ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.pharmascope.org/ijrps

Effect of bacterial serine proteinase on growth performance and nutrient digestibility in broilers

Anastasia O Koryagina*[1], Natalia L Rudakova[1], Sergey Yu Smolentsev[2], Guzel F Khadieva[1], Marat T Lutfullin[1], Ayslu M Mardanova[1], Margarita R Sharipova[1]

[1]Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan- 420008, Russia
[2]Agricultural Technology Institute, Mari State University, Yoshakar-Ola, 424002, Russia

Article History:

Received on: 10 Oct 2020
Revised on: 07 Nov 2020
Accepted on: 10 Nov 2020

Keywords:

Broiler,
Subtilisin-Like Serine Proteinase,
Bacillus Pumilus,
Food Supplement,
Nutrient Digestibility,
Productivity

ABSTRACT

We evaluated the effect of adding subtilisin-like *Bacillus pumilus* proteinase to the diet of Hubboard broiler chickens on growth, digestibility of feed nutrients, blood characteristics, meat quality and histological parameters. One hundred and eighty broiler chickens were divided into 6 groups and received 2 feed rations from 0 to 35 days. The diet was either a basic diet without additives (BD) or a basic diet supplemented with *B. pumilus* proteinase at a concentration of 10 units/kg feed. We found that the addition of proteinase did not adversely ($P < 0.05$) affect the blood counts of broilers throughout the experiment. In the balanced experiment, we showed that with the addition of the microbial enzyme, the digestibility of the organic matter of the broilers diet increases considerably ($P < 0.05$). Veterinary sanitary examination and histological studies led to the conclusion that the broiler meat of the experimental group complies with Russian state standards (GOST) for fresh meat of good quality. It was established that the addition of proteinase to the main diet for broilers enhances the digestibility of the organic and mineral components of the broiler feed ration and reduces the amount of feed consumed.

*Corresponding Author

Name: Anastasia O Koryagina
Phone: +7 843 2337814
Email: tihonovaao93@gmail.com

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v12i1.3988

Production and Hosted by

Pharmascope.org

INTRODUCTION

One of the main factors affecting the economically beneficial signs of poultry is rational feeding with maximum absorption of nutrients. Feed additives are used to balance diets, increase nutrient absorption, and reduce toxicity and bacterial contamination of feed (Borda *et al.*, 2019). The use of bacterial enzymes as a feed additive attracts more increased attention. Enzymes play an important role in the digestibility of nutrients, which helps to reduce the cost of the diet. A common group of enzymes used as nutritional supplements are proteases that catalyze protein hydrolysis. Exogenous proteases are thought to not only supplement the digestive enzymes of animals, such as pepsin and trypsin, but also destroy nutrients such as lectins and trypsin inhibitors (Cowieson and Adeola, 2005). Studies have shown that the addition of proteases (acid protease from *Aspergillus niger*, serine protease from *Bacillus licheniformis*) to poultry feeds has a positive effect, improving of the growth performance and an amino acid digestibility (Kaczmarek *et al.*, 2014; Borda *et al.*, 2019). Supplementation of food with enzymes can affect the composition of the intestinal microbiota. Recently, protease has been shown to increase the population of *Lactobacillus* species, while at the same time decreasing the presence of *Clostridium perfringens* in the ileum (Giannenas *et al.*, 2017). Thus, exogenous pro-

teases serve as prophylaxis by reducing the level of indigestible protein that causes colonization of the intestines by pathogenic bacteria (Yuan *et al.*, 2015; Toghyani *et al.*, 2017). Today, an active search is underway for new effective enzymes to expand the arsenal of commercial drugs already in use on the market. Microbial proteases are the most widely studied of all bacterial proteins due to their commercial importance and properties, since they can tolerate harsh conditions, exhibit stability and broad specificity for the substrate (Gupta *et al.*, 2002; Razzaq *et al.*, 2019). In addition, a growing number of whole genomic sequences and the advancement of genetic engineering methods keeps bringing to light new proteases, which opens doors for new prospective applications.

In this study, a new subtilisine-like *B. pumilus* serine proteinase was tested as a feed supplement for Hubbard broiler chickens (Shagimardanova *et al.*, 2014). Extracellular serine proteinase was isolated from *B. pumilus* 7P culture medium (Mikhailova *et al.*, 2009). The enzyme is inactivated by specific serine proteinase inhibitors, but is resistant to chelating agents. The proteinase has broad specificity, hydrolyzes proteins and p-nitroanilides of N-acylated tripeptides, and exhibits maximum activity in the hydrolysis of leucine and p-nitroanilides of phenylalanine. The enzyme has a molecular weight of 27kDa and an isoelectric point above 6.4. The enzyme is most active at pH 10.0-11.5 the optimum temperature is 55°C, showing about 30% of the maximum activity at 0°C. Ca^{2+}significantly increases the thermal stability of the proteinase (Mikhailova *et al.*, 2009). The kinetic characteristics (Km) of Z-Ala-Ala-Leu-pNa hydrolysis were determined to be 1,85 mM. MALDI-TOF mass spectroscopy of the purified enzyme and its enzymatic properties corresponding to subtilisin-like serine proteinase (EC 3.4.21.62).

Proteinase activity was not inhibited by natural inhibitors, such as trypsin inhibitors, thus prolonging the functioning of the enzyme in the gastrointestinal tract of chickens. Chicken bile at a concentration of 0,01-0,05 does not inhibit activity of the proteinase (Koryagina *et al.*, 2018). For effective work in the digestive tract of poultry, the enzyme must remain active under conditions of elevated temperature (40°C) and an aggressive pH of the medium, changing from acidic to alkaline. We conducted an experiment with modeling the gastrointestinal tract of chickens in terms of parameters — pH, time and temperature (Koryagina *et al.*, 2018). Proteinase retains activity and basic properties throughout the gastrointestinal tract. These characteristics drive us to recommend the enzyme as a dietary supplement. In this work, we studied the effect of the enzyme in vivo on broiler chickens. The aim of the work is to evaluate the effect of the addition of bacterial serine proteinase to diet of broiler chickens on the growth performance, nutrient digestibility, blood and histological parameters, and carcase characteristics Figure 4.

