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TOXICOLOGICAL AND BIOCHEMICAL PARAMETERS OF THE INFLUENCE OF THE NEW PROBIOTIC FOR PIGS BASED ON BACILLUS BACTERIA ON LABORATORY AND TARGET SPECIES OF ANIMALS

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Abstract. *The development of new domestic probiotics targeted at immunomodulatory effects is both timely and holds scientific and practical value. To carry out preclinical studies of the 'Combio' probiotic, which is a mixture of probiotic bacteria Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Enterococcus faecium and other substances developed by the team of authors of SSRILDVSE, under the conditions of several toxicological experiments on laboratory (white rats, rabbits) and target animals toxico-biochemical parameters were identified. The findings of the trial of the acute toxicity of the 'Combio' probiotic showed that the LD50 value could not be calculated, since the death of laboratory animals was not registered within 14 days after administration; the maximum administered dose of the preparation (by absolute weight) was 30,000.0 mg/kg of body weight, which allows it to be assigned to toxicity class VI – relatively harmless substances (LD50>15,000.0 mg/kg of body weight), and according to the degree of danger to IV class – low-risk substances (LD50>5000.0 mg/kg body weight). When applied to the skin (acute dermal toxicity) and the mucous membrane of the eye of rabbits in doses from 750.0 to 3000.0 mg/kg of body weight, the 'Combio' probiotic did not have an irritating effect, and according to the degree of danger, it can be classified as IV class – low-hazardous substances (LD50>2500.0 mg/kg of body weight). According to the results of extended oral feeding of the probiotic in doses of 1500.0; 7500.0 and 15000.0 mg/kg of feed it showed no evidence of hematological, hepatic, or nephrotoxic effects on the laboratory animals under subacute toxicological conditions. On the contrary, it showed the ability to induce metabolic responses in the bodies of white rats (according to dynamics of hematopoiesis and liver protein-synthesizing functions, especially at therapeutic doses). The dynamics of changes in metabolic indicators during extended 60-day oral feeding of probiotics to target animals and poultry in the dose range showed no evidence of hematological, immunological, or hepatoxic effects and correlated with the hematological indicators, which indicated the restoration of nonspecific resistance and the protein profile in the bodies of experimental animals and a sign of increased metabolic respond and immune responsiveness in pigs organisms. The findings regarding the effect of the new 'Combio' probiotic based on the bacteria Bacillus spp. in several experiments on laboratory and target (pigs) animals give grounds to assert that it is ecologically safe and promising for the result – improving the survival rate of target animals and poultry, increasing their body weight gain, optimizing feed conversion, and enhancing the overall quality of production.*

Keywords: toxico-biochemical parameters, probiotics, rats, rabbits, poultry, environmental safety, metabolism.

As Ukraine integrates into the international community, the main task of the agricultural sector is to increase the volume of livestock production (Kucheruk et al., 2018; Iegorov et al., 2022), what is more, poultry farming is one of the key components of livestock production. The current stance of the poultry farming industry is characterized by optimizing the cost of production by boosting the biological potential of animals and resource-saving technologies for producing quality feedstock and safe livestock products. To ensure this it is necessary to search for intensive development ways and to implement cutting-edge technologies that save resources and reduce the cost of production. To do this, it is necessary to achieve a stable epizootic situation regarding infectious diseases in poultry. Therefore,

the development and use of biologically effective, environmentally friendly, and competitive veterinary products is a critical issue of modern veterinary medicine.

Preventing bacterial diseases in poultry in Ukraine is one of the key priorities as it helps conserve the gene pool and productivity. It is a well-known fact that the greatest priority is given to environment-friendly antibacterial agents with high bactericidal properties. However, it is also known that the prolonged use of antibiotics can lead to the development of nonresponse (resistance) of microorganisms to medicines. (Landers et al., 2012; Manyi-Loh et al., 2018; Lee et al., 2019; Chechet et al., 2022; Vygovska et al., 2023; Shchur et al., 2023). In industrial poultry farming conditions, especially when organic production technologies are used, the most feasible approach is to use animal protection means based on natural substances with a high suppressive effect against infectious agents and the ability to balance the immune response. Today, the Ukrainian market can afford probiotics, which include elements of commensal microbiota (lacto- and bifidobacteria) with antibacterial and immunomodulatory properties (Milian et al., 2014; Pavlova, 2015; Pitino et al., 2010; Ashraf and Shah, 2014; Romanovych, 2018).

Thus, at the contemporary stage of antibacterial therapy development, special attention is devoted to the study of methods of neutralizing bacterial infections with non-pathogenic probiotic spore-forming microorganisms. It was found that the *Bacillus* spp. bacteria produce a wide range of biologically active compounds – antibiotics, compounds with a bacteriostatic effect, those that strengthen non-specific factors of the immune system activating macrophages, trigger the manifestation of a complex inflammatory effect, and ensure the neutralization of their competitive pathogenic microorganisms (Weiß, 2007; Chechet et al., 2021; 2023). The high antagonistic potential of *Bacillus* spp. bacteria towards other pathogens stipulates the scientific and practical significance of developing new probiotics with these bacteria as an alternative to antibiotics (Shkromada and Dudchenko, 2021; Olha Chechet et al., 2022; 2023).

