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ECOLOGY OF RUMINAL MICROORGANISMS UNDER THE INFLUENCE OF QUERCUS CORTEX EXTRACT

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ABSTRACT: The study was performed for assessing the elemental status and the bacterial composition of the rumen fluid microflora in cattle after incubation in vitro with quercus cortex extract and exogenous enzymes. The mixture of alfalfa hay, sudan grass and concentrates (60:40) was used as the substrate. The results showed that in the protozoa a decrease in such elements as p, na, ca ($p \leq 0.05$) was observed when substrate with enzyme was added. The level of essential elements in bacteria three hours after the incubation together with the enzyme changed similar to the protozoa, except for copper, the content of which increased two times ($p \leq 0.05$). The introduction of quercus cortex extract into the substrate with the enzyme preparation three hours after the incubation promoted a significant increase in iron, manganese and cobalt content in the protozoa ($p \leq 0.05$). Unlike other variants, incubation of the substrate with the enzyme together with the extract increased the number of representatives of the taxa firmicutes by 13.2%, bacteroidetes - by 13.4% ($p \leq 0.05$) against the background of a slight decrease in proteobacteria, saccharibacteria and fibrobacteres. An increase was observed in the number of microorganisms belonging to the taxa clostridia (by 23.7%; $p \leq 0.05$) and bacteroidia (by 13.4%; $p \leq 0.05$). The biosensor based on *b.subtilis* on samples with the extract reacted by quenching the luminescence by 60% on the 5th minutes and by 32% on the 60th minute contact. Thus, the chemical composition of oak bark extract significantly influenced the elemental profile and the bacterial composition of cattle rumen microorganisms.

Keywords: Quercus cortex extract, Elemental status, Rumen microorganisms, Cattle

1. INTRODUCTION

Limitations in the use of antibiotic substances for preventing diseases and increasing cattle productivity make it urgent to search for new substances with similar properties**.** Among these substances, there are plant extracts (essential oils, saponins, tannins, etc.) with positive effect on protein metabolism, production of volatile fatty acids and gases in the rumen of ruminants [1, 2, 3]. At the same time, a number of scientists identified in plant extracts a new class of substances capable of efficiently preventing development of infectious and inflammatory processes in human and animal organisms through the system of intercell chemical communication in bacteria ("quorum sensing") [4]. Thus, when screening 20 medicinal plants used by the Russian (Eastern European) folk medicine, the most pronounced ability to inhibit the "quorum sensing" system of wild and mutant strains of C. violaceum was identified in extracts of oak bark (cortex Quercus), birch buds (Betula verucosa) and eucalyptus leaves (Eucalyptus viminalis) [5]. At the same time, the need for studying the influence of these extracts directly on the microbiome, changes in its biochemical parameters may be of interest when studying intercellular chemical communication in bacteria in the rumen. The previously obtained results confirm the promising nature of these studies [6,7].

In this regard, the research aimed at studying the ability of plant extracts to inhibit microorganisms, and the influence on the elemental status of microflora of the rumen of cattle is of interest. The aim of this research is to study the effect of feeding Quercus cortex extract on the elemental status and the bacterial composition of cattle rumen fluid in vitro against the background of enzyme diet.

2. MATERIALS AND METHODS

2.1 Phytochemical Analysis

Dry Quercus cortex extract was used for the research, which was dissolved in ethanol before chromatography-mass spectrometry, and injected into the analytical cell of the chromatograph using microsyringe Hamilton 1700. Chemicals in Quercus cortex extract were identified in the gas chromatograph with mass selective detector GQCMS 2010 Plus (Shimadzu, Japan), in column HP-5MS. The research results were interpreted with the following software: GCMS Solutions, GCMS PostRun Analysis; compounds were identified with a set of spectra libraries CAS, NIST08, Mainlib, Wiley9 and DD2012 Lib. The quantitative presence

