Amino acid status and nitrogen forms of rumen contents *in vitro* when phytogenic components are added to the reaction medium

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Abstract. Phytobiotics are increasingly used in animal husbandry as an alternative to antibiotic drugs. However, before large-scale introduction of such substances into feeding practice, it is necessary to conduct a detailed analysis of their effects on physiological processes, particularly in polygastric animals where most energy is synthesized by the rumen microflora. This study aimed to investigate the indicators of nitrogen metabolism and amino acid composition of rumen fluid when quercetin, 7-hydroxycoumarin, vanillin, and trans-cinnamaldehyde were introduced into the in vitro reaction medium. The results showed that vanillin at a concentration of 49.00×10^{-5} mol/l promoted the maximum increase in the level of aliphatic, aromatic, and oxymonocarboxylic amino acids, while quercetin and 7-hydroxycoumarin at 98.0×10^{-5} and 24.50×10^{-5} mol/l, respectively, best stimulated the accumulation of methionine. Additionally, vanillin at a dose of 24.50×10^{-5} mol/l had a beneficial effect on the level of positively charged amino acids. The lowest values were found in the experiment with trans-cinnamaldehyde. These findings suggest that the presented phytobiotics, upon further consideration, can be used to regulate and manage the amino acid status of the rumen of ruminants.

1 Introduction

The ban on the prophylactic use of feed antibiotics in animal husbandry, as a factor accompanying the development and spread of multiple resistance in pathogenic strains of microorganisms, led to the fact that in research circles at the turn of the millennium, colossal work was carried out to find a suitable functional alternative. Among these, metal nanoparticles [1], antimicrobial peptides [2] and substances of plant origin – phytobiotics, or phytogenics [3] were proposed. The latter are especially interesting in the sense that they represent a virtually inexhaustible source for the isolation of biologically active components with a wide action profile and various physiological and metabolic effects.

Thus, in particular, it was previously shown that the phenolic aldehyde vanillin (the main component of the extract of *Vanilla planifolia* orchid pods) has a membrane-active potential, dissipating the gradient of potassium ions and disrupting pH homeostasis in

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Lactiplantibacillus plantarum, and also inhibits respiration in *Escherichia coli* and *Listeria innocua* [4]. It also causes mitochondrial dysfunction and oxidative stress in fungal pathogens such as Cryptococcus neoformans [5] *Alternaria alternata* [6], representatives of the genera *Aspergillus, Penicillium and Fusarium*. Another compound, already from a number of flavonoid derivatives, is quercetin, widely represented in the plant community as a component of pigments of flowers, vegetables and fruits [7], inhibits the growth and development of *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris* and *Escherichia coli*, while *Shigella flexneri* and *Lactobacillus casei* are absolutely indifferent to it [8]. O-hydroxycinnamic acid lactone, a heterocyclic compound from the class of benzopyrenes obtained from tonka beans - the seeds of Dipteryx odorata, umbelliferone (7-hydroxycoumarin) also has similar properties. At a dose of 500 μg/ml, it bacteriostatically inhibits the growth of *Escherichia coli, Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Fusarium culmorum* [9]. Finally, trans-cinnamaldehyde (the main component of the essential oil of *Cinnamomum sp.*, a monoterpene hydrocarbon) is effective against *Agrobacterium tumefaciens* [10] and uropathogenic *Escherichia coli* [11].

However, despite such a pronounced selective antimicrobial effect, before introducing such phytogenics into daily feeding practice, it is also necessary to evaluate how they affect various aspects of the digestive process, especially in ruminants, where most of the energy is synthesized through the breakdown of dietary nutrients rumen microbial ecosystem. In this regard, the amino acid status of rumen fluid is also noteworthy, as one of the most important indicators of cattle health, since the balance between different groups of amino acids (aliphatic, aromatic, sulfur-containing, positively charged and oxymonoaminocarbonic) easily changes and is coherent with such factors as diet composition, character bacterial environment, pH and enzymatic status [12]. In this case, the absolute content of amino acids is often less important than their ratio among themselves, due to the physiological irreplaceability of individual representatives of the latter. Moreover, it is also important to consider the balance between protein and non-protein nitrogen.