Bacillus spp., as well as *Saccharomyces cerevisiae*, are used as feed additives to improve digestibility and prevent diseases for industrial livestock and poultry farming and the minimization of antibiotic usage in animal husbandry, (Kei Takemura et al., 2020; Lucey et al., 2021; Satoshi Koike et al., 2021), that certainly has a positive impact on the preservation and productivity of animals and poultry. Most scientific literature confirms the effectiveness of yeast-based feed additives, but still, there is no convincing evidence of their utility in preventing various diseases (Desnoyers et al., 2009). Some scientists (Bouchicha et al., 2022), claim that probiotic (*Saccharomyces cervicae, Lactobacillus* spp.) does not have a positive effect in preventing ketosis in goats during the late stage of pregnancy. Other scientists (Ermakova et al., 2021) claim that *Bacillus subtilis* DSM 32424 probiotic with drinking water inhibits the proliferation of fecal staphylococci in laying quails and promotes the increase of the carotenoid in the yolk. There are findings (Lamari et al., 2021) regarding the effectiveness of using a probiotic feed additive to treat mastitis in cows. It has been proved that *Bacillus* spp. bacteria produce compounds that have a positive impact on specific immunity factors by increasing the levels of IgA and IgG and other components of immune response (Pavlova, 2015; Cherny and Kulak, 2016; Khariv et al., 2017; Olha Chechet et al., 2022; Lytvynenko et al., 2022).

Therefore, taking into account the existing environmental factors, the development of an effective probiotic product based on microorganisms *Bacillus* spp. was highly relevant. Following the concept of organic animal farming for the growing and housing of pigs, this product allows for efficient prevention and treatment of diseases caused by opportunistic and pathogenic microorganisms by inducing the functionality of immune-competent cells in the digestive organs as part of the overall immune reactivity of the animal's organism. However, despite the obvious achievements in the development of preventive and therapeutic products, alternatives to antibiotics, and even promising results of clinical trials, testing them for environmental safety and biological impact on the animals' organs in preclinical trials is of paramount importance.

The objective of our paper was to study the toxicological and biochemical parameters impact of the new probiotic "Combio" with a targeted immunomodulatory effect in trials on laboratory and target poultry and farming animals.

Methods and materials. To conduct preclinical studies of the probiotic "Combio," which is a mixture of *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Enterococcus faecium*, aluminosilicate bacteria, and other substances, developed by a team of scientists at the State Scientific Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Examination (SSRILDVSE; Kyiv), the number of toxicicolgical trials on laboratory animals (white rats, rabbits) and target animals (piglets) were conducted to identify toxicity parameters.

To determine the acute toxicity parameters of the "Combio" product a single intragastric

administration to 36 female non-linear white rats (4-5 months old) with a body mass ranging from 230.0 to 250.0 grams, which were kept under optimal vivarium conditions (Kotsumbas, 2005; Karkyshchenko, 2010): the room temperature was (18±2)°C, with relative humidity of the air ranging from (60–70)%, the lighting cycle during the trial was set to a day-night pattern, lasting (10–14) hours. Additionally, the air change rate in the room was 10 per hour. Before the start of the trial, each animal was weighed, and a suspension of the substance with distilled water was prepared. The doses of the "Combio" probiotic were individually calculated based on the weight of each rat. 5 experimental groups were formed under the analog principle: the rats were administered a suspension of the substance at doses of 10000.0; 15000.0; 20000.0; 25000.0 and 30000.0 mg/kg of body weight (based on absolute weight) once (dose of 10000.0 mg/kg), twice (doses ranging from 15000.0 to 20000.0 mg/kg), and three times (doses of 25000.0 and 30000.0 mg/kg of body weight) intragastrically with an esophageal-stomach tube. Rats in the control group were administered 2.0 cm^3 of distilled water following a similar procedure. The experimental and control groups had 6 rats each (n=6). The clinical conditions of trial rats were observed for 14 days. The possible appearance and development of clinical signs of poisoning, the time until death, or recovery to the normal state were observed. During the clinical examination of rats, attention was paid to their behavior, response to external stimuli, appetite, condition of the fur, color of mucous membranes, respiratory and defecation frequency, changes in the color of feces, and so on. After the death (diagnostic euthanasia) of the animals, a diagnostic necropsy was conducted. The classification of substances based on toxicity was carried out in compliance with SOU 85.2–37–736:2011 "Veterinary Preparations. Determination of acute toxicity" (SOU, 2011). The macroscopic examination method was used to find pathological anatomical changes. The diagnostic necropsy was conducted according to the following scheme (Zharov et al., 2003): in the first stage, an external examination was conducted, observing the condition of the fur and mucous membranes; in the second stage, necropsy and examination of body cavities and internal organs such as the pharynx, trachea, larynx, heart, lungs, liver, spleen, kidneys, stomach, and intestines were performed, observing changes in color, thickness, pattern, and shape of the organs.

Identification of parameters of acute dermal toxicity and irritant effect of the "Combio" probiotic on the skin and mucous membrane of rabbits' eyes. The acute dermal toxicity parameters of the probiotic were tested on 20 Chinchilla rabbits, aged (4 – 5) months, with a weight (3.0 – 3.2) kg. The rabbits were kept under standard vivarium conditions with a temperature range of (18–21) °C and a humidity level of (55–65) %. their food ration met the standard nutritional requirements. For the research, one control group and three experimental groups were formed, 5 rabbits each. A day before the start of the experiment, the fur at the intended application site on the rabbits was removed by carefully trimming it with scissors. The rabbits were fitted with protective collars to prevent them from licking the substance. Observation of the experimental animals lasted for 14 days, during which the general condition of the animals, the nature of skin lesions at the application site, as well as the time of death or recovery of the animals, were taken into account. The application of the "Combio" probiotic was carried out in the morning before feeding the animals by applying uniformly a substance soaked in distilled water on a 6x6 cm area of the rabbits' skin. The substance was applied to the skin of the rabbits in the experimental groups at the following doses (by absolute mass): Group I – 750.0 mg/kg, Group II – 1500.0 mg/kg, and Group III – 3000.0 mg/kg of body weight, respectively. The rabbits in the control group were administered distilled water under similar conditions. The irritant (harmful) effect of the "Combio" substance on the mucous membrane of the eye was tested on 3 rabbits. 0.1 grams of the substance were introduced into the conjunctival sac of the left eye of the rabbits. 0.1 ml of distilled water was introduced into the right eye of the rabbits for control purposes. The rabbits were restrained, the corner of the conjunctival sac was pulled back, and for 1 minute, the tear-nasal duct was gently blocked with a finger. After application, a thorough examination of the eyes was carried out at intervals of every one, 24; 48; 72; 96 hours, up to 14 days.

