Study of feed protein supplement with the properties of phytobiotics

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Abstract. The Russian market is oversaturated with grain raw materials in the composition of animal feed, which are characterized by protein deficiency and poor digestibility of nutrients. One of the problems of modern animal husbandry is increasing productivity, and most importantly - reducing the cost of products due to the efficient use of feed nutrients. The indiscriminate use of feed antibiotics as growth stimulants is due to the high cost of imported phytobiotics and poses an additional threat to human and animal health. Phytocompounds affect the processes of metabolism and neutralization of foreign substances, which are carcinogens and mutagens. They have the ability to bind free radicals and reactive metabolites of foreign substances, inhibit xenobiotic activating enzymes and activate detoxication enzymes. Phytobiotics have antiviral, antimicrobial, as well as immunomodelling effects. When they are included in any diet, feed intake increases, the acid-base environment normalizes. Phytobiotics are safe both for farm animals and birds, and for people, easy to use and store, and can also ensure a return on investment. The amino acid composition and antioxidant activity of the protein feed additive obtained from the leaf-stem mass of red clover plants were studied. The qualitative and quantitative composition of amino acids and the value of antioxidant activity show the feasibility of introducing a feed protein supplement into the diet of farm animals and birds.

1. Introduction
The quality of compound feed, its usefulness is the main problem of modern livestock. Since the basis of compound feeds is wheat and corn, the industry periodically faces the problem of rising grain prices. Relevant is the study of feed additives from non-traditional sources of raw materials with high biological value [1, 2].

The use of biologically active substances in feed technology that contribute to improved feed conversion, natural stimulants for the growth of animals and birds, and the rejection of feed antibiotics in order to obtain environmentally friendly products is the basis for increasing the productivity of farm animals and poultry. In this regard, it is of great interest to use natural feed additives of plant origin - phytobiotics.

Irrational use of antibiotics in animal husbandry and poultry farming as growth stimulants is not safe both for animals and poultry, and for humans, causing antibiotic resistance of pathogenic bacteria. The industry’s problems with antibiotic-resistant bacteria and the need to treat diseases caused by pathogens that do not respond to antibiotics have put enormous pressure on livestock and poultry
farming. Many governments require farmers to refuse or limit the use of feed antibiotics and seek alternatives [3, 4].

An effective alternative to feed antibiotics is phytobiotics, which have a significant and lasting positive effect on productivity and health.

Many plants are of practical interest, for example, sea buckthorn (*Hippophae rhamnoides L*), purple coneflower (*Echinacea purpurea L*), red clover (*Trifolium pratense L*), seed alfalfa (*Medicago sativa L*), amaranth (*Amaranthus cruentus L*) and others. The main advantage of feeding herbal supplements is that the animals are not exposed to any risks; the possibility of their use as flavoring, appetite-stimulating substances is fully confirmed by the EU documents regulating nutrition [5, 6].

A protein feed supplement obtained from the leaf-stem mass of leguminous plants is characterized by a high content of vitamins, vegetable proteins balanced in amino acid composition, trace elements, biologically active substances, etc. In connection with this, a promising source of protein supplement is the vegetative mass of red clover. Insufficient study of red clover as an object of feed production hinders the creation of its integrated processing and development in the direction of import-substituting technologies.

2. The purpose of the study

The important problem is to obtain a biological product with a high protein content and phytobiotic properties. As a raw material for obtaining protein feed additives according to the developed technology [7, 8], the leaf-stem mass of red clover is used. A distinctive feature of this technology is that the processing of plants proceeds under "sparing" temperature conditions, which allows saving a significant part of biologically active substances and trace elements. The aim of the study was to study the amino acid composition and antioxidant activity of a red clover feed protein supplement.

3. The object of the study

The object of the study was a protein feed supplement obtained from the leaf-stem mass of plants of red clover or meadow clover (*Trifolium pratense L*) of the late stages of vegetation collected in Voronezh region.

4. Materials and methods

4.1 The study of the amino acid composition of the feed protein supplement by high performance liquid chromatography (HPLC)

Chromatographic analysis methods are highly sensitive and allow the separation of related compounds and their metabolites, as well as hydrolysis products. The HPLC method is an arbitral method for determining the amino acid content. Determination of amino acid composition was carried out on an automatic amino acid analyzer based on a SHIMADZU Prominence LC-20 liquid chromatograph in the Centre of collective usage “Control and management of energy efficient projects” of VSUET. For analysis, a 150 × 4.6 mm Sevko & Co column TBEC.414538.005-01 was used. The separation and determination of amino acids was carried out by ion exchange chromatography with post-column derivatization with ninhydrin. The method of ion exchange chromatography is based on the interaction of the charged functional groups of the stationary phase with ionized molecules of separable substances having the opposite charge [9].

Sample preparation included the parallel conduct of acid and alkaline hydrolysis of the protein of the analyzed sample. After acid hydrolysis, 17 amino acids were determined in the hydrolyzate, and tryptophan, which is destroyed by acid hydrolysis, was determined after alkaline hydrolysis.