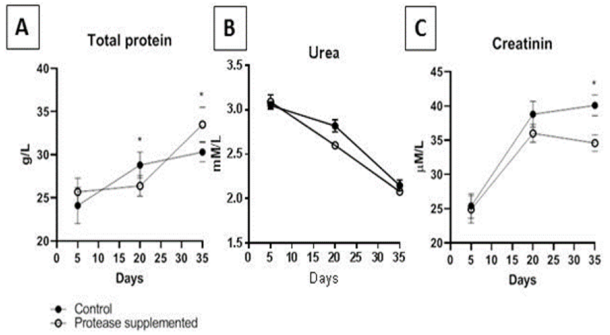

Figure 1: Biochemical indices of the blood in the control and experimental groups on days 5, 20 and 35. A - Total protein, B - Urea, C - Creatinin.

MATERIALS AND METHODS

Enzyme extraction

We used the strain *B. pumilus* 7P (obtained from the culture collection of Kazan Federal University), which has an increased ability to secrete subtilisin-like proteinase. Bacteria were initially grown on a medium of the following composition (g/L): bacteriological peptone (Sigma, USA) - 20, $CaCl_2$*$2H_2O$-0.6, $MgSO_4$*$7H_2O$ - 0.5, NaCl - 3, $MnSO_4$ - 0.1 Na_2HPO_4 - 0.35; NH_4Cl - 0.2. Cultivation was carried out in 2 L flasks with a ratio of the volume of the medium to the volume of the flask 1:5 on circular shakers (200 rpm) in a programmable thermostat "Braun", Germany at 30°C. As seeding material, we used cells of a 16-hour culture (1% v/v). Bacterial growth was determined by the optical density of the culture on an xMark spectrophotometer (Bio-Rad, USA) at λ = 600 nm. At the end of cultivation (24 h), the cells were removed by centrifugation on a Beckman Avanti JXN-26 centrifuge, Beckman Coulter, Inc., USA for 15 min at 5000 rpm, and the supernatant of the culture liquid was diluted with distilled water 10 times, the pH of the solution adjusted to 6.3. Figure 3 Carboxymethyl cellulose (Sigma, USA) equilibrated with 0.02 M Na-acetate buffer, pH 6.3 was added to the solution. The mixture was gently mixed to adsorb the enzyme on carboxymethyl cellulose (CMC) for 90 min at room temperature. The sorbent was besieged, the supernatant was drained and placed on a column (Ø 3 cm, height 20 cm). The column was washed with 0.02 M sodium acetate buffer, pH 6,3. Elution was per-

formed with 0.2 M sodium acetate, pH 6.3. Fractions with high proteolytic activity were collected. As a result of purification, a proteinase preparation was obtained with a purification degree of 20.8 times with a yield of 28.4%, the specific activity of proteinase was 0.125 units/mg of protein Table 1. Proteolytic activity was determined by the azocasein cleavage assay (Sabirova *et al.*, 2010). One unit of activity was defined as the amount of enzyme necessary to change the absorbance by 1 optical density unit per min.

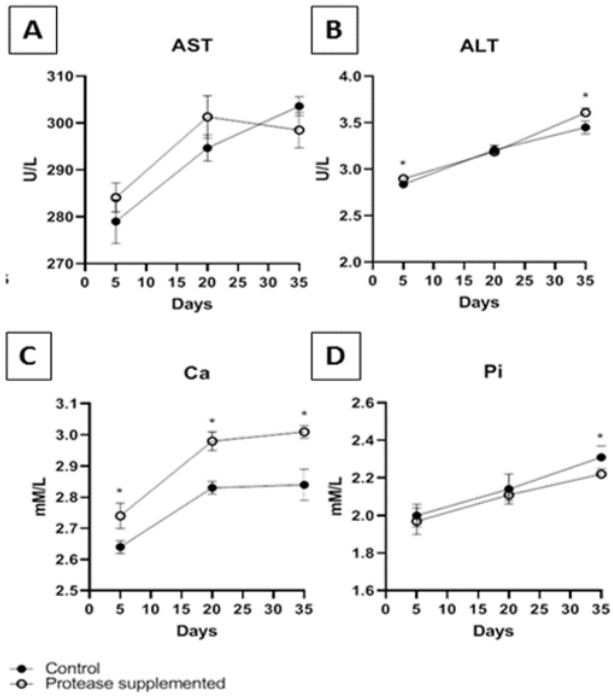

Figure 2: Biochemical indices of the blood in the control and experimental groups on days 5, 20 and 35. (A) AST -Aspartate Aminotransferase; (B) ALT - Alanine Aminotransferase; (C) Ca - Calcium; (D) Pi - inorganic phosphorus.

The purity of the product was evaluated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS electrophoresis of the purified enzyme showed the presence of a protein band with a molecular weight of 28 kDa, which corresponds to the molecular weight of *B. pumilus* subtilisin-like proteinase Figure 5. Thus, the enzyme is not a commercial product with permits and sales certificates on the market, but a product produced in the laboratory, with tests confirming its safety (Koryagina *et al.*, 2018). On the bases of preliminary experiments with poultry, we selected the concentration of the enzyme at 10 units per kg of feed as optimal. Under the described experimental conditions, we tested the addition of the enzyme at three levels: 1. 5, and 10 units per 1 kg of feed. Improvement of nutrient digestibility in the balance experiments was obtained only at a concentration of 10 units per 1 kg of feed, which corresponds to 87g proteinase/ton of feed.

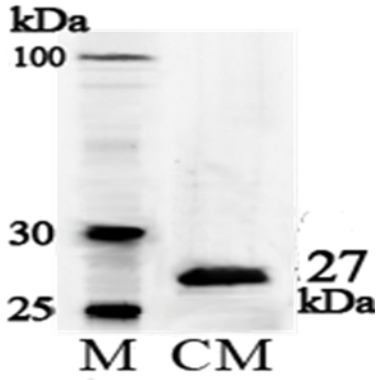

Figure 3: SDS electrophoresis of *B. pumilus* proteinase fraction after chromatography on CMC. M - protein ladder (#2610, Thermo Scientific, USA).

