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Nutrient Digestibility, Egg Productivity, and Embryo Development in Laying Hens Fed Bacterial Enzyme-Based Additives

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Abstract

The aim of the experiment was to investigate the effect of microbial enzyme supplements on egg production and the nutrient digestibility of laying hens. A total of 360 twenty-one-week-old Hisex Brown laying hens were assigned to three treatments with four replicates of 30 birds each using a completely randomized design and reared for 30 days. The treatments consisted of 1) A basal diet provided according to the recommended feeding standards (as the control group), 2) a basal diet supplemented with proteinase enzyme at a concentration of 10 U/kg, and 3) a basal diet supplemented with phytase enzyme at a concentration of 1000 FTU/kg. Supplementation of proteinase enzyme increased ($P < 0.05$) the retention percentage of dry matter and crude protein in laiyng hens compared to the control treatment. and phosphorus retention increased in laying hens by using of the enzymes. The addition of proteinase enzyme to the diet increased body weight gain of laying hens compared to other treatments. Feed convertion ratio improved in laying hens recieved enzyme supplements ($P < 0.05$). The dietary enzymes supplementation increased the egg weight and egg mass than those fed with the enzyme-free diet ($P < 0.05$). The Thickness of eggshell increased by using of phytase enzyme than other treatments. Also, ash content of eggshell increased in treatments supplemented by both of the enzymes. According to the results of the experiment, the addition of proteinase and phytase to the diet could be effective in improving the performance of laying hens and the quality characteristics of eggshell.

Introduction

Modern highly productive egg chicken crosses require balanced diets in terms of metabolic energy, complex nutrients, biologically active components, and mineral substances. An important task in poultry production is to achieve the maximum absorption of nutrients from feed mixtures. Enzyme-based feed additives help to increase the bioavailability of nutrients. Their inclusion increases the digestive system's potential, as well as declines the level of bacterial contamination of feed (Borda-Molina *et al.*, 2019). Furthermore, increasing the efficiency of nutrient uptake by birds is also beneficial since it reduces their excretion and thus has a favorable effect on the environment. This is significant, as raw protein and phosphorus (P) are the most harmful environmental wastes from poultry farming (Abdelnour *et al.*, 2018; Abbasi *et al.*, 2019). Here, feed additives based on proteinases and phytases are tested for their impact on nutrient utilization within chickens.

Exogenous phytase as a feed additive contributes to the hydrolysis of phytic acid and its salts, which are dominant in the grain component of feed (Selle *et al.*, 2012; Walk and Rao, 2019). This makes it possible to significantly increase the utilization of

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plant P by birds since poorly digested products include up to 70% of the total P contained in grains (Vieira *et al.*, 2016; Ingelmann *et al.*, 2019). In addition, phytate could bind to proteins and peptides and forming an insoluble complexes that are inaccessible for hydrolysis by the digestive enzymes of poultry (Dersjant-Li *et al.*, 2015).

Exogenous proteinases of microbial origin are an important feed additive for poultry (Angel *et al.*, 2011; Cowieson *et al.*, 2014; Olukosi *et al.*, 2015; Cowieson and Roos, 2016). They improve the digestibility of proteins and amino acids by functioning together with poultry digestive enzymes, rather than competing with them (Lee *et al.*, 2018; Mahmood *et al.*, 2018; Siegert *et al.*, 2019; Jiang *et al.*, 2020). This is especially important for young animals, in which the activity of native proteinases may be reduced (Mahagna *et al.*, 1995). Additionally, exogenous proteinase can mitigate the negative effects of thermally stable trypsin or lectin inhibitors (Cowieson and Roos, 2016). Reducing the undigested protein content in the presence of exogenous proteinase normalizes the intestinal microflora of poultry, and prevents the proliferation of pathogenic bacteria (Giannenas *et al.*, 2017; Yan *et al.*, 2017; Borda-Molina *et al.*, 2019).

Phytase and proteinase-based feed additives have been shown to improve productivity indicators for broilers as well as for laying hens (egg formation capacity, egg mass, and shell quality) (Liu *et al.*, 2007; Hassanien and Elnagar, 2011; Zyla *et al.*, 2012; Kim *et al.*, 2017).