Identification of the toxicity parameters of the "Combio" probiotic with repeated administrations in white rats (subacute toxicity via oral administration). Subacute toxicity of the probiotic substance was tested on 96 laboratory mature male rats with a weight range of (220.0–250.0) gr. The "Combio" probiotic was administered orally (mixed with the feed) for 60 days, after the administration the rats were observed for an additional 14 days. On the analog principle, one control group and three experimental groups were formed, of 24 rats each (n=24): the rats in the control group got feed without the "Combio" probiotic, Group I received the "Combio" probiotic orally with the feed at a dose of 1500.0 mg/kg of feed (therapeutic, according to the package insert), Group II received it at a dose of 7500.0 mg/kg (five times more than the therapeutic dose), and Group III received it at a dose

of 15000.0 mg/kg (ten times more than the therapeutic dose). Blood samples for hematological and biochemical tests were taken under conditions of total exsanguination under light chloroform anesthesia before feeding, on the 31st and 61st day after the start of the administration, and 14 days (on the 75th day of the trial) after the administration of the probiotic was ceased.

Identification of the toxicity parameters of the "Combio" probiotic with repeated administrations in pigs (subacute toxicity via oral administration). Subacute toxicity of the probiotic was tested in 20 piglets with a weight range of (35.0–40.0) kg. The "Combio" probiotic was administered orally for 60 days, after the administration the animals were observed for an additional 14 days. On the analog principle, one control group and three experimental groups were formed, of 6 piglets each (n=6): the animals in control group got feed without the "Combio" probiotic, Group I received the "Combio" probiotic orally with the feed at a dose of 1500.0 mg/kg of feed (therapeutic, according to the package insert), Group II received it at a dose of 7500.0 mg/kg (five times more than the therapeutic dose), and Group III received it at a dose of 15000.0 mg/kg (ten times more than the therapeutic dose). Blood samples for hematological and biochemical tests were taken next to the heart with a puncture from the subcutaneous vein of the ear before feeding. on the 31st and 61st day after the start of administration, and 14 days (on the 75th day of the study) after the substance administration was ceased.

During the toxicological experiments, the complete ration compound feed for rodents and pigs was used, respectively. The animals had free access to food and water.

Throughout the course of the subacute toxicological experiments (daily), the piglets were examined for integral indicators (behavior, external appearance, responses to external stimuli, water, and food consumption, as well as indicators that characterize organ and system functions, using commonly accepted methods).

The assessment of the functional state of the organism of experimental animals in comparison to the reference values throughout the experiments was conducted by identifying clinical and biochemical parameters in the blood samples using widely accepted methods as described in the reference materials. (Danchuk et al., 2013; Vlizlo et al., 2014). The following was determined in the stabilized blood samples: hematocrit level, erythrocytes count, leukocytes count, total hemoglobin amount; in the blood serum, the activities of indicative enzymes, such as alanine aminotransferase, were also measured (ALT activity; K. F. 2.6.1.2), aspartate aminotransferase (AST activity; K. F. 2.6.1.1) i gamma-glutamyl transpeptidase (GGT activity; K. F. 2.3.2.2), the levels of total proteins, albumins, urea, and creatinine were determined using CORMAY (Poland) reagent kits, additionally, in pigs, the levels of iron and manganese were measured respectively. The registration of biochemical parameters was done on a SHIMADZU UV-1800 (Japan) spectrophotometer.

The experiment results were processed using methods of variance statistics, with the use of a statistical analysis software package (ANOVA) StatPlus 7.6.5.0 (AnalystSoft Inc., US). The probability of the obtained results was evaluated using Tukey's (HSD) test at a significance level of 95.0% ($p<0.05$).

The manipulation action carried out on the animals complied with existing regulatory documents (Article 26 of the Law of Ukraine as of October 16, 2012 # 5456-VI; Council Directive 86/609/EEC of 24 November 1986), The organization of work involving the use of experimental animals and adherence to the principles of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986).

Results and discussions. As a result of the one-time intragastric administration of the “Combio” probiotic to female rats at doses (by absolute weight) 10000.0; 15000.0; 20000.0; 25000.0; and 30000.0 mg/kg of body weight, the following was found.

Clinical observations indicated that the one-time intragastric administration of the probiotic substance to rats in experimental Groups I, II, and III did not show acute poisoning. The rats were active and mobile, were responsive to external stimuli, and actively consumed food and water, however, rats from experimental Groups IV and V had mild depression during the first day after administration, which disappeared within 24 hours. During the 14-day- experiment there were no dead rats observed in the experimental groups.

On the 15th day of the experiment, the rats were euthanized using chloroform anesthesia, followed by a necropsy examination. The external appearance of the dead rats before dissection: the fur was white and glossy, there were no apparent changes in mucous membranes and no discharges from the oral (nasal) cavity or the anus.

The necropsy examination (compared to the control group) did not register significant changes in the mucous membranes of the oral cavity, trachea, pharynx, and esophagus; in the stomach, food residues were found; no subcutaneous tissue hyperemia was observed; the heart was not enlarged,

had a conical shape, and the myocardial consistency was elastic; the liver had a brown color, an elastic consistency, and was not enlarged; the spleen and pancreas showed no changes; the kidneys had a brown color and were not enlarged; blood vessels in the small intestine mesentery were not congested, and there were no signs of inflammation in the stomach, small intestine, or large intestine.

