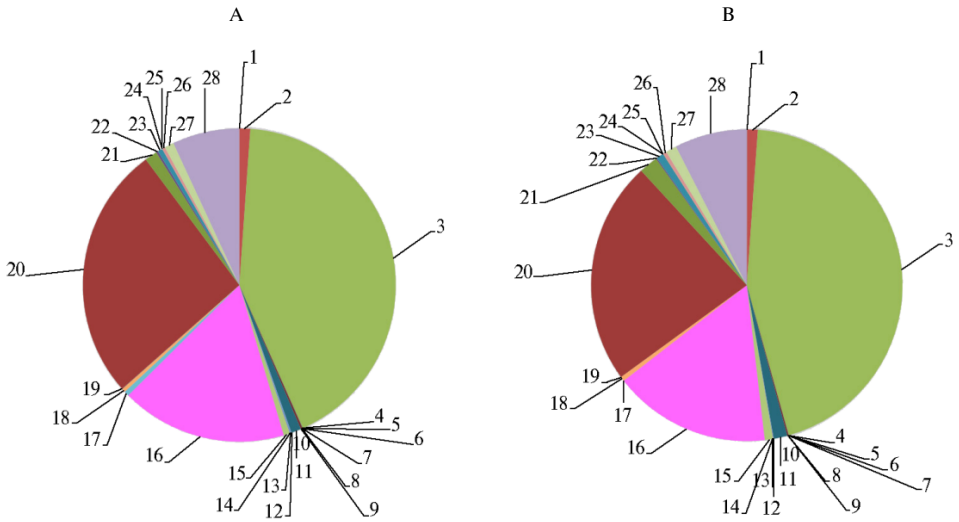

Fig. 3. Microorganisms (the phylum level) of the rumen of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) in the control (A) **and when fed dietary probiotic Cellobacterin+** (B) (data of NGS sequencing)**:** 1 — *Acidobacteria*, 2 — *Actinobacteria*, 3 — *Bacteroidetes*, 4 — *Caldiserica*, 5 — *Calditrix*, 6 — *Chlamydiae*, 7 — *Chlorobi*, 8 — *Chloroflexi*, 9 — *Chrysiogenetes*, 10 — *Crenarchaeota*, 11 — *Cyanobacteria*, 12 — *Deferribacteres*, 13 — *Elusimicrobia*, 14 — *Euryarchaeota*, 15 — *Fibrobacteres*, 16 — *Firmicutes*, 17 — *Fusobacteria*, 18 — *Nitrospirae*, 19 — *Planctomycetes*, 20 — *Proteobacteria*, 21 — *Spirochaetes*, 22 — *Synergistetes*, 23 — *Tenericutes*, 24 — *Thermi*, 25 — *Thermodesulfobacteria*, 26 — *Thermotogae*, 27 — *Verrucomicrobia*, 28 — unidentified (JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

According to estimates of taxonomic confinement of the microbiota in the rumen of the test cows, 27 phyla were found of which *Bacteroidetes* (42.2±2.9 to 44.5±3.1%), *Proteobacteria* (23.2±1.5 to 26.3±1.9%), and *Firmicutes* (16.3±0.9 to 17.2±1.2%) were dominant (Fig. 3). In the phylum *Bacteroidetes*, *Prevotella* bacteria prevailed (26.4±1.8 to 27.0±2.3%). Previously, the dominance of this genus of microorganisms in the rumen of ruminants has been repeatedly shown [27, 28]. Bacteria of the genus *Prevotella* play an important role in carbohydrate and nitrogen metabolism; succinate is one of the final products of their metabolism [29]. It was found that extracellular succinate in the rumen served as the main propionate precursor [30], the most important substrate for gluconeogenesis in ruminants [31]. As previously identified with the sheep rumen microbiome, most of the genus *Prevotella* bacteria are represented by uncultivated forms [32].

Bacteria in the rumen that did not belong to any known taxon from the

existing ones (according to the databases of the 16S RNA gene sequences) ranged from 6.9±0.5 to 7.5±0.8% (see Fig. 3).

No significant differences between the variants at the phylum level could be found (see Fig. 3). However, a detailed analysis of the rumen microbiome revealed significant differences between the groups for 13 genera of bacteria (Fig. 4).

