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ASSESSMENT OF THE BIOSAFETY OF MICROORGANISMS AND THEIR JOINT COMPOSITION

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ABSTRACT

This publication presents the results of studying the biosafety of microorganisms used for the development of a biological product-a litter destructor, both individually and in a joint composition. In the experiment, the culture of Stylonychia (*Stylonychia mytilus*) and laboratory animals (rabbits, mice, and rats) were used. Based on a series of preclinical studies, it was found that the studied microorganisms individually do not indicate any symptoms of virulence, toxigenicity, toxicity and do not give an affirmative counteraction to the formation of hemolysin. The results of the express method showed that the aqueous and acetone extracts obtained from the joint microbial composition did not have a detrimental effect on the culture of protozoa, the viability in all groups was 100.0%. The analysis of the dermonecrotic effect of the studied microbial preparation demonstrated that the studied extracts did not cause hemorrhage, hyperemia, peeling, edema, and other pathology indicating an inflammatory process on the skin of laboratory rabbits. Conducting an acute experiment confirmed the safety of the studied microbial consortium, since during the research the laboratory mice remained active, mobile, no signs of poisoning or other pathology were found, and the conducted pathoanatomical autopsy did not reveal lesions of the parenchymal organs of the laboratory test objects. In general, the results of the studies indicated that the investigated cultures of microorganisms and their joint composition in the composition of the biological product-destructor do not show a toxic impact on the body of lab test objects, which confirms their harmlessness.

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Introduction

The transition of poultry farming to an industrial basis inevitably led to the concentration of poultry stock in limited areas, which caused difficulties in storing a sufficiently large number of droppings, accompanied by an increase in the

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anthropogenic load on the environment, which entails an increase in the cost of preventing negative consequences from contamination with poultry droppings [1-3].
Data from the All-Russian Research, Design and Engineering Institute of Organic Fertilizers and Peat (VNIPTIOU) indicate that more than 600 poultry farms operate on the territory of the Russian Federation. Each bird produces an average of 100 to 150 grams of litter per day, taking into account the type, age, and method of keeping. In total, every day in the country, according to preliminary estimates, more than 50 thousand people are produced. Tons of litter and this figure is growing inexorably. As with the increased incidence of African swine fever and outbreaks of foot-and-mouth disease in cattle, poultry farming remains the most prosperous branch of agriculture for the production of safe food. Accordingly, it can be concluded that an increase in the livestock will lead to the accumulation of even more droppings, and the use of imperfect technologies and technological means, non-compliance with established environmental requirements, is a real environmental threat in the absence of an alternative solution to this problem [4-7].
In this regard, the issue of reducing the environmental burden on the environment from bird droppings, as well as the development of a unique technology for its processing with the use of biotechnology for further use in agriculture as a fertilizer with high biological activity is relevant and promising.

Materials and Methods

The research was conducted at the Department of Biotechnology, Biochemistry, and Biophysics, and also based on the research and Testing Center for Toxico-Pharmacological Research and Development of Veterinary medicines, feed additives, and disinfectants (SIC Vetfarmbiocenter), which is a structural division of the Kuban State Agrarian University named after I. T. Trubilin.
The object of research was cultures of microorganisms *Azotobacter sp*., a member of the group of aerobic gram-negative bacteria fixing molecular nitrogen and *Pseudomonas sp*., which has proteolytic activity, as well as their joint composition in the composition of a biological product-a litter destructor.
The virulence, toxigenicity, toxicity, and harmlessness of the studied cultures were studied separately according to the recommendations [8, 9]. The joint composition of the studied microorganisms in the composition of the biological product-destructor was studied by the express method, using bio testing on Stylonychia, as well as the main method for determining the total toxicity in rabbits and mice (GOST 31674-2012) [10]. The content of laboratory animals corresponded to the normative documentation (GOST 33216-2014) [11] They had free access to water and received laboratory granulated food "Chara", made according to GOST 34566-2019 [12]. Before setting up the experiment, the laboratory animals underwent a seven-day quarantine, and then acclimatization in the experimental room for five days. The creation of treatment and comparison animal groups was performed by the pairs-analogues method [13-19].