Based on the results of the acute toxicity study of the “Combio” probiotic it was not possible to calculate the LD_{50}, because no deaths of the laboratory animals were observed within 14 days following the administration of the substance. The maximum administered dose of the “Combio” veterinary product (by absolute weight) was 30,000.0 mg/kg of body weight, this places the substance into the VI class of toxicity, which signifies relatively harmless substances (LD_{50} >15000,0 mg/kg of body weight), and in terms of danger, it is classified as a class IV substance, indicating low risk (LD_{50} >5,000.0 mg/kg of body weight), according to the classification of chemical substances in SOU 85.2–37–736: 2011.

The results indicate that during the assessment of acute dermal toxicity of the "Combio" probiotic, no changes in the general condition or appetite of rabbits were observed as a result of skin application at doses (750.0 mg/kg – 3000.0 mg/kg) of body weight, this suggests the absence of a toxic impact of the product once applied to the skin of animals. It's worth noting that none of the experimental animals died during the course of the experiment. Throughout the entire trial period (14 days) in rabbits, there were no signs of erythema, skin edema, scab formation, or skin cracking observed, this indicates the absence of dermatitis and skin irritation.

The assessment of the irritating effect of the "Combio" probiotic was conducted based on the presence (absence) of conjunctival hyperemia, the red color of blood vessels, the sclera, cornea, eyelids, condition and was assessed with a scoring system according to Table 1.

During the assessment of the irritating effect of the "Combio" probiotic on the mucous membrane of the rabbit's eye, the following results were obtained (Table 2). After applying the product to the mucous membrane of the rabbit's eye, it was determined that throughout the entire observation period, it did not induce an irritating effect, the hyperemia and eye discharge within the first day after application was slight, and there was no eyelid swelling. The "Combio" probiotic when applied to the skin and the mucous membrane of the rabbit's eye at doses ranging from 750.0 to 3000.0 mg/kg of body weight, did not show an irritating effect, and according to the level of danger, it can be classified as Class IV – low-risk substances (LD_{50} > 2500.0 mg/kg of body weight) in compliance with SOU 85.2–37–736:2011.

Table 1

The irritating effect assessment of "Combio" probiotic on the mucous membrane of the rabbit's eye

A. Conjunctiva and cornea hyperemia	
1. Blood vessels are red	1 point
2. Some vessels are poorly visible	2 points
3. Diffuse deep redness	3 points
B. Eyelid swelling	
1. Mild swelling	1 point
2. Pronounced swelling with partial eyelid eversion	2 points
3. The eye is half-closed because of the swelling	3 points
4. The eye is more than half-closed because of the swelling	4 points
B. Discharges	
1. Minimal in the corner of the eye	1 point
2. The discharge moistens the eyelid	2 points
3. The discharge moistens the eyelid and the skin around it	3 points

Throughout the course of the subacute toxicological experiments (daily), the white rats were examined for integral indicators (behavior, external appearance, responses to external stimuli, water, and food consumption, as well as indicators that characterize organ and system functions, using commonly accepted methods). It was determined that during the examination of the general clinical condition of the experimental group of rats, no significant changes in behavior and external appearance were observed compared to the reference values. Throughout the entire observation period (75 days) and in the dynamics of feeding the "Combio" probiotic the animals remained active, had a satisfactory appetite, responded well to auditory and visual stimuli, and maintained responsive excitability; respiratory, urinary, or defecation

issues were not observed.

Table 2

Assessment of the irritating effect of "Combio" probiotic on the mucous membrane of the rabbit's eye in points (n=3)

Irritation	Time of observation – one day													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Assessment of the irritant effect of the probiotic on the mucous membrane of the eye of the first rabbit														
Hyperemia	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Discharges	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Assessment of the irritant effect of the probiotic on the mucous membrane of the eye of the second rabbit														
Hyperemia	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Discharges	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Assessment of the irritant effect of the probiotic on the mucous membrane of the eye of the third rabbit														
Hyperemia	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Discharges	1	0	0	0	0	0	0	0	0	0	0	0	0	0

The results of the study of hematological parameters in the blood of rats during the administration of the "Combio" probiotic are presented in Table 3. The results presented in the Table indicate that during the identification of hematological parameters in the blood of experimental rats, no pathological changes showing hepatotoxic effects of the "Combio" probiotic were registered in the animals.

However, changes in hematological parameters were observed, showing stimulation of erythropoiesis in the bodies of rats in all experimental groups. Thus, the hematocrit and leukocyte levels did not show significant deviations throughout the entire trial period, whereas the total hemoglobin amount and the erythrocytes count in the blood of animals on the 31st day of the study in the I, II, and III experimental groups exceeded the reference values by 7.8% and 6.7%; 10.3% and 8.8% ($p<0.05$), and 12.4% and 13.5% ($p<0.05$) respectively; on the 61st day, these values increased by 15.5% and 11.0% ($p<0.05$); 16.6% and 13.2% ($p<0.05$), and 18.4% and 13.5% ($p<0.05$) respectively. It should be noted that the values of total hemoglobin amount and the erythrocyte count tended to increase even after 14 days following the discontinuation of feeding the trial substance.

The results of the study of the functional parameters of the liver and kidneys in the serum of rats during the feeding of the trial probiotic are presented in Table 4. The data in the Table indicate that the values of the biochemical blood parameters in rats, both in the control and experimental groups, during the oral administration and after 14 days of discontinuation of "Combio" in doses of 1500.0, 7500.0, and 15000.0 mg/kg of feed, remained within the physiological norms.