Commercial preparations of feed phytase produced in Russia are based on fungal phytases (mostly from *Aspergillus niger*). At the same time, a large share of the domestic Russian market is made up of imported preparations based on bacterial phytases (producers of *Buttiauxella, Citrobacter braaki, B. subtilis, E. coli*) (Rosstat data). The advantage of bacterial enzymes over fungal ones is that they are less sensitive to pepsin. In our work, we tested *Pantoea sp.* 3.5.1. histidine acid 3-phytase and *B. pumilus* 7P subtilisin-like proteinase as additives to the feed for laying hens of the Hisex Brown breed. The bacterial strains were isolated from local soils and both enzymes were purified and characterized in our laboratory. The sequences of the corresponding genes were established and annotated in the GeneBank at NCBI https://www.ncbi.nlm.nih.gov/genbank/ (phytases-AN KJ783401.1; proteinases-AN AY754946.2) (Suleimanova *et al.*, 2015a), proteins were isolated, and their properties were measured in detail. The primary structure of both proteins was determined by the MALDI-TOF method (Mikhailova *et al.*, 2009a, b; Suleimanova *et al.*, 2015b). The DNA of producing strains was sequenced and entered into the GeneBank (NCBI) database (AN JHUD00000000; AN JMRT00000000.2).

In a recent study, to obtain a sufficient amount of phytase, new strains designed that produce the corresponding proteins. The *Pantoea sp.* 3.5.1 phytase gene was cloned into the commercial yeast vector *Pichia pastoris* PichiaPink (Invitrogen, USA). The purified glycosylated phytase has a molecular weight of 80 kDa, is stable at pH from 2 to 5 (with an optimal pH of 3), and has maximum thermal stability at 50°C (Troshagina *et al.*, 2018). In another study, subtilisin-like proteinase isolated from a natural strain *B. pumilus* 7P with an increased level of protein secretion. The highly purified proteinase had a molecular weight of 28 kDa and the optimal temperature for the enzyme-catalyzed reaction in the presence of 5 mM of calcium (Ca) was 50-55°C. Also, The enzyme was stable in the pH range from 7 to 10 with an optimal pH of 9.5 (Mikhailova *et al.*, 2009a, b). Both of the proteinase and phase enzymes could be active (90% for phytase and 60% for proteinase) in the presence of gastric juice (with pH of 3), but in the presence of pancreatic and intestinal juices (pH of 6-7), phytase is completely deactivated and proteinase maintains 100% of its activity. Notably, the activity of proteinase was not suppressed by a trypsin inhibitor (Koryagina *et al.*, 2018). The properties of *Pantoea sp*. 3.5.1 phytase and *B. pumilus* 7P proteinase make them a suitable option to studied as feed additives in the diets of poultry.Thus, this study aimed to investigate the effect of bacterial enzyme-based feed additives on the digestibility of nutrients and egg productivity in laying hens of the Hisex Brown breed.

Materials and Methods

Preparation of bacterial enzymes

Phytase preparation was obtained from recombinant yeast strains of *Pichia pastoris* pPINK-agpP (Troshagina *et al.*, 2018). The *P. pastoris* pPINK-agpP strain was cultured in a Biotron LiFlus SP30L bioreactor (Biotron, Inc., Korea) to obtain preparative amounts of phytase. At the end of the fermentation process, the culture fluid was drained, and the yeast cells were deposited by centrifugation. The residual supernatant containing phytase was passed through a half-fiber module UV-0.5-30-PS (Faserkraft, Russia) for enzyme concentration. As a result of the fermentation and protein concentration processes, approximately 500,000 units of recombinant phytase were obtained. The enzyme was stored at a temperature of -20°C. This phytase was added to the feed at a ratio of 1000 units per 1 kg of feed. Phytase activity was determined by the hydrolysis of sodium phytate (Sigma Aldrich, USA) using the Greiner method (Greiner, 2004). A unit of phytase activity was determined as the amount of the enzyme needed to break down sodium phytate to form 1 μm of inorganic phosphate in 1 min.

The producer of subtilisin-like proteinase is a natural isolate of *B. pumilus* 7P/3-19 from the Museum of the Department of Microbiology of the Kazan Federal University. The enzyme is secreted into the culture fluid with maximum activity at the 24th hour of growth. Purification of the enzyme was performed by ion-exchange chromatography on carboxymethyl cellulose as described by Koryagina *et al*. (2018). The amount of 7000 units of the enzyme was provided for testing within the vivarium. The concentrated solution of the enzyme was stored at -20°C. Before feed treatment, the enzyme was diluted with drinking water at room temperature. This proteinase was introduced to feed with a spray at the rate of 10 units of proteinase per 1 kg of feed. The proteinase activity was determined by hydrolysis of azocasein (Sigma, USA) using the method described by Sabirova *et al*. (2010). The amount of enzyme hydrolyzing 1 µg of substrate per 1 min was measured as a unit of proteinase activity.