Specification of the Virulence of Individual Cultures: The culture of the 2nd passage, grown on a dense nutrient medium, was cleansed with 0.9% sodium chloride dilution. The condensation of microbial cells in the resulting suspension was determined by the optical turbidity criterion. Some tenfold solutions were developed from the obtained mixture. The obtained mixture of different condensations (doses) of microbes was applied orally. In the test, minimally ten animals in the group were used. The applied dose was 1.0 ml for mice and 10.0 ml for rats. Mice and rats were injected with the test suspension of microorganisms fractional during the day. The follow-up duration was 14 days. The animals were examined every day, considering the protocol of the experiment and the number of living and dead animals [20]. At the end of the follow-up period, a half-year dose (LD50) was calculated.

Determination of Toxigenicity of Individual Cultures: The toxigenicity of microorganisms was studied similarly to virulence. To determine the toxigenicity, the culture of the tested microflora was sown on a liquid nutrient medium, kept in a thermostat at the desired temperature for growth during ten days for the accumulation of toxin in it, if it is produced by the strain. Then it was cleared by a bacterial filter. The obtained transparent filtrate was introduced undiluted. Each dose of the filtrate was examined on ten lab animals with intraperitoneal administration, at the same time [21-24].

Determination of the Toxicity of Individual Crops: The toxicity of the examined strains was investigated by intraperitoneal application of a suspension of the tested microorganism killed by heating at a temperature of 100 °C for 30 minutes (at the maximum concentration of microbial cells). The heated culture, native, was administered to ten animals in a volume of 1.0 ml for mice and 10.0 ml for rats. The LD50 or maximum endured dose was determined. The animals were controlled for 14 days [25].

The Harmlessness of Individual Crops: 5.0% of defibrinated sheep blood was added to the molten and cooled to 48-50 ºC nutrient medium. Following the filling of the medium in Petri dishes with a thin layer (1.5-2.2 mm) on the frozen and dried surface, the 18-hour culture was seeded with a stroke to achieve isolated colonies. The cups were incubated at a temperature of (37 ± 1) °C for 24-48 h, after which the findings were recorded [26, 27].

Specification of the Toxicity of the Joint Composition: Among the biological objects used in the experiments were the simplest genera of gastropod infusoria (*Stylonychia mytilus*) and laboratory animals (mice, rabbits).

The rapid method for determining the total toxicity was carried out on a daily culture of *Stylonychia mytilus*, which is in the logarithmic growth phase. To do this, the culture of *Stylonychia mytilus* was exposed to an aqueous and acetone extract containing various fractions of toxic substances extracted from the analyzed composition with water and acetone. The result of the express method was evaluated by the number of dead Stylonychia in five repetitions. Non-toxic is considered a joint composition in which, with the simultaneous parallel use of water and acetone extracts, 70-100% of the infusoria did not die. A 1.0% acetone solution (1.0% Ar) and a Lozin-Lozinsky solution (rl) were used as controls. The reaction of the viability of infusoria (N, %) was calculated by the formula (1):

$$N = N_2 / N_1 \times 100 \quad (1)$$

N_1 is the arithmetic mean (out of five tests) value of the number of Stylonychia at the beginning of the experiment;
N_2 is the arithmetic mean (out of five tests) value of the number of Stylonychia at the end of the experiment after 1 hour of exposure;

The method for determining the total toxicity of a microbial preparation in laboratory animals involves its bioassay in parallel on rabbits (skin test) and mice (acute experience), which makes it possible to take into account both the dermonecrotic effect of toxins and their effect on the digestive system of warm-blooded animals.

To conduct a skin-resorptive study, water and acetone extracts were applied to the cut areas of the skin of two laboratory rabbits (on one side) with a plastic spatula. As a control field for evaluating the results of the studies, we used a trimmed area of skin on the symmetrical side of the rabbits, which was not subjected to treatment. To prevent the licking of extracts, a collar was put on the neck of the animals. The duration of the experiment is 72-120 hours. The toxicity of the drug was assessed by the presence or absence of an inflammatory reaction on the exposed skin areas of rabbits with applied extracts in two parallel studies.

