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Preclinical Studies of Microbial Feed Additives on Laboratory Animals

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Abstract

The object of research was a microbial feed additive SBT-Lacto, containing a dry culture of lactic acid bacteria: *Bifidobacterium lactis* (2.5-3.0 %), *Lactobacillus acidophilus* (2.5–3.0%), *Streptococcus thermophilus* (2.5–3.0%), *Lactobacillus delbrueckii ssp. Bulgaricus* (2.5–3.0 %), and fillers: edible citrus fiber (14.0-15.0 %), maltodextrin (76.0–84.0%), to improve the digestibility of mixed feed, increase the safety, growth, and productivity of farm animals, including poultry. The work aimed to determine the acute and chronic toxicity, as well as the irritating effect of the SBT-Lacto feed additive on laboratory animals (mice, rats, rabbits). In the course of research work, clinical, pathoanatomic, biochemical, and morphological studies of the blood of experimental animals were carried out. As a result of the research, it was found that the feed additive SBT-Lacto at a dose of 7500 mg/kg of body weight does not have a toxic effect on the body of laboratory animals. According to the degree of exposure to the body of warm-blooded animals, the feed additive refers to substances that are not very dangerous. The microbial feed additive SBT-Lacto can be recommended for use in practical animal husbandry, including poultry farming.

Disciplinary: Veterinary, Zoology, Biotechnology.

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1 Introduction

In the modern world, industrial poultry farming is unthinkable without the use of feed additives in poultry diets, which contribute to the intensification of the industry by increasing the productivity and safety of livestock, as well as obtaining high-quality products, which is consistent with the "Concept of state policy in the field of healthy nutrition of the population in the Russian Federation", which imposes high requirements for the balance of mixed feeds and diets, which determine the quality of food products, and, consequently, the health of the nation [1-5].

2 Literature Review

A balanced diet of poultry is the basis for obtaining high-quality poultry products. This can be achieved through the introduction of new technologies in the feeding system of modern highly productive crosses. Using high-quality feed and feed additives, it is possible to obtain a greater growth rate and development of poultry [6-10].

However, a limiting factor in the intensification of the poultry industry is diseases of various origins that can cause disorders of the gastrointestinal tract. These circumstances and other stress factors reduce the safety, growth, productivity, and accordingly, the quality of products. To solve these problems, microbial feed additives are used in the world practice that are as safe as possible for the poultry body and consumers of poultry products. They reduce the negative impact of environmental stress factors on the bird's body, normalize the functioning of the gastrointestinal tract, prevent diseases of both bacterial and viral etiology [11-16].

In this regard, the development and use of new modern approaches in poultry feeding with the use of feed additives, in particular of microbial origin, is a promising and relevant direction for solving problems related to providing the population of the Russian Federation with environmentally biosafety and high-quality poultry products, and the study of the preliminary harmlessness of such additives on laboratory animals is a mandatory measure for their further registration and application on an industrial scale.

3 Method

Research experiments were carried out at the Scientific and Research Center for Toxico-Pharmacological Research and Development of Veterinary Medicines, Feed Additives and Disinfectants (SRC Vetfarmbiocenter), which is a structural subdivision of the Kuban State Agrarian University named after I. T. Trubilin (Krasnodar, Russia).

The purpose of this research was to study the general toxic and irritating effect of the microbial feed additive "SBT-Lacto" on laboratory animals.

Within the framework of this goal, the following tasks were solved:

1. Determination of the tolerable and toxic doses of the SBT-Lacto feed additive (acute toxicity).

2. Identification of the most sensitive organs and systems of the body to the studied feed additive, the nature and degree of pathological changes in them, as well as to investigate the reversibility of the damage caused (chronic toxicity).