Scheme and conditions for experiments on laying hens

All birds were kept in accordance with the Guide for the Care and Use of Experimental Animals of Kazan Federal University and commercial poultry farm regulations, 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 experiment was conducted on 21-week-old laying hens of the Hisex Brown breed under vivarium conditions at the KFK Alimshoeva Z.I. farm (Russia, Mari El Republic, Medvedevsky District, the village of Middle Azyakovo). A total of 360 21-week-old Hisex Brown hens were studied. Fowls were individually weighed, labelled using plastic rings of different colors, and randomly divided into three treatments, with 4 replicates of 30 birds each. The treatments consisted of 1) basal diet without proteinase or phytase (control treatment); 2) basal diet supplemeted with proteinase enzyme at a concentration of 10 U/kg; 3) basal diet supplemeted with phytase enzyme at a concentration of 1000 FTU/kg. The ingredients and nutrient contents of the basal diet are shown in Table 1. The birds were kept in three-tier cages made of galvanized wire mesh (1.63 m × 0.98 m × 0.68 m per 5 birds) under controlled climate conditions with temperature maintained at 18°C, 65% relative humidity, lighting period 16L:8D, and light intensity of 12 lux. Feed and water were available *ad libitum*. The experiment lasted 30 days.

Table 1. Composition of laying hens' basal diet

Ingredients	Basal diet (as %)
Wheat	48.00
Corn	10.40
Barley	30.00
Sunflower cake	2.00
Table salt	0.40
Grass meal	6.00
Meat and bone meal	2.00
Chalk	1.20
Chemical composition	
Metabolizable energy (kcal/kg diet)	2390
Crude protein (%)	17.00
Crude fat (%)	4.55
Crude fiber (%)	3.20
Calcium (%)	3.30
Total phosphorus (%)	0.50

Nutrients retention coefficients

After sampeling from the diets and birds' droppings, chemical analysis of them was carried out according to the methods described in GOST 31640-2012 (2012), GOST 32933-2014 (2014), GOST 31675-2012 (2012), GOST R 51417-99 (1999), and GOST 23042-86 (1086) for determination of dry matter, ash, crude fibre, crude protein, and crude fat, respectivly. Total Ca and P were determained according to the GOST R 50852-96 (1996). Total tract retention coefficients (TRC) of nutrients in the test diets were calculated using the following equation (Mutucumarana *et al*., 2014):

TRC (%) = (total nutrient ingested − total nutrient excreted)/(total nutrient ingested).

Productive performance and eggshell quality

Body weight changes of the birds was assessed by monitoring the live weight. Hen-day egg producttion was determined by daily counting of laid eggs in each treatment. Also, the feed intake were recorded daily and feed conversion ratio (FCR) was calculated at the end of experiment. The thickness of the eggshell was measured using a micrometer. The content of ash and Ca in eggshells was measured using the methods described in GOST 26570-95 (1995) and GOST P 50852-96 (1996), respectively.

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's test for multiple pairwise comparisons of quantitative indicators of the different groups. The results were presented as the mean ± SD, considering *P*-value < 0.05 as a level of significancy.

Results and Discussion

The effect of dietary phytase and proteinase enzymes on the apparent total tract retention of nutrients in laying hens is presented in Table 2. Dry matter retention was higher in birds fed with the diet containing proteinase enzyme than control group ($P < 0.05$). The highest protein retention coefficient was observed in group received the proteinase enzyme, which indicates the contribution of the supplemented enzyme to the hydrolysis of the diet proteins. In the phytase group, the digestibility coefficients remained at the level of the control group.

Phytase can degrade phytate-protein complexes that are inaccessible to the native enzymes of the laying hens' gastrointestinal tract (Ravindran *et al.*, 2001; Nourmohammadi *et al.*, 2010; Walk and Rao, 2019). Likely, the activity of phytase, in this case, is not enough to raise the corresponding indicators. There was no statistical difference in the retention coefficient of crude fiber ($P > 0.05$). Also, the values of crude fat retention coefficients was not significant among the experimental treatment, indicating that the enzymes did not affect the processes of crude fat hydrolysis within the gastrointestinal tract of laying hens. None of the treatments had significant effet on retention of crude fiber and crude fat in laying hens.