However, it should be noted that the use of the probiotic at doses of 1500.0, 7500.0, and 15000.0 mg/kg with feed led to a tendency of increased levels of total proteins and albumins (on average by 4.5% and 8.0%) in the serum of the rats on the 31st day of the trial, and an increase in urea content (on average by 14.0%; $p<0.05$) compared to the reference values. On the 61st day after the start of administering the probiotic, there was also a tendency towards an increased amount of total proteins (on average by 5.1%) and an increased number of albumins and urea (on average by 13.0% and 12.8%; $p<0.05$) in the serum respectively. The same tendency continued 14 days after discontinuing the administration of the product.

Table 3

The level of hematological blood parameters in rats during subacute oral administration of the "Combio" probiotic: M±m; n=6; *– p<0.05 – compared to reference values

Experimental groups	Time of observation – one day			
	Before feeding	31st-day since administration	61st-day since administration	After 14 days following the discontinuation of administration
Total hemoglobin (HGB), g/dm^3				
Reference	85.49±2.32	87.51±2.35	84.25±2.56	90.32±2.42
1500,0 mg/kg	86.57±2.63	94.32± 2.52*	96.46± 2.71*	94.55±2.51
7500,0 mg/kg	85.19±2.44	96.49± 2.76*	98.21± 2.56*	93.41±2.62
15000,0 mg/kg	86.56±2.30	98.37± 2.30*	99.75± 2.10*	93.02±2.77
Hematocrit (HCT), %				
Reference	35.10±0.71	36.99±0.82	37.41±0.72	37.14±0.51
1500,0 mg/kg	37.02±0.84	36.54±0.76	37.54±0.85	36.91±0.85
7500,0 mg/kg	36.21±0.78	37.35±0.55	38.65±0.78	36.81±0.63
15000,0 mg/kg	35.19±0.74	38.41±0.83	39.60±0.81	36.93±0.76
Red blood cells (RBC), 10^{12}/dm^3				
Reference	9.30±0.32	9.11±0.35	8.99±0.32	9.20±0.31
1500,0 mg/kg	9.29±0.34	9.72± 0.22*	9.98± 0.28*	9.51±0.24
7500,0 mg/kg	9.26±0.21	9.91± 0.27*	10.18± 0.31*	9.42±0.34
15000,0 mg/kg	9.27±0.24	9.95± 0.31*	10.20± 0.26*	9.47±0.25
White blood cells (WBC), 10^9/dm^3				
Reference	13.40±1.12	13.36±0.52	13.99±0.75	13.20±1.35
1500,0 mg/kg	13.32±1.35	13.34±0.76	13.98±0.62	13.18±0.69
7500,0 mg/kg	13.40±0.62	13.33±0.84	13.27±0.56	13.16±0.83
15000,0 mg/kg	13.27±0.78	13.34±0.61	13.25±0.81	13.21±0.42

Therefore, the obtained results show that the "Combio" probiotic has an inducing effect on hematopoiesis activity and protein synthesis function of the liver in the bodies of white rats, especially at the therapeutic dose. It can be concluded that repeated oral administration of the "Combio" probiotic at doses of 1500.0, 7500.0, and 15000.0 mg/kg of feed does not exhibit hematotoxic, hepatotoxic, or nephrotoxic effects on the bodies of laboratory animals under conditions of the subacute toxicological experiment, on the contrary, it contributes to the induction of metabolic reactions in the bodies of white rats.

Throughout the course of the subacute toxicological experiments (daily), the pigs were examined for integral indicators (behavior, external appearance, responses to external stimuli, water, and food consumption, as well as indicators that characterize organ and system functions, using commonly accepted methods). During the examination of the general clinical condition of the experimental group of pigs, no significant changes in behavior and external appearance were observed compared to the reference values. Throughout the entire observation period (75 days) of the "Combio" probiotic the animals remained active, had a satisfactory appetite, responded well to auditory and visual stimuli, and maintained excitability; respiratory, urinary, or defecation issues were not observed. However, it should be noted that there was an increase in the motor activity of pigs in all experimental groups, starting from the 9th to the 16th day after the initiation of probiotic administration.

Table 4

The dynamics of basic biochemical values in rats' serum at subacute oral administration of the "Combio" probiotic: M±m; n=6; *– p<0.05 – compared to reference values

