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METABOLIC PARAMETERS AND METHANOGENESIS IN THE RUMEN LIQUID IN in vitro TESTING EXPERIMENTAL DIETS SUPPLEMENTED WITH PHYTOBIOTICS AND $CoCl_2$

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Abstract

Dietary bioactives that increase the efficiency of feed nutrient use can provide sustainable and safe livestock products. Some bioactives are modifiers of rumen function in ruminants. These compounds are mostly administered separately. This paper is the first to describe the metabolic changes during in vitro incubation of the Kazakh white-headed bull ruminal liquid (RL) with feed compositions (biosubstrates) containing phytopreparations and cobalt chloride. The most effective combinations and dosages of these additives are evaluated. It was found out that *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) increases digestibility of feed dry matter (by 2.1 %), the activity of digestive enzymes and the concentration of metabolites in the RL while decreases methane production by 33.9 %. *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) provide the maximum reduction in methane emissions (by 46.3 %). Plant preparations increase the activity of RL amylase 2.6-4.0-fold and RL proteases 3.6-fold compared to control. Our goal was to reveal the effect of herbal preparation and cobalt chloride combination on the metabolic changes assessed in RL by in vitro technique. The experiments were carried out in 2021 at the BST RAS (Orenburg). Rumen liquid (RL) was sampled from four Kazakh white-headed bulls (*Bos taurus taurus*) weighing 250-265 kg at the age of 9-10 months. The samples were collected 12 h after feeding, through a chronic rumen fistula. The control ration (variant I) was 70 % coarse feed (mixed meadow hay) and 30 % concentrated feed (crushed barley). Test rations II was added with $CoCl_2$ (1.5 mg/kg DM; OOO NPK Ascont+, Russia), III with *Salviae folia* (1.6 g/kg DM), IV with *Artemisiae absinthil herba* (2.0 g/kg DM), V with *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), and VI with *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM). Each RL sample was tested 4-fold (n = 16). Feed samples weighing 500 mg in polyamide bags were incubated for 48 h at 39.5 °C in a mixture of buffer solution with RL. At the end of incubation, the samples were rinsed and dried at 60 °C to a constant mass. The coefficient of digestibility in vitro of dry matter was calculated. Air samples were taken separately from each container to determine the methane content by gas chromatography (a Crystallux-2000M device, OOO NPF Meta-chrome, Russia). The amount of volatile fatty acids (VFA) in the RL was determined by gas chromatography with flame ionization detection (a gas Crystallux-4000M chromatograph). The concentration of various forms of nitrogen was determined by the Kjeldahl method (the Millab company equipment, Italy). Amylase activity was measured by Smith-Roy method modified by Anosone for high activity enzymes in the pancreatic juice. Proteolytic activity was assessed colorimetrically (λ = 450 nm) by destruction of Hammarsten Grade casein. The dry matter of biosubstrates was determined by drying to a constant mass at 60 °C. It was found that *Salviae folia* and *Artemisiae absinthil herba* shifted the fermentation during incubation towards propionate and butyrate. *A. absinthil herba* increased the intensity of nitrogen metabolism in RL during incubation, while total nitrogen content increased by 11.6 %, non-protein nitrogen by 144.3 %, ammonia by 71.4 %, and urea by 31,7 % ($p < 0.05$). Phytomaterials significantly increased the activity of amylase, proteases, and the concentration of VFA, but also increased the methane emission. Combinations of phytomaterials and cobalt chloride had a positive effect on the fermentation processes in the "artificial rumen". The maximum effect was revealed when using *A. absinthil herba* and cobalt chloride. There was an increase in the digestibility of dry matter with a decrease in methane formation by 2.1 %, and an increase in the

activity of digestive enzymes and the volatile fatty acid concentration.

Keywords: *Artemisiae absinthil herba*, *Salviae folia*, phytobiotics, cobalt chloride, nitrogen, volatile fatty acids, methane, digestive enzymes, "artificial rumen", beef cattle

The emergence of bacterial resistance and the abandonment of the use of feed antibiotics as growth promoters [1] have led to the need for searching natural and safe alternatives, such as probiotics, prebiotics, mineral components or phytobiotics [2, 3].