of individual identified components was assessed by relative value (%), which correlated the area of the peak to the total area of the extract. In the extract of oak bark 35 substances were identified (according to IUPAC): propanetriol-1,2,3*; decane*; 2-furancarboxylic acid*; 1,3,5-triazine-2,4,6-triamine*; pentadecane*; 2,3-dihydroxypropyl*; butanedioic acid*; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one*; 2-amino-9-[3,4-dihydroxy-5-(hydroxymethyl) oxolan-2-yl] -3H-purine-6-one*; cyclopentane-1,2-diol**; 1,2:5,6-dianhydrogalaxytole**; 5-hydroxymethylfurfural*; acetylcysteine, -2-acetamido-3-mercaptopropionic acid*; 1-methylundecyl ether 2-propenoic acid**; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one**; 1- (2-hydroxyethyl) -4-methylpiperazine**; 6- (4-hydroxy-6-methoxy-2-methyl -, tetrahydro-pyran-3-yloxy) -2-methyl-dihydro-pyran-3-one**; 1,2,3-trihydroxybenzene* (pyrogallol); 2-methyl-5-nitro-pyrimidine-4,6-diol**; 4-hydroxy-3-methoxybenzaldehyde*(vanilla); 2-amino-9- [3,4-dihydroxy-5- (hydroxymethyl) oxolan-2-yl] -3H-purine-6-one*; 1,6-anhydro-β-D-glucopyranose*; 1- (β-D-arabinofuranosyl) -4-O-trifluoromethyl uracil**; 4-hydroxy-3-methoxy benzoic acid**; 1,6-anhydro-β-D-glucofuranose*; 4-propyl-1,3-benzenediol* (propylresorcinol); 1,2,3,4,5-cyclohexanol*; 4-(hydroxymethyl)-2,6-dimethoxyphenol*; 4- (3-hydroxy-1-propenyl) -2-methoxyphenol*(coniferyl alcohol); 9 -[(2R, 3R, 4S, 5R) -3,4-dihydroxy-5- (hydroxymethyl) oxolan-2-yl]-3H-purine-2,6-dione*; 7-hydroxy-6-methoxy-2H-1-benzopyran-2-one*(coumarine); methyl-α-D-glucopyranoside*; 2H-1-benzopyran-2-one*(scopoletin); 2 ethoxy-6-(methoxymethyl) phenol**; 3,4,5-trimethoxyphenol**(antiarol) (*components identified with the probability of over 90%; ** components identified with the probability of at least 90%).

2.2 Scheme of the Experiment

Reference - ruminal fluid + ethanol (0.5% of volume), variant I – ruminal fluid + exogenous enzyme + ethanol (0.5% of volume), variant II - scar liquid + phytoextract in ethanol (0.5% of total) + exogenous enzyme. The exogenous enzyme preparation contained glucoamylase and related cellulosolytic enzymes (xylanase, β-glucanase, cellulase). The preparation was dosed in the amount of 0.05% of dry ingredients. Experimental studies were performed at the scientific equipment common use center of Federal Research Center of Biological Systems and Agro-technologies of the Russian Academy of Sciences.

2.3 Ruminal Digestion in Vitro

The previously used method of studying ruminal fermentation in vitro [8] was considered to be an acceptable method for assessing the influence of phytocompounds on ruminal microflora. Two heads of cattle (males) with chronic rumen fistula were kept on a diet consisting of 60% coarse forage (alfalfa hay and Sudan grass) and 40% pellets containing barley grain, wheat bran, wastes of vegetable oil, limestone, salt, vitamins and a premix. The chemical composition of the diet: (g/kg of dry matter) was 153 non-decomposing protein, 485 neutral detergent fiber, 47 crude fat, 85 ash and 10.00 MJ/kg exchange energy of dry matter. The fodder was supplied twice a day, and animals had free access to drinking water. The animals were used as donors of ruminal fluid.

Two hundred milligrams of dry matter in the forage consisting of hay and concentrates (pellets) in the ratio of 60:40 were used as substrate for fermentation in vitro. The incubation medium was prepared as described by Menke and Steingass [9], and 30 ml of it were anaerobically placed into a 100 ml syringe. The mixture of substances was dissolved in ethanol; the substrate based on dry matter (9 mg) was introduced into each syringe at the concentration of 4.5% (by weight). The final concentration of ethanol in each syringe was 0.5%. The reference was substrate with 0.5% ethanol. The syringes were incubated at 39° C for 24 hours. Degradability of the dry matter, quantitative assessment of the rumen microorganisms' population, and their elemental composition were measured. The total of nine syringes were used for each research.

2.4 Assessment of the Effect on Luminescent Bacterial Biosensors

Strains of Escherichia coli MG1655 pXen7, Salmonella typhimurium LT2 pACXen and Bacillus subtilis EG168-1 were used as test objects. Cells were grown within 24 hours on LB agar (Sigma-Aldrich, USA) with a selectable marker, and then suspended in a 0.5% solution of sodium chloride to OD450 = 0.50 rel. units in transparent wells at a volume of 200 µl using StatFax 303+ photometer (Awareness, USA). Two-fold dilutions from 100% to 1.56% were prepared in wells of a 96-well plate with a volume of 50 µl from samples of ruminal fluid, then 150 µl of biosensor cell suspension were added. The luminescence of bacteria was registered in a kinetic mode using LM-01T luminometer (Immunotech, Czech Republic) for 60 minutes [10].

