Impact of developed ruminant feed products on the surrounding ecosystem

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Abstract. The use of feed additives based on the so-called "phytobiotics" and a complex of trace elements is a promising direction in the nutrition of farm animals and birds. However, along with the positive characteristics associated with the increase in body weight, environmental friendliness of production, etc., these biologically active additives may have a negative effect on animals and humans associated with the toxicity of the used components. In this regard, the purpose of this study was to determine the toxic effect of such feed additives as phytochemicals and trace element metals using a recombinant luminescent strain *Escherichia coli* K12 MG1655 (pXen7) and a representative of ciliated protozoa *Stylonychia mytilus*. During the studies, the absolute toxicity of the Cu biocomplex and *Scutellaria baicalensis* was recorded in relation to the test organisms used. Against this background, *Artemisiae absinthil herba* and the Digestarom agent showed the smallest toxic effect. The toxic effect of other tested bio-additives was in high doses (0.625-10 mg/mL) and disappeared with a decrease in the concentration of the tested feed additive.

1 Introduction

In recent years there is a growing interest in the problem of the transition to a highly productive and environmentally friendly agricultural farming. Currently, research scientists working in the field of animal husbandry and feed production are actively developing and introducing new feeding schemes for farm animals, which make it possible to obtain large volumes of meat products [1]. Special attention is focused on the creation of feed additives, which are characterized by high indicators of the body weight gain, environmental friendliness, as well as minimum production costs [2]. However, along with the positive characteristics of the use of such bio-additives, there may be negative effects associated with the toxic effect of the used components on animals and humans [3]. Thus, there are data on high hepatotoxicity of plant extracts *Frangula alnus* [4], *Mentha pulegium* [5], *Chelidonium majus* associated with liver damage [6]; cardiovascular toxicity caused by *Aconitum carmichaeli* [7], *Curcuma longa* [8]; with respect to the digestive system – by *Mentha pulegium* [9]; neurotoxicity – by *W. somnifera* [10]. In turn, trace element metals used in animal husbandry and poultry farming also have a number of limitations for use, since these compounds have a dose-dependent effect [11]. All of the above paves the way for a more in-depth study of such bio-additives when used in the feeding schemes for farm animals and birds.

In this regard, the purpose of this study was to determine the toxic effect of feed additives – phytochemicals and trace element metals using recombinant luminescent strain *Escherichia coli* K12 MG1655 (pXen7) and a representative of ciliated protozoa *Stylonychia mytilus*.

2 Materials and Methods

2.1 Feed additives

The objects of study included the so-called "phytobiotics", namely *Artemisiae absinthil herba* (Phytopharm), *Salviae folia* (Krasnogorskleksredstva JSC), *Oríganum vulgare* (Production Company KIMA Company LLC), *Scutellaria baicalensis* (Belovodye LLC), as well as *Inulae rhizomata et radices* (Krasnogorskleksredstva JSC): a microelement biocomplex represented by manganese (Mn), copper (Cu) and cobalt (Co); and Digestarom, which is a food additive improving the taste of feed and increasing the appetite of farm animals, including birds (Biomin LLC).

The samples were ground to a powdered state and dissolved in sterile distilled water for further biological analyses.

2.2 Bacterial strains and protista

The toxicity of the test feed additives was evaluated using two test objects. The first was represented by a recombinant luminescent strain *Escherichia coli* K12 MG1655 (pXen7) with a constitutive glow type. This biosensor was obtained by transforming the cells of the host strain with a hybrid plasmid *pUC18* with an embedded EcoRI DNA fragment of about 7 thousand bp

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containing structural bioluminescence genes of the soil microorganism *Photorhabdus luminescens* ZM1 [12].

Another test organism for determining the toxic effect of the selected feed additives was the representative of the ciliated protozoa *Stylonychia mytilus*, which is a standard test object for determining the general toxicity of feed [13].

2.3 Toxicity study using *E. coli* K12 MG1655 (pXen7)

The *E. coli* K12 MG1655 (pXen7) biosensor was cultivated for a day on LB agar (Sigma, USA) at 37 °C, and the selective marker – ampicillin (100 μg/mL) – was added to the medium. The cells were then suspended in NaCl solution (0.9%) to an absorbance of 0.5 relative units at 450 nm, these measurements were made in plastic transparent wells using an Infinite 200 microplate photometer (Tecan, Austria). Thereafter, a 500 μL bacterial suspension was added to 1000 μL of LB broth. Then, two-fold dilutions of the test bio-additives were prepared at the final concentration of 0.001 mg/mL to 10 mg/mL. After that, 100 μL of diluted substances were added to the test wells, and 100 μL of water was added to the control wells, then 100 μL of the finished bacterial suspension was added to all wells. The plate was placed in the Infinite 200 Pro luminometer measuring unit (Tecan, Austria). The measurement was performed in a kinetic mode for 180 minutes at 37°C. The obtained results were primarily processed using the Magellan™ plate luminometer software, and further processing of the obtained data was carried out using the Excel 2010 computer program (Microsoft Inc.). The normalized luminescence was calculated using Formula 1:

$$I_{norm} = \frac{I_{\pi}^{test} \cdot I_0^{control}}{I_0^{test} \cdot I_{\pi}^{control}},$$

where I_{norm} – normalized luminescence at n-minute;