Through secondary metabolism, plants produce a variety of organic compounds that can be beneficial to animals. Phytobiotics have been shown to exhibit high biological activity. They have been investigated as modifiers of rumen function in ruminants [4-6]. Thus, the addition of a mixture of plant extracts had a positive effect on ruminal fermentation and growth performance in bulls consuming large amounts of feed concentrates [7]. Another study [8] found a positive effect of neomycin and oregano leaves on the severity of gastrointestinal diseases, as well as the mortality of newborn calves.

Plants and their bioactive compounds with antimicrobial properties have been found to improve feed utilization and animal productivity by altering microbial fermentation in the rumen [9]. However, currently plant products are used in the feed industry mainly as additives, flavorings and appetite stimulants [10]. Despite many studies, mostly in vitro, on the potential use of phytobiotics [6, 7], there is little information on their use in combination with other substances to improve metabolic processes and stimulate growth.

Previously, in in vitro experiments, we tested herbal remedies *Salviae folia*, *Inulae rhizomata et radices*, *Artemisiae absinthil herba*, *Scutellaria baicalensis*, *Origanum vulgare* and selected samples, the sage leaves and wormwood grass that showed the greatest functional activity [11]. Bioactive substances of herbal preparations, such as alkaloids, flavonoids, saponins, tannins, phenolic compounds, terpenoids and essential oils, optimize protein metabolism, reduce methane production and acidosis, which ultimately improves fermentation in the rumen [12]. To correct the effect of herbal preparations on methane production and fermentation in the rumen, it is necessary to investigate the effectiveness of using their compositions with chemical elements.

Cobalt is a promising chemical element because it is important for the microbial population in the rumen of ruminants, in particular for cellulolytic microorganisms. In addition, the production of vitamin B_{12}, vital for the host and protozoa, increases with the amount of cobalt available through bacterial synthesis [13-15]. A mixture of essential plant ingredients and organic cobalt in small ruminants]16] helped reduce the formation of methane and ammonia in the rumen and improve fermentation, and the form and amount of cobalt had a toxic effect on the number of methanogenic bacteria [17].

This paper describes for the first time changes in metabolic parameters in rumen fluid in vitro when herbal remedies and cobalt chloride were added to diet samples (biosubstrates), and the most effective combinations and dosages of these additives were determined. When using the *Artemisiae absinthil* herba complex (2.0 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM), an increase in the digestibility of dry matter, the activity of digestive enzymes and the content of metabolites in the rumen fluid, as well as a decrease in methane formation were revealed. The use of the *Salviae folia* complex (1.6 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM) led to the maximum reduction in in vitro methane formation.

The purpose of the work is to study in vitro the effect of herbal preparations and cobalt chloride (separately and in combination) on changes in metabolic parameters in the rumen fluid of bulls ("artigicial rumen method").

Materials and methods. The experiments were carried out in 2021 at the Shared Use Center of the BAT RAS (Orenburg). The material for the study was obtained from bulls (*Bos taurus taurus*) of the Kazakh white-headed breed ($N = 4$),

with an average weight of 250-265 kg at the age of 9-10 months. The animals were kept individually on a leash in standard cages, fed twice a day, access to water was unlimited. Feeding was carried out with regards to the recommendations of A.P. Kalashnikov et al. [18].

Rumen fluid (RF) was collected 12 h after feeding, through a chronic rumen fistula (d = 80 mm; Ankom Technology Corp., USA) with a rubber hose (outer diameter 40 mm) into a 3-liter thermos. Transportation was carried out at 4-8 °C for 20-30 min. RF, preheated to 39 °C, was used immediately upon arrival at the laboratory.