As a result of CMC protein purification, we obtained 6834 units proteinases Table 1, which is sufficient for treating about 600 kg of feed with the enzyme. The enzyme was stored in 0.2 M Na-acetate buffer pH 6.3 at a temperature of -20°C. Before adding the enzyme to the feed, the protein was thawed, diluted to the required concentration (10 units/kg feed) with drinking water at room temperature. The resulting enzyme solution was then used to process the chicken feed.

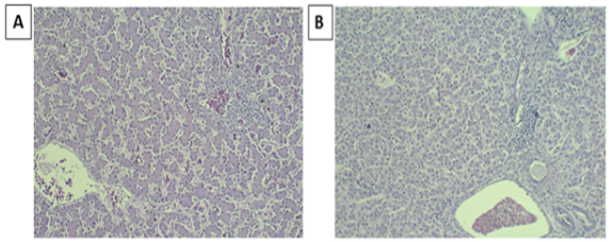

Figure 4: Histology of liver cells: A -control group, B - experimental group. Stained with hematoxylin and eosin ×100.

Management of birds

All birds were kept in accordance with the Guide for the Care and Use of Experimental Animals of Kazan Federal University and the commercial poultry farm (KFK Alimchueva Z.I., Russia, Mari El Republic, Medvedevsky District, the village of Middle Azyakovo); as well as the Directive of the European Parliament and Council on the protection of animals used for scientific purposes dated September 22, 2010 (Directive 2010/63/UE on the protection of animals used for scientific purposes). The total number of 180 day-old broiler chickens (Hubbard Classic) kept in an industrial incubator

were purchased, with initial average weight of 22 $\pm$ 0.21 g. Fowls were individually weighed, labeled using plastic rings of different colors and randomly divided into 2 groups (control and experimental) with 3 replicates (30 birds per replicate). The birds were kept in three-tier cages made of galvanized wire mesh (0.5 x 0.5 m x 0.35 m per 5 birds) with controlled temperature conditions. The initial temperature was 32°C in the first week and gradually decreased with age by 2°C per week until reaching approximately 18°C at the end of the experiment. The illumination period of 23 hours per day was provided throughout the experiment. Chicks were provided with free access to water and feed during the experimental period.

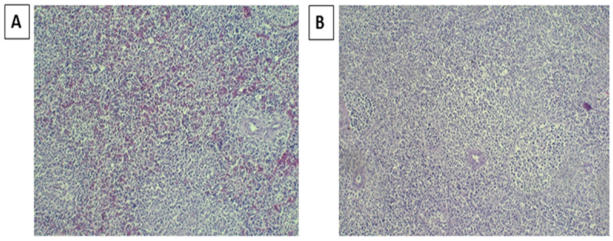

Figure 5: Histology of spleen cells: A -control group, B - experimental group. Stained with hematoxylin and eosin $\times$100.

Experimental design and diet

Two treatments: I (control group) and II (experimental group) were carried out simultaneously under identical conditions, having 90 birds in each group. The control group (group I) received a basic diet (BD) without enzyme additives. The experimental group (group II) consumed a BD supplemented with subtilisin-like proteinase (10 U/kg) from day six. Broilers were fed in abundance using dry compound feeds with nutrition parameters corresponding to the recommended feeding standards (GOST 18221-99 "Compound feed for poultry. Technical conditions"). The chemical composition of the rations is shown in Table 2.

Growth indicators

Body weight (BW) of birds was measured to the nearest gram prior to the commencement of the experiment and on subsequent days: 1, 8, 15, 22, 28 and 35. The safety of the food additive for poultry was determined by monitoring the daily mortality of birds in each group, taking into account the reasons. To study the effect of proteinase supplements on the digestibility and absorption of nutrients, a balance experiment was performed. For this, a comparative quantitative and chemical analysis of feed and the droppings of birds was carried out using the methods described in GOST 31640-2012, 32933-2014 and 31675-2012 (GOST 31640-2012 "Feed. Methods for determining the dry matter content" GOST 32933-2014 "Feed. Compound feed. Method for determining crude ash", GOST 31675-2012 "Feed. Methods for determining crude fiber content with intermediate filtration", Kjeldahl nitrogen determination method, Soxhlet extraction method for determining fat). The digestibility coefficient was calculated as the ratio of digestible to absorbed nutrients, expressed as a percentage. Feed conversion ratio (FCR) was obtained on the 35^{th} day of age, and the European productivity index (EPI) of broilers was calculated by the formula:

$$\mathrm{EPI} = \frac{\mathrm{Viability(\%)} \times \mathrm{BW(kg)} \times 100}{\mathrm{Age(d)} \times \mathrm{FCR(kg\ feed\ intake/kg\ gain)}}$$

Blood parameters

Blood samples were taken from the axillary vein of 5 randomly sampled birds at the age of 5, 20 and 35 days in each group. For blood sampling, disposable sterile vacuum systems Venosafe (Terumo, Belgium) were used. For hematological studies, vacuum systems with an anticoagulant Trilon B were used. A blood sample was thoroughly mixed before testing, moving the tube up and down and rotating it for 5 minutes. For biochemical studies, vacuum systems without an anticoagulant were used; blood serum was taken, obtained by settling in it at room temperature for 30 minutes until a clot formed completely. Hematological blood tests were performed on a MicroCC-20 Plus analyzer (USA) and biochemical blood tests on a BioChem SA semi-automatic biochemical analyzer (USA).

