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Research Article
Impact of Dietary Enzymes Prepared at Ensiling (ZADO®) on Productivity, Blood Metabolites and Enzymes Activity in Commercial Laying Hens

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Abstract

Background and Objective: An Egyptian patented product (ZADO®) consists of exogenous enzyme mixture prepared from anaerobic bacterium, improves performance of broilers but not examined in laying hens. Therefore, this study was conducted to assess the impacts of ZADO® on productivity, blood metabolites and enzyme activity in laying hens. **Materials and Methods:** A total of 280 Hisex Brown laying hens (48 week of age) were used to evaluate the impacts of exogenous xylanases, cellulases, protease and α-amylase enzyme preparations at ensiling (ZADO®) on the productive performance and enzymes activity up to 64 week of age. Hens were divided randomly into 5 treatment groups (basal diets supplemented with 0.00, 0.25, 0.50, 1.00 or 2.00 g kg^{-1} diet with ZADO®) and housed in individual cages in an open house system under the same managerial conditions. Hens' performance traits were measured every 4 weeks and blood metabolites and enzymes activity parameters for protease and α-amylase were measured at 54 week of age. **Results:** No significant effects of dietary ZADO® levels on productive performance of laying hens were detected. However, numerically, a slight increase in egg production rate (92.1, 92.5, 93.9, 93.1 and 93.2%), egg mass (55.8, 57.0, 57.0, 56.8 and 57.2 g) and feed consumption (116.3, 115.8, 117.8, 118.2 and 115.9 g hen^{-1} day^{-1}) were noted for birds fed diet supplemented with 0.00, 0.25, 0.50, 1.00 or 2.00 g kg^{-1} diet with ZADO®, respectively. Dietary enzymes cocktail increased total protein level in favor of globulin ($p<0.05$) and dietary 1 g kg^{-1} ZADO® or more increased total cholesterol level ($p<0.05$) with no significant impact on neither LDL- nor HDL-cholesterol levels in plasma. Moreover, enzymes supplementation increased enzymes activity in digesta of both proventriculus and ileum ($p<0.05$). For example, α-amylase activity records were 0.70, 1.50, 2.26, 19.08 and 11.96 g kg^{-1} ($p = 0.037$) in proventriculus digesta and 90, 108, 282, 407 and 287 g kg^{-1} ($p = 0.013$) in ileum digesta for the 5 treatment groups, respectively. **Conclusion:** Laying hens' diets supplemented with ZADO® might increase plasma protein and enhance enzymes activity which might reflect into slight increase in hens' productivity.

Key words: Cellulases, enzymes activity, laying hens, plasma protein and cholesterol, productive performance, protease, xylanases, ZADO® enzyme complex, α-amylase

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Recently, the addition of enzymes to poultry diets has gained increasing attention because of economical and environmental aspects. Their prospect is to stimulate a better utilization of the diet, because less feed is needed to produce a certain amount of meat or egg and fewer nutrients end up in the litter[1,2]. Freitas *et al.*[3] stated that enzyme products can be categorized into single (monocomponent) enzyme, blended of mono-component enzymes and fermented products from wild type microorganism strains expressing a spectrum of enzyme activities[4,5]. In addition, multi-enzymes application has been increased as the numbers of formulated ingredient, enzyme type and their products have increased[6,7].

Exogenous enzymes have been used to improve the feeding value of cereal-based diets such as wheat and rye-based diets. These cereals are high in soluble non-starch polysaccharides that induce viscosity[8-10]. In addition, enzyme cocktail (carbohydrase and protease) improve egg mass and reduce viscosity especially of corn and soybean meal (SBM), which induce less viscosity[11] or at least did not cause any adverse effect on egg production, egg quality or immunity status[12]. Moreover, Amerah *et al.*[13] observed a synergetic effect of xylanase and amylase on broiler performance and nutrient digestibility.

The efficacy of many commercial enzyme products has been well stated but there is still some vagueness in their mode of action[14]. Moreover, Wen *et al.*[15] stated that enzyme supplementation to laying hens' diets improved nutrient retention and tended to increase enzyme activity in the intestinal digesta. In this respect, the authors hypothesized that the improvement of productivity and enzyme activity might be reflected in modifying blood metabolites profile especially, if these enzymes are prepared at ensiling from anaerobic bacterium[5].