The studies were carried out in vitro using an Ankom DaisyII incubator (Ankom Technology Corp., USA) according to a special technique [19, 20]. Before use, RF samples were filtered through 4 layers of gauze and mixed with a buffer solution (1:4). The chemical composition of the buffer solution corresponded to saliva and maintained the pH of the "artificial rumen" close to physiological range (pH 6.0-6.5). Before mixing, the buffer solution was heated to 39 °C and saturated with CO_2.

Control diet (sample I) was 70% roughage (mixed-grass meadow hay) and 30% concentrated feed (crushed barley). Five test samples were added with phytosubstances and chemical elements, sample II with $CoCl_2$ (1.5 mg/kg DM; NPK Askont+ LLC, Russia), sample III with *Salviae folia* (1.6 g/kg DM), sample IV with *Artemisiae absinthil herba* (2.0 g/kg DM), sample V with *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), sample VI with *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM). The choice of dosages of herbal preparations and chromium chloride was based on previous studies and recommendations [21, 22]. Each RF sample from 4 animals was tested 4 times (n = 16). *Salviae folia* (LSR-005376/07, Krasnogorskleksredstva JSC, Russia) contained 1.3-2.5% essential oil, consisting of D-α-pinene, cineole (~ 15%), α- and β-thujone, D-borneol and D-camphor. Alkaloids, flavonoids, tannins, oleanolic and ursolic acids were also found in the leaves of this plant [21]. *Artemisiae absinthil herba* (LSR-000171/08, LLC PKF FITOFARM, Russia) contained sesquiterpene lactones, bitter glycosides (absinthine, anabsinthine, artabsin, etc.), which give the plant a peculiar bitter taste, saponins, flavonoids, phytoncides, ascorbic acid, resinous and tannin substances, potassium salts, artemisetin, essential oil (0.2-0.5%), carotene, organic acids (malic, succinic) [21].

Feed samples (500 mg) were placed in polyamide bags and incubated in a mixture of a buffer solution with rumen fluid for 48 h at 39.5 °C. At the end of incubation, the samples were washed and dried at 60 °C to constant weight.

The in vitro dry matter digestibility coefficient was calculated as the difference in the feed sample mass with the bag before and after incubation:

$$K = (A - B)/C \times 100\%,$$

where K is the digestibility coefficient of the feed dry matter, %, A is the weight of the feed sample with bag before incubation, mg, B is the weight of the food sample with the bag after incubation, mg, C is initial weight of the food sample without the weight of the bag before incubation, mg.

After incubation, air samples were collected separately from each incubator container into special glass 200 ml^3syringes with rubber stoppers to determine the methane content by gas chromatography (a Kristallyuks-2000M device, OOO NPF Meta-Chrome, Russia). The amount of volatile fatty acids (VFA) in the RF was determined by gas chromatography with flame ionization detection (a Kristallyuks-4000M gas chromatograph). The content of various forms of nitrogen was assessed (a Millab equipment, Italy) according to Kjeldahl.

Amylase activity was measured by the Smith-Roy method modified by Anoson to determine high enzyme activity in pancreatic juice [23] and was expressed in mg of digested starch · ml^{-1} · min^{-1}. Amylase activity estimates were based on the hydrolysis of starch paste. By measuring the color intensity of a starch solution with

iodine reagent on KFK-3-01 (JSC ZOMZ, Russia), the rate of hydrolysis of the paste (amylase substrate) was determined. The activity of proteolytic enzymes was assessed by the amount of digested purified Hammersten casein with colorimetric control (λ = 450 nm) [24]. The technique was based on the colorimetric determination of casein concentration on KFK-3-01. The dry matter (DM) of biosubstrates was determined by drying to constant weight at 60 °C.

Animal care and experimental studies were carried out in accordance with the instructions and recommendations of regulations, the Order of the USSR Ministry of Health No. 755 of August 12, 1977 "On measures to further improve organizational forms of work using experimental animals" and Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). During the experiments, measures were taken to minimize animal suffering and reduce the number of prototypes.