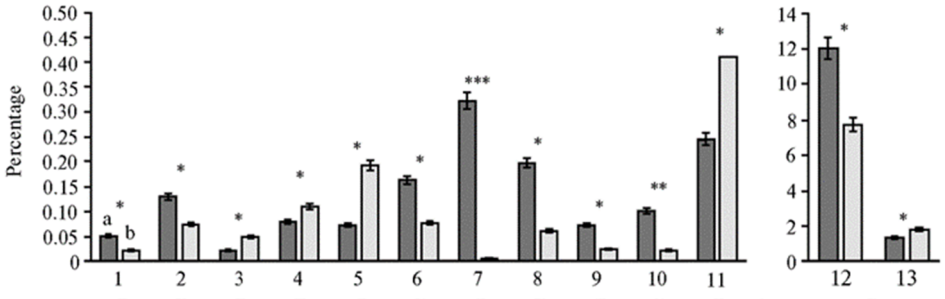

Fig. 4. Microorganisms (the genus level) of the rumen of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) in the control (a) **and when fed dietary probiotic Cellobacterin+** (b) (data of NGS sequencing)**:** 1 — *Anaerofilum*, 2 — *Anaerostipes*, 3 — *Anaerovibrio*, 4 — *Bdellovibrio*, 5 — *Bifidobacterium*, 6 — *Campylobacter*, 7 — *Fusobacterium*, 8 — *Gemella*, 9 — *Mycoplasma*, 10 — *Odoribacter*, 11 — *Pseudobutyrivibrio*, 12 — *Shewanella*, 13 — *Lachnospira* (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).
*, **, *** Differences between groups are statistically significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0{,}001$, respectively.

In particular, in the rumen of cows fed Cellobacterin+ as compared to the control group, we detected a lower proportion of representatives of the order *Clostridia*, bacteria of the *Anaerofilum* sp. (2.3 times lower, $p \leq 0.05$) and *Anaerostipes* sp. (1.8 times lower, $p \leq 0.05$). Representatives of these genera produce lactate as the final product of glucose metabolism [33, 34]. Our observations may indicate a positive role of the probiotic in the health control of cows, since, during high-concentration feeding in animals, dysbiotic disorders of the cicatricial microflora often occur with a shift in metabolism towards the lactate synthesis [35]. Lactate excess correlates with decreased rumen pH and lactate acidosis [35]. Associated with acidosis, as a resulting suppression of pH-sensitive producers of volatile fatty acids, such as *Selenomonas ruminantium* and *Megasphaera elsdenii* [36], the beneficial metabolite synthesis in the rumen decreases. Similarly, bacteria synthesizing cellulases decrease which leads to disruption of the feed non-starch polysaccharide digestion [35]. The obtained results are consistent with those of Goto et al. [37] who showed that the nutritional intervention of a multistrain bacterial probiotic for cows with induced subacute rumen acidosis caused a decrease in lactic acid in the rumen fluid.

In the rumen of animals treated with the probiotic, we found a decrease in the representativity of genera *Campylobacter*, *Gemella*, *Mycoplasma*, *Shewanella* ($p \leq 0.05$), and *Fusobacterium* ($p \leq 0.001$), among which pathogens are often found. Data on a decrease in the abundance of the genera *Campylobacter* and *Fusobacterium* in the test group animals are consistent with the above results on a decrease in somatic cells in milk, since it has been proven [38, 39] that these microorganisms are associated with mastitis in cattle. The 60.5-fold increase ($p \leq 0.001$) in abundance of *Fusobacterium* bacteria, represented mainly by *F. necrophorum*, observed in the control group cows, could be associated with an increase in the abundance of lactate-producing microorganisms in the rumen. The thing is, low acidity values are optimal for the *F. necrophorum* pathogen development for which lactic acid is the main nutrient substrate. *F. necrophorum* is an opportunistic pathogen causing necrotic rumen lesions (necrobacteriosis), laminitis, and liver

abscesses [40]. The presence of genus *Campylobacter* bacteria in milk can be dangerous to humans, as *C. jejuni* and *C. coli* can initiate gastrointestinal campylobacteriosis. *C. fetus* bacteria are associated with infertility and abortion in cattle [41].