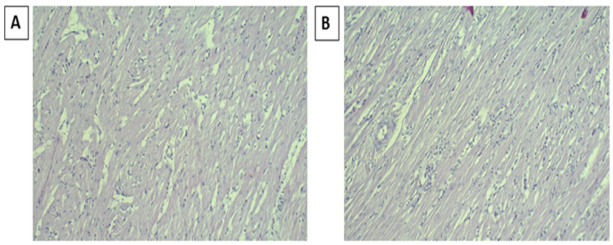

Figure 6: Histology of myocard cells: A- control group, B - experimental group. Stained with hematoxylin and eosin $\times$100.

Histomorphological studies

Ten birds per feeding group were subjected to anatomical and pathological studies on the 36th day of the experiment. After slaughtering the fowls, spleen, liver and heart were examined. To study the histological structure samples of internal organs were taken at an average size of 5*5*1.5 cm and fixed in a 10% aqueous solution of neutral formalin for three days. Fixed pieces of organs were dried, treated with chloroform, placed in blocks of paraffin

Table 1: Chromatography of subtilisin-like proteinase of B. *pumilus* 7p.

Purification stage	Volume (mL)	Protein (A_{280})	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Degree of purification	Yield ()
Culture fluid	10920	370	2.2	24024	0.006	1	100
CMC* chromatography	510	107	13.4	6834	0.125	20.8	28.4

* Carboxymethyl cellulose.

wax, cut off with a thickness of 5 μm, mounted on slides and stained with hematoxylin and eosin (OH). Histological sections were examined in transmitted light using a Leica DM 1000 microscope (Leica Microsystems, Germany) under oil immersion.

Veterinary and sanitary assessment of broiler meat

For veterinary and sanitary assessment of broiler meat, birds were humanely killed via electric damping on the 36th day of the experiment. To assess the effect of proteinase supplements on veterinary and sanitary indicators of broiler meat quality, a complex of organoleptic and laboratory studies of 3 carcasses from each group of broiler chickens killed at 36 days of age was carried out. Organoleptic research was carried out in accordance with the protocols of GOST 7702.0-74 (GOST 7702.0-74 "Poultry meat. Methods of sampling. Methods of organoleptic quality assessment"). The appearance, smell, color, texture of muscle tissue and fat, the condition of the cut muscles, transparency and aroma of chicken broth were determined. Bacteriological study of muscle tissue was performed to identify aerobic and facultative anaerobic microorganisms: *Escherichia coli*, *Salmonella*, *Clostridia*, *Proteus*, *Staphylococcus aureus* according to GOST 7702.2-74 (GOST 7702.2-74 "Poultry meat. Methods of bacteriological analysis"). Microscopic analysis of smears and physico-chemical studies of meat were performed in accordance with GOST 7702.1-74 (GOST 7702.2-74 "Poultry meat. Methods of chemical and microscopic analysis of meat freshness"). The analysis included Gram staining, pH, amino nitrogen, peroxidase, benzidine, reaction with copper sulfate, reaction with ammonia and ammonium salts, as well as acid and peroxide value of fat. The safety and biological value of meat was determined using the paramecium *Tetrahymena pyriformis* in accordance with the Guidelines for the toxicological biological assessment of meat, meat products and milk using *T. pyriformis* (1997). We examined 10 meat samples in 10 fields of view of the microscope. Analysis of toxic elements Pb, Cd, Ar, Hg was carried out by the atomic-emission method recommended by GOST 30538-97.

Statistical Analyses

Analyses of data were performed using Microsoft Excel (2016). Values were expressed as mean $\pm$ SEM of the results of three independent repetitions. Statistical significance was determined using Student's t-test with a significance value set to $P < 0.05$.

RESULTS AND DISCUSSION

Productivity and growth

To study the effect of proteinase as a feed additive on the digestibility and absorption of nutrients in broiler feed, we performed a balance experiment. For this study, we used compound feed, the nutritional value of which is presented in Table 2. The chemical composition of the complete mixed feed of broilers was fully consistent with the nutritional ration of this group of test subjects (GOST 18221-99 "Mixed full-ration feeds for poultry. Specifications"). The digestibility of nutrients entering the body through food depends on the activity of enzymes of the endocrine glands, the gastrointestinal tract, and individual organs. We calculated the digestibility coefficients of the nutrients of broiler chickens for the control group and the group with the addition of exogenous proteinase Table 3. The increase in the digestibility of the organic matter of the diet of broiler chickens of the experimental group was mainly due to protein digestion. The maximum digestibility of protein in the experimental group was 81.1$\pm$0.5%, which is 7% higher in comparison to the control group, while the digestibility of fiber and fat increased by an average of 2% ($P<0.01$). The introduction of an additional proteinase enzyme into the diet improved digestion of feed nutrients, as evidenced by an increase in digestibility ratios in the experimental groups relative to the control. Our results are consistent with data presented on the addition of *Nocardiopsis prasina* serine protease to feed the monocomponent enzyme had a beneficial effect on a feed to gain ratio and improved crud protein and fat digestibilities, and this effect was more pronounced in the high

Table 2: Composition of the basal diet fed to broilers.

Ingredient	Days 0-10	Days 11-21	Days 22-35
Wheat (%)	45	40	26
Barley (%)	5	12	10
Peas (%)	-	4	5
Corn (%)	10	-	20
Sunflower cake (%)	8	14	18
Extra Soy (%)	9	24	18
Soybean meal (%)	17	3	-
Monocalcium phosphate (%)	0.8	0.3	0.2
Salt (%)	0.3	0.3	0.3
ME (kcal/100g)	305	307	311
Crude protein (%)	23.00	21.70	18.00
Crude fiber (%)	3.55	3.73	4.77
Lysine (%)	1.43	1.32	1.08
Methionine and cysteine (%)	1.08	1.01	0.86
Threonine (%)	0.98	0.90	0.77
Calcium (%)	1.00	0.91	0.91
Potassium (%)	0.76	0.71	0.60
Sodium (%)	0.17	0.20	0.15
Chlorine (%)	-	-	0.20
Vitamin A (IU*103/kg)	14.40	12.00	12.00
Vitamin D3 (IU*103/kg)	4.80	4.00	4.00
Vitamin E (mg/kg)	72.00	60.00	60.00
Vitamin K3, mg/kg	2.40	2.00	2.00
Vitamin B1 (mg/kg)	2.40	2.00	2.00
Vitamin B2 (mg/kg)	9.60	8.00	8.00
Vitamin B3 (mg/kg)	36.00	30.00	30.00
Vitamin B4 (mg/kg)	600.00	500.00	500.00
Vitamin B5 (mg/kg)	12.00	10.00	10.00
Vitamin B6 (mg/kg)	3.60	3.00	3.00
Vitamin B12 (mg/kg)	0.03	0.025	0.025
Vitamin Bc (mg/kg)	0.60	0.50	0.50
Vitamin H (mg/kg)	0.12	0.10	0.10
Iron (mg/kg)	30.00	25.00	25.00
Copper (mg/kg)	12.00	10.00	10.00
Zinc (mg/kg)	96.00	80.00	80.00
Manganese (mg/kg)	96.00	80.00	80.00
Cobalt (mg/kg)	1.20	1.00	1.00
Iodine (mg/kg)	0.84	0.70	0.70