205 Analysis of the Rumen microbial Population

Assessment of the microbial biodiversity included: sampling, isolation, purification, measuring DNA concentration, PCR, validation and

normalization of libraries followed by sequencing at the platform of high-performance sequencer MiSeq Illumina (USA). Bioinformatical analysis of the results was made in application PEAR (Pair-End AssembeR, PEAR v0.9.8, April 9, 2015) [11].

206 Analysis of the Substrates' Elemental Status

The elemental composition of the extract, animalculines and bacteria (liquid samples) was determined by atomic emission and mass spectrometry (AES-ISP and MS-ISP) at the test laboratory of Autonomous Nonprofit Organization"Biotic Medicine Centre",Moscow (Registration Certificate of ISO 9001: 2000, Number 4017-5.04.06). Biological substrates were ashed using a microwave decomposition system MD-2000 (USA). Elements' content in the obtained ash was assessed using a mass spectrometer Elan 9000 (Perkin Elmer, USA) and an atomic emission spectrometer Optima 2000 V (Perkin Elmer, USA). Elemental composition of Quercus cortex extract, mg/g was the following: Ca - 246; P - 8.22; K - 124; Mg - 32.5; Na - 85.0; Zn - 1.75; Mn - 5.8; Cu - 0.05; Fe - 2.01; Co - 0.018; Se - 0.02; I - 0.037; Al - 0.46; Sr - 0.7; Cr - 0.11; Cd - 0.001; Pb - 0.008.

2.7 Statistical Processing

Statistical processing was made in application IBM "SPSS Statistics Version 20", calculating the mean value (M), the standard deviation (σ), the standard deviation error (m). The level of significance was considered veracious with $p<0.05$. The student's t-criterion was used for statistical processing.

3. RESULTS AND DISCUSSION

According to the research results, the level of essential elements in protozoa after incubation of variant I decreased, except for copper, the value of which increased 2 times, compared to the reference. Incubation of the II variant contributed to a significantly increased amount of substances; this was particularly evident for iron (21.8%), manganese (8.8%) and cobalt (16.5 times). Similar pattern was observed in studying the composition of bacteria: for copper, the increase was 11.3 times, for zinc – 3.3 times, and for iron – 2.4 times (Table 1). Due to the fact that the topic has been poorly studied, and to the lack of direct data in the literature, one can see the need for using indirect facts in the discussion. Thus, it is necessary to consider the fact that bacteria are in some ways "food" for the animalculines, and increasing the amount of macro- and essential elements in them may be explained by ingestion, or by normal digestion process. Besides, it should be noted that microelements' concentration in plants largely depends on the environment, the climate, the age, as well as the composition of diets, and other factors [12, 13, 14, 15, 16]. It is known that metal ions are cofactors for a number of enzymes, including microorganisms and protozoa [17]; therefore, the probability of their influence on the activity of the enzyme systems of the ruminal microflora is quite high.

At the same time Quercus cortex, which is positioned as the source of tannins, has no adverse effect on fermentation in the rumen of cattle, positively influences energy exchange and protein utilization in the rumen [18,19], the number of protozoa [20], although this requires further research [21,22].

Table 1 The mean values of essential elements' content in protozoa and bacteria, μg/g

Diet	Zn	Mn	Cu	Fe	Co	Se	I
Protozoa							
reference	1.6 ±0.24	1.6 ±0.25	0.14 ±0.028	7.5 ±1.12	0.003 ±0.00094	<0.0039	0.007 ±0.00229
Protozoa (after 3 hours)							
MD+E	1.2 ±0.18	0.7 ±0.148	0.31 ±0.063	4.0 ±0.61	0.002 ±0.00067	<0.0039	0.006 ±0.00198
MD+E+extract	5.7 ±0.85	6.2 ±0.93	1.6 ±0.24	87.4 ±13.11	0.033 ±0.008	0.017 ±0.004	0.027 ±0.007
Bacteria							
reference	1.3 ±0.19	0.5 ±0.108	0.2 ±0.039	2.6 ±0.39	0.004 ±0.00133	<0.0039	0.005 ±0.00158
Bacteria (after 3 hours)							
MD+E	1.2 ±0.18	0.5 ±0.099	0.3 ±0.06	2.9 ±0.44	0.004 ±0.0013	<0.0039	0.008 ±0.00245
MD+E+extract	3.9 ±0.6	0.7 ±0.148	3.4 ±0.51	7.0 ±1.06	0.007 ±0.00218	<0.0039	0.012 ±0.003