The data were processed using the SPSS Statistics 20 program (IBM, USA). Mean values (*M*) and standard errors of means (±SEM) were calculated. The statistical significance of differences between the experimental and control groups was determined by Student's *t*-test; differences were considered significant at $p \leq 0.05$ and $p \leq 0.01$.

Results. The composition and nutritional value of the diet of the animals from which ruminal fluid were sampled are shown in Table 1.

1. Composition and nutritional value of the daily diet (per 1 animal) **of the Kazakh white-headed bulls (*Bos taurus taurus*) producing rumen liquid for in vitro tests** (Center for shared equipment use BAT RAS, 2021)

Ingredient	Amount
Mixed grass hay, kg	4.5
Legume hay, kg	3.2
Concentrates, kg	1.8
Table salt, kg	0.035
Vitamin A, thousand IU	30.0
Vitamin D, thousand IU	2.7
The diet contained:	
dry matter, kg	8.78
exchange energy, MJ	70.0
crude protein, kg	0.52
crude fiber, kg	2.46
neutral detergent fiber, kg	0.55
acid detergent fiber, kg	0.41
hemicellulose, kg	0.15
crude fat, kg	0.24
organic matter, kg	8.20
calcium, g	45.0
phosphorus, g	33.0

The digestibility of the dietary DM with the inclusion of phytochemicals increased in sample III by 1.3% ($p \leq 0.05$) and decreased in sample IV by 2.9% vs. control (Fig. 1). Cobalt chloride increased the digestibility of dry matter by 1.5%. The best digestibility was haracteristic of composition *Artemisiae absinthil herba* + $CoCl_2$, being 2.1% higher vs. control ($p \leq 0.05$). For *Salviae folia* + $CoCl_2$, the value was 1.3% higher than the control. These findings are indirectly supported by previous studies that found that essential oils from certain *Artemisiae* species improved in vitro rumen fermentation and dry matter digestibility [25]. This fact is explained by the likely increase in the content of terpenes, which are present in significant quantities in the wormwood extract. Feed intake is inversely related to dietary terpene concentrations, and ruminants cannot consume terpenes above a threshold [26]. Terpenoid extracts of wormwood suppress rumen microbiota and reduce the rate of cellulose digestion [27]. Additionally, J.P. Wu et al. [28] noted a change in rumen fermentation and better absorption of substances when adding a mixture of oregano (*Origanum vulgare* L., *Lamiaceae*) essential oils and cobalt lactate. In our experiment, the similar

effects occurred for *S. folia* (*Lamiaceae*) and *A. absinthil herba*.

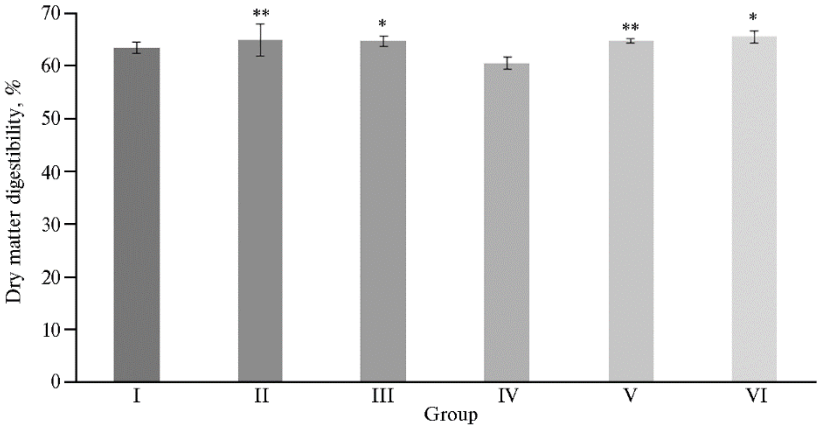

Fig. 1. Dry matter in vitro digestibility of diet composition added with herbal remedies and $CoCl_2$ after 48-hour incunation in the rumen fluid collected from Kazakh white-headed bulls (*Bos taurus taurus*): I — control, II — $CoCl_2$ (1.5 mg/kg DM), III — *Salviae folia* (1.6 g/kg DM), IV — *Artemisiae absinthil herba* (2.0 g/kg DM), V — *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), VI — *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021).
* and ** Differences from control are statistically significant at $p \le 0.05$ and $p \le 0.01$, respectively.