Egyptian patented product (ZADO®) consists of exogenous enzyme mixture prepared from anaerobic bacterium, improves performances of broilers[5], dairy cows fed diets containing Egyptian by-product feeds[16], sheep and goats fed diets contained wheat straw[17]. Moreover, dietary serine protease derived from fermentation of *Bacillus licheniformis* in broiler diets' based on corn and SBM resulted in improved body weight (BW)[18], feed efficiency and digestibility of fat, protein[3] and amino acids[19]. Therefore, the aim of this study was to evaluate the impacts of ZADO® on productivity, blood metabolites and enzyme activity of laying hens.

MATERIALS AND METHODS

The experiment was conducted at the commercial laying hens' farm of Poultry Services Center and the biochemical analyses were completed at the laboratory of biochemistry, Faculty of Agriculture, Cairo University, Giza, Egypt. The study was conducted under the guidelines of the institutional Ethics of Animal Use in Research Committee (EAURC), Cairo University, Egypt.

Husbandry and Experimental Design: A total of 280 Hisex Brown laying hens (48 week of age) were selected at random, weighed individually and divided randomly into 5 treatments (basal diets supplemented with 0.00, 0.25, 0.50, 1.00 or 2.00 g kg^{-1} diet with ZADO®). ZADO® is an Egyptian patented product manufactured by the Academy of Scientific Research and Technology, Egypt and contains a mix of anaerobic bacteria and their enzymes of xylanases (2.3 unit g^{-1}), cellulases (7.1 unit g^{-1}), α-amylase (61.5 unit g^{-1}) and protease (29.2 unit g^{-1}) in a powder form obtained through an anaerobic fermentation process[16,17]. Each treatment replicated 4 times (2 cages each of 7 hens per replicate). Hens housed in battery cages at an open house system under the same managerial conditions. Birds fed layer diet (15.5% crude protein and 2800 ME kcal kg^{-1}) during the experimental period up to 64 week of age (Table 1). Diets were formulated to meet or exceed the nutritional recommendations of National Research Council (NRC)[20] for laying hens. Room temperature was maintained at 21±2.6°C during the experimental period and the light program consisted of 16.5 h of light/day throughout the experiment (48-64 week of age). Feed and water were provided *ad libtum*.

Productive performance: The body weight was recorded at the beginning and at the end of the experiment. All the eggs produced were individually weighed and egg number was recorded per each replicate. Feed consumption was recorded by replicate every 4 week and mortality was recorded daily. Feed wastage was observed to be negligible and was not measured. From these data BW gain (BWG), egg production rate (ER), egg mass (EM), average daily feed intake (ADFI) and feed conversion ratio by kilogram of eggs (FCR, g feed: g egg) corrected for mortality were calculated cumulatively.

Blood metabolites: At the same day of slaughter, 3 mL of blood samples were collected from each slaughtered hen (4 hens per each replicate) and obtained in heparinized tubes. Blood samples were centrifuged at 3000 rpm min^{-1} for 10 min. Clear plasma samples were separated into Eppendorf tubes and kept in the deep freezer at -20°C until chemical

Table 1: Ingredient composition and calculated nutrient analysis of laying hens diet (% as-fed basis, unless stated otherwise)

Items	Layer diets (48-64 week of age)
Ingredients	
Yellow corn	60.00
Soybean meal, 44% CP	25.00
Soybean oil	3.82
Met-hydroxy, 88%	0.17
Sodium chloride	0.30
Sodium bicarbonate	0.06
Vitamin and mineral premix[1]	0.40
Fine limestone	10.00
Dicalcium phosphate	0.25
Calculated analysis[2]	
AMEn (kcal kg^{-1})	2.80
CP	15.50
Total Lys	0.77
Total Met	0.42
Total Thr	0.59
Total Trp	0.18
Total ash	12.00
Ca	4.00
Total P	0.45
Available P	0.35

