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The Effect of Bacterial Enzyme-Based Feed Additives on the Productivity, Digestibility and Assimilation of Nutrients in Young Laying Hens

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Abstract: The work aimed to study the effect of feed additives based on proteinase and phytase on the productivity, digestibility of nutrients and the development of laying hens up to 18 weeks of age. A total of 360 1-day-old Hisex Brown chickens were assigned to a completely randomized design composed of 3 diets with 4 replicates of 30 birds each. Dietary treatments were: (1) Control group: Basic diet with nutritional parameters consistent with recommended feeding standards, without enzymes additives, (2) experimental group A: Basic diet with the addition of proteinase at a concentration of 10 U/kg, (3) experimental group B: Basic diet with the addition of phytase at a concentration of 1000 FTU/kg. It has been shown that by adding microbial enzyme is increased digestibility of organic matter of diet of laying hens ($p<0.05$): The use of proteinases and phytase had a favourable influence on the absorption of calcium, phosphorus and nitrogen. The addition of enzymes in the feed resulted in an increased in body weight and weight gain in absolute hens with a decrease in the total amount of feed consumed by birds ($p<0.05$). The inclusion of proteinase and phytase in the diet of laying hens increases the digestibility of nitrogen, phosphorus and calcium, leads to a decrease in the amount of feed consumed and also does not adversely affect histomorphological and biochemical blood parameters.

Keywords: Hisex Brown Laying Hens, Feed Additives, Proteinase, Phytase, Balance Experience, Digestibility

Introduction

The use of feed enzymes is an actively developing area in the nurturing of farm birds and has shown active growth over the past decade. The need for the use of enzymes is rooted in the development of new advanced technologies of modern poultry farming, the increased need for productive crosses and the importance of reducing the cost of the final product.

Enzymes play a key role in the digestion processes of animals. Their activity determines the dietary indicator referred to as digestibility, i.e., the degree of nutrient assimilation of the feed. An important indicator for the productivity of laying hens is egg production, which largely depends on the diet. Balancing the contents of protein, amino acids, minerals and vitamins is essential for high productivity in this category of birds. The feed mixture for laying hens is based on grain containing an increased amount of poorly digestible phytates, which accommodate up to 80% of the total phosphorus content in the grain (Vieira *et al*., 2016).

Chickens remain deficient in phosphates from phytic acid due to the low activity of their intestinal phytases, thus the bulk of ingested phytates transits through the gastrointestinal tract of chickens unassimilated. The addition of inorganic phosphorus to the diet leads to the accumulation of excess phytin phosphorus in excreta, which in turn causes environmental pollution and eutrophication of water bodies (Abd El-Hack *et al*., 2018).

To enhance the efficacy of grain-based compound feeds, phytases are added to the diet for phytate hydrolysis and the release of phosphates. Besides improving the phosphorus nutrition of animals, which cuts back its accumulation in droppings, the addition of phytases to feed increases the bioavailability of many minerals and proteins that would be otherwise bound to phytate (Dersjant-Li *et al*., 2015). Successful application of feed phytases in poultry farming has been reported for broilers, turkeys and geese (Abd El-Hack *et al*., 2018).

A positive effect of phytase addition on feed intake, digestibility of minerals and nutrients, egg production and weight, as well as eggshell quality has been established in laying hens (Hassanien and Sanaa, 2011; Kim *et al*., 2017).

In addition to phytases, proteinases, which ensure the most efficient use of the protein component of feed, are actively gaining popularity. It is crucial that proteinases do not cause self-proteolytic inactivation in the body, but synergize in their modus operandi with digestive proteases (Yuan *et al*., 2017; Jiang *et al*., 2020). Several studies have shown that supplementing poultry feed with proteinases improves growth indicators and amino acid digestibility (Dozier III *et al*., 2010; Angel *et al*., 2011; Lee *et al*., 2018; Mahmood *et al*., 2018; Siegert *et al*., 2019). Exogenous proteinases serve prophylactic purposes and reduce the level of undigested protein and thus, hampering colonization of intestines by pathogenic bacteria (Timbermont *et al*., 2010; Yan *et al*., 2017). Proteinases boost the number of probiotic species including *Lactobacillus* and decrease the population of pathogenic bacteria such as *C. perfringens* in the ileum of broiler chickens Ross 308 (Giannenas *et al*., 2017; Borda-Molina *et al*., 2019).

We studied the prospective application of histidine acid 3-phytase of *Pantoea sp*. 3.5.1 and subtilisin-like proteinase of *B. pumilus* 7 p as dietary supplements for laying hens. An important aspect of the novelty of this work is that the producing strains were obtained from local soils and the bacterial enzymes were first isolated and described in detail in our laboratory. The MALDI-TOF method was used to establish primary structures of the enzymes. Genes of the corresponding proteins were cloned and the DNA of producer strains were sequenced (AN JHUD00000000; AN JMRT00000000.2) (Suleimanova *et al*., 2015a; 2015b; Mikhailova *et al*., 2009a).

For preliminary purification, a sufficient quantity of enzymes was obtained from media cultured with the microbial strains. The phytase of *Pantoea sp*. 3.5.1. was cloned in *Pichia pastoris* (Troshagina *et al*., 2018). Effective secretion by the yeast and glycosylation modification properties were noted for the enzyme. The obtained highly purified phytase (80 kDa) was stable at a pH range of 2.0-5.0 with maximum activity at pH 3.0 (authentic protein at pH 4.5), which conforms to the acidic environment in the chicken stomach. For the recombinant phytase, the optimum temperature range was found to be from 37 to 50°C. With an increased thermostability, the recombinant enzyme retained more than 95% activity at 70°C, while the native enzyme lost more than 50% activity at 50°C (Suleimanova, in press). Increased thermal stability is an important factor in the production of food additives. The optimum temperature of the purified proteinase was 37°C; for the purpose of diet supplementation, it is important that calcium ions at a final concentration of 5 mM increase the temperature optimum of the enzyme to 50°C (Mikhailova *et al*., 2009a; 2009b). The optimum pH of the proteinase was pH 9.5; the proteinase is stable in a pH range of 7-10. More than 90% of phytase activity was retained in the presence of gastric juice of chickens (pH 3.0), whereas pancreatic and intestinal juice inhibited its activity by 10%. The bacillary proteinase retained up to 60% activity in the presence of gastric juice of chickens (pH 3). In the presence of pancreatic and intestinal juice, the proteinase's activity was fully preserved. At bile concentrations from 0.01 to 0.05% for 1 h, the proteinase's activity remained at the control level. With an increase in concentration to 1%, a 10% decrease in enzyme activity was observed. At a bile concentration of 5%, the residual activity of the enzyme was 60%. Its activity was not suppressed by natural inhibitors, such as trypsin inhibitor, which allows it to function in the gastrointestinal tract of chickens (Koryagina *et al*., 2018). Grounding on the abovementioned properties, the obtained enzymes were concluded to be prospective feed supplements for poultry.