Artemisiae absinthil herba increased the content of total nitrogen by 11.6% ($p \le 0.05$) vs. control. In sample II, the total nitrogen decreased by 10.6%, in III by 22%, in V by 19.7%, in VI by 14.4% ($p \le 0.05$). A direct relationship was found between protein nitrogen and the metabolism of total nitrogen (Table 2). The content of non-protein nitrogen in the test samples, on the contrary, was higher than in the control, by 76.7% in sample III ($p \le 0.05$), by 144.3% in samples IV ($p \le 0.05$) by 28.3% in samples V, by 16.7% in samples VI.

2. Nitrogen content after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021)

Treatment	N, mg%				
	total	non-protein	ammonia	urea	protein
I (control)	92.4±1.71	21.0±1.13	4.2±0.90	4.1±1.51	71.4±2.02
II ($CoCl_2$, 1,5 мг/кг СВ)	82.6±1.12	19.6±1.42	3.5±0.91	6.4±1.20*	63.0±1.33
III (*Salviae folia*, 1,6 г/кг СВ)	72.1±1.83*	37.1±1.31*	5.9±0.51*	6.0±0.81	35.0±1.21*
IV (*Artemisiae absinthil herba* 2.0 g/lg DM)	103.1±1.61*	51.3±1.71*	7.2±1.81	5.4±1.92	51.8±1.43
V (*Salviae folia* 1.6 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	74.2±1.41*	26.9±1.32	3.9±0.73	4.5±1.13	47.3±1.71*
VI (*Artemisiae absinthil herba* 2.0 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	79.1±1.22*	24.5±1.53	6.7±0.82*	4.5±1.31	54.6±1.51*

* Differences from control are statistically significant at $p \le 0.05$.

The content of various bioactive substances in plants [29, 30] which are eaten by animals, contributes to changes in enzymatic processes in the rumen. There may also be a dependence on the dosage of the administered components. Thus, a high content of wormwood in the diet of sheep led to an increase in the amount of ammonia and nitrogen in the rumen [31]. Cobalt is necessary for enzymes of rumen microorganisms involved in nutrient metabolism [32, 33].

The likely mechanism of action of herbal substances is their ability to inhibit the activity of ammonia-producing bacteria in the rumen, with a corresponding change in the nitrogen content of the rumen fluid [34]. In addition, a decrease in the concentration of nitrogenous substances may be due to an increase in the proteolytic activity of microorganisms, which we will discuss hereinbelow.

The content of ammonia nitrogen turned out to be maximum with *Artemisiae absinthil herba* used both separately and with $CoCl_2$, 7.2±1.81 and 6.7±0.82 mg%, respectively (see Table 2).