It is known that the genus *Gemella* bacteria which decreased by 3.3 times ($p \leq 0.05$) as a result of the use of Cellobacterin+ are associated with respiratory tract infections and bacteremia [42]. Similarly, *Mycoplasma* representatives, in particular *M. bovis*, cause chronic bronchopneumonia with caseous and coagulative necrosis, as well as arthritis in cattle and calves [43]. Genus *Shewanella* (*S. haliotis* and *S. upenei*) bacteria were isolated from the lung tissue of people with respiratory infection and bacteremia [44]. An increase in the pulmonary pathogen pool in the rumen of control group animals may indicate an intercommunication between microbiomes with different localizations in the host organism and the existence of the rumen—respiratory tract axis, as well as the possible rumen microbiome interference during respiratory diseases. Previously, it was shown in rats that fecal transplantation of the microbiome induced changes in the lung microbiota [45].

The data obtained indicate the role of probiotic bacterial strains in not only the microbiota homeostasis but also the macroorganism health.

A decrease in the abundance of undesirable forms of microorganisms as a result of probiotic exposure could be associated with direct antagonism through the production of antimicrobial metabolites (bacteriocins, organic acids) [46], as well as with modulation of the indigenous microbiota composition and activity under the influence of a strain in the biological product. So, as a result of the Cellobacterin+ use, in the rumen, the number of *Bifidobacterium* and *Bdellovibrio* increased. *Bifidobacterium* is widely known in response to pronounced antimicrobial properties against a wide range of pathogens [47]. Representatives of the genus *Bdellovibrio*, i.e., *B. bacteriovorus*, are predatory microorganisms that control such pathogens as *Salmonella* sp. and *Escherichia coli* [48].

The obtained results demonstrating the modulating effect of the probiotic on the microflora, which was expressed in a decrease in the pathogenic forms and an increase in the abundance of microorganisms with antimicrobial activity, are consistent with the data obtained on calves [49]. The use of boluses based on *Pediococcus acidilactici*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* helped to reduce diarrhea in animals.

An increase ($p \leq 0.05$) in bacteria of the families *Lachnospiraceae* (*Pseudobutyrivibrio* sp. and *Lachnospira* sp.) and *Selenomonadaceae* (*Anaerovibrio* sp.) in the rumen of cows from the test group could also make a positive contribution to the activation of metabolic processes. Genus *Pseudobutyrivibrio* bacteria were represented by the species *P. xylanivorans* which has a potent xylanolytic enzyme system with at least seven different xylan hydrolases (27-145 kDa) [50]. In this regard, it can ferment xylan polysaccharide in feed. The final product of its metabolism is volatile fatty acids, which are important for the metabolism, health, and productivity of animals, as well as bacteriocin-like inhibitory substances that are active against pathogens. Genus *Lachnospira* microorganisms were represented by the species *L. pectinoschiza* which shows a pronounced ability to ferment pectin by extracellular pectin methylesterase and Ca^{2+}-dependent exopolygalacturonate lyase [51]. The final product of the metabolism of bacteria of the genus *Lachnospira* is acetic acid as the main substrate for de novo lipid synthesis, in particular in the mammary glands of lactating cows.

The results of measuring the lipolysis rate with *Anaerovibrio* sp. pure cultures including *A. lipolytica* [52] showed that these bacteria played an important role in the ruminal digesta lipolytic activity. In this case, the fermentation products

include such important compounds as propionate, which is produced along the path of the dicarboxylic acid conversion to succinate. An increase in propionate biosynthesis can be associated with an increase in milk production in cows treated with the probiotic [30]. In addition, short-chain fatty acids produced by bacteria have other important properties. For example, they are involved in the epigenomic regulation of interactions between the microbiota and the host macroorganism [53]. It has long been known that epigenetic modifications can regulate gene expression, affecting its intensity and duration, without changes in the DNA sequence.

These study results are logical since we have previously described the mechanisms of the positive effect of Cellobacterin+ on the rumen and intestine microbiota [14]. These mechanisms are expressed in the ability of bacterial strains in a biological product to produce low molecular weight organic acids and other biologically active substances including antimicrobial factors. We have shown that the synthesis of xenobiotic biodegradation enzymes in the rumen results in the detoxification of feed mycotoxins with antimicrobial activity against the normal biota [14]. Consequently, the appearance of new metabolites in the rumen due to the introduction of a probiotic strain leads to changes in microorganisms.

Using the PICRUSt2 and MetaCyc software packages, the authors reconstructed and predicted the functional content of the metagenomic community of the bovine rumen. Changes in the taxonomic structure of rumen microorganisms under the influence of the biological product were associated with metabolic changes. The predicted functional potential of seven metabolic pathways was enhanced in cows fed Cellobacterin+ (Fig. 5).