The present work aimed to study the effect of feed additives based on bacterial phytase and proteinase on the productivity, digestibility and assimilation of nutrients of young laying hens until 18 weeks of age.

Materials and Methods

Ethics Statement

The experimental protocol was designed according to the guidelines of the current European Directive (2010/63/EU) on the care and protection of animals used for scientific purposes and approved by the Ethical Committee of the Kazan Federal University (Russian Federation, Kazan, accession number No 459/06/04/2020).

Extraction and Purification of Bacterial Enzymes

The phytase preparation was obtained on the basis of the recombinant strains of the yeast *Pichia pastoris* pPINK-agpP that we developed (Troshagina *et al*., 2018). To obtain desired amounts of phytase, the *P. pastoris* pPINK-agpP strain was cultured in a Biotron LiFlus SP30L bioreactor (Biotron, Inc., Korea).

Following the fermentation process, yeast cells were precipitated by centrifugation and the culture fluid was collected. The residual phytase-containing supernatant was passed through a UV-0.5-30-PS hollow fiber module (Faserkraft, Russia) to concentrate the enzyme. As a result of fermentation and concentration processes, about 500 thousand units of recombinant phytase were obtained, which were then stored at -20°C.

Phytase activity was determined by the amount of phosphorus released during hydrolysis of the substrate sodium phytate (Sigma Aldrich, USA) according to Greiner's method (Greiner, 2004). A unit of phytase activity was taken as the amount of enzyme necessary for the cleavage of sodium phytate to form 1 μM inorganic phosphate in 1 min.

Subtilisin-like proteinase was isolated from the *B. pumilus* 7 p strain and purified as previously described by (Koryagina *et al*., 2018). *B. pumilus* 7 p is a natural soil isolate, which shows an increased level of subtilisin-like proteinase secretion. The bacterium was cultured in a medium with 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_4-0.2, NH_4Cl-0.2. A preliminary study on the dynamics of proteolytic activity showed that maximum activity occurs at the 24th h of growth. The supernatant was obtained by centrifugation (Beckman Avanti JXN-26 centrifuge, Beckman Coulter, Inc.) of the culture fluid for 15 min at 15,000 rpm. For maximum protein sorption, the supernatant was diluted (1:10) with water prior to the addition of Carboxymethylcellulose (CMC), the pH of the solution was adjusted to 6.3. CMC, (Sigma, USA) was equilibrated with 0.02 M Na-acetate buffer (pH 6.3), added to the enzyme solution and gently stirred for 90 min at room temperature. After the natural deposition of the sorbent, the supernatant was discarded. Proteolytic activity in the supernatant did not exceed 0.005 u/mL. The sorbent was placed on a glass column (Ø 3 cm, height 20 cm) and washed with 0.02 M sodium acetate buffer, (pH 6.3). Elution was performed with 0.2 M sodium acetate, (pH 6.3). Fractions with high proteolytic activity were collected and pooled. With the aid of CM chromatography, a proteinase preparation was obtained with a purification degree of 20 and with a yield of 29.3% the specific activity of proteinase was determined to be 0.1 u/mg protein (Table 1). Proteinase activity was determined by the hydrolysis of azocasein (Sigma, USA) according to the method described in (Sabirova *et al*., 2010).

The unit of activity was taken as the amount of the enzyme hydrolyzing 1 μg of the substrate in 1 min under the experimental conditions.

SDS-PAGE electrophoresis (Laemmli, 1970) of the protein fraction following the CMC purification phase showed the presence of a protein with a molecular weight of 28 kDa, which corresponds to the molecular weight of *B. pumilus* subtilisin-like proteinase (Appendix, Fig. S1).

Management of Layer Birds

A total of 360 1-day-old Hisex Brown hens were weighed individually and randomly assigned to 3 treatments with 4 replicates of 30 birds each. The treatments consisted of 3 distinct diets: (1) Control group: Basic diet without proteinase and phytase, (2) experimental group A (Proteinase group): Basic diet with the addition of proteinase at a concentration of 10 U/kg, (3) experimental group B (Phytase group): Basic diet with the addition of phytase at a concentration of 1000 FTU/kg. Feed pellets were sprayed with aqueous solutions of enzymes at the desired concentrations at room temperature. During the first 5 days of age, chickens of all groups exclusively received the basic diet. From the 6th day of age, birds of the experimental groups were fed with their respective experimental diets. The ingredient and nutrient contents of basic diet are shown in Table 2. Each 30-birds group in each repeat of the experiment was placed in a separate cages (1630×980×680 mm) under controlled climate conditions with temperature maintained at 18°C, 65% relative humidity, alternating lighting period (16L:8D) and light intensity of 12 lux. Feed and water were available *ad libitum*. The experiment lasted for a total of 112 days.

Hematological and Biochemical Analysis of Chicken Blood

For hematological and biochemical analysis of venous blood collection was carried out in 5 birds, randomly sampled from each group on days 30, 60 and 90 of age. Whole blood heparinized and stabilized in Trilon B was used. With the aid of MicroCC-20 Plus (USA) and BioChem SA (USA) analyzers, blood parameters such as erythrocytes, leukocytes, hemoglobin, hematocrit, total protein, urea, creatinine, calcium, inorganic phosphorus, as well as aspartate, alanine and aminotransferase activities were measured.