Experimental groups	Time of observation – one day			
	Before feeding	31st-day since administration	61st-day since administration	After 14 days following the discontinuation of administration
The activity of ALT μmol/h·cm³				
Reference	3.86±0.25	3.85±0.24	3.88±0.29	3.90±0.18
1500,0 mg/kg	3.87±0.19	3.88±0.20	3.87±0.27	3.92±0.20
7500,0 mg/kg	3.84±0.30	3.87±0.23	3.90±0.26	3.92±0.22
15000,0 mg/kg	3.88±0.21	3.89±0.27	3.87±0.17	3.91±0.26
The activity of AST μmol/h·cm³				
Reference	7.02±0.35	7.09±0.24	7.10±0.27	7.11±0.28
1500,0 mg/kg	7.05±0.20	7.10±0.26	7.07±0.38	7.10±0.38
7500,0 mg/kg	7.00±0.37	7.04±0.30	6.95±0.25	7.02±0.24
15000,0 mg/kg	7.10±0.22	6.98±0.24	7.10±0.28	7.05±0.13
Total proteins g/dm³				
Reference	72.10±1.27	73.00±1.90	73.90±1.50	73.25±1.10
1500,0 mg/kg	71.50±1.70	75.27±1.23	76.25±1.91	75.60±1.50
7500,0 mg/kg	73.20±1.26	76.70±1.60	76.94±1.76	75.28±1.15
15000,0 mg/kg	73.10±1.60	76.91±1.10	79.91±1.06	75.20±1.39
Albumine, g/dm³				
Reference	36.71±0.91	33.82±1.45	33.13±1.90	35.80±1.36
1500,0 mg/kg	35.02±0.88	36.62±1.60	37.54± 1.14*	37.90±1.00
7500,0 mg/kg	35.58±0.77	36.83±1.73	37.42± 1.08*	37.98±1.05
15000,0 mg/kg	37.15±0.75	36.16±1.87	37.34± 1.62*	36.62±1.20
Creatinine, μmol/cm³				
Reference	97.25±2.43	96.93±2.72	97.73±2.50	98.32±2.19
1500,0 mg/kg	98.07±2.62	97.02±2.40	98.47±2.41	97.05±2.30
7500,0 mg/kg	96.20±2.48	98.01±2.36	99.10±2.33	98.94±2.28
15000,0 mg/kg	98.36±2.31	98.31±2.18	99.80±2.25	100.08±2.14
Urea, μmol/dm³				
Reference	5.18±0.15	5.22±0.16	5.17±0.12	5.20±0.12
1500,0 mg/kg	5.12±0.12	5.78± 0.22*	5.63± 0.16*	5.41±0.18
7500,0 mg/kg	5.20±0.12	6.01± 0.22*	5.95± 0.20*	5.37±0.10
15000,0 mg/kg	5.18±0.16	6.07± 0.13*	5.91± 0.17*	5.40±0.17

The results of the study of hematological parameters in the blood of pigs during the administration of the "Combio" probiotic are presented in Table 5. The results indicate that the hematological parameters (hematocrit and leukocyte count) in pigs did not significantly change before and after the administration of the trial probiotic. However, a probable increase in the total hemoglobin content in the blood of pigs was observed on the 31st day after the oral administration of the "Combio" probiotic at doses of 1500.0, 7500.0, and 15000.0 mg/kg of feed, for the I, II, and III experimental groups, respectively, this increase amounted to 12.2%, 12.6%, and 12.2% ($p<0.05$), on the 61st day, the values were 16.2%, 14.3%, and 13.4% ($p<0.05$) for the respective groups compared to the reference values. The red blood cell count showed similar dynamics: on the 31st day, the increase in the red blood cell count in the I, II, and III experimental groups was 9.4%, 8.5%, and 8.0%, respectively ($p<0.05$), on the 61st day, it was 14.4%, 10.9%, and 10.2% ($p<0.05$) respectively. It should be noted that the dynamics of changes in these parameters remained consistent even 14 days after discontinuing the administration of the "Combio" probiotic. The observed changes in hematological parameters show stimulation of erythropoiesis in the organisms of the trial animals in all experimental groups and the

absence of hematotoxic effects of the "Combio" probiotic.

Table 5

Level of hematological parameters in piglets' peripheral blood at subacute oral administration of the "Combio" probiotic: M±m; n=5; *– p<0.05 – compared to reference values

Experimental groups	Time of observation – days			
	Before feeding	31st-day since administration	61st-day since administration	After 14 days following the discontinuation of administration
Total hemoglobin (HGB), g/dm^3				
Reference	136.50±2.32	127.20±2.68	125.32±2.15	131.62±2.30
1500,0 mg/kg	134.86±2.63	142.73± 2.08*	145.64± 2.24*	137.12±2.36
7500,0 mg/kg	135.05±2.44	143.21± 2.76*	143.23± 2.37*	135.50±2.78
15000,0 mg/kg	137.52±2.30	142.68± 204*	142.15± 2.78*	135.84±2.32
Hematocrit (HCT), %				
Reference	42.05±0.93	41.10±0.84	41.45±0.71	41.93±0.62
1500,0 mg/kg	41.55±0.85	41.31±0.73	41.42±0.95	41.81±0.83
7500,0 mg/kg	41.61±0.77	41.95±0.78	42.00±0.96	42.10±0.98
15000,0 mg/kg	42.09±0.84	41.03±0.95	42.34±0.82	42.01±0.86
Red blood cells (RBC), 10^{12}/dm^3				
Reference	9.11±0.20	9.08±0.21	8.91±0.23	9.10±0.21
1500,0 mg/kg	9.20±0.35	9.93± 0.27*	10.19± 0.29*	9.69±0.32
7500,0 mg/kg	9.10±0.22	9.85± 0.28*	9.88± 0.22*	9.58±0.23
15000,0 mg/kg	9.18±0.24	9.81± 0.32*	9.82± 0.27*	9.53±0.28
White blood cells (WBC), 10^9/dm^3				
Reference	11.40±0.93	11.26±0.93	10.98±0.86	11.21±0.76
1500,0 mg/kg	11.32±0.87	11.30±0.85	10.93±0.93	11.18±0.78
7500,0 mg/kg	11.50±0.82	11.33±0.81	11.17±0.67	11.16±0.94
15000,0 mg/kg	11.27±0.95	11.38±0.72	11.25±0.92	11.24±0.83

The results of the study of the functional parameters dynamics of the liver and kidneys in the serum of animals during the feeding of the trial probiotic are presented in Table 6. As a result of 60-day oral administration of "Combio" probiotic in doses of 1500.0, 7500.0, and 15000.0 mg/kg of feed, an increase in the values of the total protein in the serum of pigs compared to their reference level was recorded by 7.8, 7.6, and 7.3%; Iron – by 10.8, 6.0, and 6.3% ($p<0.05$); Manganese – by 10.2, 6.8, and 13.6% ($p<0.05$), respectively, and the concentration of urea – by 6.6, 7.6, and 8.1%, respectively.