[1]Provided the following (per kg of diet); Vitamin A (trans-retinyl acetate): 8,000 IU, Vitamin D3 (cholecalciferol): 1,750 IU, Vitamin E (all-rac-tocopherol acetate): 5 mg, Thiamine (thiamine mononitrate): 1 mg, Riboflavin: 3 mg, Pyridoxine (pyridoxine.HCl): 1 mg, Vitamin B12 (cyanocobalamin): 0.01 mg, Vitamin K (bisulfate menadione complex): 1 mg, Nicotinic acid: 16 mg, Pantothenic acid (D-calcium pantothenate): 7 mg, Mn ($MnSO_4.H_2O$): 70 mg, Zn (ZnO): 50 mg, Fe ($FeSO_4.H_2O$): 30 mg, Cu ($CuSO_4.5H_2O$): 4 mg, I (KI): 1 mg, Co: 0.2 mg, Se (Na_2SeO_3): 0.1 mg, Choline (choline chloride): 240 mg, Canthaxanthin (carophyll red): 200 mg, Phytase: 300 FTU, Ethoxyquin: 110 mg. [2]Calculated according to NRC [20] with a modification of increasing Ca level to 4% according to Safaa *et al.* [21]

analyses. Total protein (mg dL^{-1}), albumin (g dL^{-1}), globulin (g dL^{-1}), total cholesterol, low density lipoprotein cholesterol (LDL cholesterol) and high density lipoprotein cholesterol (HDL cholesterol) were determined and calculated as described by Safaa[5].

Enzymes activity: At 54 week of age, 16 hens from each treatment (4 hens per replicate, 2 hens per each cage) were chosen at random, weighted, slaughtered and eviscerated. The same located segments of their digestive tract (proventriculus and ileum) were emptied by gentle squeezing, contents of individual segments were taken and mixed and about 1 g of the mixed content was immediately diluted with 10 mL of distilled water. All samples were centrifuged for 10 min. The supernatant fluid was taken and stored in sealed bottles at -20°C until analyzed. After thawing at room temperature, the digesta samples were homogenized (1:9, wt/vol) with ice-cold 154 mmol L^{-1} sodium chloride solution and then centrifuged at 6000 rpm min^{-1} for 15 min at 4°C. Aliquots of the supernatant were collected for enzyme activity assay. All determinations were carried out in duplicate. Protease activity was assayed according to a modified method of Brock *et al.*[22] using casein as substrate and reacting it with Folin reagent. One protease unit is defined as the amount of enzyme that hydrolyses casein to form 1 μmol product per min. α-amylase activity was determined using the iodometric method of Somogyi[23] and one α-amylase unit is defined as the amount of enzyme that hydrolyses 10 mg of starch in 30 min. Enzyme activity was expressed as units per gram of digesta for proventriculus and ileum samples.

Statistical analysis: The completely randomized design was used with 5 treatments and 4 replications. The experimental unit (replicate) was consisted of a group of 14 hens (7 hens/cage). For enzymes activity and blood metabolites traits, the experimental unit was consisted of 4 birds, selected at random from each replicate. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for comparisons among means. Statistical Analysis System (SAS) software[24] was used to analyze the data. Differences between treatment means were considered significant at $p<0.05$ and $p<0.10$ were considered as a trend. Orthogonal polynomial contrasts were performed to study the linear and quadratic effects of dietary ZADO® levels on all traits. Results are presented as least square means±SEM.

RESULTS

Productive performance: No mortality was detected during the experimental period (48-64 week of age). Productive performance traits of Hisex lying hens in response to different dietary levels of ZADO® are presented in Table 2. Results indicated no significant differences in BW at 48 week of age. In addition, average BWG from 48-64 week of age was not affected in response to dietary ZADO® levels comparing with the control group. Moreover, dietary ZADO® levels significantly affected ER, EM, ADFI and FCR of hens. But, numerical favorable effects might be noted. No linear or quadratic effects were noted for all productive traits.

Blood metabolites: The values of plasma constituents in lying hens fed different levels of ZADO® at 54 week of age are presented in Table 3. Enzyme supplementation significantly increased plasma total protein ($p = 0.0320$) and globulin ($p = 0.0235$). Regarding cholesterol profile, total plasma cholesterol was increased in response to dietary 1 or 2 g ZADO® kg^{-1} diet when compared to chicks fed 0, 0.25 or

Table 2: Effect of dietary ZADO® on productive performance traits in laying hens from 48-64 weeks of age