Table 2. Effect of different treatments on total tract retention of nutrients (as %) in laying hens

Item	Treatments[1]			*P*-value
	Control	Proteinase	Phytase	
Dry matter	$65.00^{b} \pm 2.02$	$69.40^{a} \pm 1.18$	$68.70^{ab} \pm 1.64$	0.035
Crude protein	$81.50^{b} \pm 1.04$	$86.80^{a} \pm 1.86$	$83.10^{b} \pm 1.55$	0.014
Crude fiber	17.90 ± 1.22	18.90 ± 0.57	18.50 ± 0.40	0.380
Crude fat	90.30 ± 1.81	91.50 ± 1.04	90.60 ± 1.70	0.617
Calcium	$51.60^{c} \pm 0.03$	$55.80^{a} \pm 0.02$	$53.50^{b} \pm 0.03$	<0.0001
Phosphorus	$52.60^{c} \pm 0.08$	$55.80^{a} \pm 0.10$	$53.10^{b} \pm 0.08$	<0.0001

[a,b] Values in the same row with different letters are significantly different ($P < 0.05$).

[1] Mean ± Standard error

The retention coefficient of Ca and P was improved in birds under proteinase and phytase treatments compared to control group. Furthermore, the retention of these minerals were higher in birds fed with proteinase enzyme than phytase treatment. Calcium and P are two important components in the diets of laying hens (Bello and Korver, 2019; Adhikari *et al.*, 2019). Calcium increases the egg-laying capacity and contributes to the formation of a strong shell, as well as strengthens the bone structure of laying hens. So, the absorption rate of Ca is important in laying hens, as they lose Ca daily during the formation of the eggshell. Phosphorus is also important for the productivity of laying hens, more than 70% of which in plant diets are present in the non-digestible form of phytic acid and its salts as phytates (Abbasi *et al.*, 2019).

Phytate-forming complexes present in the composition of plant- based diets bind divalent metal ions, as well as proteins, oligopeptides, and amino acids. The cleavage of phytate-binding complexes occurs with the participation of enzymes, and the release of calcium. An increase in crude protein retention with the use of proteinase indicates an increase in the proportion of protein digestibility and assimilation of diet nutrients (Mahmood *et al.*, 2018). It is reported that dietary addition of proteinase enzyme obtained from *B. lichiniformes* incease the protein digestibility and amino acid uptake in laying hens (Vieira *et al.*, 2016). Phytase enzyme increase P retention with increasing the levels of phytase from 300 to 1500 FTU / kg of diet (Taylor *et al.*, 2018).

The effect of adding phytase and proteinase enzymes to the diet of laying hens, on the body weight changes is presented in Table 3. The use of proteinase enzyme significantly increased body weight gain of laing hens after 30 days than other treatments. Also, the same trend was found for daily weight gain. A part of these effects could be due to the improvement of the digestibility and retention of nutrients in the digestive system.

Table 3. Effect of dietary enzyme supplementation on live body weight changes of laying hens during days 1 to 30 of experimet

Item	Treatments [1]			*P*-value
	Control	Proteinase	Phytase	
Live body weight at d 1 (g)	1421.80 ± 13.50	1410.70 ± 10.40	1416.10 ± 11.20	0.548
Live body weight at d 30 (g)	1467.90 ± 12.0	1465.60 ± 11.30	1463.20 ± 10.10	0.878
Body weight gain (g)	46.10^{b} ± 1.50	54.90^{a} ± 0.90	48.10^{b} ± 1.01	0.0002
Daily weight gain (g)	1.54^{b} ± 0.05	1.83^{a} ± 0.06	1.60^{b} ± 0.03	0.0008

[a,b] Values in the same row with different letters are significantly different ($P < 0.05$)
[1] Mean ± standard error

Egg production is the main indicator of the productivity of laying hens, which is largely determined by important factors such as the availability of Ca and P, as well as their ratio in the feed mixture. Therefore, to ensure the high productivity of laying hens, targeted feed additives are developed. Several studies have previously shown a positive effect of phytase on the production of eggs in the diet of chickens (Mohammed *et al.*, 2010; Ponnuvel *et al.*, 2016; Englmaierová *et al.*, 2017), however, there is contradicting evidence indicating no effect in the presence of phytase (Musapuor *et al.*, 2005). Interestingly, in a study conducted by Kim *et al.* (2017), the use of phytase in high concentrations (10.000, 20.000, and 30.000 FTU / kg) in laying hens had a positive effect on egg production (Kim *et al.*, 2017).