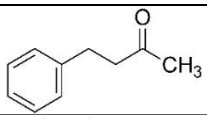
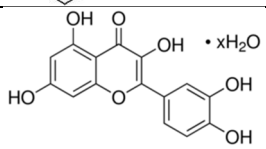
In this connection, the purpose of the presented work was to study the indicators of nitrogen metabolism and amino acid composition of ruminal fluid when such phytobiotic substances as quercetin, 7-hydroxycoumarin, vanillin and trans-cinnamaldehyde are introduced into the reaction medium *in vitro*.

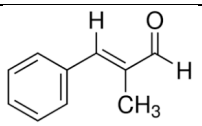
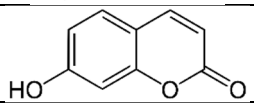
2 Materials and methods

2.1 Phytogenics, or small molecules of plant origin

The phytobiotics used in the work were represented by their chemically synthesized analogues produced by Acros Organics (Table 1).

Table 1. Tested phytobiotic agents (phytogenics, small molecules of plant origin).

Name: Trivial (CAS)	Gross formula	Structural formula	Molar mass
vanillin (121-33-5)	$C_8H_8O_3$		152.1
quercetin dihydrate (6151-25-3)	$C_{15}H_{10}O_7 \times 2H_2O$		338.3

trans-cinnamaldehyde (104-55-2)	C_9H_8O		132.2
7-hydroxycoumarin (93-35-6)	$C_9H_6O_3$		162.1

2.2 *In vitro* installation simulating rumen digestion.

Native rumen fluid for analysis was taken from fistulated Kazakh white-headed bull calves with an average weight of 266±1.53 kg and 11-12 months of age, whose diet included 30% concentrates and 70% roughage. Transportation was carried out within 30 minutes, maintaining a temperature of 38.5-39.5 ˚C. Before use, the rumen fluid was thoroughly shaken and filtered through 4 layers of gauze, mixing with a previously prepared buffer solution. Next, the resulting imitation was placed in the containers of an ANKOM Daisy II incubator (USA) with a basic digestible substrate (wheat bran) and the additive under study, setting the program: 48 hours at 39.5 °C. After the time had elapsed, experimental samples were taken. Small molecule dosages were selected based on previously conducted trials [13, 14].

2.3 Analysis of the amino acid composition of rumen fluid, nitrogen form

The analysis was performed in accordance with GOST 55569-2013 on a capillary electrophoresis system Kapel-105M (Lumex, Russia). A total of five experiments (Table 2). The content of 5 groups of amino acids in rumen fluid was studied: oxymonoaminocarboxylic, aliphatic, sulfur-containing, aromatic and positively charged. The control sample was tested without additives. Nitrogen forms were determined according to GOST 26180-84, GOST 13496.4-2019.

Table 2. Scheme of experimental work.

Groups of amino acids studied in the work (AMA)	Experiment, #				
	#1 (vanilline VN)	#2 (quercetin QR)	#3 (trans-cinnamal dehyde, CA)	#4 (7-hydroxycoumarin CM)	# 5 (mixture, VN+QR+CA+CM)
Aliphatic AMA	Glycine, Alanine, Valine*, Leucine* + Isoleucine*, Proline				
Aromatic AMA	Phenylalanine*, Tyrosine**				
Sulfur-containing AMA	Methionine*				
Positively charged AMA	Lysine*, Arginine, Histidine				
Oxymonoamino-carbonic AMF	Threonine*, Serine				

* - irreplaceable AMA
** - this AMA is indispensable for phenylalanine deficiency

During the research, all necessary measures were taken to minimize animal suffering and reduce the number of samples used.

Statistical processing of the research results was carried out using Microsoft Excel (Microsoft Corporation, USA) and Statistica 10.0 RU.