	ZADO® (g kg^{-1})						Effects		
Traits	0.0	0.25	0.50	1.00	2.00	SEM[1]	p-value	Linear	Quadratic
Body weight at 48 wk (g)	1957.000	1966.000	1954.000	1962.000	1971.000	19.7000	0.2112	0.1750	0.5409
Body weight gain (g)	59.200	56.800	56.500	61.500	55.600	2.9700	0.1297	0.1643	0.6142
Egg production rate (%)	92.100	92.500	93.900	93.100	93.200	1.4700	0.2195	0.3623	0.7122
Egg weight (g)	60.600	61.600	60.700	61.000	61.400	2.9700	0.1302	0.1654	0.6123
Egg mass (g/bird/d)	55.800	57.000	57.000	56.800	57.200	2.9700	0.1297	0.1542	0.6141
Feed intake (g/bird/d)	116.800	115.800	117.800	118.200	115.900	3.3400	0.1304	0.3425	0.6884
Feed conversion ratio (g:g)	2.093	2.032	2.067	2.081	2.025	0.0651	0.3752	0.2900	0.4810

®Exogenous xylanases, cellulases, protease and α-amylase enzyme preparations at ensiling, [1]Standard error of the mean (n = 4 replicates with 2 cages with 7 hens per each), No significant differences ($p \leq 0.05$) were detected among treatments for all traits

Table 3: Effect of dietary ZADO® on blood metabolites in plasma of laying hens at 54 weeks of age

	ZADO® (g kg^{-1})						Effects		
Traits	0.0	0.25	0.50	1.00	2.00	SEM[1]	p-value	Linear	Quadratic
Plasma protein metabolites									
Total protein (mg dL^{-1})	4.43^{b}	4.96^{a}	5.17^{a}	5.47^{a}	5.37^{a}	0.103	0.0320	0.1185	0.7481
Albumin (mg dL^{-1})	2.91	2.90	2.98	3.01	3.03	0.109	0.1427	0.1076	0.6639
Globulin (mg dL^{-1})	1.52^{b}	2.06^{a}	2.19^{a}	2.46^{a}	2.34^{a}	0.212	0.0235	0.3148	0.7102
Plasma cholesterol profile									
Total cholesterol (mg dL^{-1})	223.70^{b}	224.90^{b}	227.70^{ab}	229.20^{a}	230.50^{a}	2.410	0.0356	0.0345	0.8352
LDL-cholesterol (mg dL^{-1})[2]	128.50	128.00	129.10	131.30	131.70	2.030	0.4150	0.4897	0.6582
HDL-cholesterol (mg dL^{-1})[2]	89.70	89.40	87.80	86.70	85.80	1.910	0.1053	0.1198	0.7657

®Exogenous xylanases, cellulases, protease and α-amylase enzyme preparations at ensiling, [1]Standard error of the mean (n=4 replicates with 5 samples per each), [2]LDL: Low density lipoprotein, HDL: High density lipoprotein, [a-d]Means within rows with different superscripts are significantly different ($p \leq 0.05$)

Table 4: Effect of dietary ZADO® on enzymes activities (unit)* in proventriculus and ileum digesta of laying hens at 54 weeks of age

	ZADO® (g kg^{-1})						Effects		
Items	0.0	0.25	0.50	1.00	2.00	SEM[1]	p-value	Linear	Quadratic
Proventriculus digesta									
α-amylase	0.70^{c}	1.50^{c}	2.26^{c}	19.08^{a}	11.96^{b}	0.368	0.0371	0.0412	0.0354
Protease	20.4^{c}	27.10^{c}	52.60^{b}	131.90^{a}	55.00^{b}	9.070	0.0102	0.0231	0.0126
Ileum digesta									
α-amylase	89.6^{d}	107.90^{c}	282.30^{b}	407.40^{a}±	287.00^{b}	23.410	0.0134	0.0354	0.0302
Protease	23.6^{c}	30.10^{b}	48.70^{b}	86.20^{a}	54.70^{b}	9.280	0.0102	0.0254	0.0196

®Exogenous xylanases, cellulases, protease and α-amylase enzyme preparations at ensiling, *Enzyme activity for α-amylase unit is defined as the amount of enzyme that hydrolyses 10 mg of starch in 30 min and for protease unit is defined as the amount of enzyme that hydrolyses casein to form 1 μmol product per min, [1]Standard error of the mean (n = 4 replicates with 4 samples per each). Each sample was duplicated, [a-d]Means within the same row with different superscripts are significantly different ($p \leq 0.05$)

0.50 g ZADO® kg^{-1} diet. In addition, linear effects were noted for this trait. However, no significant effects were observed for the other plasma metabolites levels in response to dietary ZADO® levels. Moreover, no quadratic effects of dietary ZADO® levels were observed for all plasma metabolites traits.