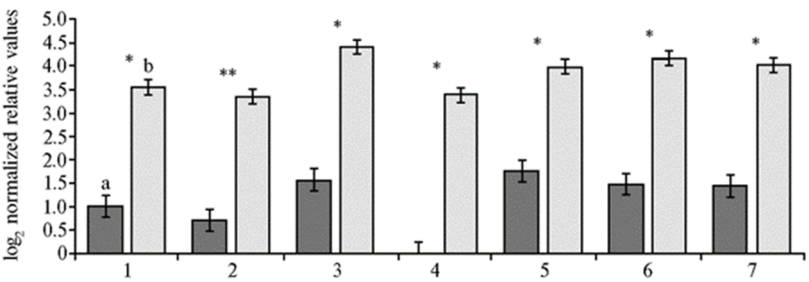

Fig. 5. Functional annotation of metabolic pathways in the rumen metagenomic community of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*): 1 — glyoxylate synthesis, 2 — urea synthesis, 3 — γ-amino-N-butyrate synthesis, 4 — peptidoglycan synthesis, 5 — propionate synthesis, 6 — succinyl-CoA synthesis, 7 − succinate synthesis (PICRUSt2 and MetaCyc processing; *M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).
*, ** Differences between groups are statistically significant at $p \leq 0.05$ и $p \leq 0.01$, resprctively.

In particular, in cows from the test group, as compared to the control, the predicted metabolic capabilities of the microbiome increased 3.5 times ($p \leq 0.05$) that was associated with the synthesis of glyoxylate from allantoin by allantoinase (EC 3.5.2.5), allantoin amidohydrolase (EC 3.5.3.9), ureidoglycine aminohydrolase (EC 3.5.3.26), and ureidoglycolic lyase (EC 4.3.2.3). The initial substrate of the cycle is allantoin, a product of purine catabolism. Allantoin is rich in nitrogen, and many microorganisms can process it. The glyoxylate as a result of allantoin transformations serves as a substrate for the glyoxylate cycle (two-carbon acid cycle). The principal possibility of the glyoxylate cycle in the rumen is associated with the catalytic activity of key enzymes, i.e., isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) [54]. These enzymes allow glucose synthesis which is deficient for physiologically hypoglycemic ruminants from acetic acid produced in high concentrations in the rumen. Compared to the tricarboxylic acid cycle, the pathway for the bicarboxylic acid oxidation is less energy-consuming and more

efficient, since it is a shorter cycle that can function as a tricarboxylic acid cycle excluding the rate-limiting reactions with isocitrate dehydrogenase and α-keto-glutarate dehydrogenase [54]. The ability of cattle gastrointestinal microorganisms to carry out the glyoxylate cycle can be considered as a factor contributing to metabolism intensification and an increase in productivity. Another reaction to the nutritional intervention of the probiotic Cellobacterin+ detected by bioinformatic data processing is the activation (4.8 times, $p \leq 0.01$) of the allantoin conversion through ureidoglycolate into urea. It is well known [55] that in ruminants, endogenous urea is partially recirculated in the body and used for the synthesis of a high-value microbial protein absorbing in the host small intestine.

In animals from the experimental group, there was a 2.8-fold activation ($p \leq 0.05$) of the potential of the rumen microflora associated with the synthesis of γ-amino-N-butyrate from L-ornithine previously studied in detail by Kurihara et al. [56]. γ-Amino-butyrate is the main inhibitory neurotransmitter in the mammal central nervous system, has an etiotropic effect on the health and growth rate of calves [57], and has a protective effect against neurotoxicant-induced cell death [58]. It is well known that genus *Bifidobacterium* bacteria actively produce γ-amino-N-butyrate from L-ornithine [59] the abundance of which increased in the current experiment in the rumen of animals fed dietary probiotic Cellobacterin+.

With the nutritional intervention of Cellobacterin+, the metabolic capabilities of the microbiome associated with the propionate biosynthesis from L-glutamate increased 2.3 times ($p \leq 0.05$). This pathway was first described for two members of the family *Veillonellaceae — Anaeromusa acidaminophila* and *Barkera propionica* [60, 61]. We detected bacteria of the family *Veillonellaceae* in the rumen of cows from the control and test groups; however, no significant differences in their content could be identified. Propionic acid being involved in gluconeogenesis becomes the main glucose source in the blood of ruminants [62]. Dietary Cellobacterin+ also activated synthesis of the important compounds, e.g., succinate through L-arginine, putrescine, and γ-amino-N-butyrate. Succinate is involved in the tricarboxylic acid cycle and serves as the main propionate precursor produced in the rumen [30].