3 Results

In the first experiment, the effect of the following concentrations of vanillin on the amino acid status of the rumen contents of cattle was studied: 12.25 × 10^{-5} mol/l (minimum), 24.50 × 10-^{5} mol/l (average), 49.00 10^{-5} mol/l (maximum). The total change in amino acid content as a percentage of control by group is presented in Table 3.

Table 3. Dynamics of the amino acid composition of rumen fluid with the introduction of vanillin in comparison with the control.

Amino acid groups	Vanillin concentration, mol/l		
	12.25×10^{-5}	**24.50×10^{-5}**	**49.00×10^{-5}**
Aliphatic AMA, %	↓-8,0	↑+173,4	↑+203,0
Aromatic AMA, %	↑+50,0	↑+125,0	↑+150,0
Sulfur-containing AMA, %	↑+50,0	↑+50,0	↑+50,0
Positively charged AMA, %	↑+7,5	↑+145,0	↑+139,2
Oxymonoaminocarbonaceous AMA, %	↓-25,0	↑+28,6	↑+26,8

The total amount of aliphatic amino acids increased in proportion to the proportion of vanillin in the reaction medium, exceeding the control values by 203.00% at the maximum dosage. At the same time, the level of leucine and isoleucine increased by 50.00%, valine – by 42.86%, proline – by 40.00%, alanine – by 27.00% and glycine – by 42.86%. The total content of aromatic phenylalanine and tyrosine changed similarly (by 100.00 and 50.00%, respectively, at 49.00 10^{-5} mol/l vanillin). The amount of sulfur-containing methionine at all doses exceeded the control by 50.00%. The total level of positively charged amino acids reached its maximum with an average dosage of vanillin of 24.50 × 10^{-5} mol/l, while the proportion of arginine increased by 20.00%, lysine by 25.00%, histidine by 100.00%. The hydroxymonoaminocarbonic compounds threonine and serine showed a minimal upward trend. It is important that at a low concentration of vanillin, a leveling of the level of essential lysine by 12.50% was observed, which was not detected in relation to other AMAs, as well as at higher doses. In the second experiment, the effect of the following concentrations of quercetin on the amino acid status of the rumen contents of cattle was studied: 24.5×10^{-5} mol/l (minimum), 49.00×10^{-5} mol/l (average), 98.00×10^{-5} 5 mol/l (maximum). The total change in amino acid content as a percentage of control by group is presented in Table 4.

Table 4. Dynamics of the amino acid composition of rumen fluid with the introduction of quercetin in comparison with the control.

Amino acid groups	Vanillin concentration, mol/l		
	24,5×10^{-5}	**49,0×10^{-5}**	**98,0×10^{-5}**
Aliphatic AMA, %	↑+71,5	↓-33,7	↓-1,3
Aromatic AMA, %	↑+0,0	↓-10,0	↓-11,1
Sulfur-containing AMA, %	↑+0,0	↑+100,0	↑+133,3
Positively charged AMA, %	↑+18,3	↓-28,3	↓-3,3
Oxymonoaminocarbonaceous AMA, %	↑+8,3	↓-25,8	↓-25,8

In contrast to vanillin, in the presented experiment, an inverse relationship was observed with respect to aliphatic amino acids: the maximum values were recorded with a minimum dose of quercetin. At the same time, the content of essential leucine + isoleucine increased by 18.52%, valine - by 7.14%, proline - by 37.50%, glycine - by 8.33%, the level of alanine did not change, as did the amount of tyrosine and phenylalanine. The latter in all other cases decreased. Similar dynamics were observed in the series with positively charged and oxymonoaminocarboxylic AMAs: the proportion of lysine and arginine with minimal addition of quercetin increased by 10.00 and 8.33%, respectively, while the level of histidine did not change, the amount of threonine also increased by 8.33%. At higher dosages, a significant decrease in the proportion of noted AMAs was recorded. On the contrary, the concentration of methionine exceeded the control values by 133.33% at 98.0 × 10^{-5} mol/L quercetin.