In turn, the use of enzyme-containing diet could not influence the microflora in view of the numerous data indicating their efficient use in feeding cattle [23, 24, 25]. In studying bioavailability of microelements in the gastrointestinal tract of ruminants, it is necessary to veraciously consider their bonding with the coarse fiber part of the fodder, with undigested fodder

components and newly formed complexes insoluble in acidic environment [26, 27, 28]. The results of the research show low bioavailability of selenium for both protozoa and bacteria. This is determined by the results of previous experiments that showed low digestion of selenium by these animals, and the transition of its available form into a less available one directly in the ruminal medium [29]. Some authors [30] indicate that high levels of calcium in the diet result in low selenium absorption, which was also observed in this experiment.

The results of the research show the prevalence of iron concentration over the same value of copper in the experiment in both protozoa and bacteria, especially against the background of enzyme diet. This increase is also due to the high iron content in Quercus cortex extract. Several researchers in their studies indicated a decreased level of copper in the organism of ruminants with increasing the level of iron in the diet [31]. Zinc concentration in protozoa and bacteria in the reference and after the incubation virtually did not change, probably due to the relatively high absorption capacity of this element in the rumen, and the availability of the phytaze activity of the microflora [32]. Manganese is absorbed insignificantly in the rumen of ruminants [33]. This may explain its low content in the bacteria after fermentation of the fodder, but after the extract had been included into the diet, the concentration of the element in animalculines increased.

Analysis of the microbiocenosis of ruminal content showed that before incubation it had been represented by bacteria (82.1%) and microscopic fungi (17.9%) (Table 2).

Table 2 – Taxonomic diversity of the bacterial composition of the ruminal fluid

Group	Taxon			
	phylum	class	family	genus
MD	Firmicutes (32.4%)	Bacilli (17.3%)	Lactobacillaceae (13.9%)	Lactobacillus (13.9%)
		Clostridia (11.1%)	Lachnospiraceae (6.93%)	-
			Clostridiaceae (4.2%)	Faecalibacterium (4.2%)
		Negativicutes (3.99%)	Acidaminococcaceae (3.99%)	Succiniclasticum (3.99%)
	Proteobacteria (12.8%)	Gammaproteobacteria (12.8%)	Moraxellaceae (2.52%)	Acinetobacter (2.52%)
			Enterobacteriaceae (10.3%)	Escherichia (5.04%)
				Enterobacter (5.25%)
	Bacteroidetes (30.1%)	Bacteroidia (30.1%)	Prevotellaceae (19.4%)	Prevotella (18.1%)
			Porphyromonadaceae 3.15%)	-
			Bacteroidaceae (7.56%)	-
	Fibrobacteres (5.04%)	Fibrobacteria (5.04%)	Fibrobacteraceae (5.04%)	Fibrobacter (5.04%)
	Saccharibacteria (18.7%)	-	-	-
	Other* (1%)	Other* (19.7%)	Other*(23%)	Other* (42%)
MD+Enzyme+extract	Firmicutes (43.5%)	Clostridia (34.8%)	Ruminococcaceae (17.4%)	-
			Lachnospiraceae (13%)	-
			Clostridiaceae (4.35%)	-
	Saccharibacteria (13%)	-	-	-
	Bacteroidetes (43.5%)	Bacteroidia (43.5%)	Bacteroidaceae (17.4%)	-
			Prevotellaceae (26.1%)	Prevotella (26.1%)
	-	Other* (21.7%)	Other* (21.7%)	Other* (73.9%)
MD+Enzyme	Firmicutes (2.11%)	Clostridia (2%)	-	-
	Bacteroidetes (85%)	Bacteroidia (83.5%)	Prevotellaceae (59%)	Prevotella (58%)
	Saccharibacteria (5.32%)	-	-	-
	Other* (7.6%)	Other* (14.5%)	Other* (41%)	Other* (42%)

* This group combines the taxa, with number of each not exceeding 2% of the total