To conduct an acute experiment on mice, toxic substances were extracted from the microbial composition with acetone and water and injected into the stomach of laboratory animals. In the experiment, 20 white mice with a live weight of 18-22 g were used, which were previously kept on a starvation diet for 6 hours. Thus, four groups of animals were formed: control group 1-animals were injected with vegetable oil in the stomach for comparison with the group that was injected with acetone extract in a volume of 0.5 ml; the 2nd control group-mice were injected with distilled water in the stomach for comparison with the group that was injected with an aqueous extract in the volume of 0.5 ml; the 1st experimental group-laboratory animals were injected with an acetone extract in the volume of 0.5 ml; the 2nd experimental group-laboratory biological objects were injected with an aqueous extract in the volume of 0.5 ml. The laboratory mice were monitored for 3 days. The result of the experiment was evaluated based on the analysis of the state of the gastrointestinal tract, liver, spleen, and kidneys during the pathoanatomical autopsy of laboratory mice. The biopreparation was considered non-toxic if all laboratory animals remained viable, and no pathoanatomical changes were recorded during their autopsy [28, 29].

Results and Discussion

Determination of the Virulence of Individual Cultures

The findings of the research of the virulence of the investigated microorganisms are presented in **Tables 1 and 2**.

Table 1. Findings of the Study of the Virulence of *Azotobacter sp*. in Mice and Rats (n = 10)

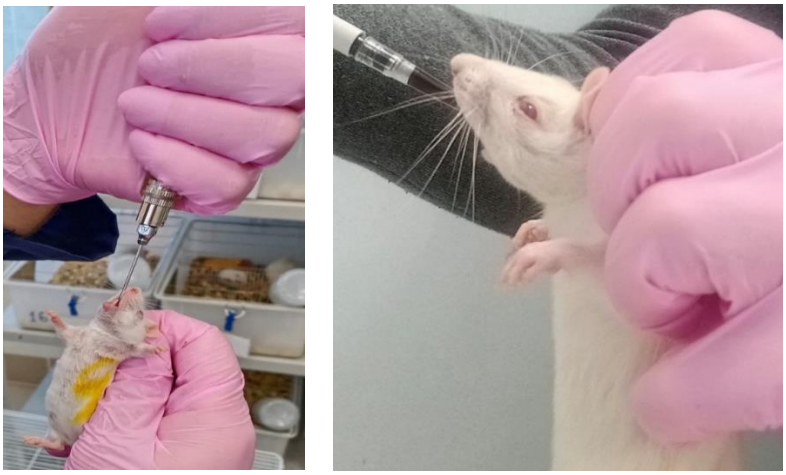
Group	Bio-object	The volume of the injected liquid, method of administration	Strain dose, CFU/ml	Test result		
				sick	dead	alive
1	Mice	1,0 ml *Azotobacter sp., per os*	$2,1\times10^9$	0	0	10
	Rats	10,0 ml *Azotobacter sp., per os*				10
2	Mice	1,0 ml *Azotobacter sp., per os*	$2,1\times10^8$	0	0	10
	Rats	10,0 ml *Azotobacter sp., per os*				10
3	Mice	1,0 ml *Azotobacter sp., per os*	$2,1\times10^7$	0	0	10
	Rats	10,0 ml *Azotobacter sp., per os*				10

Table 2. Results of the Study of the Virulence of *Pseudomonas sp*. in Mice and Rats (n = 10)

Group	Bio-object	The volume of the injected liquid, method of administration	Strain dose, CFU/ml	Test result		
				sick	dead	alive
1	Mice	1,0 ml *Pseudomonas sp., per os*	$1,1\times10^9$	0	0	10
	Rats	10,0 ml *Pseudomonas sp., per os*				10

2	Mice	1,0 ml *Pseudomonas sp., per os*	$1,1\times10^8$	0	0	10
	Rats	10,0 ml *Pseudomonas sp., per os*				10
3	Mice	1,0 ml *Pseudomonas sp., per os*	$1,1\times10^7$	0	0	10
	Rats	10,0 ml *Pseudomonas sp., per os*				10

The method of introducing microorganisms to laboratory animals is shown in **Figure 1**.



a) b)

Figure 1. Oral Administration (per os) of the Test Suspension:
a) mice, b) rats

The results of the study of the virulence of the microorganisms used in the work indicated that ignoring the condensation of the cultures entered into the body of laboratory animals, no death of treated animals was observed, there were no symptoms of health disorders and body weight loss at the end of the observation period. LD50 could not be determined. Therefore, the investigated cultures do not indicate virulence.