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

Purification phase	V, mL	Protein, A_{280}	Activity, U/mL	Total activity, U	Specific activity, U/mg	Purification degree	Yield, %
Culture fluid	12200	410	2.09	25498	0.005	1	100
CM-cellulose	570	131	13.10	7467	0.100	20	29.3

Table 2: Diet composition of laying hens

	Age, weeks		
Ingredients	1-8	8-16	16-18
Corn, %	48.4	36	10.4
Wheat, %	20.3	30.2	48
Barley, %	-	-	30
Soybean meal, %	12.8	2	-
Fish powder, %	1	-	-
Sunflower meal, %	12.8	19.5	2
Common salt, %	0.3	0.3	0.4
Chalk, %	3.0	2.4	1.2
Methionine, %	0.1	-	-
Lysine, %	0.1	0.2	-
Monocalcium phosphate, %	1.2	1	-
Extruded soy, %	-	8.4	-
Herbal flour, %	-	-	6
Meat and bone meal, %	-	-	2

Assessment of Productivity, Digestibility and Nutrient Absorption in Birds

Poultry growth was assessed, by monitoring live weight indicators at the beginning and end of each age period. The absolute (BW) and average daily weight gain were calculated. The safety of food additives for birds was determined by monitoring the safety in each group of birds, taking into account the cause of death. To study the effect of the addition of proteinase and phytase on the digestibility and assimilation of nutrients by birds, a balance test, involving two periods (a preliminary duration of 6 days and an accounting duration of 5 days) was carried out. The birds were kept in cages with a meshed floor, under which frames made of plastic film were installed to collect droppings. In each group of birds, the quantity and chemical composition of the consumed feed and the collected litter were taken into account. Chemical analysis of feed and litter 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 8th and 16th weeks of age and the European Productivity Index (EPI) of layers was calculated using the formula:

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

The Effect of Enzymes on the Structure of Internal Organs

To study the effect of enzymes on the structure of internal organs after 112 days of growth, 3 randomly sampled birds each from the control and experimental groups were slaughtered. After the slaughter of laying hens, the spleen, liver and heart were collected and tissue samples (5×5×1.5 cm in size) of the internal organs were taken for the histological analysis. The material was 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 wax, cut off with a thickness of 5 μm, mounted on slides and stained with hematoxylin and eosin. Histological sections were examined in transmitted light using a Leica DM 1000 microscope under oil immersion. Photographs were taken with a Nikon coolpix 4500 digital camera.

Statistical Analysis

Statistical processing of the results was performed in Graph Pad Prism, Graph Pad Software, version 8.0 (LA Jolla, CA, USA) using a two-way ANALYSIS Of Variance (ANOVA) and Tukey test for multiple pairwise comparison of quantitative indicators of the different groups. The results were presented as the mean ± SD, considering P value <0.05 as significant.

Results and Discussion

To study the effect of phytase and proteinase on the digestibility and assimilation of nutrients (calcium, phosphorus and nitrogen) in layer feed, balance tests were performed on young laying hens at 8 and 16 weeks of age. The daily ingested and excreted amounts of elements were compared and the digestibility of the nutrients was calculated based on the nutritional value of the feed (Table 3).

The chemical composition of the complex feed corresponded to the nutritional value for this type of poultry in accordance with GOST 18221-99 "Complete feed for full-time poultry. Technical conditions". The digestibility of nutrients entering the body depends on the enzymatic activity of secretory glands of the gastrointestinal tract. The introduction of exogenous phytases and proteinases with retained catalytic function, under optimal conditions, in the gastrointestinal tract is aimed at increasing the digestibility of nutrients in the feed mixture and reducing the cost of feed for growing young birds. From our data, the final feed mixture contained cereal components with phosphates at an amount of 6 mg/g of feed.

In birds aged 1 to 8 weeks old in the experimental group that received bacterial proteinase (Proteinase group) the protein digestibility coefficient was 89.1±2.34%. This was 7.9% more in comparison to the control group (Control group) and 6% more in comparison to the phytase receiving experimental group (Phytase group) [F (2.6) = 8.264, p = 0.018] (Table 4). This trend was continually observed in young laying hens likewise from the 8th to 16th weeks of age: The protein digestibility coefficient remained the highest in the Proteinase group and amounted to 67.3±2.06% as against 61.1±1.60% and 64.2±2.14% in the Control and Phytase groups, respectively [F (2.6) = 10.688, p = 0.02] (Table 4). No significant statistical difference (p > 0.05) was detected in the digestibility of dry matter and fat among the three groups between the 1st and 16th weeks of age. From the obtained data, we can infer that phytase and proteinase do not take a significant part in fat metabolism due to their specificity in function. Interestingly, the influence of phytase and proteinase on the absorption of fiber by birds decreases with age. We suppose that this is due to the changes in the structure of the gut microbiota of the birds, which depends more on the age of the bird than on the diet (Ballou *et al*., 2016). The phyla *Bacteroides* and *Firmicutes*, which are dominant in the intestines of adult birds play an important role in the metabolism and degradation of complex polysaccharides (Magnúsdóttir *et al*., 2017; Medvecky *et al*., 2018). Thus, their influence on the absorption of fibers is expected. Under the conditions of a well-formed microbiome (8-16 weeks), exogenous phytase and proteinase may show a minimal effect on the absorption of fiber. However, in young birds (1-8 weeks), they can contribute to more effective fiber absorption.

The digestibility coefficient of fat was higher by a 5% range in birds of the experimental groups [F (2.6) = 7.050, p = 0.026]. According to our data, a steady rise in the digestibility coefficient of protein and organic compounds is observed in birds that received additional bacterial enzymes to basic feed mixture. Throughout the experiment, the addition of exogenous proteinase at a concentration of 10 U/kg to the diet contributed to an increase in the digestibility of proteins due to a more effective breakdown of the protein components of the feed mixture.