Table 3: Effect of the proteinase supplementation on nutrient digestibility.

Digestibility coefficient (%)	Control group (BD[1])	Experimental group (BD[1]+proteinase)	SEM[2]	P=
Protein	74±0.6	81.1±0.5	3.629	0.0005
Dry matter	72.6±0.9	75.5±1.1	1.602	0.0003
Fiber	10.3±0.4	12.4±0.2	1.150	0.0001
Fat	57.4±0.9	60.1±1.1	1.446	0,0001

BD[1]-basal diet; SEM[2]-standard error of the mean.

Table 4: The effect of proteinase on calcium, phosphorus and nitrogen utilization within the first 5 weeks of growth.

Indicator	I (BD[1])	II (BD[1]+proteinase)	SEM[2]	p =
Calcium				
Ingested (g)	1.20±0.01	1.21±0.008	0.0075	0.741
Excreted (g)	0.52±0.007	0.49±0.004	0.017	0.00079
Digested (g)	0.68±0.005	0.72±0.01	0.019	0.001
Digestibility coefficient (%)	56.6±0.002	59.5±0.03	1.598	0.000005
Phosphorous				
Ingested (g)	0.75±0.003	0.71±0.004	0.022	0.0008
Excreted (g)	0.53±0.002	0.53±0.005	0.0018	0.121
Digested (g)	0.22±0.006	0.18±0.007	0.021	0.0016
Digestibility coefficient (%)	29.3±0.015	25.3±0.005	2.19	0.0000001
Nitrogen				
Ingested (g)	3.17±0.04	3.10±0.02	0.029	0.063
Excreted (g)	1.48±0.005	1.35±0.008	0.070	0.00002
Digested (g)	1.69±0.008	1.75±0.01	0.029	0.00034
Digestibility coefficient (%)	53.3±0.006	57.4±0.04	2.245	0.0000001

BD[1]-basal diet; SEM[2]-standard error of the mean.

Table 5: Growth performance based on change in broiler weight (g).

Indicator	Control group (BD[1])	Experimental group (BD[1]+proteinase)	SEM[2]	P=
0 w[3]	22.1±0.21	22.0±0.18	0.089	1
1 w[3]	306.8±0.36	295.8±0.31	6.08	0.00008
2 w[3]	373.6±1.02	382.9±0.98	4.967	0.00001
3 w[3]	591.1±2.20	580.5±2.14	6.283	0.0095
4 w[3]	926.0±4.55	985.1±3.61	32.82	0.00018
5 w[3]	1488.3±8.67	1550.7±6.52	34.45	0.0003
Absolute gain (g)	1465.9±37.4	1528,7±42.0	54.86	0.004
Feed intake (g)	2521±7.48	2440±8.01	44.14	0.0083
Feed conversion ratio	1.75±0.078	1.61±0.041	0.075	0.05
European productivity index (EPI)	242.4±5.10	275.06±2.84	17.98	0.0016

BD[1]-basal diet; SEM[2]-standard error of the mean; w[3]-week.

protein diets (Freitas *et al.*, 2011). In carrying out our experiments, we determined the balance and absorption coefficients of nitrogen, calcium and phosphorus, taking into account their intake with an average daily amount of feed and excretion from the body with litter. Nitrogen containing feed substances, entering the gastrointestinal tract of an animal, are hydrolyzed into free amino acids, which are used for the growth and development of a growing organism. Features of the digestive system and urinary organs in birds enable assessment of the total nitrogen excreted from the body, in conjunction with the amount of nitrogen deposited in the body. A positive effect when using proteinases was obtained by the balance of nitrogen and calcium Table 4. According to our data, a higher coefficient of calcium digestibility (59.5%) was registered in the experimental group, as against 56.6% in the control group. The nitrogen balance assessment showed that in the experimental group the digestibility coefficient was 57.4%, which exceeded that of the control group by 3.1% (P<0.01). Evidently, the use of exogenous proteinase led to an increase in protein assimilation, which was reflected in the nitrogen balance. Other authors have also shown that the addition of *Aspergillus niger* protease to feed resulted in reduc-

Table 6: Blood and serum parameters of the control and experimental group on days 5, 20 and 35.

Parameters	Group I (BD[1])	Group II (BD[1]+proteinase)	SEM[2]	p=
		Day 5		
Hemoglobin (g/dL)	104.7±1.05	109.1±1.14	2.6	0.0001
Red blood cells ($\cdot 10^{12}$/L)	2.2±0.04	2.3±0.02	0.26	0.69
Hematocrit (%)	9.1±0.45	8.7±0.31	0.409	0.038
		Day 20		
Hemoglobin (g/dL)	115.3±0.95	116.0±1.08	1.213	0.09
Red blood cells ($\cdot 10^{12}$/L)	2.4±0.02	2.5±0.04	0.061	0.01
Hematocrit (%)	10.6±0.37	9.4±0.30	0.723	0.001
		Day 35		
Hemoglobin (g/dL)	127.8±1.17	129.3±1.10	1.306	0.0007
Red blood cells ($\cdot 10^{12}$/L)	2.6±0.05	2.4±0.04	0.117	0.0008
Hematocrit (%)	9.3±0.55	9.6±0.50	0.498	0.009

BD[1]-basal diet; SEM[2]-standard error of the mean.