The bacteriological composition was represented by phyla such as Firmicutes (32.4%), Saccharibacteria (18.7%), Proteobacteria (12.8%), Fibrobacteres (5.04%), and others (1% from the reference), where the dominant classes were Bacilli (17.3%), Gammaproteobacteria (12.8%), Bacteroidia (30.1%), and Clostridia (11.1%). Species diversity was represented by bacteria belonging to genera such as g. Lactobacillus (13.9%), g. Prevotella (18.1%), g. Escherichia (5.04%), g. Enterobacter (5.25%), g. Fibrobacter (5.04% of the reference), etc. The most abundant in the samples were Lactobacillus salivarius (13%), and Prevotella ruminicola (2.94%). The use of the enzyme in the diet was accompanied by a significant decrease in the number of bacteria in the rumen, which related to the taxa Firmicutes by 30.3% (P≤0.05), Saccharibacteria - by 13.4%, and Proteobacteria - up to 2% of the total, and by an increase in the number of the phylum Bacteroidetes by 54.9% (P≤0.05) from the reference, which was reflected in the change in the percentage of representatives of classes Clostridia, Bacteroidia, and Bacilli in the microbiocenosis.

For taxa Firmicutes and Proteobacteria, a decreased number was observed for bacteria of classes Clostridia (9.1%), Bacilli (less than 2% of the total) and Gammaproteobacteria (less than 2% of the total). At the same time, an increased number of bacteria of class Bacteroidia by 53.4% (P≤0,05) was observed within the Bacteroidetes taxon, which was mainly due to the increase in representatives of g. Prevotella (by 39.9%; P≤0.05). On the contrary, the use of the enzyme together with the extract in the diet increased the number of representatives of taxon Firmicutes by 13.2%, and taxon Bacteroidetes by 13.4% (P≤0.05), but decreased the number of bacteria that belong to phyla Proteobacteria, Saccharibacteria, and Fibrobacteres up to 2% of the total number in the sample. The similar effect when using tannin-containing substances was observed in recent experiments by other researchers as well [34].

An increase was observed in the number of microorganisms belonging to the taxa Clostridia (by 23.7%; P≤0.05) and Bacteroidia (by 13.4%; P≤0.05). Analysis of the microbiocenosis showed an increase, compared to the reference, in the number of bacteria of g. Prevotella (by 8% of the reference), and a decrease in the number of bacteria of other genera that were the most numerous in the reference. Since there is currently no accurate method for describing changes in microorganisms in a complex system such as rumen, this study has been focused on bacteria. Quercus cortex extract in the composition of the fodder contributed to decreasing the number of cellulosolytic microorganisms (Fibrobacteres). This result is consistent with the previous studies [35,36,37], where a similar process was also observed in sheep and rats after introducing plants (extract) containing tannins into the diet. The decreased levels of Proteobacteria and Fibrobacteres may be explained by different molecular weights of the substances in the extract, including tannins [38]; this fact was not considered in this experiment.

Assessment of acute toxicity with luminescent bacteria showed that during the first five minutes of contact with the whole ruminal fluid the luminescence in the control samples is inhibited by no more than 25% for E. coli pXen7 and up to 20% for *S. typhimurium* pACXen, andindicators remain at the level of control for I and II variants (Figure 1). Nevertheless, the use of samplesdiluted two or more times for these strains removes toxic effect and leads to an increase in luminescence, most likely associated with a change in membrane permeability and activation of proton systems due to acidification of the medium. On the other hand, the biosensor based on *B. subtilis* EG168-1 also reacted on the control samples with a drop in luminescence up to 15%, however, samples of the I and II variants led to quenching by 65% and 60%, respectively. A similar effect was registered earlier [6], it was established that after contact of native ruminal fluid with bioluminescent E. coli strain, a dose-dependent inhibition of luminescence was registeredon the first seconds of contact.

Assessment of luminescence on the 60th minute of contact showed that it exceeded the control by 35%for E. coli pXen7 in the case of samples of the second variant, indicating a restoration of luminosity and no bactericidal effect,. In general, similar effects were recorded for *S.typhimurium* pACXen, - luminescence was restored in control variants, in the samples with the extract, a luminescence was observed, only variants with a vervum were characterized by a 19.5% drop. Studies are known [39] with positive effects of inhibition against E. coli isolated from cattle are found against the background of Caryocar brasiliense plant extracts. In this case, the role of tannins in the manifestation of antibacterial effects was registered. In our case, the results of studies using ruminal fluid of cattle are presented. A high inhibitory effect of Quercus infectoria gall extracts against Staphylococcus aureus was also observed in the study of antibacterial activity in vitro [40].