Table 3: Nutritional value of the combined feed used in the balance experiment, g

Component	Amount per 100 g
Weeks 1- 8	
Crude protein	19.5
Crude fats	5.00
Crude fiber	3.22
Calcium	1.0
Phosphorus	0.44
Weeks 8 - 16	
Crude protein	18.3
Crude fats	4.1
Crude fiber	3.6
Calcium	0.9
Phosphorus	0.40

Table 4: Digestibility coefficients of compound feed nutrients, %, ± SD

	Groups			
Indicators	Control group	Proteinase group	Phytase group	p =
Weeks 1-8				
Protein digestibility	81.2±2.11	89.1±2.34	83.4±1.95	0.018
Dry matter	62.7±2.25	67.6±2.12	63.5±2.55	0.083*
Fiber	15.3±0.77	18.4±0.63	16.9±0.70	0.02
Fat	85.3±2.91	92.4±3.25	89.4±3.18	0.087*
Weeks 8-16				
Protein digestibility	61.1±1.60	67.3±2.06	64.2±2.14	0.02
Dry matter	52.9±2.76	55.8±1.84	54.1±3.11	0.45*
Fiber	10.1±0.66	11.9±0.81	11.6±0.79	0.055*
Fat	62.7±3.14	68.7±3.90	67.7±3.47	0.16*

*Not significant value (p>0.05)

A high dietary protein content remains essential for young birds, owing to the inadequate secretion of proteases by their pancreas and a subsequent reduction in the level of protein hydrolysis and the absorption of amino acids (Angel *et al*., 2011). Proteinases are enzymes that hydrolyze high polymer proteins to amino acids, thus added feed enzymes are most effective precisely during the early development of poultry. This paves way for an increase in digestible protein indicators by up to ~4% (Kononenko, 2016).

Data analysis of the balance of calcium, phosphorus and nitrogen in the three groups of young laying hens aged 1 and 16 weeks was conducted. In birds aged 8 weeks, the digestibility coefficient of calcium in both experimental groups was higher, relative to the control and amounted to 57.1±0.2 for the Proteinase group and 52.8±0.3 for the Phytase group [F (2.6) = 36.5, p = <0.0001] (Table 5). The digestibility coefficient of phosphorus for the selected period was highest in the Phytase group and amounted to 65.5±0.4%, which exceeded the Control group by 22.2% and the Proteinase group by 13.8% [F (2.6) = 170.59, p = 0.000005] (Table 5). The digestibility coefficient of nitrogen in the experimental group with proteinase exceeded the indicators of the control group by 7.5% and that of the Phytase group by 5.6% [F (2,6) = 103, p = 0.00002] (Table 5).

Table 5: Calcium, phosphorus and nitrogen balance in birds from weeks 1 to 8, ± SD

	Groups			
Feed parameters	Control group	Proteinase group	Phytase group	p =
Calcium				
Amount ingested, g	0.55±0.03	0.56±0.03	0.53±0.03	0.5*
Amount excreted, g	0.33±0.02	0.24±0.02	0.25±0.02	0.002
Amount digested, g	0.22±0.01	0.32±0.01	0.28±0.01	<0.0001
Digestibility coefficient, %	40.0±0.1	57.1±0.2	52.8±0.3	<0.0001
Phosphorus				
Amount ingested, g	0.30±0.02	0.29±0.01	0.29±0.01	0.63*
Amount excreted, g	0.17±0.007	0.14±0.009	0.10±0.007	<0.0001
Amount digested, g	0.13±0.08	0.15±0.06	0.19±0.05	0.545*
Digestibility coefficient, %	43.3±0.5	51.7±0.5	65.5±0.4	<0.0001
Nitrogen				
Amount ingested, g	1.36±0.04	1.38±0.03	1.37±0.04	0.809*
Amount excreted, g	0.90±0.02	0.81±0.02	0.83±0.03	0.008
Amount digested, g	0.46±0.01	0.57±0.01	0.54±0.02	0.0002
Digestibility coefficient, %	33.8±0.01	41.3±0.01	39.4±0.01	<0.0001

*Not significant value (p>0.05)

Table 6: Phosphorus and nitrogen balance in birds from weeks 8 to 16, ± SD

	Groups			
Feed parameters	Control group	Proteinase group	Phytase group	p =
Calcium				
Amount ingested, g	0.60±0.02	0.61±0.03	0.60±0.04	0.903*
Amount excreted, g	0.36±0.03	0.32±0.02	0.33±0.02	0.182*
Amount digested, g	0.24±0.02	0.29±0.01	0.27±0.01	0.02
Digestibility coefficient, %	40.0±0.01	47.5±0.01	45.0±0.01	<0.0001
Phosphorus				
Amount ingested, g	0.25±0.01	0.24±0.02	0.25±0.01	0.63*
Amount excreted, g	0.16±0.006	0.13±0.008	0.09±0.004	0.435
Amount digested, g	0.09±0.03	0.11±0.05	0.16±0.05	0.218*
Digestibility coefficient, %	36.0±0.3	45.8±0.4	64.0±0.3	<0.0001
Nitrogen				
Amount ingested, g	1.68±0.02	1.69±0.01	1.69±0.03	0.813*
Amount excreted, g	0.52±0.04	0.43±0.02	0.48±0.02	0.022
Amount digested, g	1.16±0.01	1.26±0.02	1.21±0.03	0.0039
Digestibility coefficient, %	69.0±0.01	74.5±0.01	71.6±0.02	<0.0001

*Not significant value (p>0.05)

In young laying hens aged 8 to 16 weeks, the calcium digestibility coefficient in the Proteinase and Phytase groups surpassed the Control group by 7.5 and 5%, respectively [F (2.6) = 12, p<0.0001] (Table 6). In the case of phosphorus, its digestibility remained the highest in the Phytase group and amounted to 64.0±0.3%, as against 36±0.3 and 45.8±0.4% for the Control and Proteinase groups, respectively [F (2.6) = 198.3, p = 0.000003]. At this stage of growth, the digestibility coefficient of nitrogen in the Proteinase group was 74.5±0.01%, which was 5.5 and 2.9% higher than the Control and Phytase groups, accordingly [F (2.6) = 17.85, p<0.0001] (Table 6).