Buffer solutions were supplied to the degasser, then, depending on the given program, they were mixed in a vortex chamber and then they were pumped through an ammonia trap to the autosampler unit, where sampling and injection took place automatically. The sample was mixed with buffer solutions and entered the ion-exchange column, in which the separation of amino acids occurs. Amino acids came out in accordance with their isoelectric points with a smooth change in pH of the mobile
phase. After separation, the amino acids entered the reactor, where they interacted with ninhydrin to form colored derivatives, which were detected by a spectrophotometric detector at $\lambda = 570$ nm and 440 nm.

4.2 Determination of antioxidant activity
The basis of this technique is the amperometric method for determining the content of antioxidants (CA), which consists in measuring the electric current generated during the oxidation of the test substance on the surface of the working electrode at a certain potential and comparing the received signal with a standard signal (quercetin), measured in the same criteria. The amperometric method is the only one directly measuring the content of all antioxidants in a sample [10]. The measurement was performed on an antioxidant activity analyzer “Tsvet Yauza - 01 – AA”. The working electrode is made of glassy carbon, which is the most multipurpose in determining polyphenolic compounds.

Calculation of SA, mg / g of the test sample was carried out according to the calibration graph of quercetin and formula 1. When calculating the result for a liquid sample, dilution of the sample (N) was taken into account. The calculation was carried out according to the formula:

$$CA = \frac{CA_{gr} \cdot V_s \cdot N}{m_s \cdot 1000},$$

where $CA_{gr}$ is the content of antioxidants found from the calibration graph, mg/dm$^3$; $V_s$ – the volume of the solution (extract) of the analyzed sample, cm$^3$; $m_s$ – sample of the analyte, g; N – is the dilution of the analyte.

The content of natural flavonoids, in particular, catechins; quercetin, rutin, dehydroquercetin; as well as vitamins and other compounds that can bind free radicals determine the value of CA samples.

5. Discussion of the results
Figure 1 and table 1 shows the amino acid composition of the feed protein supplement of red clover.

![Chromatogram](image-url)

**Figure 1.** Chromatogram
Table 1. Amino acid content in the sample

| Amino acid name | Mass fraction in dry matter, % |
|-----------------|-------------------------------|
| ASP+ASN         | 3.71                          |
| THR             | 1.66                          |
| SER             | 1.43                          |
| GLU+GLN         | 2.99                          |
| PRO             | 2.07                          |
| GLY             | 2.08                          |
| ALA             | 1.22                          |
| CYS             | 1.98                          |
| VAL             | 1.11                          |
| MET             | 1.50                          |
| ILEU            | 1.14                          |
| LEU             | 2.11                          |
| TYR             | 1.45                          |
| PHE             | 0.86                          |
| HYS             | 1.43                          |
| LYS             | 1.38                          |
| ARG             | 4.49                          |
| TRP             | 2.86                          |

According to the research, 18 amino acids are identified, 8 essential acids are present in the product. The qualitative and quantitative composition of amino acids shows the feasibility of introducing a feed protein supplement into the diet of animals and birds.

One of the methods for determining the biological value of proteins is the determination of the Essential Amino Acid Index (EAAI). The method is a modernization of the chemical score method and allows taking into account the amount of all essential acids.

\[ EAAI = \left( \frac{\text{LYS}_p \times \text{ARG}_p \times \text{TRP}_p \times \ldots}{\text{LYS}_r \times \text{ARG}_r \times \text{TRP}_r \times \ldots} \right)^{1/n}, \]

where: \( n \) is the number of amino acids; indices \( p, r \) are the amino acid content in the studied protein and in the reference, respectively.

EAAI feed protein supplement is 0.7. It is established that the index of essential amino acids of the product tends to unity; the amino acid composition is close to the standard.

Determination of antioxidant activity. The aim of the study was to compare the total antioxidant activity in the feed protein supplement from the vegetative mass of red clover, vitamin grass flour (GOST R 56383-2015), the “Extrafit” phytobiotic (TU No. 9296-001-99904284-2012 based on amaranth plant materials) and dry sea buckthorn. The results of the calculations for quercetin are shown in table 2.

As it can be seen from table 2, the feed protein supplement from the leaf-stem mass of red clover is not inferior in terms of antioxidant activity to products used as phytobiotics. They have antiviral, antimicrobial, as well as immunomodulatory effects. When they are included in any diet, feed intake increases, the acid-base environment normalizes.
Table 2. Results of calculating total antioxidant activity (AOA) for quercetin.

| Product                      | AOA, mg/g |
|------------------------------|-----------|
| Feed protein supplement      | 1.85      |
| Vitamin grass flour          | 1.28      |
| “Extrafit”                   | 1.96      |
| Dry sea buckthorn            | 1.44      |

6. Conclusion
The amino acid composition and antioxidant activity of the protein feed additive obtained from the leaf-stem mass of red clover plants were studied. The qualitative and quantitative composition of amino acids and the value of antioxidant activity show the feasibility of introducing a feed protein supplement into the diet of farm animals and birds.

The main advantage of feeding additives of plant origin is that farm animals and birds are not exposed to any risks, as well as the possibility of its use as a taste and stimulating appetite [11, 12]. It is assumed that the use of additives from the vegetative mass of red clover will stimulate growth, reproduction, weight gain and will have an antibacterial and immune stimulating effect.

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