In the third experiment, the effect of the following concentrations of cinnamaldehyde on the amino acid status of the rumen contents of cattle was studied: 1.52×10^{-5} mol/l (minimum), 3.05×10^{-5} mol/l (average), 6.10×10^{-5} mol/l (maximum). The total change in amino acid content as a percentage of control by group is presented in Table 5.

Table 5. Dynamics of the amino acid composition of rumen fluid with the introduction of trans-cinnamaldehyde in comparison with the control.

Amino acid groups	**Aromatic AMA, mol/l**		
	1,52×10^{-5}	**3,05×10^{-5}**	**6,10×10^{-5}**
Aliphatic AMA, %	↓-4,5	↑+11,5	↑+36,9
Aromatic AMA, %	↑+3,3	↑+16,7	↑+16,7
Sulfur-containing AMA, %	↑+40,0	↑+20,0	↑+20,0
Positively charged AMA, %	↓-41,7	↓-43,1	↑+23,6
Oxymonoaminocarbonaceous AMA, %	↓-29,2	↓-12,5	↑+0,0

The total amount of aliphatic amino acids increased most strongly when cinnamaldehyde was added to the reactor at the maximum concentration: by 36.92%. At the same time, the level of essential valine and leucine + isoleucine increased by 37.50% and 10.53%, respectively, the level of non-essential glycine decreased by 11.11%.

Aromatic AMA and methionine consistently exceeded the control at the average and maximum dose by 16.67% and 20.00%, however, if in the first case these were peak values, then in the second they shifted to the minimum volume of cinnamaldehyde in the reaction medium. On the contrary, the first two dosages led to a decrease in the level of positively charged and oxymonoaminocarboxylic amino acids, while in the third concentration the amount of arginine increased by 11.11%, and lysine by 12.50%, the proportion of threonine did not change.

In the fourth experiment, the effect of the following concentrations of 7-hydroxycoumarin on the amino acid status of the rumen contents of cattle was studied: 12.25×10^{-5} mol/l (minimum), 24.50×10^{-5} mol/l (average), 49.00× 10^{-5} mol/l (maximum). The total change in amino acid content as a percentage of control by group is presented in Table 6.

Table 6. Dynamics of the amino acid composition of rumen fluid with 7-hydroxycoumarin in comparison with control.

Amino acid groups	Oxymonoaminocarbonaceous AMA, mol/l		
	$12{,}25\times10^{-5}$	$24{,}50\times10^{-5}$	$49{,}00\times10^{-5}$
Aliphatic AMA, %	↑+92,7	↑+121,8	↑+83,6
Aromatic AMA, %	↑+53,3	↑+20,0	↑+36,7
Sulfur-containing AMA, %	↑+40,0	↑+140,0	↑+60,0
Positively charged AMA, %	↑+63,3	↑+5,0	↑+99,2
Oxymonoaminocarbonaceous AMA, %	↑+0,0	↓-1,8	↑+1,8

In the presented experiment, most of the AMAs showed a clear upward trend, with the exception of oxymonoaminocarbonic AMAs. Thus, the content of aliphatic AMA was maximum when 24.50×10^{-5} mol/l 7-hydroxycoumarin was added, which included an increase in the level of essential leucine+isoleucine and valine by 17.65% and 62.50%, respectively. The amount of proline exceeded the control by 33.33%, alanine – by 8.33%, the concentration of glycine did not change. The methionine content increased similarly.

The minimum proportion of 7-hydroxycoumarin in the medium provided the greatest increase in the level of aromatic AMA - by 53.33% (the amount of essential tyrosine and phenylalanine increased by 20.00 and 33.33%, respectively).

The amount of positively charged arginine, lysine and histidine was maximum in the medium with 49.00×10^{-5} mol/L 7-hydroxycoumarin: an increase of 20.00%, 12.50% and 66.67% relative to the control, respectively. However, at the minimum dose, the highest amount of lysine was noted (25.00% higher than control). It is also important that, despite the general decrease in oxymonoaminocarboxylic AMA, at an average dosage there was an increase in the content of essential threonine by 12.50%.