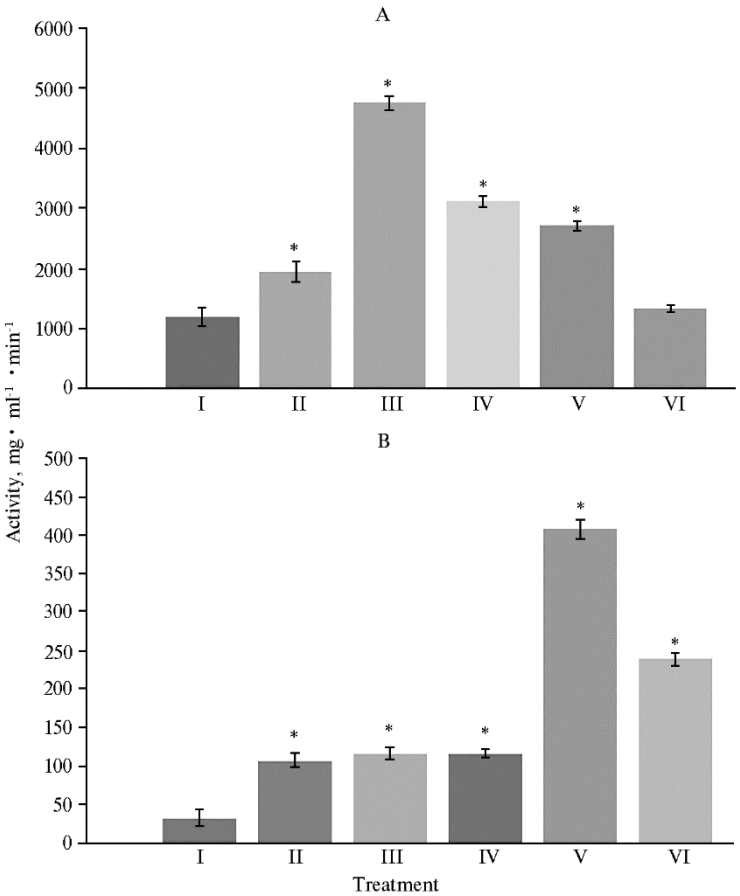

Fig. 2. Activity of amylase (A) **and proteolytic enzymes** (B) **after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*):** I — control, II — $CoCl_2$ (1.5 mg/kg DM), III — *Salviae folia* (1.6 g/kg DM), IV — *Artemisiae absinthil herba* (2.0 g/kg DM), V — *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), VI — *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021).
* Differences from control are statistically significant at $p \leq 0.05$.

The introduction of phytonutrients into the micro-diet led to an increase in the activity of digestive enzymes, amylase and proteases in RF in vitro (Fig. 2, 3). Thus, amylase activity was higher vs. control in sample II by 64.5% ($p \leq 0.05$), in sample III by 303.7% ($p \leq 0.05$), in sample IV by 164.6% ($p \leq 0.05$), in sample V by 130.7% ($p \leq 0.05$), in sample VI by 11.9%. The maximum amylolytic activity was recorded for *S. folia*. Our results confirm previous data, according to which decoctions of the aerial parts of *Salvia aegyptiaca* and *Salvia verbenaca* showed lower activity towards α-amylase [35]. There is also an opposite opinion [36] that an aqueous solution of *Salvia eriophora* inhibited the enzyme α-amylase due to the presence of phenolic substances (fumaric and caffeic acid, epicatechin) in the extract.

In the control, protease activity was 32.7±0.12 mg · ml^{-1} · min^{-1}. When *S. folia* and $CoCl_2$ were added together, the protease activity showed maximum (12.4 times higher than the control, $p \leq 0.05$). In sample VI, proteolytic activity was 7.3 times higher than in the control ($p \leq 0.05$). Cobalt chloride increased the protease activity

by 3.2 times ($p \leq 0.05$), and the phytosubstances *S. folia* and *A. absinthil herba* by 3.4 times ($p \leq 0.05$).

In the available literature, data on this issue are contradictory. Thus, salvianolic acid, the most common bioactive component of *Salvia miltiorrhiza*, can inhibit metalloproteinase [37]. High proteolytic activity could be associated with the dominant effect of cobalt. It is known that aqueous metal complexes with cobalt enhance proteolytic activity [38]. As for Artemisiae, these grass species contain nitrogenous metabolites [39] which probably contribute to the activation of proteases of rumen microorganisms.

Our studies have shown that the herbal substances of *S. folia* and *A. absinthil herba* can shift rumen fermentation towards propionate and butyrate prodution. When using *A. absinthil herba*, we noted an increase in nitrogen metabolism in the RF with an increase in total nitrogen by 11.6%, non-protein nitrogen by 144.3%, ammonia nitrogen by 71.4%, and urea nitrogen by 31.7% ($p \leq 0.05$). It has previously been shown that herbal substances shift rumen fermentation towards propionate and reduces ammonia concentrations and methane production due to effects of bioactive substances such as terpenoids [40, 41], essential oils [42, 43] and tannins [44] on rumen microorganisms. The increase in the amount of nitrogenous substances may also be associated with the presence of similar metabolites in Artemisiae [39].