3. Determination of the irritating effect of the feed additive by the method of skin applications on laboratory rabbits.

The object of research was a microbial feed additive SBT-Lacto containing a dry culture of lactic acid bacteria: strain Bifidobacterium lactis (2.5-3.0 %), strain Lactobacillus acidophilus (2.5–3.0%), strain Streptococcus thermophilus (2.5–3.0%), strain Lactobacillus delbrueckii ssp. Bulgaricus (2.5-3.0 %), and fillers: edible citrus fiber (14.0-15.0 %), maltodextrin (76.0-84.0 %). The moisture content is 10.0-12.0 %. The total number of lactic acid microorganisms is not less than 1×106 CFU/g. The feed additive is a powder from white to beige color, odorless. It is packaged in multi-layer paper bags with a polyethylene liner with a capacity of 0.3; 0.5; 1.0; 2.0; 25.0 kg. The feed additive is stored in a sealed manufacturer's packaging in a clean, well-ventilated room protected from direct sunlight and precipitation, at a temperature of minus 25-35°C and relative humidity of no more than 75.0 %. The shelf life is 24 months from the date of manufacture, subject to storage conditions.

The mechanism of action of the feed additive SBT-Lacto is due to the ability of lactic acid bacteria included in its composition to enhance the activity of the intestinal microbiota Synthesized substances (enzymes, amino acids, and other biologically active substances) restrain the development of pathogenic and conditionally pathogenic microflora, activate metabolism, as a result of which the digestibility of feed improves, the safety, growth, and productivity of farm animals, including poultry increases. The feed additive SBT-Lacto promotes the early formation of normal intestinal microbiocenosis in young animals from the first days of life, reduces mortality and positively affects the productivity of young animals and poultry. SBT-Lacto is used to normalize the intestinal microflora and increase productivity in farm animals and poultry. The additive is added to mixed feed at feed mills or in feed mills of farms, using existing mixing technologies.

All experiments were carried out in compliance with the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes [17].

The toxicological properties of the additive samples were studied based on generally accepted methods and regulatory documents [18-21].

The study of the irritating effect of the feed additive SBT-Lacto was carried out by the main method for determining the total toxicity in rabbits according to GOST 31674-2012 [22].

During the experiments, clinically healthy animals raised in the vivarium of the research and testing center were used after a 14-day quarantine. Laboratory animals have not previously participated in experiments. During the quarantine, a daily examination of each animal was carried out to control the manifestation of deviations in the state of health (behavior and general condition, morbidity, and mortality).

Laboratory animals were kept in conditions corresponding to the sanitary rules for the design, equipment, and maintenance of experimental biological vivariums, in rooms with a

temperature of 22-24°C, with a relative humidity of 40-50 %, under natural light lighting conditions, according to the requirements for the maintenance of laboratory animals [23].

The obtained digital values of the research results were processed by mathematical statistics methods [22] using the standard Microsoft Office Excel 2013 program in the Windows 10 operating system. The results were considered reliable at the probability level $P \leqslant 0.05$.

4 Result and Discussion

4.1 Acute Toxicity Research

Determination of the acute toxicity was studied on 20 mongrel white mice (males and females). The age of the animals is 11 weeks, the average body weight is 33.5 ± 0.35 g.

Laboratory mice were kept in standard polycarbonate cages with five animals of the same sex per cage. From above, the cages were closed with a mesh metal lid, on which a drinking bowl was mounted. The animals were watered using standard drinking bowls with tap water. Feeding is a complete feed for rodents, consisting of wheat, corn, wheat bran, sunflower meal, fish flour, mineral, and vitamin supplements. Litter – sawdust "Zveryo Moyo" ("Ideal" LLC, Russia).

The animals were divided into experimental and control groups of 10 heads each (two cages of 5 female heads, two cages of 5 male heads) (Table 1).

Table 1: Dynamics of body weight of mice in the study of acute toxicity of the feed additive SBT-Lacto with intragastric administration

No., gender	Body weight, g			
	Before the introduction of a feed additive		14 days after the introduction of SBT-Lacto	
	Control group	Experimental group	Control group	Experimental group
1,female	31.7	32.7	33.5	33.2
2, female	34.4	34.9	35.8	35.8
3, female	32.6	35.6	33.2	36.7
4, female	31.0	34.2	31.8	35.3
5, female	30.7	33.8	31.2	34.9
6, male	36.1	35.1	37.4	36.2
7, male	35.8	32.0	36.2	33.4
8, male	33.7	33.7	34.1	34.2
9, male	34.5	32.8	35.2	33.9
10, male	32.1	32.6	33.0	33.4
Mean, ±	33.3±0.61	33.7±0.38	34.1±0.63	34.7±0.4