I_n^{test} – luminescence index of the test sample at n minute of the experiment;

I_0^{test} – luminescence index of the test sample at 0 minute of the experiment;

$I_n^{control}$ – luminescence index of the control sample at n minute of the experiment;

$I_0^{control}$ – luminescence index of the control sample at 0 minute of the experiment.

The graphs reflecting the dependence of normalized luminescence (relative units) on the concentration of test substances (mg/mL) were plotted on the basis of the obtained data.

2.4 Toxicity study using *S. mytilus*

The toxic effect of the test feed additives was also studied on the cell culture of the freshwater infusoria *S. mytilus*. It was prepared as follows. A day before the analysis, the mass of stylonychia was transplanted into a new Lozin-Lozinsky nutrient solution with the addition of yeast (*Saccharomyces cerevisiae*) as feed and placed in a thermostat at 25°C. Thus, the protist cells were in the exponential growth phase at the time of the experiment. The studied test functions included: survival, abundance (biomass).

The study was conducted in a concentration range of 0.039 to 10 mg/ml. Each sample of the test sample was studied five times. Transplantation and counting of stylonychia were performed using a light microscope (MT 5300L). The analysis was performed as follows. An automatic pipette was used to take 20 μl of the medium with stylonychia, which were placed in each of the five microaquariums. Then, 20 μL of the aqueous extract of the test feed additive prepared for biotesting was added with an automatic pipette. After 2 minutes, the number of stylonychia was counted in each microaquarium. After counting, 200 μl of the aqueous extract of the test sample was poured into each microaquarium and the start time of the test was marked. The toxicity of the test feed additives was determined by the survival of stylonychia after 1 and 3 hours of exposure, manifested as the movement cessation and change in the shape of cells from ellipsoid to rounded or violation of the integrity (lysis) of cells.

The survival rate of N stylonychia (%) was calculated using Formula 2:

$$N = \frac{N_2}{N_1} \cdot 100,$$

where N_2 – arithmetic mean (out of five tests) of the number of stylonychia at the end of the test after 1 hour of exposure, pcs;

N_1 – arithmetic mean (out of five tests) of the number of stylonychia at the beginning of the test, pcs;

100 – coefficient of conversion of the result into percentages.

The toxicity of the test feed additives was determined based on the following:

- from 70% to 100% survival of stylonychia – non-toxic sample;
- from 40% to 69% survival of stylonychia – slightly toxic sample;
- from 0% to 39% survival of stylonychia – toxic sample.

2.5 Statistical processing of study results

All experiments were performed in at least five replicates. The obtained results were processed by the methods of variation statistics in Excel for Windows 10.

3 Results

3.1 Evaluation of the toxic effect of test feed additives on recombinant luminescent strain *E. coli* K12 MG1655 (pXen7)

The toxicity assessment using a constitutively luminescent biosensor showed different activity of the test feed additives. Among all studied compounds, the *Inulae rhizomata et radices*, Digestarom, *Artemisiae absinthil herba*, *Salviae folia* and *Oríganum vulgare* were not toxic, as evidenced by a slight level of luminescence relative to the control.

The biological complex of trace elements, as well as the *Scutellaria baicalensis* showed some toxic effect.

The Cu biocomplex completely inhibited the glow of a microorganism at maximum concentrations ranging from 1.25 to 10 mg/mL, with an increase in dilution from 0.625 to 0.313 mg/mL, the percentage of survived cells increased from 64% to 76% relative to the control, respectively. Further reduction in concentration did not cause a toxic effect on the test strain.

The Co biocomplex at the same concentrations as Cu had the toxic effects observed at concentrations of 5-10 mg/mL causing 100% quenching of bacterial luminescence. Lower concentrations of 1.25 and 2.5 mg/mL inhibited the biosensor glow by 64% and 86%, respectively, relative to the control, and with a decrease in concentration the effect was offset.

Table 1. EC_{20} and EC_{50} of the analyzed bio-additives for recombinant luminescent strain *E. coli* K12 MG1655 (pXen7), mg/ml.