Table 7: Physical-chemical parameters of broiler chicken meat.

Indicator	Control group (BD[1])		Experimental group (BD[1]+proteinase)		SEM[2]	p=
	Leg muscle	Breast muscle	Leg muscle	Breast muscle		
pH	5.70±0.04	5.71±0.09	5.81±0.03	5.8±0.11	$0.068^a/0.103^b$	$0.003^a/0.02^b$
Peroxidase reaction	Positive	Positive	Positive	Positive	-	-
Reaction to products of primary protein breakdown	Negative	Negative	Negative	Negative	-	-
Reaction to ammonia and ammonium salts	Negative	Negative	Negative	Negative	-	-
Amino ammonia nitrogen mg	0.77±0.02	0.83±0.02	0.75±0.04	0.86±0.02	$0.03^a/0.02^b$	$0.62^a/0.33^b$
Bacterioscopy of fingerprints	4.35±0.12	5.09±0.60	4.44±1.45	4.55±0.34	$0.92^a/0.53^b$	$0.92^a/0.07^b$

BD[1]-basal diet; SEM[2]-standard error of the mean; [a]means for leg muscle; [b]means for breast muscle.

ing dietary protein without compromising broiler performance, and thus lowering protein loss, and levels of excreted nitrogen (Ghazi *et al.*, 2003; Murugesan *et al.*, 2015). Live weight and average daily gain are important zootechnical indicators. Monitoring changes in live weight can reveal the avoidable disadvantages of growing young animals. In our studies, the measurement of the live weight of broilers was carried out throughout the experiment with a frequency of seven days Table 5. At the beginning of the experiment, the average live weight of the birds in the control and experimental groups were similar and amounted to 22.1±0.21g. On day 8, the average live weight of the fowls in the control was 3.5% higher than in the experimental group (P < 0.05). However, in subsequent weeks, the live weight of the birds in the experimental group significantly (P<0.05) exceeded the control indicators by 9g (day 15), and 59g (day 28). By the end of the experiment, at 35 days, the live weight in the experimental group was 1550.7±6.52g (P < 0.05). Absolute growth indicators were 1528.7±42g and 1465.9±37.4g in the experimental and control groups, respectively (P<0.05). Feed conversion ratio (FCR) of broilers by the 35th day was 1.61±0.041 in the experimental group, while the control group registered a higher value at 1.75±0.078 (P=0.05). In addition to the fact that the use of proteinase contributed to an increase in bird growth rate, the viability of birds in both groups was 100%. Consequently, the EPIs of the control and experimental birds were 242.93±5.1 and 275.06±2.84 (P<0.05), respectively. Thus, it can be completed the use of the proteinase could

be prospective in reducing cost of the final product. Exogenous proteases can enhance the action of endogenous peptidases due to the hydrolysis of protein anti-nutritional factors such as lectins, trypsin inhibitors and antigenic proteins (Murugesan *et al.*, 2015). The gastrointestinal tract quickly adapts to changes in diet, as the activity of endogenous digestive enzymes is modulated in response to physiological needs (Wang *et al.*, 2005). It has been reported that the addition of protease into the diet of broiler can significantly improve the digestibility of amino acids (Angel *et al.*, 2011).

Adding exogenous protease (commercial product from *Bacillus licheniformis*) to the diet of broiler chickens was beneficial in improving the digestibility of amino acids, the body weight gain and feed conversion ratio (Mohammadigheisar and Kim, 2018). On the other hand new fungal serine proteases supplementation did not improve broiler growth performance or N digestibility above that of a nutrient adequate control diet (Walk *et al.*, 2012, 2019). The response to protease supplementation is not equally always beneficial depending on the origin of the enzymes, the conditions of their use and biochemical characteristics. These findings suggest that addition of new protease always requires testing and further justification for use in mono gastric diets. Seemingly, the improvement in the digestibility of amino acids in the diet, originating from any exogenous proteases, depends on the composition of the protein ingredients used in the feed formulation (Angel *et al.*, 2011).

Biochemical parameters of blood and serum

Poultry farming in modern industry implicates a large physiological burden on the organism of birds. Errors in technology and formula of feeding can cause irreversible changes in the metabolism of birds and lead to disease or death. To prevention such errors and disorders the assessment of hematological and biochemical parameters of blood are evaluated during life. We measured the blood parameters of broiler chickens on days 5, 20, and 35. With age, the concentration of hemoglobin, erythrocytes, leukocytes and hematocrit in the blood of broilers of the control and experimental groups increased, at the end of the experiment the concentration of hemoglobin remained within the physiological norm and amounted to 127.8 $\pm$ 1.17 g/L and 129.3$\pm$1.10 g/L in the control and experimental groups, respectively ($P < 0.05$). The hemoglobin content reflects the metabolic rate, increasing with the growth and development of chickens. The level of hematocrit on day 35 relative to day 5 was higher by 0.2% and 0.9% in the control and experimental groups, respectively ($P < 0.05$) Table 6. Biochemical blood tests are important when developing and evaluating the effect of new feed additives on the bird's organism. The total serum protein content is an important indicator of growth intensification, which determines the state of natural resistance and physiological activity of broiler chickens. Throughout the experiment, this indicator did not exceed normal values in both groups, however, the level of total protein on day 5 was 24.1$\pm$2.1 g/L in the control, and 25.7$\pm$1.6 g/L in the experimental group. On day 35, protein content in the blood serum of the birds was 10.5% higher in the experimental group Figure 1 a.