One of the most important elements of the mineral nutrition for laying hens is calcium. In addition to its vital functions as the main component of bone structure, participation in the acid-base balance and many enzymatic systems, calcium is also the main component of the eggshell. It is estimated that each egg contains ~2.2 g of calcium, which is mainly present in the eggshell (Pelicia *et al*., 2009). The positive values resulting from the calcium balance indicates that using each of the studied enzymes, increases the coefficient of calcium digestibility, especially to a greater extent during the 1st to 8th weeks of age (Table 5 and 6).

Phytic acid, being a primary grain feed component (50 to 80%) (Selle and Ravindran, 2007), exhibits chelating properties by binding metal ions and thus, reducing the bioavailability of calcium, magnesium and other minerals. The release or breakdown of phytic acid occurs during phytase-mediated hydrolysis (Rao *et al*., 2009; Yao *et al*., 2012). With regard to the more elevated digestibility coefficient of calcium in the presence of proteinase, we suggest a possible stimulating effect of enzyme on the system of cells responsible for calcium metabolism. On the whole, both enzymes portray a beneficial effect on the digestibility of calcium in laying hens, which is vital for this category of bird.

For the entire period of growth, data on phosphorus digestibility coefficient showed that supplementing layer diet with bacterial phytase at a concentration of 1000 U/kg of feed facilitated the utilization of feed compounds with inaccessible phosphorus (phytate) and led to a reduction in the amount of undigested phosphate in the litter. Without the addition of phytase to the diet (control group), the digestibility coefficient of phosphorus was almost halved; a substantial amount of the phosphorus was excreted with litter under these conditions (p<0.05) (Table 5 and 6).

Analysis of the nitrogen balance showed that, the digestibility coefficient of nitrogen for the Proteinase group was higher in all cases in comparison to corresponding values recorded for the Control and Phytase group (p<0.05). The digestibility of feed protein in the presence of enzyme additives and in particular proteinase, exerted a positive effect on the nitrogen balance in the bird (Tables 5 and 6). With equal amounts of nitrogen available to birds in all experimental variants, its loss through the litter decreased with the use of enzyme supplement. This was due to an elevated bioavailability of amino acids as a result of effective hydrolysis of plant proteins. In addition, bacterial proteinases are stable, nonspecific, exhibit high molecular activity and can reduce the negative effects of animal proteinase inhibitors, allergens and other digestive blockers (Cowieson *et al*., 2005).

When developing and evaluating the effect of new feed additives on the physiological functioning of a bird, systematic blood tests are important for diagnosing metabolic disorders. The hematological (the number of red blood cells, leukocytes, hemaglobin and hematocrit) and biochemical (total protein, urea, creatinine, total calcium, inorganic phosphorus, aspartate aminotransferase and alanine aminotransferase) parameters were analyzed on days 30, 60 and 90. The results showed that blood parameters varied within the normal range in control and experimental birds. The ratio of calcium to phosphorus (Ca/P) in the blood serum remained above 1, in accordance with the physiological norm of laying hens in all variants (Appendix, Tables S1, S2). Based on the obtained data, we concluded that the digestive enzymes used by us at the studied concentration do not exert a negative influence on the metabolism and gastrointestinal tract functioning in the birds.

Feed additives in the diet of laying hens had an impact on the dynamics of live weight of poultry. Taking into account the starting live weight and at the final for each age period, the absolute and average daily growth indices were estimated. Notwithstanding the comparable starting live weights in all variants (~51.6-52.9 g), live weights of 506.1±6.18 g, 520.9±5.6 g and 519.3±5.11 g were recorded for the Control, Proteinase and Phytase groups on the 8th week, respectively (Table 7). Thus, a higher increase in live weight of young layers in the experimental groups was achieved [F (2.6) = 8.385, p = 0.04]. The difference in absolute live weight gain of the experimental birds relative to that of the Control group is as a result of a higher average daily gain (p<0.05) (Table 7).

In birds aged 16 weeks, the live weights were 1015.7±7.41 g, 1036.5±5.1 g and 1030.8±4.5 g for the Control, Proteinase and Phytase groups, respectively [F (2.6) = 11.166, p = 0.012]. The absolute weight gain in the Control group was 509.6±0.02 g, while 520.6±0.02 g and 518.5±0.04 g were recorded for the in the Proteinase and Phytase groups [F (2.6) = 35.393, p<0.001]. The average daily weight gain was higher in the Proteinase group (11.56±0.02 g) as compared to the Control (9.11±0.02 g) and Phytase groups (11.52±0.04 g), respectively [F (2.6) = 8.375, p<0.001].

Table 7: Live weight of young chickens at different ages, ± SD

	Groups			
Measured parameter	Control group	Proteinase group	Phytase group	p =
Weeks 1 to 8				
Starting live weight, g	52.9±1.15	51.6±1.23	51.8±1.55	0.48*
Live weight at the end of the period, g.	506.1±6.18	520.9±5.6	519.3±5.11	0.04
Absolute weight gain, g.	453.2±5.1	469.3±4.5	467.5±4.1	0.0096
Average daily weight gain, g.	9.23±0.12	9.57±0.1	9.54±0.11	0.02
Amount of feed spent per bird, kg	1.843±0.01	1.750±0.01	1.760±0.01	<0.001
Feed Conversion Ratio (FCR)	4.06±0.05	3.73±0.045	3.76±0.04	0.0002
European Productivity Index (EPI)	22.77±0.06	25.66±0.08	25.34±0.04	<0.001
Weeks 8 to 16				
Starting live weight, g	506.1±6.18	515.9±5.6	512.3±5.11	0.18*
Live weight at the end of the period, g.	1015.7±7.41	1036.5±5.1	1030.8±4.5	0.012
Absolute weight gain, g.	509.6±0.02	520.6±0.02	518.5±0.04	<0.001
Average daily weight gain, g.	9.11±0.02	11.56±0.02	11.52±0.04	<0.001
Amount of feed spent per bird, kg	3.797±0.01	3.572±0.01	3.581±0.01	<0.001
Feed Conversion Ratio (FCR)	7.45±0.02	6.86±0.02	6.9±0.02	<0.001
European Productivity Index (EPI)	12.2±0.03	13.49±0.04	13.32±0.01	<0.001

*Not significant value (p>0.05)

Bird safety for the entire experimental period was 100%. The obtained differences in the live weight of young laying hens was probably due to the digestibility of nutrients in the diet (Table 4). At 8 weeks of age, the digestibility of dry matter was 62.7±2.25% for the control group, whereas in proteinase and phytase fed birds it increased to 67.6±2.12% and 63.5±2.55 (p<0.05), respectively. A similar pattern was observed in the digestibility of organic matter (Table 4).