In accordance with the route of administration intended for introduction into animal husbandry practice, the feed additive was administered through an oesophageal (nutrient) probe (a syringe with a curved injection needle and a soldered olive at the end) (Figure 1). It was taken into account that the maximum volume of liquid recommended for injection into the stomach for this type of animal and with a bodyweight of more than 30.0 g is 1.0 ml. The amount of feed additive was calculated per unit of body weight of the animal. We used the principle of reducing the injected volume of feed additives from the maximum possible (predicted mortality of 100 %) to the volume that does not cause the death of animals in the experimental group. According to the literature data on the low toxicity of feed additives with a similar composition, the introduction of the studied feed additive SBT-Lacto began with a volume of 1.0 ml of feed additive per animal.

Figure 1: Intragastric administration of a mouse feed additive with an oesophageal probe

For intragastric administration, the additive was previously dissolved in distilled water. The resulting aqueous suspension of the feed additive in a 50% concentration was administered to the experimental group using a probe once, in the morning, on an empty stomach, after a 12-hour fasting diet, at a dose of 1.0 ml/head. The control group, in a similar way, was injected with distilled water at a dose of 1.0 ml/head.

All experimental animals were monitored for 14 days, and the first day after administration, the animals were under continuous observation. The toxic effect of the additive was judged by the results of monitoring the general condition of the animals.

The results of monitoring the general condition of animals in the study of acute toxicity of the feed additive SBT-Lacto are presented in tables 2 and 3.

Table 2: Monitoring of the general condition of animals in the study of acute toxicity of feed additives and SBT-Lacto, experimental group (n=10)

Index	A day after the start of the feed additive administration					
	0	1	4	7	10	14
safety (lost/survived)	0/10	0/10	0/10	0/10	0/10	0/10
general condition	stable, no changes					
features of behavior	without features					
intensity and nature of motor activity	expressed					
the presence and nature of seizures	not observed					
impaired coordination of movements	not observed					
skeletal muscle tone	within the physiological norm					
reaction to tactile, painful, sound and light stimuli	-slightly reduced during the first two hours after the introduction of the supplement, -significant for the rest of the observation period					
frequency of respiratory movements	within the physiological norm					
heart rate	within the physiological norm					
the condition of the coat and skin	stable, no changes					
coloration of the mucous membranes	pale pink					
tail position	physiological, without deviations					
the amount and consistency of fecal masses	within the physiological norm					
frequency of urination and color of urine	without deviations from the norm, straw-yellow					
feed and water consumption	appetite is pronounced, water consumption has not increased					
change in body weight	stable					

Table 3: Monitoring of the general condition of animals in the study of acute toxicity of the feed additive SBT-Lacto, control group (n=10)

Index	A day after the start of the feed additive administration					
	0	1	4	7	10	14
safety (lost/survived)	0/10	0/10	0/10	0/10	0/10	0/10
general condition	stable, no changes					
features of behavior	without features					
intensity and nature of motor activity	expressed					
the presence and nature of seizures	not observed					
impaired coordination of movements	not observed					
skeletal muscle tone	within the physiological norm					
reaction to tactile, painful, sound and light stimuli	-slightly reduced during the first two hours after the introduction of the supplement, -significant for the rest of the observation period					
frequency of respiratory movements	within the physiological norm					
heart rate	within the physiological norm					
the condition of the coat and skin	stable, no changes					
coloration of the mucous membranes	pale pink					
tail position	physiological, without deviations					
the amount and consistency of fecal masses	within the physiological norm					
frequency of urination and color of urine	without deviations from the norm, straw-yellow					
feed and water consumption	appetite is pronounced, water consumption has not increased					
change in body weight	stable					

As a result of the conducted studies, it was found that intragastric administration of the SBT-Lacto feed additive at a dose of 7500 mg/kg of body weight in the body of white mice does not cause a visible clinical picture of poisoning and death of laboratory animals. After the introduction of the feed additive, there were no changes in the general condition of the animals (behavioral features, intensity and nature of motor activity, the presence and nature of seizures, impaired coordination of movements, skeletal muscle tone, reaction to tactile, painful, sound and light stimuli, frequency of respiratory movements, heart rate, condition of the coat and skin, color of the mucous membranes, position of the tail, number and consistency of fecal masses, frequency of urination and color of urine, consumption of feed and water, changes in body weight). The changes in the body weight of the experimental and control mice at the end of the experiment were insignificant (Table 1).