Indicator	Co	Cu	Mn	Scutellaria baicalensis
EC_{50}	1.094	0.781	–	0.059
EC_{20}	0.729	0.273	7.5	0.004

*Note: The threshold concentration (EC_{20}) is defined as the concentration resulting in the smallest significant decrease in luminescence, i.e. suppression by 20%, and EC_{50} – suppression by 50%.

In the group of studied trace elements the Mn biocomplex showed the least toxic effect. Its maximum concentration had a minor toxic effect. The percentage of survived cells reached 80% (EC_{20}).

The evaluation of the concentration dependence of *Scutellaria baicalensis* showed its toxicity in a wide range of concentrations (0.005-10 mg/mL), and with a dose-dependent effect. Maximum concentrations were characterized by the greatest decline in bioluminescence, which was 88% (5 mg/mL) and 92% (10 mg/mL). With a subsequent decrease in concentrations (from 2.5 to 0.078 mg/mL), the toxic effect was reduced to 60%. At lower concentrations of 0.039-0.005 mg/mL, toxicity decreased to 25% and disappeared at subsequent dilutions (Table 1).

3.2 Biological effect of the test feed additives on the *S. mytilus* cell population

The analysis of the obtained data showed that the greatest toxic effect, as in the case of the *E. coli* K12 MG1655 (pXen7) strain, was the Cu biocomplex and *Scutellaria baicalensis*, the toxic effect of which was manifested after 1 hour of exposure in the entire range of studied concentrations of these substances, characterized by cell lysis.

In turn, the Mn biocomplex and *Salviae folia* showed a toxic effect in the concentration range of 1.25-10 mg/ml, and in this case, the cells changed shape from ellipsoid to rounded, and when exposed to *Salviae folia* – cell lysis occurred.

Significant toxicity was observed in the *Inulae rhizomata et radices*, which appeared in the concentration range of 2.5-10 mg/mL and was accompanied by lysis of all cells of the test object.

Against this background the *Artemisiae absinthil herba* and the Digestarom had a minor toxic effect, which manifested itself only in the concentration of 10 mg/ml for the *Artemisiae absinthil herba* and 5-10 mg/ml for the Digestarom agent, and in the concentration of 5 mg/ml the survival of test organisms corresponded to 50%.

The *Oríganum vulgare* and the Co biocomplex were characterized by a high level of survival of stylonychia manifested in the concentration range of 0.039-2.5 mg/mL (Table 2).

4 Discussion

The study of phytochemical substances and metals of trace elements as feed additives allows referring to some features, as well as restrictions on their use in feeding farm animals and birds. Thus, the Cu biocomplex and *Scutellaria baicalensis* had absolute toxic activity against the biosensors used, which confirms the results of the studies carried out on other test organisms [3]. Moreover, if we analyze the causes of such action of these bio-additives in the present study, the toxicity of the Cu biocomplex can be explained by the participation of this trace element in biological processes in the cells of bacteria and protozoa (interaction with enzymes or allosteric effectors during metabolism, interaction with the luciferase complex responsible for luminescence, etc.) [15]. As for the *Scutellaria baicalensis*, this effect can be associated with its well-known antibacterial effect on many pathogenic bacteria, and in the case of stylonychiae, with the large-needle structure of the solid parts of the plant, which when swallowed cause the cell lysis. It is also necessary to take into account the effect of substances contained in extracts [16, 17].

Analyzing the effect of the remaining feed additives, it should be noted that their use should be strictly regulated in the feed of farm animals and birds, since exceeding the safe dose of these bio-additives may lead to negative consequences [3].

5 Conclusion

Thus, the results of the study indicate the need for a detailed study of the above feed additives in multicellular organisms, since the data obtained in single-celled biotests cannot guarantee the absolute safety of their use in animal husbandry and poultry farming.

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Table 2. Biological effect of the test feed additives on the *S. mytilus*.

Feed additive	Exposure time, h	Concentration, mg/ml								
		0.039	**0.078**	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
Oríganum vulgare	1	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox
	3	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox
Salviae folia	1	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox	Tox	Tox
	3	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox	Tox	Tox
Inulae rhizomata et radices	1	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox	Tox
	3	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox	Tox
Artemisiae absinthil herba	1	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
	3	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox
Scutellaria baicalensis	1	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
	3	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
Digestarom	1	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	LOEC	Tox
	3	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	LOEC	Tox
Cu	1	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
	3	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox
Co	1	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox
	3	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox
Mn	1	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox	Tox	Tox
	3	NOEC	NOEC	NOEC	NOEC	NOEC	Tox	Tox	Tox	Tox

Note: Tox – 0-39% survival rate of the test object; LOEC – 40-69% survival rate of the test object; NOEC – 70-100% survival rate of the test object [14].

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