Determination of the Toxigenicity of Individual Crops
The results of the toxigenic study of the studied strains are presented in **Table 3**.

Table 3. Results of Toxigenic Studies of the Investigated Cultures on Lab Animals (n = 10)

Group	**Bio-object**	**The volume of the injected liquid, method of administration**	**Test result**		
			sick	**sick**	**sick**
1	Mice	1.0 ml of *Azotobacter sp*. filtrate, intraperitoneal	0	0	10
	Rats	10.0 ml of *Azotobacter sp*. filtrate, intraperitoneal			10
2	Mice	1.0 ml of *Pseudomonas sp*. filtrate, intraperitoneal	0	0	10
	Rats	1.0 ml of *Pseudomonas sp*. filtrate, intraperitoneal			10

The absence of animal deaths, symptoms of health disorders, and body weight loss in mice and rats by the end of the test were established, which shows the lack of a toxic factor in the filtrate of the test cultures. Therefore, *Azotobacter sp*. and *Pseudomonas sp*. do not have toxigenic properties.

Determination of Toxicity of Individual Crops
The findings of the research of the toxicity of the investigated lactobacilli are presented in **Table 4**.

Table 4. Findings of the Toxicity Study of the Studied Cultures on Lab Animals (n = 10)

Group	**Bio-object**	**The volume of the injected liquid, method of administration**	**Test result**		
			sick	**пало**	**sick**
1	Mice	1.0 ml of the killed suspension of Azotobacter sp., intraperitoneal	0	0	10
	Rats	10.0 ml of a killed suspension of Azotobacter sp., intraperitoneal			10
2	Mice	1.0 ml of killed suspension of Pseudomonas sp., intraperitoneal	0	0	10
	Rats	1.0 ml of a killed suspension of Pseudomonas sp., intraperitoneal			10

Due to investigating the toxicity of the strains in the treatment groups, all the treatment animals survived where no symptoms of health disorders and weight loss in mice and rats were recorded by the end of the test [30-32]. Hence, the studied microorganisms are not toxic.

Harmlessness of Individual Crops

An extra indicator of the harmlessness of the tested cultures of microorganisms is a test for the creation of hemolysin. Some microorganisms generate hemolysins (materials that eradicate red blood cells), which are related to pathogenicity agents. In this regard, the generation of hemolysin in many cases is an indicator of virulence. On blood agar, the grown colonies of such microorganisms surround the zones of enlightenment. The hemolytic features of microbes were investigated on nutrient media with defibrinated sheep blood. As a result of the test, when cultivating the studied strains on 5.0 % blood agar, the hemolysis zone (enlightenment) around the colonies was not seen, the result of hemolytic specifications was negative. Therefore, the cultures used are *Azotobacter sp*. and *Pseudomonas sp*. that are safe.

Determination of the Toxicity of the Joint Composition

Since today there is no clear regulation on the toxicity (safety) of the microbial composition used in research work on the territory of the Russian Federation, it was decided to carry out these experiments referring to the methods for determining the total toxicity according to GOST 31674-2012. These methods are qualitative and make it possible to assess the overall toxicity of feed, compound feed, feed raw materials, and feed additives.

The first stage of the research was to conduct an accelerated or preliminary bioassay of the analyzed microbial consortium, which allows for a short period (1.5–3 hours) to determine the toxicity of the biological product on *Stylonychia mytilus*. The results of the express method on *Stylonychia mytilus* are presented in **Table 5**.

Table 5. Results of the Express Method on *Stylonychia mytilus*

Group	Number of stylonychia	Repetitions					N, %
		1	2	3	4	5	
Control (L-L solution)	N1	19,0	20,0	18,0	18,0	17,0	100,0
	N2	19,0	20,0	18,0	18,0	17,0	
Control (1,0 % A solution)	N1	18,0	18,0	18,0	17,0	19,0	100,0
	N2	18,0	18,0	18,0	17,0	19,0	
Water Extract of Supplements	N1	20,0	19,0	19,0	18,0	17,0	100,0
	N2	20,0	19,0	19,0	18,0	17,0	
Acetone Extract of Supplements	N1	19,0	19,0	18,0	19,0	21,0	100,0
	N2	19,0	19,0	18,0	19,0	21,0	

As a result of the conducted studies, it was found that in the control, where Lozin-Lozinsky liquid was used as a solution, no changes in the number of stylonychia were recorded in any of the repetitions at the end of the experiment, the viability index was equal to 100.0 %. In the control group, where a 1.0% solution of acetone was used, the viability index of the infusoria was similar to the same group of 100.0 %. When analyzing the number of *Stylonychia mytilus* at the end of the experiment in groups where water and acetone extracts of microbial suspension were used, the studied indicator in all five replicates remained similar at the beginning of the experiment. In general, the viability index in these groups was also 100.0 %.