The concentration of urea (the end product of protein metabolism in birds) in blood serum depends on the rate of protein breakdown in the body. At the age of 20 days and 35 days, broilers showed a decrease in urea concentration by an average of 7% in the experimental group compared to the control group ($P < 0.05$) Figure 1 b. Creatinine, being the end product of creatine metabolism, plays an important role in tissue energy metabolism. At days 20 and 35, creatinine level was 7.2% and 13.7% lower in the experimental group in comparison to the control ($P < 0.05$) Figure 1 c. The creatinine (an indicator of catabolism) levels recorded showed a predominance of anabolic processes over catabolic activities in birds when an exogenous protease was added to feed. The levels of calcium and inorganic phosphorus in the blood serum of broilers in the control and experimental groups increased during the experiment, but remained within the physiological norm (not exceeding 4.5 mmol/L). In this case, the calcium level in the experimental group exceeded the calcium level in the control throughout the experiment, in contrast to the phosphorus level, which in the experimental group did not vary significantly from values recorded in the control group Figure 2 a, b, c, d. The difference in calcium content provides metabolic benefits to the experimental birds and is a result of improved bone structure.

The effect of protease on the veterinary sanitary assessment of broiler meat

Veterinary sanitary examination of broiler chicken meat was carried out in accordance with Russian state standards 7702.0-74, 7702.1-74 and 7702.2-74 Table 7. The carcasses of broilers in the control group and the tested groups examined 24 h after the slaughtering had a similar appearance: dry surface, whitish-yellow skin with a pink tint, dense muscles, elastic in texture, and slightly moist at the sites of cut. The chicken breast muscles and dark meat samples appeared normal. The odor on the surface and

on cut muscles corresponded to the scent of standard fresh meat. Chicken fat was pale yellow, elastic without extraneous odors. Analysis of the parameters of adipose tissue (peroxide and acid number) of both groups corresponded to Russian state standards for human consumption without any restrictions. The activity of the muscle tissue enzyme (peroxidase) is preserved in fresh and meat of good quality. Using a slight acidic reaction of the medium, its determination is an important indicator of the sanitary state of meat quality. The reaction to peroxidase of meat samples collected in both groups was positive. On the contrary, the reaction to the products of the primary decomposition of proteins, ammonia, and ammonium salts was negative for samples of both broiler groups Table 7.

The content of volatile fatty acids is another important indicator of the sanitary assessment of meat quality. In accordance with GOST 7702.1-74, the content of volatile fatty acids for fresh, high-quality poultry meat should not exceed 4.5 mg KOH/g (GOST 7702.1-74 "Poultry meat. Methods of chemical and microscopic analysis of meat freshness"). The analysis showed that for meat samples collected from both groups, this indicator was in the range of 1.92-2.08 mg KOH (Potassium hydroxide)/g. The acid content in fats collected from chilled and frozen poultry carcasses should not exceed 1 mg KOH/g according to GOST (GOST 7702.1-74 "Poultry meat. Methods of chemical and microscopic analysis of meat freshness"). For broiler fat from the control group and the proteinase treated group, acid numbers were 0.55±0.02 KOH/g and 0.57±0.02 KOH/g, respectively. Analysis of the muscle surface in samples collected from both groups showed the presence of isolated cocci and gram-positive rod-shaped bacteria under the microscope, which is an indicator of the freshness of meat (SanPin 2.3.2.1078-01, Hygiene requirements for food safety and nutritional value, rules and standards Health and Hygiene, 2002).

According to the relative biological value of meat, the experimental and control groups did not have significant differences (99.0-99.2%). Meat from any of the groups was non-toxic for Tetrahymena pyriformis, the number of altered cell forms ranged from 0,1 to 1.0% (Guidelines for toxicological evaluation of meat, meat products and milk using *Tetrahymena pyriformis*, 1997). Analysis of meat and feed contamination with heavy metals (lead, cadmium, mercury and arsenic) was negative for samples taken from both groups of birds. Thus, the meat of broiler chickens, which additionally received proteinase in the diet, met the standards for human consumption and met the state standards of Russia (GOST) for fresh meat of good quality in terms of organoleptic, physico-chemical and bacterioscopic characteristics. We performed a histological examination of tissue samples of the liver, spleen and myocardium of carcasses of birds after using bacterial proteinase as a dietary supplement in experimental and control variants. For the study, tissue samples of 10 spontaneously selected bird carcasses from each experiment were used.

In all cases histological studies showed that the proteinase-based supplement did not cause pathological changes in the parenchymal organs of broiler chickens Figure 6. We tested a subtilisin-like serine proteinase *B. pumilus* as a feed additive, which we developed and obtained in the laboratory in the amount necessary for the test. Our results showed that the addition of proteinase in chickens in the amount of 10 units per 1 kg of feed leads to an improvement in indicators such as body weight gain (BWG) and feed conversion ratio (FCR). These results may be due to the fact that the enzyme is stable and retains activity in the digestive tract of birds, where, together with endogenous digestive enzymes, it breaks down raw feed protein. This conclusion is consistent with the data on the results of the balance sheet experiment. The results of our study are consistent with other studies where feeding broiler chickens with commercial serine protease supplementation had a positive effect on growth and absorption of raw feed protein (Lemme *et al.*, 2004; Angel *et al.*, 2011; Mohammadigheisar and Kim, 2018).

It has also been reported that the addition of protease to the diet has a positive effect on the digestibility of amino acids, which leads to an improvement in BWG and FCR (Angel *et al.*, 2011; Liu *et al.*, 2013). The authors suggested that the positive effect of exogenous protease on the digestibility of amino acids could be additionally due to the ability to target protease inhibitors (Liu *et al.*, 2013; Mahmood *et al.*, 2018). Another beneficial effect of adding proteinase is improved digestibility of calcium and nitrogen, which will reduce the level of Ca in the diet. Proteinases are added to feed with the purpose of increasing dietary protein hydrolysis and thus enabling improved nitrogen utilization. The use of proteinase can contribute significantly to current efforts to reduce nitrogen emissions from livestock production. This may affect the reduction of environmental pollution (Oxenboll *et al.*, 2011; Alagawany *et al.*, 2018).