Histological studies of parenchymal organs (liver, spleen and myocardium) in the control and experimental groups of young laying hens did not reveal any abnormalities (Appendix, Fig. S2-S4,).

The most important indicator during a comprehensive assessment of the effectiveness of animal feed is the cost of feed. This is due to the fact that, in bird management, feed accounts for more than 70% of production costs. To manage birds for the first 8 weeks, we spent 1.843±0.01 kg of feed per head for the control group, while 1.750±0.01 kg and 1.760±0.01 kg were spent per head in the Proteinase and Phytase groups, respectively. For the subsequent 8 to 16 weeks, the minimal feed cost was registered in Proteinase group (3.572±0.01 kg per bird), which was 5.9% less in comparison to the control group. With regard to the Phytase group, the cost of feed per head amounted to 3.581±0.01 kg, which surpassed the Control group by 5.6% [F (2.6) = 580.40, p<0.001].

At the final stage of the study, we assessed feed conversion and the European productivity index, which is used to evaluate the economic feasibility of using the studied feed additive.

The results demonstrated that the addition of proteinase (10 U/kg) and phytase (1000 FTU/kg) to diet of laying hen enhances the total and daily weight gain, while decreasing the FCR. The absolute weight gain of the in the Proteinase group birds was higher than that of the control group by 3.4% (p<0.001) for the first 8 weeks and 1.1% (p<0.001) at the end of the experiment. The Feed Conversion Ratio (FCR) for the entire 16 week period was lower in the experimental groups (6.86±0.02 for Proteinase group; and 6.9±0.02 for the Phytase group) in contrast to the Control group (7.45±0.02) [F (2.6) = 612.25, p = 0.001].

Conclusion

Commercial phytases and proteinases from diverse sources are checked and confirmed for their prospective use as probiotics for all types of farm birds and animals. We have demonstrated that the new enzymes, subtilisin-like serine protease of *B. pumilus* 7 p and histidine acid 3-phytase of *Pantoea sp.* 3.5.1, also show good results. Adding phytase at a concentration of 1000 FTU/kg of feed contributes to better absorption of calcium and phosphorus. The inclusion of proteinase in the feed at a concentration of 10 U/kg increases the digestibility of calcium, nitrogen and crude protein. As a result, both enzymes reduce feed cost per experimental fowl by more than 5%. On the whole, the daily weight gain of the birds increased by an average of 3% during the study. The enzymes used in the study were secreted by natural bacterial strains isolated from the soils of the Tatarstan region, where their intended use is expected. *B. pumilus* 7 p subtilisin-like proteinase has a naturally high proteolytic activity, as well as increased stability under changing pH conditions. This allows the enzyme to not only "survive" in the aggressively acidic environment of the poultry stomach, but also completely restore its catalytic activity when it enters the more alkaline environment of the poultry intestine. The bacterial phytase from *Pantoea sp*. 3.5.1 is glycosylated during

expression in cells of the methylotrophic yeast *Pichia pastoris* and becomes more resistant to elevated temperature and acidic pH values: The pH-optimum of the enzyme changes from pH 4.5 to pH 3.0, which causes a more effective hydrolysis of its substrate in the acidic environment of the poultry stomach. Both enzymes were isolated and studied in detail in our laboratory. They have convincingly shown their effectiveness as feed additives for laying hens, providing an increase in the main important indicators of poultry productivity.

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Author Contributions

The authors of the manuscript have made a significant contribution to the work and fully agree with the content of the manuscript.

Smolentsev Sergei Yur'evich: Development of experimental design, control of work with poultry in the vivarium, analysis of the results obtained and histological studies.

Rudakova Natalia Leonidovna: Preparation of proteinase (isolation and purification) and writing of the manuscript (proofreading, author for correspondence).

Koryagina Anastasia Olegovna: Preparation of proteinase (isolation and purification).

Bulmakova Daria Sergeevna: Preparation of phytase (isolation and purification).

Suleymanova Aliya Damirovna: Preparation of phytase (isolation and purification).

Mardanova Ayslu Mirkasimovna: Supervision of experimental work, analysis of the results obtained, participation in writing the article (working with sources).

Sharipova Margarita Rashidovna: Project head, development of the experiment plan, Manuscript design and writing.

Conflict of Interest

The authors declare no conflicts of interest.

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Appendix

Table S1: Hematological parameters of young laying hens, ± SD

	Sampling time, day		
Group	30th	60th	90th
Erythrocytes, $\cdot 10^{12}$/L			
Control	1.78±0.03	1.88±0.02	1.84±0.03
Proteinase group	1.81±0.02	1.95±0.05	1.93±0.04
Phytase group	1.75±0.04	1.91±0.01	1.96±0.03
Leucocytes, $\cdot 10^{9}$/L			
Control	25.2±0.05	27.9±0.07	26.0±0.08
Proteinase group	26.3±0.04	25.4±0.05	27.8±0.06
Phytase group	26.1±0.06	27.2±0.03	26.5±0.04
Hemoglobin, g/L			
Control	75.7±0.99	71.0±0.67	73.4±0.39
Proteinase group	80.0±1.01	76.1±0.44	75.3±0.70
Phytase group	77.8±0.65	74.5±0.80	76.0±0.41
Hematocrit, %			
Control	26.5±0.25	25.8±0.26	26.4±0.31
Proteinase group	25.5±0.33	26.1±0.20	27.3±0.15
Phytase group	26.8±0.18	25.5±0.25	26.2±0.31

