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Enzymatic Association of Phytase and Xylanase in Diets for Cage Free Laying Hens

Filipe A. Moreno[a*], Carlos Eduardo da Silva Soares[b], Fabiano Dahlke[c], Marcus VN Alvarez[d], Valdir Silveira de Ávila[e], Everton Luis Krabbe[e], Alex Maiorka[a]

[a]*Department of Veterinary Science, Federal University of Paraná, Curitiba, PR, 80035-050, Brazil.*

[b]*Department of Food Science and Technology, Federal University of Santa Catarina, Florianópolis, SC, 88034-000, Brazil.*

[c]*Department of Animal Science and Rural Development, Federal University of Santa Catarina, Florianópolis, SC, 88034-000, Brazil.*

[d]*Department of Genetics, São Paulo State University, Botucatu, SP, 18618-68, Brazil.*

[e]*Brazilian public agricultural research corporation, Concórdia, SC, 897000-000, Brazil.*

Abstract

The objective of this study was to evaluate the influence of an enzymatic association between phytase and xylanase on the production performance of commercial laying hens reared in cage free system during a complete cycle. A total of 840 Hy-Line Brown laying hens were used from 23 to 88 weeks of age and distributed in a completely randomized design, with 4 treatments and 5 replicates of 42 hens each. Hens The dietary treatments were formed according to the enzymatic levels and the nutritional matrix (Conventional: used by Brazilian poultry industries; and overvalued) and were as follows: Positive control: 300 units of phytase (FTU)/kg + 8,000 units of xylanase (BXU)/kg + Conventional Matrix (102 Kcal/kg AME; 0.17% Ca; 0.15% available P; 0.04% Na; 0.02% digestible Lysine); Superdosing (1,500 FTU/kg + 8,000 BXU/kg) + Conventional; Negative control: no enzymes + Overvalued Matrix (120 Kcal/kg AME; 0.22% Ca; 0.20% available P; 0.05% Na; 0.05% digestible Lysine); and Superdosing + Overvalued Matrix. Analyzed variables included productive performance, internal and external egg quality variables. Data were submitted to ANOVA to assess the effect of treatments and analysis of regression to assess the effect of treatments over time. The association of phytase and xylanase increased egg production rate, egg weight, egg mass, feed conversion per egg mass and feed conversion per dozen eggs ($P<0.001$), regardless of the nutritional matrix valorization. Egg quality was not affected by the enzymatic association. As a conclusion, the association of xylanase and phytase in diets for cage free layers can enhance the production performance, increasing the quantity of albumen and egg weight throughout the whole cycle of production

Keywords: egg production; egg quality; exogenous enzymes; nutritional matrix

INTRODUCTION

The last few decades were marked by an increase of egg consumption across all Latin America, especially in Brazil. The laying hens population in Brazil was 124 million birds in 2020, which produced approximately 54 billion eggs and raised the *per capta* egg consumption to 251 eggs/year/person (ABPA, 2021). This increase can be attributed to the competitive price of eggs and the development of marketing campaigns describing eggs as a healthy and nutritious food.

An increase in the number of layers reared in non-conventional systems – or cage-free systems - was also observed throughout the years, pushed forward by a new demand from consumers that are increasingly aware and concerned about animal welfare, thus leading to important changes in the production system (Weeks et al., 2016). These systems allow the birds to express their natural behavior of environment exploration and foster social interactions between individuals, but also impose severe restrictions on some types of ingredients included in the diet, especially animal by-products and antibiotics (Savory et al., 2006; Zeltner & Maurer, 2009).

A current concern around these new production systems is to meet the high nutritional requirements of modern laying hens. However, diets for laying hens are mainly based on vegetable ingredients, which contain considerable amounts of antinutritional factors and substances that are not readily digested by the endogenous digestive enzymes of the bird, thus diminishing dietary nutrient utilization (Munir & Maqsood, 2013). Therefore, the use of exogenous enzymes becomes an important strategy to increase diet utilization, lower feeding costs and reduce the excretion of potentially pollutant components (Bedford & Schulze, 1998). Significant attention is given to enzymes that can hydrolyze non-starch polyssacharides (NSP) abundantly found in cereals (e.g. arabinoxylans) and have antinutritional properties that increase the digesta's viscosity and reduces nutrient digestibility through a process of encapsulation (Kim et al., 2005; Zhang et al., 2014). Xylanase enzyme, for instance, can break down the long-chain structure of arabinoxylans into small xylo-oligomer chains, releasing nutrients for digestion and countering the high viscosity of the digesta (Zhang et al. 2014).

Another important enzyme is phytase, a key enzyme for the release of phytic phosphorus (P) from the phytate molecule, which is the main form of P storage in plants (Silversides et al., 2006). Phytic-P is majorly unavailable for digestion and absorption by monogastric animals, and phytate is considered a strong antinutrient due to its capacity of complexing not only P, but also other minerals, protein, and amino acids,

consequently reducing their utilization (Ravindran et al., 1995). Phytase belongs to the group of phosphatases, enzymes that act by hydrolyzing phosphate esters and removing them from the substrate – all nutrients bound to these phosphates have their bioavailability increased, including P, Ca and other minerals and amino acids (Ravindran et al., 2001). The recommended level of inclusion of exogenous phytase in layers' diets is 300 units of phytase (FTU)/kg (Dersjant-Li et al., 2015), but there's a rising interest in the use of greater doses of phytase (also known as superdosing) to further improve the production performance. Greater levels of phytase may potentialize the release of P, energy and amino acids (Cowieson et al., 2011), enhance mineral bioavailability (Kies et al., 2006), and reduce the antinutritional effect of phytate by reducing it concentration in the intestinal lumen (Lee and Bedford, 2016; Woyengo et al., 2012).