It should also be noted that the addition of small molecules of plant origin in all experiments led to a decrease in the content of non-protein nitrogen in the rumen fluid (Figure 1), while the protein concentration increased only in the experiment with vanillin (49.00×10^{-5} mol/l) and trans-cinnamaldehyde (1.52×10^{-5} mol/l) by 9.57 and 6.79%, respectively.

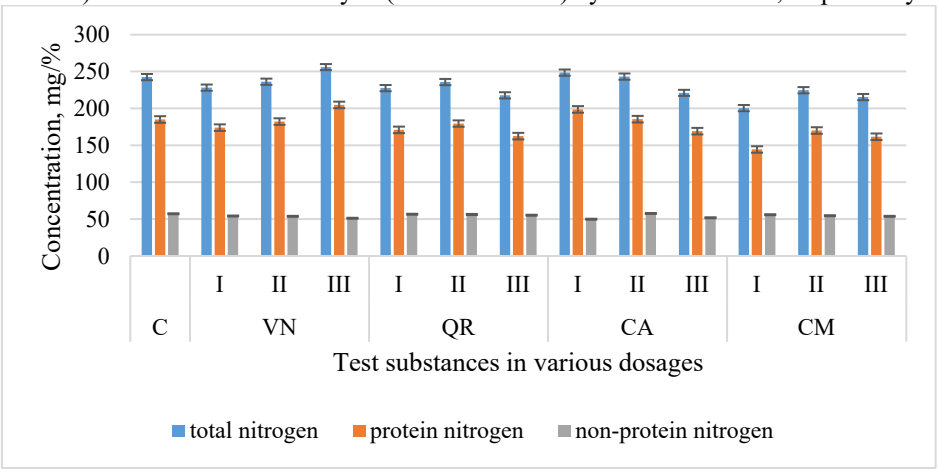


Fig. 1. Concentration of nitrogen forms in rumen fluid when adding test substances. *Note: I – minimum concentration, II – average concentration, III – maximum concentration; K – control, VN – vanillin, QR – quercetin, CA – trans-cinnamaldehyde, CM – 7-hydroxycoumarin.*

Thus, vanillin in a concentration of 49.00×10^{-5} mol/l promotes the maximum increase in the level of aliphatic, aromatic and oxymonocarboxylic AMAs (Figure 2), while quercetin and 7-hydroxycoumarin in an amount of 98.0×10^{-5} and 24.50×10^{-5} mol/l, accordingly, best stimulates the accumulation of sulfur-containing methionine.

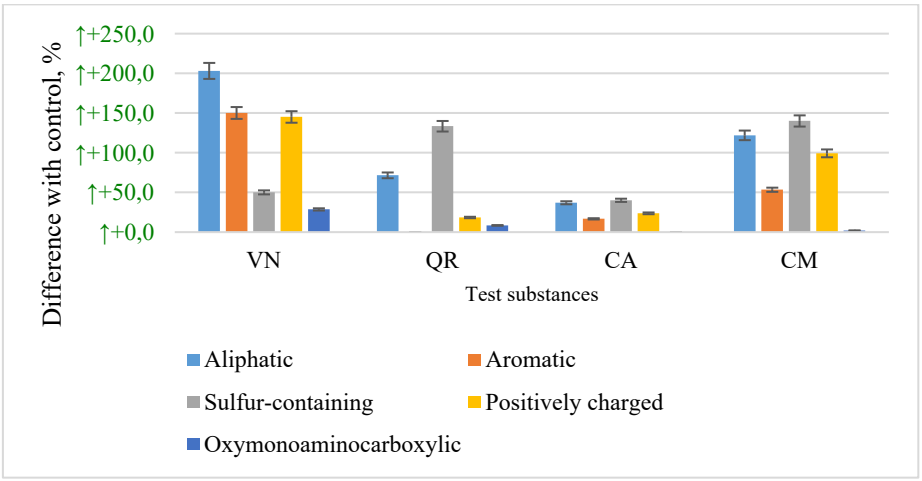


Fig. 2. Maximum increase in AMA level in rumen fluid

Vanillin at a dose of 24.50×10^{-5} mol/l has a beneficial effect on the level of positively charged AMA. The lowest values were found in the experiment with trans-cinnamaldehyde. In other words, the presented phytogenics, upon further consideration, may well be used to regulate and manage the amino acid status of the rumen of ruminants.