Enzymes activities: Table 4 shows enzymes activity enhancement of α-amylase and protease in the digesta of proventriculus and ileum of laying hens at 54 week of age in response to different dietary levels of ZADO® for 6 wk. Linear and quadratic effects for these traits were also noted ($p<0.05$). Results showed that dietary multi-enzymes up to 1 g kg^{-1} diet improved the enzyme activity of either α-amylase and protease in the digesta of both proventriculus and ileum in linear pattern then the level reduced when hens fed diets supplemented with 2 g ZADO® kg^{-1} diet indicated the quadratic pattern.

DISCUSSION

Productive performance: Results of this trial proved that ZADO® enzyme cocktail, which prepared at ensiling and contains xylanases, cellulases, α-amylase and protease, has numerical but not significant effects on the productive performance of hens when birds fed a corn-SBM based diet, which might reflected economic benefits for producers. These results are in agreement with Bigge *et al.*[25] who reported no significant effect of xylanases supplementation to the diet of

Bovan White Leghorns from 23-43 or from 43-58 week of age on productive performance of hens. Moreover, these results are also supported by Khan *et al.*[12] who reported that 40-week-old Hy-line W-98 lying hens fed diets supplemented with 2.0 g Phytezyme kg^{-1} during 70 days did not appear to cause any adverse effect on ER, EM and FCR (80.8%, 52.4 g and 2.24 g:g vs. 81.0%, 52.6 g and 2.18 g:g for control and treated hens, respectively). According to Khan *et al.*[12] phytezyme is a microbial multi-enzyme produced by *Saccharomyces cerevisiae* which contains a minimum amount of 1,500,000 unit of amylase kg^{-1}, 50,000 units of xylanase kg^{-1}, 30,000 units of b-glucanase kg^{-1}, 40,000 units of cellulase kg^{-1}, 30,000 units of portease kg^{-1}, 90,000 units of phytase kg^{-1}, 11,000,000 units of acid phosphates kg^{-1} and 500 unit of lipase kg^{-1}. In addition, Wen *et al.*[15] stated that diets supplemented with enzyme which contains phytase, xylanase, cellulase, α-amylase and acidic protease did not affect the productive performance of Isa-Brown laying hens from 60-68 weeks of age. They recorded ER (85.2 vs. 85.8%) and EM (53.6 vs. 53.8 g hen^{-1} day^{-1}) for control and supplemented groups, respectively. In the same context, in broilers, Marsman *et al.*[26] showed no improvement in FCR or BWG when mixed-enzyme preparation (carbohydrases and proteases) was added to the broiler diets from 7-25 days of age. Also, Barekatain *et al.*[27] observed that an admixture of xylanase and protease to broiler corn-SBM based diets up to 21 days of ages did not result in further improvement in productive performance represented by BWG, feed intake and FCR.