CONCLUSIONS

Based on our results, the addition of proteinase to the diet led to improved growth rates reduced feed intake. Inclusion of proteinase based supplements contributed to efficient digestion and increased digestibility of nutrients. The biochemical parameters of blood serum remained within the physiological norm. The experimental broiler meat meets the Russian state standards (GOST) for fresh meat of good quality in terms of organoleptic and physical-chemical parameters. Thus, subtilisin-like *B. pumilus* proteinase can be recommended as a feed additive for broiler chickens at a concentration of 10 U / kg of feed without harmful effects on bird health.

Funding Support

This work was performed in accordance with the Russian Government Program of Competitive Growth of Kazan Federal University and supported by the Russian Science Foundation (Project No. 16-16-04062).

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

REFERENCES

Alagawany, M., Elnesr, S. S., Farag, M. R. 2018. The role of exogenous enzymes in promoting growth and improving nutrient digestibility in poultry. *Iranian Journal of Veterinary Research*, 19(3):157–164.

Angel, C. R., Saylor, W., *et al.* 2011. Effects of a monocomponent protease on performance and protein utilization in 7- to 22-day-old broiler chickens. *Poultry Science*, 90(10):2281–2286.

Borda, M. D., Zuber, T., *et al.* 2019. Effects of protease and phytase supplements on small intestinal microbiota and amino acid digestibility in broiler chickens. *Poultry Science*, 98(7):2906–2918.

Cowieson, A. J., Adeola, O. 2005. Carbohydrases, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. *Poultry Science*, 84(12):1860–1867.

Freitas, D. M., Vieira, S. L., *et al.* 2011. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono-component protease. *Journal of Applied Poultry Research*, 20(3):322–334.

Ghazi, S., Rooke, J. A., Galbraith, H. 2003. Improvement of the nutritive value of soybean meal by protease and a-galactosidase treatment in broiler cockerels and broiler chicks. *British Poultry Science*, 44(3):410–418.

Giannenas, I., Bonos, E., *et al.* 2017. Effects of Protease Addition and Replacement of Soybean Meal by Corn Gluten Meal on the Growth of Broilers and on the Environmental Performances of a Broiler Production System in Greece. *PLOS ONE*, 12:e0169511–e0169511.

Gupta, R., Beg, Q., Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, 59(1):15–32.

Kaczmarek, S. A., Rogiewicz, A., *et al.* 2014. The effect of protease, amylase, and nonstarch polysaccharide-degrading enzyme supplementation on nutrient utilization and growth performance of broiler chickens fed corn-soybean meal-based diets. *Poultry Science*, 93(7):1745–1753.

Koryagina, A. O., Rudakova, N. L., *et al.* 2018. Gene construct-based serine protease of Bacillus pumilus as a feed additive for poultry farming. *Agricultural Biology*, 53(6):1274–1284.

Laemmli, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227(5259):680–685.

Lemme, A., Ravindran, V., Bryden, W. L. 2004. Ileal digestibility of amino acids in feed ingredients for broilers. *World's Poultry Science Journal*, 60(4):423–438.

Liu, S. Y., Selle, P. H., Cowieson, A. J. 2013. The kinetics of starch and nitrogen digestion regulate growth performance and nutrient utilisation of broilers fed coarsely ground, sorghum-based diets. *Animal Production Science*, 53(10):1033–1033.

Mahmood, T., Mirza, M. A., *et al.* 2018. Exogenous protease supplementation of poultry by-product meal-based diets for broilers: Effects on growth, carcass characteristics and nutrient digestibility. *Journal of Animal Physiology and Animal Nutrition*, 102(1):e233–e241.

Mikhailova, E. O., Balaban, N. P., *et al.* 2009. Purification of a subtilisin-like serine proteinase from recombinantBacillus subtilis during different phases of growth. *Annals of Microbiology*, 59(2):301–307.

Mohammadigheisar, M., Kim, I. H. 2018. Addition of a protease to low crude protein density diets of broiler chickens. *Journal of Applied Animal Research*, 46(1):1377–1381.

Murugesan, G. R., Syed, B., *et al.* 2015. Phytogenic feed additives as an alternative to antibiotic growth promoters in broiler chickens. *Frontiers in*

Veterinary Science, 2(21):1–6.

Oxenboll, K. M., Pontoppidan, K., Fru, F. 2011. Use of a Protease in Poultry Feed Offers Promising Environmental Benefits. *International Journal of Poultry Science*, 10(11):842–848.

Razzaq, A., Shamsi, S., *et al.* 2019. Microbial Proteases Applications. *Frontiers in Bioengineering and Biotechnology*, 7(110):1–20.

Sabirova, A. R., Rudakova, N. L., *et al.* 2010. A novel secreted metzincin metalloproteinase from Bacillus intermedius. *FEBS Letters*, 584(21):4419–4425.

Shagimardanova, E. I., Toymentseva, A. A., *et al.* 2014. Draft Genome Sequence of Bacillus pumilus 7P, Isolated from the Soil of the Tatarstan Republic, Russia. *Genome Announcements*, 2(3):599–614.

Toghyani, M., Wu, S. B., *et al.* 2017. Performance, nutrient utilization, and energy partitioning in broiler chickens offered high canola meal diets supplemented with multicomponent carbohydrase and mono-component protease. *Poultry Science*, 96(11):3960–3972.

Walk, C. L., Bedford, M. R., McElroy, A. P. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. *Poultry Science*, 91(6):1371–1378.

Walk, C. L., Juntunen, K., *et al.* 2019. Evaluation of novel protease enzymes on growth performance and nutrient digestibility of poultry: enzyme dose response. *Poultry Science*, 98(11):5525–5532.

Wang, Z. R., Oiao, S. Y., *et al.* 2005. Effects of enzyme supplementation on performance, nutrient digestibility, gastrointestinal morphology and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. *Poultry Science* , 84:875–881.

Yuan, C., Ding, Y., *et al.* 2015. L-arginine upregulates the gene expression of target of rapamycin signaling pathway and stimulates protein synthesis in chicken intestinal epithelial cells. *Poultry Science*, 94(5):1043–1051.