Thus, it was not possible to determine the half-year dose (LD_{50}) of the microbial feed additive SBT-Lacto.

4.2 Chronic Toxicity Research

The main task of research to determine the chronic toxicity of the feed additive SBT-Lacto was to determine its possible toxic effect on organs and tissues with repeated use. When determining the parameters of chronic toxicity of the SBT-Lacto feed additive, three groups of rats were formed at the age of 2.5-3 months, with an average body weight of 145.8±1.59 g (two experimental and one control), 10 animals each, 5 females and males. Laboratory animals were kept in standard wooden cages on a wooden-chip litter. From above, the cages were closed with a mesh metal lid, on which a drinking bowl was mounted. All experimental animals were kept in conditions similar to the studies when determining the acute toxicity of the additive.

Since LD_{50} was not established in the acute experiment, the starting point for choosing doses in the first experimental group of animals was 1/10 of the maximum possible volume of introduction into the stomach of the additive samples for this species and body weight of animals (3.0 ml/head) - 0.3 ml/head, the second experimental group 1/20 of the maximum possible volume – 0.15 ml/head. The third group of laboratory rats served as biological control.

The studied sample of the additive was administered intragastrically to the animals of the experimental groups through an oesophageal (nutrient) probe (Figure 2). The feed additive was set daily and individually for 28 days. The control group was on the usual feed diet.

Figure 2: Intragastric administration of a feed additive to a rat with an oesophageal probe

The toxic effect of the drug during its long-term use was evaluated by the following parameters: the clinical condition of the animals, the possible picture of intoxication, behavioral reactions, the number of fallen animals and the timing of death, the effect of the feed additive on the overall blood parameters and the biochemical composition of the blood serum, pathoanatomic changes. When evaluating the changes observed in animals in a toxicological experiment, the possibility of the influence of all side factors not related to taking the supplement (animal diseases, changes in diet, content, etc.) was excluded.

At the beginning and end of the experiment, experimental and control animals were weighed to control the dynamics of body weight, at the end of the experiment, blood was taken from animals of all groups (n=5) under anesthesia for morpho-biochemical studies according to the method described by Demchenkov et al. (2021) [24].

Morphological blood tests were performed on an automatic hematological analyzer Abacus Junior Vet (DIATRON, Austria). Biochemical studies of blood parameters were carried out on a semi-automatic biochemical analyzer BS-3000P (Sinnova, China) with a set of biochemical reagents for veterinary medicine DiaVetTest (Diakon-DS, Russia).

As a result of the experiments, it was found that the feed additive SBT-Lacto in the tested doses does not have a pronounced toxic effect on the body of laboratory animals. No significant

deviations from physiological norms in behavior, general condition, and appetite were recorded throughout the experiment. The rats were mobile, their reactions and reflexes were preserved. There were no changes in the functions of digestion and urination. Changes in the body weight of the experimental and control rats at the end of the experiment were not significant (Table 4).

Table 4: Dynamics of body weight of rats in the study of the chronic toxicity of the feed additive SBT-Lacto with intragastric administration

No., gender	Body weight, g					
	Before the introduction of a feed additive			Before the introduction of a feed additive		
	Group					
	Control	Experimental#1	Experimental#2	Control	Experimental#1	Experimental#2
1,female	142	152	154	159	172	161
2, female	137	146	157	151	164	176
3, female	138	138	142	162	156	154
4, female	136	141	148	149	167	164
5, female	146	156	149	158	168	152
6, male	151	153	147	166	171	162
7, male	151	141	150	173	169	168
8, male	158	135	159	174	164	174
9, male	143	147	148	163	159	163
10, male	151	153	137	166	172	161
Mean, ±	145.3±2.32	146.2±2.28	149.1±2.08	162.1±2.61	166.2±1.72	163.5±2.41

At the end of the experiment, blood was taken from five animals from each group for hematological and biochemical studies, and then they were killed for pathoanatomic examination of internal organs and tissues. Changes in morpho-biochemical parameters of blood with repeated use of the feed additive SBT-Lacto are reflected in tables 5 and 6.