A different picture was developed for *B. subtilis* EG168-1, where the luminescence of the control sample exceeded the initial level 8 times, in the first variant - 1.6 times, and the II variant inhibited the luminescence by 32%. Moreover, the second variant in the case of *B.subtilis* biosensor was characterized by the absence of a luminescence dependence on the sample concentration. The data obtained are in agreement with the studies [41], where positive results were registered when evaluating the antibacterial activity of the oak extract against B. subtilis.

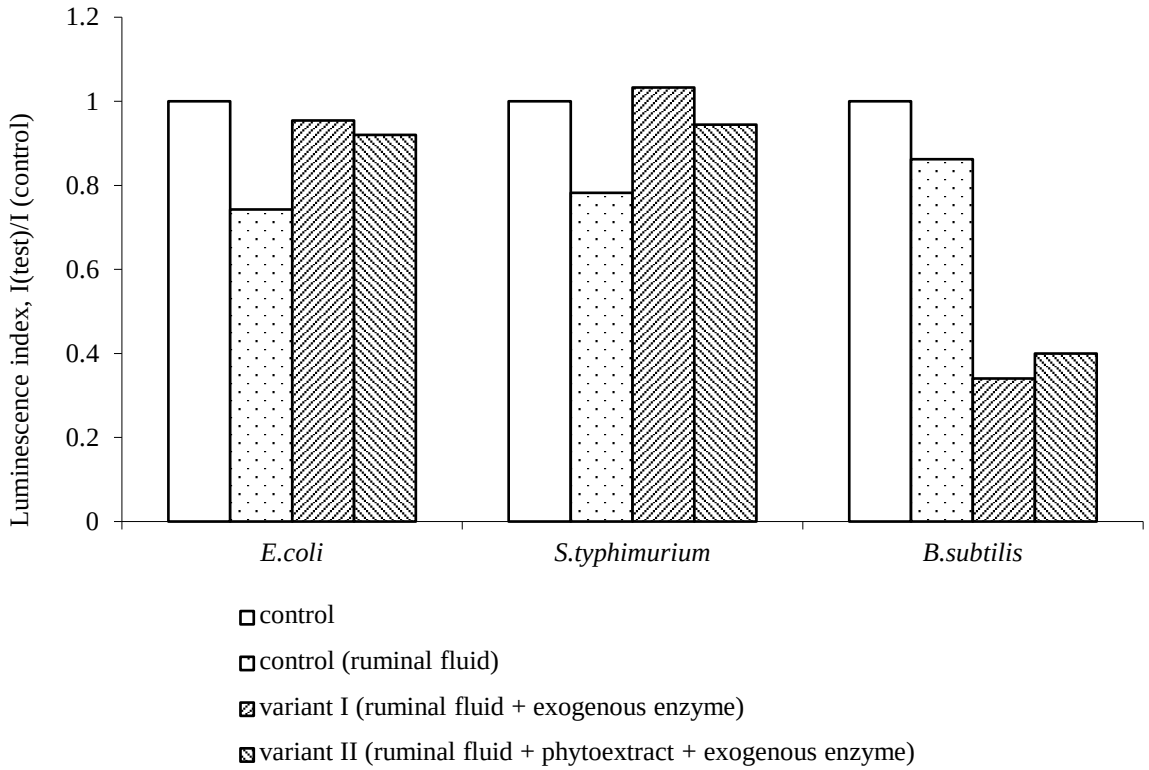

Fig. 1 Luminescence of bacterial biosensors on the fifth minute in the presence of ruminal fluid from cows with various feeding factors.

4. CONCLUSIONS

The chemical composition of Quercus cortex extracthas a significant effect on the elemental profile of cattle rumen microorganisms. At the same time, the introduction of the extract into the diet does not change the overall bacterial structure of rumen microflora. The ratio of phyla Firmicutes and Bacteroidetes, the parameter associated with the energy accumulation function, was increased, same as the number of microorganisms that belonged to the taxon Clostridia. Further study is required for assessing the influence of plant extracts on ruminal microorganisms and changing the products of fermentation associated with the productivity of animals. The biosensor based on *B. subtilis* EG168-1 reacted more actively to the introduction of the extract into ruminal fluid. Further work is required to assess the effect of individual components of plant extracts on ruminal microorganisms and changes in fermentation products associated with animal productivity.

5. ACKNOWLEDGEMENTS

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