3. Volatile fatty acid concentration after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021)

Treatment	Volatile fatty acids, mmol/l				
	acetic	propionic	butyric	valerian	nylon
I (control)	0.070±0.0002	0.010±0.0001	0.020±0.0001	0.030±0.0004	0.008±0.0006
II ($CoCl_2$, 1.5 mg/kg DM)	0.250±0.0050**	0.014±0.0003	0.008±0.0020	0.020±0.0011	0.006±0.0010
III (*Salviae folia*, 1.6 g/kg DM)	0.070±0.0020	0.050±0.0040*	0.060±0.0030*	0.041±0.0050	0.014±0.0003
IV (*Artemisiae absinthil herba* 2.0 g/lg DM)	0.080±0.0010	0.020±0.0010	0.170±0.0020*	0.063±0.0040	0.030±0.0010
V (*Salviae folia* 1.6 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	0.250±0.0030	0.260±0.0020**	0.160±0.0030*	0.190±0.0020**	0.140±0.0020**
VI (*Artemisiae absinthil herba* 2.0 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	0.460±0.0060**	0.36±0.00400*	0.180±0.0050*	0.030±0.0001	0.050±0.0002**

* and ** Differences from control are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

The VFA content in the control and test treatmrnts with the addition of phytochemicals turned out to be quite low (Table 3). Cobalt chloride increased the concentration of acetic acid when testing samples II and V by 3.6 times ($p \leq 0.01$), sample VI by 6.6 times ($p \leq 0.01$) vs. control. The concentration of propionic acid also increased in all test variants with $CoCl_2$, in the presence of *S. folia* by 26 times ($p \leq 0.01$), *A. absinthil herba* by 36 times ($p \leq 0.05$). Results suggest that cobalt chloride may increase microbial methylmalonyl-CoA mutase activity, thereby increasing the amount of propionic acid in rumen contents [45].

The amount of butyric acid was higher copared to control in all variants with phytosubstances, in sample III by 3.0 times, in sample IV by 8.5 times, in sample V by 8.0 times, in sample VI by 9.0 times ($p \leq 0.05$). Propionic acid is the only gluconeogenic VFA produced in the rumen, which is absorbed and metabolized to succinate through a series of reactions [46, 47]. However, cobalt chloride at a dosage of 1.5 mg/kg DM had no effect on the production of propionate and contributed to a decrease in the amount of butyrate and an increase in methane formation by 51.2% ($p \leq 0.05$). Vitamin B_{12} serves as a growth factor for ruminal bacteria such as *Prevotella ruminicola* and *Methanomicrobium mobile*. *Prevotella ruminicola* increases the content of propionic acid in the rumen, which contributes to an increase in the methane concentration.

Combinations of phytosubstances and cobalt chloride had a positive effect on in vitro fermentation processes in RF. The best effect ocurred when using *A. absinthil herba* and cobalt chloride, that is, the digestibility of DM increased, while methane production decreased by 33.9%, the activity of digestive enzymes increased, as well as the concentration of VFAs in the rumen fluid (see Tables 3, 4).

Controlling greenhouse gas emissions is critical to industrial beef production. Thus, polyphenols of phytobiotics can significantly influence the microbiome of the digestive system of ruminants, including reduced abundance of methanogenic archaea [48]. Due to their natural origin and safety, essential oils are increasingly used to modify the microbiome, especially to reduce methane production in ruminants [34].

The methane concentration in our tests increased vs. control by 51.2% with cobalt chloride, and by 16.5% ($p \leq 0.05$) with *Salviae folia*. Phytosubstances + cobalt chloride contributed to a reduction in methane synthesis in samples V and VI by 46.3 and 33.9%, respectively ($p \leq 0.05$) (see Table 4).