Compared to the control group, the use of Cellobacterin+ increased 2.8 times ($p \leq 0.05$) the microbiome metabolic capabilities associated with the biosynthesis of succinyl-CoA from phenylacetate, which is a thioester of dicarboxylic succinic acid and coenzyme. The existence of a similar pathway in bacteria was reported as early as 1955 [63]. Ring dearomatization occurs through the conversion of phenylacetyl-CoA to 2-(1,2-epoxy-1,2-dihydrophenyl) acetyl-CoA with the participation of phenylacetyl-CoA 1,2-epoxidase (EC 1.14.13.149). Further, the reactive non-aromatic epoxide is isomerized to the seven-membered o-heterocyclic enol ether (2-oxepin-2(3H)-ylideneacetyl-CoA), as a result, the ring is cleaved. The rest of the pathway consists of β-oxidative steps leading to the formation of succinyl-CoA [64]. It is well known that succinyl-CoA is involved in many biochemical pathways, in particular, it serves as the Krebs cycle intermediate [65] and a precursor for the synthesis of α-aminolevulinic acid, a specific intermediate in the porphyrin synthesis.

An increase in the predicted metabolic capabilities of the microbiome associated with the synthesis of glyoxylate, γ-amino-N-butyrate, propionate, urea, peptidoglycan, succinyl-CoA, and succinate, identified in cows fed dietary Cellobacterin+, confirms the important role of the biological product for maintaining the metabolism homeostasis, health, and productivity of animals. This is a valuable scientific and practical conclusion since modern intensive livestock farming methods require the inclusion of a significant amount of starch in the diet, which puts

the animal at the risk of metabolic disorders, the occurrence of diseases, and a decrease in product longevity. The explanation of the data obtained on the increase in the potential of physiological and biochemical processes in the bovine rumen also requires an in-depth analysis of the complex interactions between microbiota and the macroorganism. Cellobacterin+ may also be useful for immunobiological effects in breast diseases, including mastitis, but further testing is needed to confirm this assumption.

Thus, biopreparations based on microorganisms effectively modulating the microbial community expand the list of tools for modifying the microbiome structure. In Russia, since the entry into force in 2020 of Law No. 280-FZ of August 3, 2018 "On Organic Products and Amendments to Certain Legislative Acts of the Russian Federation", interest in such natural supplements has sharply increased due to the restriction on the use of antibiotics (except for drugs permitted by the national, interstate, and international standards in the field of organic production in force in the Russian Federation).

So, the nutritional intervention of the probiotic Cellobacterin+ for dairy cows led to a significant ($p = 0.049$) increase in milk yield, as well as to a decrease in somatic cells in milk (by 38 thousand $\cdot ml^{-1} \cdot head^{-1}$, $p = 0.003$). According to the results of NGS sequencing, the biological product had a beneficial effect on the microbial community restoration. A detailed analysis of the rumen microbiome revealed significant differences in 13 bacterial genera. In particular, in the rumen of cows fed Cellobacterin+, there is a decrease in abundance of the genera *Anaerofilum* sp. (2.3-fold, $p \leq 0.05$) and *Anaerostipes* sp. (by 1.8-fold, $p \leq 0.05$), producing lactate as the final product of glucose metabolism, and taxa among which pathogens are often found, namely, *Campylobacter*, *Gemella*, *Mycoplasma*, *Shewanella* ($p \leq 0.05$) and *Fusobacterium* (including *F. necrophorum*) ($p \leq 0.001$). A decrease in the counts of somatic cells in milk was associated with a decrease in mastitis pathogens in the rumen. Based on bioinformatics data processing, the authors described in detail the metabolic changes in the cicatricial microbiota at the gene level as a result of the probiotic strain introduction and changes in the microbiome structure. The predicted functional potential of seven metabolic pathways was enhanced in cows fed with Cellobacterin+. It seems interesting to further study the beneficial effect of introduced bacteria on the host, in particular, the assessment of the viability, adhesive potential, and survival of the bacterial strain as part of a biological product in the digestive tract conditions.

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