Table S2: Biochemical blood parameters of birds, ± SD

	Sampling time, day		
Group	30th	60th	90th
Total protein, g/L			
Control	34.8±1.7	41.5±2.0	49.4±1.5
Proteinase group	36.7±1.1	44.2±1.6	51.1±1.2
Phytase group	35.4±1.4	42.9±0.9	48.6±1.8
Urea, mmol/L			
Control	2.53±0.05	2.60±0.02	2.40±0.07
Proteinase group	2.41±0.03	2.52±0.06	2.33±0.02
Phytase group	2.46±0.06	2.64±0.04	2.35±0.04
Creatinine, μmol/L			
Control	30.4±1.4	41.9±1.5	44.0±0.8
Proteinase group	26.0±1.8	39.2±1.2	41.5±1.1
Phytase group	28.3±1.4	40.5±1.3	42.8±1.5
Total calcium, mmol/L			
Control	2.05±0.04	2.75±0.03	5.08±0.05
Proteinase group	2.10±0.02	2.80±0.05	5.14±0.03
Phytase group	2.08±0.03	2.78±0.02	5.18±0.04
Inorganic phosphorus, mmol/L			
Control	1.94±0.02	2.15±0.03	1.78±0.04
Proteinase group	2.01±0.01	2.20±0.05	1.85±0.02
Phytase group	2.18±0.03	2.33±0.02	1.93±0.04
Aspartate aminotransferase, U/L			
Control	4.88±0.64	3.45±0.80	5.44±0.72
Proteinase group	5.05±0.55	3.33±0.62	4.90±0.48
Phytase group	5.73±0.39	4.12±0.44	5.02±0.53
Alanine aminotransferase, U/L			
Control	11.7±0.91	8.5±0.95	10.4±0.77
Proteinase group	12.6±0.88	9.0±0.79	9.8±0.65
Phytase group	10.5±0.76	8.2±0.61	11.3±0.53

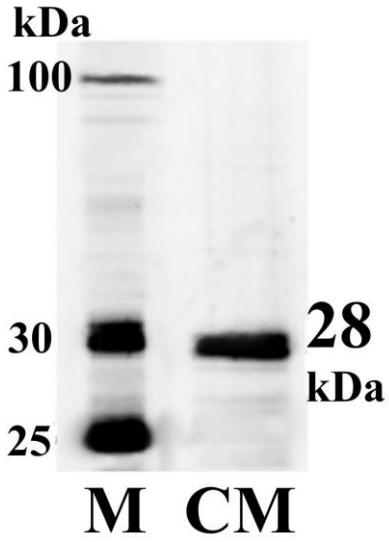
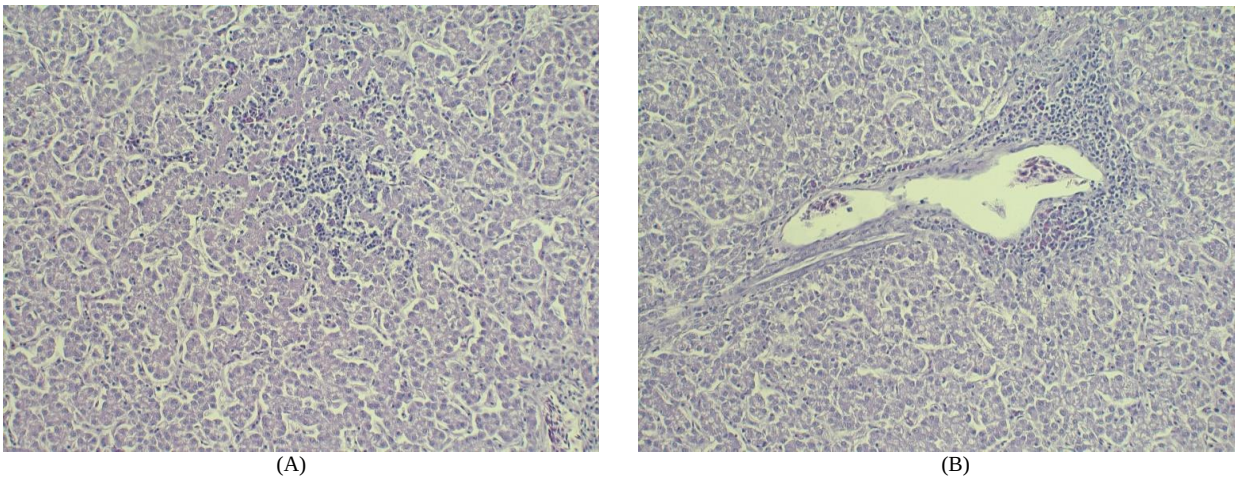


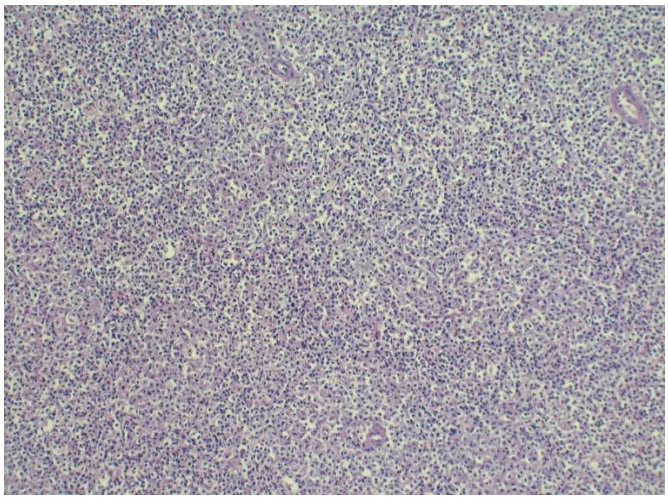
Fig. S1: SDS electrophoresis of *B. pumilus* proteinase fraction after carboxymethyl cellulose chromatography. M -protein ladder (#2610, Thermo Scientific, USA)