4. Methane emission after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*) (n = 16, M±SEM, Center for shared equipment use SAT RAS, 2021)

Treatment	Methane concentration	
	CH_4, g/m^3	CO_2 equivalent/g
I (control)	12.1±2.2	302.5±1.1
II ($CoCl_2$, 1.5 mg/kg DM)	18.3±2.3*	457.5±3.1*
III (*Salviae folia*, 1.6 g/kg DM)	14.1±1.9*	352.5±2.4
IV (*Artemisiae absinthil herba* 2.0 g/kg DM)	9.4±2.4	235.0±1.9*
V (*Salviae folia* 1.6 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	6.5±1.2*	162.5±2.3
VI (*Artemisiae absinthil herba* 2.0 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	8.0±1.4*	200.0±2.5

* Differences from control are statistically significant at $p \leq 0.05$.

Some papers have reported that phytochemicals are effective in reducing ruminal acetate and ammonia concentrations and methane production in small ruminants and beef cattle [49-51]. In our experiments, the amount of methane decreased only with *A. absinthil herba* by 22.3%, and with *S. folia*, on the contrary, it increased by 16.5% ($p \leq 0.05$).

A likely mechanism for reducing methanogenesis may be inhibition of the enzymes responsible for this process. Methyl-CoM reductase is known to play a significant role in methanogenesis [51]. Finding target molecules to inhibit this enzyme in ruminants is an important task. There is evidence that phytochemicals have better affinity for hydrogen bonds and can be used to reduce methanogenesis [52].

It is possible that a decrease in methanogenesis also results from changes in the populations of methanogenic bacteria in the rumen fluid. It was previously found that *A. capillaris* extract reduced methane emissions by 14% ($p < 0.05$) after 48 h of incubation with a reduce in the abundance of methanogen communities (ciliates and methanobacteria populations) [53]. A similar effect was observed under the influence of a mixture of essential oils and cobalt carbonate [16]. In addition, some sources and dosages of cobalt had toxic effects on the abundance of methanogenic bacteria [17].

The amount of CH_4 and CO_2 equivalent were maximally reduced when using the *Salviae folia* and $CoCl_2$ composition ($p \leq 0.05$). The molar fractions of acetate, propionate and butyrate in this variant increased significantly compared to the control, while the intensity of nitrogen metabolism in RF was high. It was found that the introduction of cobalt into a micro-diet without herbal substances did not reduce methane formation and worsened the DM digestibility of the diet. It has previously been noted that the total content and individual quantity of some short-chain fatty acids, the acetate:propionate ratio, pH and overall gas production in the rumen of ruminants are significantly dependent on certain plant substrates (wormwood, chamomile, fumitory and mallow) [54], which was also observed in our experiments.

Thus, when using herbal preparations and cobalt chloride (individually and in

combination) as part of biosubstrates, changes in metabolic parameters in the rumen fluid of bulls in vitro ("artificial rumen") were not uniform. Phytosubstances significantly increased the activity of amylase, proteases, the concentration of volatile fatty acids in the rumen fluid and activated nitrogen metabolism, but also increased the methane emission. The combination of *Artemisiae absinthil herba* and *Salviae folia* with $CoCl_2$ reduced methane production and enhanced metabolization. For the complex of *Artemisiae absinthil herba* (2.0 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM), the activity of digestive enzymes and the content of metabolites in the rumen fluid in vitro increased and methane emission decreased by 33.9%, while DM digesability was 2,1% higher compared to control. The complex of *Salviae folia* (1.6 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM) maximally reduced methane production (by 46.3%). Herbal preparations contributed to an increase in amylase activity in the rumen fluid by 2.6-4.0 times and protease activity by 3.6 times when compared to the control. However, we did not identify patterns of dose-dependent changes in metabolic parameters in the rumen fluid when combining herbal preparations and cobalt chloride, so we plan to continue studying the effect of herbal substances (individually or in combination with other substances) on the ruminant rumen microbiome.

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