4 Discussion

Amino acids, as the building blocks of protein macromolecules, are one of the key nutrients necessary for the normal development of any organism. In the rumen fluid of ruminants, they are present, as a rule, in three forms - in free form, as part of peptides, and as a structural part of the body of prokaryotes and protozoa [15]. Of course, with an identical diet, health status and housing conditions, changes in the amino acid spectrum and distribution of nitrogen forms are determined by the functioning of the microbiota. And since the antibiotic effect of the presented phytobiotics has already been mentioned, it is possible to assume that they change the ratio of individual representatives of the bacteriome, and, as a consequence, the general direction of the flow of biochemical reactions. The latter is consistent with previous studies. For example, quercetin reduced the total population of protozoa and methanogens in rumen fluid *in vitro* without having any negative effect on microbial fermentation [16]. Similarly, vanillin, while suppressing protozoa, stimulated reproduction of *Ruminococcus flavefaciens, Prevotella bryantii, Butyrivibrio fibrisolvens, Clostridium aminophilum, Ruminobacter amylophilus* and *Prevotella ruminicola.* The latter, thanks to the production of dipeptidyl peptidase, is actively involved in the breakdown of feed peptides [17], which causes the accumulation of amino acids in the rumen fluid, shown in the first experiment. Vanillin inhibits ammonia production [18], which is expressed in a dose-dependent decrease in the proportion of the non-protein form of nitrogen.

The addition of cinnamaldehyde to the diet of lambs, on the contrary, promoted the activation of enzymes, which was reflected in an increase in the total level of volatile fatty

acids [19]. It is also known that various coumarin derivatives, including umbelliferone, are widely used to control a number of serine proteases [20]. In general, phytogenics as modulators of ruminal metabolism act in two main ways: either by changing the structure of the microbiome or by regulating enzymatic activity. The latter is due to their small size, interaction with a large number of bacterial cellular targets and, as a rule, lipophilic properties [21]. In particular, many phytochemicals have been shown to be able to increase the permeability of the cytoplasmic membrane, interfere with protein- or enzyme-dependent reactions such as electron transfer, maintenance of ion gradients, translocation, phosphorylation and ATP production, or even cause cytoplasmic coagulation and cell lysis. Aldehydes also directly interact with nucleic acids and proteins [22]. For example, cinnamaldehyde, due to the presence of a carbonyl group, is involved in membrane disintegration, enzyme inactivation and depletion of intracellular ATP reserves, while phenolic derivatives actively interact with protein transporters [22, 23]. Substances from the flavonoid class, in turn, are able to bind to the hydrophobic catalytic pockets of enzymes involved in bacterial DNA replication, such as the Rep A helicase from *Streptomyces sp.* [24]. 7-hydroxycoumarin, quercetin, trans-cinnamaldehyde and vanillin also inhibit quorum sensing systems, disrupting communication between microorganisms [25].

At the same time, phytogenics are characterized by pronounced antioxidant activity. These compounds can inhibit lipid peroxidation, chelate metals, and stimulate superoxide dismutase, catalase, peroxidase, and glutathione reductase. Moreover, previous *in vitro* and *in vivo* studies have shown that plant-derived small molecules have positive effects on protein metabolism, VFA production, fiber digestion, and methane and ammonia production in the rumen [26], prompting further analysis of their mechanisms of action.

5 Conclusion

Phytochemicals, due to their abundance and diversity, currently act as a very attractive alternative to antibiotic drugs. Moreover, they usually have associated effects aimed at maintaining immunity and regulating metabolic processes. The obtained data on the effect of phytogenics on the amino acid status and distribution of nitrogen forms in the rumen fluid of cattle in vitro can be further used for the targeted development of premixes with a selective type of action.

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