A similar pattern was observed on the 61st day of the experiment: the values of the total proteins were higher than the reference by 8.1%, 7.6%, and 5.8% ($p<0.05$); for Iron, it was higher by 12.0%, 6.6%, and 10.2% ($p<0.05$); for Manganese, it was higher by 14.5%, 9.7%, and 17.7% ($p<0.05$); and for urea concentration, it was higher by 7.4%, 7.7%, and 9.2% ($p<0.05$), respectively. It should be noted that even 14 days after the discontinuation of the probiotic administration, there was a tendency towards an increase in the above-mentioned values.

Table 6

Dynamics of basic biochemical values in piglets' peripheral blood at subacute oral administration of the "Combio" probiotic:
M±m; n=5; *– p<0.05 – compared to reference values

Experimental groups	Time of observation – days			
	Before feeding	31st-day since administration	61st-day since administration	After 14 days following the discontinuation of administration
The activity of ALT μmol/h·cm³				
Reference	0.93±0.04	0.95±0.01	0.94±0.02	0.94±0.03
1500,0 mg/kg	0.92±0.05	0.93±0.03	0.92±0.04	0.93±0.05
7500,0 mg/kg	0.93±0.02	0.94±0.05	0.92±0.02	0.92±0.04
15000,0 mg/kg	0.92±0.04	0.93±0.05	0.94±0.05	0.93±0.02
The activity of AST μmol/h·cm³				
Reference	1.25±0.15	1.24±0.02	1.25±0.05	1.25±0.04
1500,0 mg/kg	1.24±0.05	1.25±0.08	1.26±0.08	1.27±0.01
7500,0 mg/kg	1.24±0.02	1.25±0.03	1.26±0.03	1.22±0.02
15000,0 mg/kg	1.25±0.04	1.26±0.02	1.25±0.04	1.27±0.05
The GGT activity μmol/h·cm³				
Reference	0.30±0.02	0.34±0.02	0.31±0.01	0.32±0.06
1500,0 mg/kg	0.32±0.02	0.30±0.01	0.28±0.02	0.29±0.03
7500,0 mg/kg	0.28±0.01	0.35±0.02	0.33±0.02	0.30±0.06
15000,0 mg/kg	0.30±0.01	0.37±0.03	0.28±0.03	0.31±0.03
Total proteins g/dm^3				
Reference	59.01±1.72	60.14±1.08	60.55±1.02	60.21±1.50
1500,0 mg/kg	58.91±1.70	64.85± 1.21*	65.47± 1.70*	62.32±1.02
7500,0 mg/kg	59.11±1.23	64.73± 1.05*	65.15± 1.05*	61.94±1.04
15000,0 mg/kg	58.99±1.56	64.51± 0.90*	64.05± 1.84*	61.05±1.91
Iron, μmol/dm^3				
Reference	23.62±0.65	22.81±0.45	23.27±0.48	23.26±0.65
1500,0 mg/kg	23.58±0.52	25.29± 0.48*	26.07± 0.55*	24.95±0.54
7500,0 mg/kg	23.02±0.63	24.18± 0.56*	24.81± 0.46*	24.73±0.52
15000,0 mg/kg	23.21±0.56	24.25± 0.58*	25.65± 0.57*	24.28±0.45
Manganese, μmol/dm^3				
Reference	0.63±0.03	0.59±0.02	0.62±0.02	0.58±0.02
1500,0 mg/kg	0.61±0.03	0.65± 0.03*	0.71± 0.03*	0.63±0.03
7500,0 mg/kg	0.61±0.02	0.63± 0.03*	0.68± 0.03*	0.60±0.03
15000,0 mg/kg	0.62±0.01	0.67± 0.03*	0.73± 0.04*	0.63±0.02
Urea, μmol/dm³*				
Reference	6.18±0.16	6.20±0.13	6.22±0.17	6.17±0.16
1500,0 mg/kg	6.12±0.13	6.61± 0.14*	6.68± 0.15*	6.53±0.17
7500,0 mg/kg	6.20±0.13	6.67± 0.11*	6.70± 0.11*	6.52±0.15
15000,0 mg/kg	6.18±0.11	6.70± 0.14*	6.79± 0.14*	6.50±0.13

The dynamics of changes in metabolic indicators correlate with the dynamics of hematological parameters, indicating the restoration of non-specific resistance and the protein profile in the bodies of experimental target animals, this is a sign of enhanced metabolic response and immune reactivity in the animal organism.

The values of these indicators, even after the discontinuation of the 60-day consumption of the "Combio" veterinary probiotic remained within their reference range, this shows the absence of hematotoxic, immunotoxic, and hepatotoxic effects of the experimental probiotic on the bodies of the trial piglets. The results of the research are consistent with the data we obtained previously testing

similar probiotics "Biomagn" and "Biozapin" using a model with test microorganism strains, animalculines, trial animals, and poultry (Chechet at al., 2021; 2022; 2023), that proved that these probiotics are highly effective in broiler chicken farming technology when used in combination with disinfectants. They can induce the functional endogenous detoxification system (by reducing the formation of toxic metabolites in purine metabolism: uric acid and creatinine levels, normalization of processes of transamination, and reducing alkaline phosphatase activity) and immunobiological responses (such as enhanced erythropoiesis, hemoglobin formation; induction of lysozyme, bactericidal, and phagocytic activities), these findings indicate that the probiotic product used in this series of experiments on laboratory and target (piglets) animals is environmentally safe and promising for result – improving the survival rate of pigs, increasing their body weight gain, optimizing feed conversion, and enhancing the overall quality of pork production. Thus, the development of new domestic probiotics targeted at immunomodulatory effects is both timely and holds scientific and practical value.