The effect of adding phytase and proteinase enzymes to the diet of laying hens, on the productive performance is presented in Table 4. Supplementation of diet with phytase enzyme significantly increased the total egg number and hen-day- egg production compared to other treatments. Egg weight, egg mass, and FCR was improved in laying hens fed with proteinase and phytase ($P < 0.05$). However, feed intake was not affected by the treatments. In addition to the fact that the use of proteinase and phytase contributed to an increase in bird growth rate, the survival rate of birds in both groups was 100% (data not shown).

Table 4. Effect of different treatments on productive performance of laying hens

Item	Treatments [1]			*P*-value
	Control	Proteinase	Phytase	
Total eggs numbers (per treatment/ 30 d)	756.00 ± 2.00^{b}	762.00 ± 4.00^{ab}	768.00 ± 3.00^{a}	0.0094
Total eggs numbers (per hen/treatment)	25.20 ± 0.20^{b}	25.40 ± 0.10^{ab}	25.60 ± 0.10^{a}	0.037
Egg weight (g)	64.70 ± 0.14^{b}	66.10 ± 0.36^{a}	66.80 ± 0.28^{a}	0.047
Hen-day-egg production (%)	84.00 ± 0.30^{b}	84.60 ± 0.20^{ab}	85.30 ± 0.30^{a}	0.002
Egg mass (g/d per hen)[2]	54.34 ± 0.15^{b}	55.92 ± 0.30^{a}	56.98 ± 0.34^{a}	<0.0001
Feed intake (g/d per hen)	120.21±3.08	118.97±2.47	120.08±3.08	0.851
Feed conversion ratio (g/g)[3]	2.15 ± 0.05^{b}	2.06 ± 0.03^{a}	2.05 ± 0.03^{a}	0.033

[a,b] Values in the same row with different letters are significantly different ($P < 0.05$)
[1] Mean ± standard error
[2] Egg mass = hen-day-egg production (%) × mean egg weight (g).
[3] Feed conversion ratio was calculated by by dividing the feed intake by egg mass.

The effect of dietary treatments on the eggshell quality of laying hens is showed in Table 5. Eggshell thickness in hens fed with phytase enzyme increased than to the control treatment ($P < 0.05$). The ash content of the eggshells increased by the enzyme supplementing ($P < 0.05$). The Ca content of eggshell was higher in the enzyme-supplemented treatments. Also, the use of proteinase significantly increased the Ca content of eggshell than phytase treatment. This effect may be due to the fact that during the hydrolysis of phytic complexes by phytase, Ca is released, which affects the quality of the shell and contributes to an increase in egg production.

Table 5. Effect of dietary enzyme supplementation on eggshell quality in laying hens

Item	Treatments [1]			*P*-value
	Control	Proteinase	Phytase	
Thickness (µM)	348.10^{b} ± 4.51	355.60^{ab} ± 5.84	364.00^{a} ± 6.73	0.04
Ash (%)	92.00^{b} ± 0.12	92.60^{a} ± 0.12	92.60^{a} ± 0.11	0.012
Calcium (%)	31.00^{c} ± 0.12	32.60^{a} ± 0.11	32.00^{b} ± 0.10	<0.0001

[a,b,c] Values in the same row with different letters are significantly different ($P < 0.05$)
[1] Mean ± standard error

Conclusion

Based on the results of the peresent study, due to the improvement of nutrient digestability and retention, which makes it possible to use diets more efficiently, productive performance and eggshell quality could be positively affected by supplementing of proteinase and phytase enzymes in the diets of laying hens. Also, increasing the digestibility and assimilability of P, saving of inorganic P sources justify the benefits of using these environmentally friendly biological additives. Thus, subtilisin-like proteinase of *B. pumilus* and phytase of *P. pastoris* can be used as feed additives for laying hens at a concentration of 10 units/kg of feed and 1000 units/kg of feed, respectively, without harmful effects on production performance of laing hens.

Conflict of interest: The authors declare no conflicts of interest.

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