On the other hand, Lee *et al.*[11] noted that Natuzyme [multiple enzyme preparation composed of xylanase (10,000,000 unit kg^{-1}), cellulase (5,000,000 unit kg^{-1}), β-glucanase (1,000,000 unit/kg), pectinase (140,000 unit kg^{-1}) from *Trichoderma reesei* and *Trichoderma longibrachiatum*, protease (6,000,000 unit kg^{-1}), phytase (500,000 unit kg^{-1}) from *Aspergillus niger* and α-amylase (1,800,000 unit kg^{-1}) from *bacillus subtilis*)] supplementation into low energy and protein diets increased EM (61.3 vs. 63.5 g hen^{-1} day^{-1}) but did not affect ER (90.4 vs. 92.8%) and EW (67.8 vs. 68.3 g) in Hy-Line brown laying hens from 43 to 51 wk of age. In broilers, Kocher *et al.*[28] reported that using an enzyme cocktail containing pectinase, amylase and protease in corn–SBM-based diets for chicks resulted in improved performance. Also, Cowieson *et al.*[29] indicated that exogenous xylanase, amylase, protease and phytase (Avizyme) can be used successfully in a strategically formulated low nutrient density diet to maintain performance to that of birds fed on a nutritionally adequate diet. In addition, Cowieson and Ravindran[30] stated that supplementing corn-SBM-based broiler diets with an enzyme product containing xylanase, amylase and protease improved BWG and feed efficiency compared with the un-supplemented diets but feed intake did not affected. They also, reported that the energy and amino acid values of corn-based diets for broilers can be enhanced by supplementation with an enzyme cocktail of xylanase, amylase and protease, offering potential economic benefits to producers. The mode of action of enzymes in corn-based diets has been linked to improved starch digestibility associated with augmentation of endogenous α-amylase or improved digestion of resistant starches, improved access to cell contents via a reduction in cell wall integrity, modification of the intestinal microbial communities, improved protein solubility and digestibility and a reduction in the inimical effects of maize and/or soy-derived anti-nutritive factors. In the same context, Saleh *et al.*[31] reported that the commercial enzymes, which are mostly comprised of carbohydrases and contain small amount of protease activity (Energex) improved significantly the productivity (BWG and FCR) of broilers fed corn-SBM based diets in compare with pure carbohydrases (cellulase, hemicellulase and pectinase) supplementation, which tended to affect in compare to control group (without enzyme supplementation). However, they noted that feed intakes were not affected by dietary enzymes. Similar results have been found earlier by Zanella *et al.*[32] when they supplemented a corn-SBM diet with Avizyme (commercial enzyme). They found that BWG and FCR were significantly improved by Avizyme and demonstrated that the energy and amino acid digestibility of a corn-SBM-based diet for broilers could be improved by around 3% when supplemented with xylanase, amylase and protease allowing performance to be maintained on a diet with a lower nutritional plane. In addition, Kalmendal and Tauson[1] observed that the combination of xylanase and serine protease improved FCR, compared with the control diet but, BW and FI were not affected by enzyme addition sole or mixed. Moreover, Amerah *et al.*[13] suggested that xylanase and amylase had a synergetic effects on broiler FCR from 1-42 days of age.

It is well known that laying hens had active physiological cycle for producing eggs which might play an important role in decline of nutrient additives in small amount in response to increase the transit time of feed in the Gastro-Intestinal Tract (GIT)[14]. In addition, diversity of the quality of diets (protein and energy content) among trials might affect differently in response to enzyme supplementation. From these points of view, the authors suggesting that the accumulation of the additive effect of the enzymes, the effect of short period enzyme supplementation (from 48-64 week of age) and the nature of the diets used in the current trial might explain, at least in part, the differences among the above mentioned findings and the results of laying hens productivity in the current trial.

Blood metabolites: The values of plasma constituents in laying hens at 54 week of age (Table 4) were within the normal ranges for plasma total protein and albumin[5]. Moreover, the values of plasma lipid metabolites (total-, LDL- and HDL-cholesterol and their ratios) are within the normal ranges[11,12]. The present study showed that ZADO® supplementation to corn-based diets significantly increased the protein and globulin levels in plasma. It is well stated that gama-globulin is the main component of anti-body production, which presents the humoral immune response. So, findings of globulin levels in plasma in the current study are supported by Gao *et al.*[33], who suggested that xylanase supplementation, to wheat-based diets for cockerels from 7-21 days of age enhanced the humoral immune response. Enzyme supplementation of chicken diets is employed in order to increase the availability of starch, protein and other macronutrients that are entrapped by intact cell wall structures or viscous polymers that are resistant to digestion by endogenous host enzymes[34].