Conclusions

1. Based on the results of the acute toxicity study of the “Combio” probiotic it was not possible to calculate the LD_{50}, because no deaths of the laboratory animals were recorded within 14 days following its administration. The maximum administered dose of the “Combio” veterinary product (by absolute weight) was 30,000.0 mg/kg of body weight, this places the substance into the VI class of toxicity, which signifies relatively harmless substances (LD_{50} >15000,0 mg/kg of body weight), and in terms of danger, it is classified as a class IV substance, indicating low risk (LD_{50} >5,000.0 mg/kg of body weight) according to the classification in SOU 85.2–37–736:2011.
2. The "Combio" probiotic did not exhibit irritant effects when applied to the skin (acute dermal toxicity) or the mucous membrane of the eye of rabbits at doses ranging from 750.0 to 3000.0 mg/kg of body weight. According to the degree of hazard classification, it can be categorized as class IV, signifying it is relatively harmless, with an (LD_{50} > 2500.0 mg/kg of body weight) according to the classification in SOU 85.2–37–736:2011.
3. The results of the extended oral administration of the "Combio" probiotic at doses of 1500.0, 7500.0, and 15000.0 mg/kg of feed showed no evidence of hematological, hepatic, or nephrotoxic effects on the laboratory animals under subacute toxicological conditions. On the contrary, it showed the ability to induce metabolic responses in the bodies of white rats, (according to dynamics of hematopoiesis and liver protein-synthesizing functions, especially at therapeutic doses).
4. The dynamics of metabolic changes during the extended 60-day oral administration of “Combio” probiotic to piglets in the dose range showed the absence of hematotoxic, immunotoxic, and hepatotoxic effects of it, and it correlates with the hematological indicators, indicating the restoration of nonspecific resistance and the protein profile in the bodies of experimental target animals. This shows increased metabolic response and immune responsiveness in pigs’ organisms.
5. The findings regarding the impact of the new "Combio" probiotic based on *Bacillus* spp. bacteria in a series of experiments on laboratory and target (piglets) animals suggest that it is ecologically safe and promising for the result – improving the survival rate of target animals (pigs), increasing their body weight gain, optimizing feed conversion, and enhancing the overall quality of pork production.

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ТОКСИКО-БІОХІМІЧНІ ПАРАМЕТРИ ВПЛИВУ НОВОГО ПРОБІОТИЧНОГО ЗАСОБУ ДЛЯ ОСНОВІ БАКТЕРІЙ РОДУ BACILLUS НА ЛАБОРАТОРНИХ І ЦІЛЬОВИХ ВИДАХ ТВАРИН

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Резюме. *Питання розроблення нових вітчизняних пробіотичних засобів спрямованої імуномодулюючої дії є на часі та викликає науковий і практичний інтереси. З метою проведення доклінічних досліджень пробіотичного засобу «Комбіо», що являє собою суміш пробіотичних бактерій Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Enterococcus clausii, алюмосилікату та інших речовин розробленого колективом авторів ДНДІЛДВСЕ, у за умов низки токсикологічних експериментів на лабораторних (білі щури, кролі) і цільових тваринах (підсвинки) визначали токсико-біохімічні параметри. За результатами дослідження гострої токсичності пробіотичного засобу «Комбіо» показник LD_{50} розрахувати не вдалося, оскільки загибелі лабораторних тварин не було виявлено протягом 14-ти діб після введення; максимальна введена доза засобу (за абсолютною масою) становила 30000,0 мг/кг маси тіла, що дозволяє його віднести до VI класу токсичності – речовини відносно нешкідливі (LD_{50}>15000,0 мг/кг маси тіла), а за ступенем небезпечності до IV класу – малонебезпечних речовин (LD_{50}>5000,0 мг/кг маси тіла). Пробіотичний засіб «Комбіо» при нанесенні на шкіру (гостра дермальна токсичність) та на слизову оболонку ока кролів у дозах від 750,0 до 3000,0 мг/кг маси тіла не чинить подразнювальної дії, а за ступенем небезпечності його можна віднести до IV класу – малонебезпечних речовин (LD_{50}>2500,0 мг/кг маси тіла). За результатами тривалого перорального згодовування пробіотичного засобу в дозах 1500,0; 7500,0 і 15000,0 мг/кг корму встановлено відсутність гемо-, гепато- та нефротоксичної дії на організм лабораторних тварин за умов підгострого токсикологічного експерименту, а, навпаки, здатність впливати індукуючим чином на метаболічні реакції в організмі білих щурів (за динамікою показників активності гемопоезу та білоксинтезуючої функції печінки, особливо у терапевтичній дозі). Динаміка змін метаболічних показників під час тривалого 60-добового перорального згодовування пробіотику підсвинкам у діапазоні доз свідчить про відсутність гемо-; імуно- та гепатотоксичного його впливу та корелює із такою гематологічних показників, що вказує на відновлення стану неспецифічної резистентності та білкового профілю у організмі експериментальних тварин, та є ознакою посилення метаболічних реакцій та імунної реактивності в організмі свиней. Отримані результати щодо впливу нового засобу «Комбіо» на основі бактерій Bacillus spp. у серії експериментів на лабораторних і цільових (підсвинки) видах тварин дозволяють стверджувати про екологічну безпечність досліджуваного пробіотику та перспективність його використання у кінцевому результаті з метою підвищення рівня збереженості цільових тварин (свиней), підвищення приросту маси їх тіла, поліпшення конверсії корму та оптимізації виробництва свинарської продукції покращеної якості.*

Ключові слова: токсико-біохімічні параметри, пробіотичний засіб, щури, кролі, підсвинки, екологічна безпечність, метаболізм.

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