Table 5: Morphological parameters of the blood of experimental groups in the study of the chronic toxicity of the feed additive SBT-lacto with intragastric administration

Animal No	Erythrocytes, 10^{12}/l	Hemoglobin, g / l	Leukocytes, 10^{9}/l	Hematocrit, %
Control group				
1	6.2	127	7.6	38
2	6.7	142	7.8	41
3	6.9	131	7.3	39
4	7.2	154	7.1	42
5	7.4	129	8.4	40
Mean, ±	6.9±0.21	136.6±5.07	7.6±0.23	40.0±0.71
Experimental group 1				
6	6.4	129	8.0	35
7	6.9	113	7.7	36
8	6.7	136	8.1	41
9	7.8	147	7.8	42
10	6.3	141	7.9	38
Mean, ±	6.8±0.27	133.2±5.85	7.9±0.07	38.4±1.36
Experimental group 2				
11	7.1	134	7.8	37
12	7.5	136	7.6	39
13	6.3	145	7.4	40
14	6.2	147	7.5	42
15	6.4	139	7.3	38
Mean, ±	6.7±0.26	140.2±2.52	7.5±0.09	39.2±0.86

Table 6: Biochemical blood parameters of experimental groups in the study of the chronic toxicity of the feed additive SBT-Lacto with intragastric administration

Animal No	Total protein, g/l	Albumin, g/l	AST, units/l	ALT, units/l	Alkaline phosphatase, units/l	Glucose, mmol/l	Cholesterol, mmol/l
Control group							
1	78	27	54	73	442	4,7	1,7
2	67	34	61	78	389	5,1	1,8
3	84	31	48	69	345	4,2	2,0
4	69	35	45	63	377	4,1	1,9
5	65	39	57	81	368	4,3	1,7
Mean, ±	72,6±3,61	33,2±2,01	53,0±2,94	72,8±3,2	384,2±16,15	4,5±0,19	1,8±0,06
Experimental group 1							
6	83	32	63	89	387	5,2	2,1
7	77	29	67	85	401	5,0	1,8
8	76	26	59	77	357	4,8	1,9
9	68	38	54	71	369	4,0	2,3
10	71	34	51	68	364	4,1	2,4
Mean, ±	75±2,59	31,8±2,06	58,8±2,91	78,0±4,0	375,6±8,06	4,6±0,24	2,1±0,11
Experimental group 2							
11	68	37	66	85	354	4,0	1,7
12	75	31	61	89	366	4,7	1,5
13	84	25	58	77	378	4,8	2,0
14	91	29	55	79	391	4,9	1,9
15	85	34	64	81	367	5,0	1,8
Mean, ±	80,6±4,06	31,2±2,07	60,8±1,99	82,2±2,15	371,2±6,24	4,7±0,18	1,8±0,09

With prolonged administration of the feed additive to laboratory animals at these doses, no significant differences between hematological parameters (erythrocytes, hemoglobin, leukocytes, hematocrit) were recorded relative to the control group animals. All indicators were within the physiological norm for this type of animal.

In the biochemical assessment of blood serum, the difference between the groups in the content of total protein, albumins, glucose, cholesterol, as well as the activity of AST, ALT and alkaline phosphatase enzymes was insignificant, the level of the studied indicators corresponded to the parameters of the physiological norm.

Pathomorphological studies confirmed the absence of toxic effects on the organs and body systems of experimental rats, no visible changes in the macroscopic structure were detected, the organs of both experimental and control rats corresponded to the physiological norm. In all the animals studied, the esophageal mucosa was shiny, smooth, and pale in color. The stomach is of the usual size and shape, filled with food contents. The gastric mucosa of the animals receiving the feed additive and the control group had no differences and were folded, pink, shiny. The local irritating effect of the feed additive after administration was not revealed.