The current experiment showed a favorable effect of enzyme addition to laying hens' diets for 6 week (from 48-54 week of age) on increasing the cholesterol level in plasma, suggesting that enzyme supplementation might play a role in lipid metabolism. Unfortunately, little information has been published on the effects of enzyme supplementation in laying hens' diets on blood lipid metabolites. However, Onilude and Oso[35] reported that the supplementation of enzyme mixture including amylase, cellulase and pectinase to broiler fiber-containing diets from hatch to 42 days of age reduced blood lipid metabolites including plasma cholesterol level from 246-136 mg dL^{-1} at 42 day of age. Also, Cowieson *et al.*[36] reported that phytase addition to broiler diets reduced total cholesterol concentration in the blood of chickens fed the positive control diet (adequate in P and Ca) but increased cholesterol concentrations in the blood of chickens fed the negative control diet (with P and Ca levels reduced by 0.12 and 0.14%, respectively) however, no effect of phytase on total- and HDL-cholesterol was noted. They hypothesized that enzyme addition with adequate minerals levels (Ca and P) might reduce the cholesterol content in the plasma, which might explain, at least in part, the reduction of cholesterol level in plasma in response to dietary ZADO® by providing improvement of feed digestion and enhancement of mineral absorption. In contrast, Sarica *et al.*[37] reported that xylanase supplementation in broiler diets based on wheat-corn-SBM did not affect cholesterol content in plasma (169.4 vs. 180.6 mg dL^{-1} for treated and control groups, respectively). Frigard *et al.*[33] noted a higher serum cholesterol level in broilers at 21 days of age fed rye-corn-SBM based diet supplemented with commercial enzyme (2 g kg^{-1} diet; GP-5000, based on β-glucanases and xylanases) than those of birds fed the corresponding un-supplemented diet and attributed that to the elimination of the dietary fiber effect on reducing cholesterol content in the serum by the enzyme supplementation. In conclusion, the response to enzyme supplementation is based not only on ingredient 'quality' but also on bird age, environmental conditions, managerial conditions, enzymes preparations, duration of enzyme supplementations and the dose of supplementation.

Enzyme activity: The additive effect of endogenous and exogenous enzyme affected the enzyme activities in intestinal digesta represent the digestive capacity of poultry because most of amylase[38] and protease[15] activities are distributed in duodenal, jejunal and illeal sections of the small intestine. The present results suggested that enzyme supplementation increased the enzyme activity of α-amylase and protease in the digesta of both proventriculus and ileum in linear and quadratic patterns might affirm regulatory effect of enzyme supplementation on pancreatic enzymes indicating that the optimal level of supplementation is 1 g ZADO® kg^{-1} diet for brown laying hens. The increase in ileum protease activity may be attributed to an additive effect of exogenous and endogenous enzymes[39]. These changes suggest that enzyme activity might affect digestive enzyme activity by different mechanisms in different regions of the intestine. In addition, it has been reported that amylase and protease supplementation increased chymotrypsin activity in the pancreas of laying hens fed a diet based on corn-soybean[15] and decreased them in broiler chicks fed a diet based on sorghum[40]. Also, Jiang *et al.*[41] found a negative response of pancreatic amylase production to exogenous amylase addition in broilers. The regulatory effect of enzyme supplementation on pancreatic enzymes in laying hens in the present study is in agreement with studies conducted with broilers. In the broiler studies, enzyme supplementation started immediately after hatch, when the digestive tract is not well-developed and has to adapt gradually to the presence of exogenous enzymes. However, laying hens have a more mature digestive system that is more prepared to confront the impact of enzyme supplementation. Moreover, if the enzymes were additive in their effect, it would be expected that the sum of the effect attributed to each enzyme individually should not be different from the effect attributed to the use of the enzymes in combination[42]. This point of view might explain, at least in part, the linear effect of enzyme supplementation on enzyme activity of in the proventriculus and ileum of laying hens.

CONCLUSION

Laying hens' diets supplemented with 0.00, 0.25, 0.50, 1.00 or 2.00 g kg^{-1} diet ZADO® had slight numerical but not significant impact on laying hens productivity including egg production rate, egg mass and feed consumption. Dietary enzymes cocktail increased total protein level in favor of globulin and dietary 1 g kg^{-1} ZADO® or more increased total cholesterol level with no significant impact on neither LDL- nor HDL-cholesterol levels in plasma. Moreover, enzymes supplementation increased enzymes activity in digesta of both proventriculus and ileum. Therefore, it could be concluded that laying hens' diets supplemented with ZADO® up to 2.00 g kg^{-1} diet might increase plasma protein and enhance enzymes activity without any negative impacts on hens' productivity.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect of exogenous xylanases, cellulases, protease and α-amylase enzyme preparations at ensiling that can be beneficial for improving enzymes activity without any negative impacts on laying hens productivity. Thus, a new research area is opened to discover the mode of actions and possible mechanisms of enzyme mixture interactions and its impacts on hens' performance. Moreover, this study will guide the researchers to do more studies on the impacts of laying hens' diets supplemented with ZADO® levels more than 2.00 g kg^{-1} diet on laying hens' productivity and breeders' ones.

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