Thus, the rapid assessment showed that in all experiments the viability index was 100.0 %, which, according to GOST 31674-2012, confirms the non-toxicity of the studied microbial composition.

The next important stage of research, which gives the most reliable results, is to study the safety of the biological product by conducting the main method for determining the total toxicity in rabbits and mice according to GOST 31674-2012.

Preparation for the dermonecrotic study on a test bio-object included cutting the hair in the area of the rabbits' thighs and shoulder blades on one side and on the other side, only on the side of the skin area measuring 6.0 x 6.0 cm, further applying the extracts to the areas under study. At the same time, to not get distorted research results when visualizing the reaction, the skin of the experimental rabbits was without damage and pigmentation. The toxicity of the microbial preparation was determined by the presence of an inflammatory reaction on the cut area of the skin in contact with the studied extract.

As a result of the conducted study, there were no hemorrhages, hyperemia, peeling, edema, or other pathology indicating an inflammatory process. The animals remained active, willingly consumed food and water, and there were no pain signs when pressing lightly on the studied skin areas. Thus, the results of two parallel bioassays on rabbits demonstrated the absence of toxicological properties of the studied microbial biological product.

When conducting an acute experiment on mice, pre-test biological objects in the amount of five heads in each group were deposited in separate cells without giving food for 6 hours. Then, according to the research plan, mice of all groups were once injected with *per os* analyzed extracts and control solutions using a special oral-esophageal (nutrient) probe. The mice were

monitored for 72 hours, providing them with unlimited access to feed and drinking water. The findings of the viability of experimental lab animals are presented in **Table 6**.

Table 6. Findings of Acute Experiment in Mice (n = 5)

Group	Bio-object	The volume of the injected liquid, method of administration	Test result	
			dead	alive
Control 1	mice	0.5 ml, vegetable oil, *per os*	0	5
Control 2		0.5 ml, distilled water, *per os*	0	5
Experimental 1		0.5 ml, acetone extract of microbial suspension, *per os*	0	5
Experimental 2		0.5 ml, water extract of microbial suspension, per os	0	5

The results of the experiment when the mice were injected with the studied biological product showed that after three days, the death of laboratory animals was not observed. The experimental biological objects remained active, mobile, and no signs of poisoning or other pathology were found. To confirm the safety of the microbial consortium, laboratory mice were killed in compliance with the requirements of humanity for laboratory animals, while the state of individual organs of biological objects (gastrointestinal tract, liver, spleen, kidneys) was studied by pathoanatomical autopsy. The results of the studies showed that the mice of all groups had no hemorrhagic inflammation of the gastrointestinal tract, no degeneration of the liver, kidneys, and spleen, and no hemorrhages in the parenchymal organs. The location of the examined organs in the cavity was anatomically correct.

Conclusion

Thus, based on a series of preclinical studies, it was seen that the studied microorganisms individually do not indicate symptoms of virulence, toxigenicity, toxicity and do not give an affirmative counteraction to the generation of hemolysin. The results of the express method showed that the aqueous and acetone extracts obtained from the joint microbial composition did not have a detrimental effect on the culture of protozoa (*Stylonychia mytilus*), the viability in all groups was 100.0 %. The analysis of the dermonecrotic effect of the studied microbial preparation demonstrated that the studied extracts did not cause hemorrhage, hyperemia, peeling, edema, and other pathology indicating an inflammatory process on the skin of laboratory rabbits. Conducting an acute experiment confirmed the safety of the studied microbial consortium, since during the research the laboratory mice remained active, mobile, and there were no signs of poisoning or other pathology. In general, it can be concluded that the studied microbial composition (*Azotobacter sp*. and *Pseudomonas sp*.) according to GOST 31674-2012 is safe and can be used without restrictions.

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Conflict of interest: None

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Ethics statement: None

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