4.3 Study of the Irritating Effect of a Feed Additive

The method for determining the total toxicity of a feed additive in laboratory animals implies its bio-testing on rabbits (skin test), which makes it possible to take into account the dermonecrotic effect of toxins that may be present in a microbial additive.

To conduct a skin-resorptive study, water and acetone extracts of feed extract were applied to the trimmed skin areas of two laboratory rabbits (on one side) with a plastic spatula. As a control field for evaluating the results of the studies, a trimmed area of skin on the symmetrical side of the rabbits, which was not subjected to treatment, was used. 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 microbial additive 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.

Preparation for the dermonecrotic study of the additive on a test bio-object included cutting the wool cover in the area of the thigh of rabbits and the shoulder blade on one side and on the other side only on the side of the skin area measuring 6.0×6.0 cm and further applying the extracts studied to the areas (Figure 1). At the same time, in order not to get distorted research results when visualizing the reaction, the skin of the experimental rabbits was without damage and pigmentation. The irritating effect of the additive was determined by the presence of an inflammatory reaction on the trimmed area of the skin in contact with the studied extract.

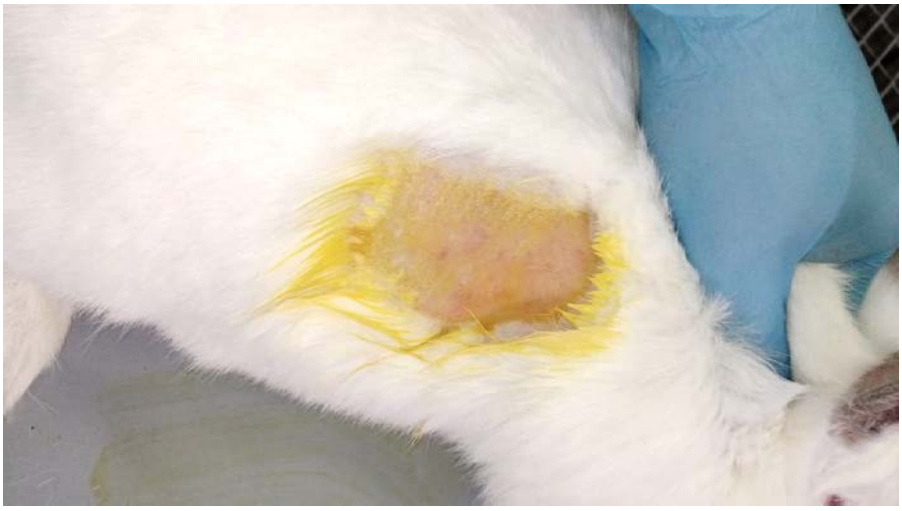

Figure 3. Study of the dermonecrotic properties of the feed additive (application of the additive extract)

As a result of the conducted studies, there were no hemorrhages, hyperemia, peeling, edema, and other pathologies indicating an inflammatory process. Laboratory animals remained active, willingly consumed food and water, no pain signs were detected when pressing on the studied skin areas.

Thus, the results of two parallel biotests on laboratory albino rabbits demonstrated the absence of an irritating property of the studied microbial additive.

5 Conclusion

As a result of the conducted studies, it was found that with intragastric administration of the SBT-Lacto feed additive to mice in the volume of 1.0 ml per animal (7500 mg/kg of body weight), lethal effects could not be achieved. Monitoring of the general condition of the animals of the experimental groups did not reveal any deviations from physiological norms. The feed additive SBT-Lacto refers to low-hazard substances.

When studying the chronic toxicity of the SBT-Lacto feed additive, it was found that daily administration of doses 1/10 and 1/20 of the maximum administered to animals in acute experience does not have a negative effect on the body of white rats, does not cause death, and does not have a pathological effect on the morphological and biochemical parameters of animal blood. The difference in the body weight of the experimental and control animals in quantitative terms was insignificant. During necropsy of experimental animals, no visible changes were found in the macroscopic structure of organs and tissues.

The local irritating effect on the mucous membranes of the digestive organs at the injection site, as well as the dermonecrotic properties of the feed additive SBT-Lacto does not show.

6 Availability of Data And Material

Data can be made available by contacting the corresponding authors.

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