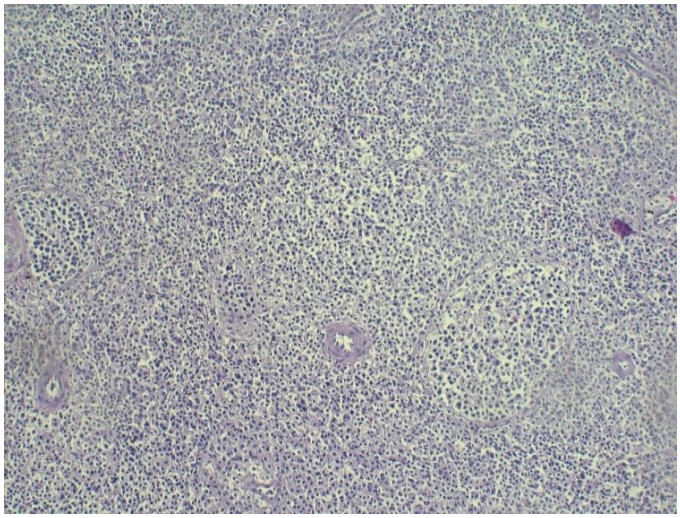
(A) (B)

(C)

Fig. S2: Digital micrographs of liver tissues extracted from the control group (A), Proteinase group (B), Phytase group (C) (100x, Hematoxylin and eosin (H&E) staining)



(A)



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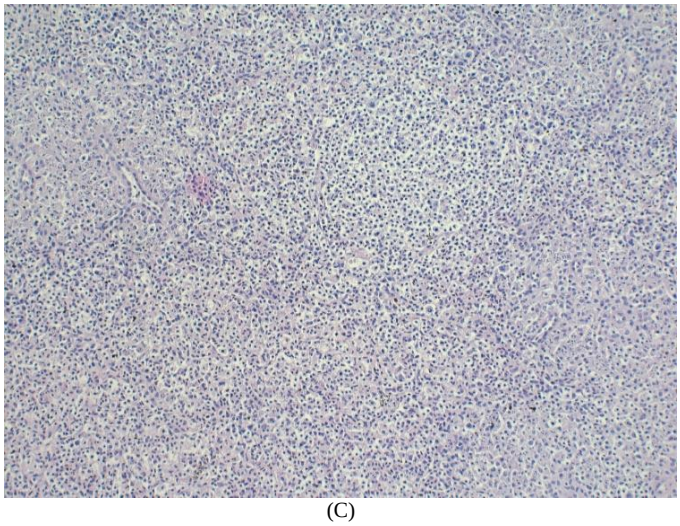
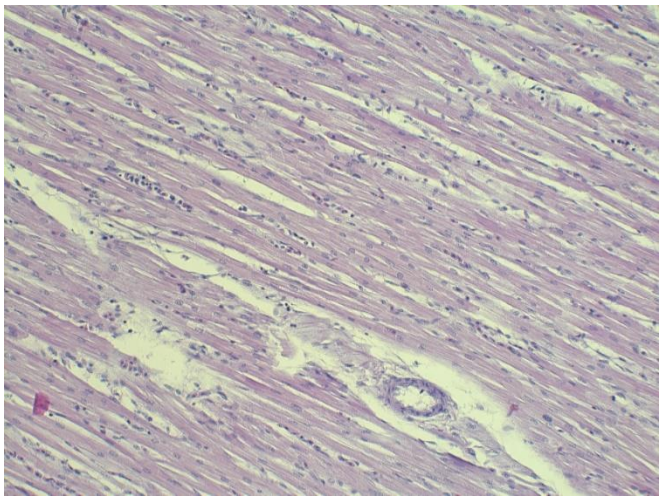
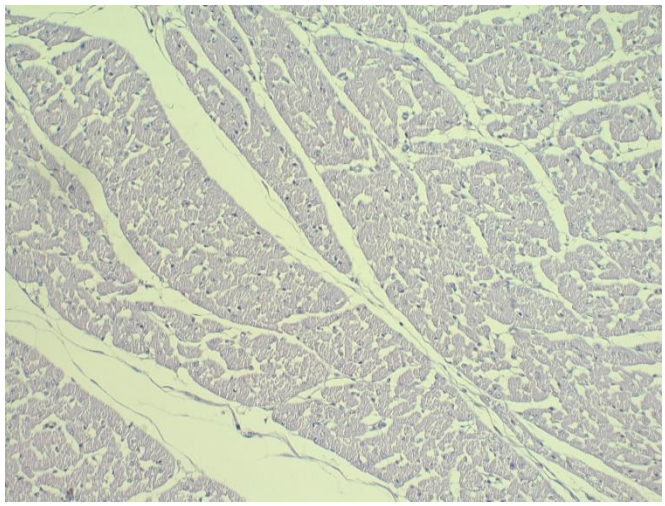


(C)

Fig. S3: Digital micrographs (100x) of H&E stained segments of spleen harvested from birds of the Control group (A), Proteinase group (B), Phytase group (C) (100x, H&E staining)



(A)



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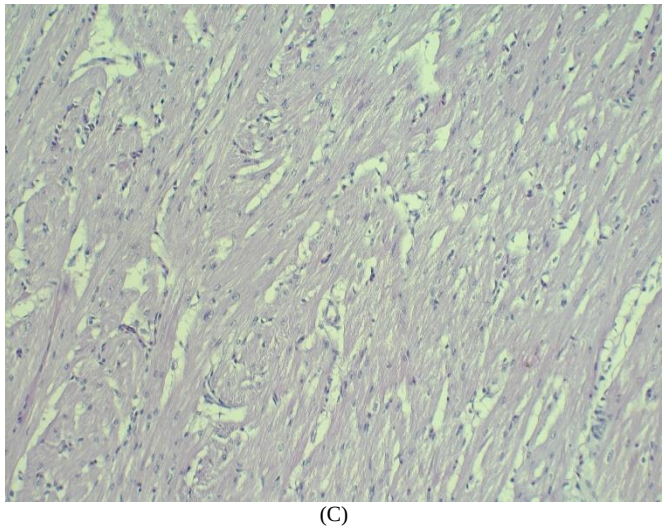

(C)

Fig. S4: Digital photographs of myocardium obtained from layer birds of the Control group (A), Proteinase group (B), Phytase group (C) (100x, H&E staining)