The combined action of exogenous phytase and xylanase may have complementary effects, such as a greater production of the intestinal hormone Peptide YY that increases the digesta's retention time, allowing for a better nutrient digestion and hydrolysis of NSPs by xylanase. This in turn may result in a greater efficiency of phytase in hydrolyzing phytate (Taylor et al., 2018). Ultimately, the association of both enzymes allows for the application of new feed formulation strategies for laying hens, with nutritional matrices that consider greater utilization a sophisticated utilization of Ca, P, crude protein, amino acids and metabolizable energy by the birds (Nagashiro, 2007).

Based on the above considerations, the objective of this study was to evaluate the effect of the association of different doses of exogenous xylanase + phytase and different values of dietary nutritional matrices on characteristics of egg production and egg quality throughout the entire productive period of laying hens reared in cage-free systems.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics Committee on the Use of Animals of Embrapa Swine & Poultry, nº 015/2018.

Animals, facilities, and handling procedures

A total of 840 Hy-line Brown layers with 13 weeks of age were obtained from a commercial farm. At 16 weeks of age, the birds were transferred to an experimental farm. Prior to the transfer, a sample weighing of the flock (10%) was done to obtain the average weight and coefficient of variation. Only birds with a body weight of 1400 ± 50 g were selected.

The birds were housed in an open shed, divided into 20 pens with an area of 5.6m². The pens were equipped with a perch (15 cm per bird) and a nest (5 birds per opening) covered with new wood shavings, pendular feeders with a 16 kg capacity and nipple drinkers. Feeding management (114 g/bird/day) and lighting program were provided according to the lineage management guideline.

Experimental design, treatments, and diets

The layers were distributed in a completely randomized design with 4 treatments and 5 replicates of 42 birds each. Dietary treatments varied according to the inclusion of phytase and xylanase and different doses, and 2 different dietary nutritional matrices stablished from the producer's recommendations (AB Vista; Table 1). Phytase was included either in dosages of 300 FTU/kg (conventional) or 1,500 FTU/kg (superdosing), whereas xylanase had an unique dose of 8000 units of xylanase (BXU)/kg. The treatments were: Positive control diet (PC): 300 FTU/kg + 8,000 BXU/kg + Conventional Matrix; Conventional matrix with superdosing phytase (CS) diet: 1,500 FTU/kg + 8,000 BXU/kg + Conventional matrix; Negative control diet (NC): no enzymes + Overvalued matrix; Overvalued matrix with superdosing phytase (OS) diet: 1,500 FTU/kg + 8,000 BXU/kg + Overvalued matrix.

The diets were formulated to meet the nutritional requirements of the lineage guide in a single phase according to the farm's routine. The utilized phytase was Quantum Blue (AB Vista, Marlborough, UK – IUB: 3.1.3.26) and xylanase was Econase-XT25 (AB Vista Wiltshire, UK – IVB: 3.2.1.8). Experimental diets were based on corn, soybean meal and wheat barn (Table 2).

Table 1. Nutritional requirements and nutrient valorization from the combination of exogenous phytase and xylanase using for laying hens from 16 to 88 weeks of age, using two methods of valorization (conventional and overvalued).

Nutrients	Nutritional requirements (weeks of age)	Nutritional valorization	
	16 to 88	Conventional	Overvalued
AME, Kcal/kg	2,850	102	120
Ca,%	3.90	0,165	0,220
Av. P, %	0.43	0,150	0,200
Na, %	0.17	0,035	0,045
Dig. Lys., %	0.8	0,021	0,050
Dig. Met+Cis, %	0.7	0,038	0,050
Dig. Met., %	0.41	0,004	0,016

Table 2. Ingredients composition and nutrient content of experimental diets fed from 16 to 88 weeks of age.

Treatments	NC	PC	CS	OS
Xylanase (BXU/kg)	0		8000	
Phytase (FTU/kg)	0	300	1500	1500
Ingredient (%)				
Corn	54.175	54.175	54.175	54.175
Soybean meal (45% CP)	24.842	24.842	24.842	24.842
Limestone	9.158	9.128	9.128	9.158
Wheat bran	7.500	7.500	7.500	7.500
Soybean oil	2.481	2.663	2.663	2.481
Dicalcium phosphate	0.598	0.869	0.869	0.598
Sodium chloride	0.291	0.316	0.316	0.291
Layer premix[1]	0.300	0.300	0.300	0.300
DL-methionine	0.108	0.120	0.120	0.108
Lysine	0.019	0.056	0.056	0.019
Caulin	0.518	0.012	0.000	0.498
Butylhydroxytoluene (BHT)	0.010	0.010	0.010	0.010
Nutritional composition (%)				
Crude protein	16.209	16.240	16.240	16.209
Crude fat	5.115	5.295	5.295	5.115
Calcium	3.680	3.735	3.735	3.680
Available phosphorus	0.230	0.280	0.280	0.230
Total phosphorus	0.480	0.530	0.530	0.480
Crude fibre	2.965	2.965	2.965	2.965
Sodium	0.125	0.135	0.135	0.125
Digestible lysine	0.750	0.779	0.779	0.750
Digestible methionine	0.394	0.406	0.406	0.394
Digestible methionine + cystine	0.650	0.662	0.662	0.650
Digestible isoleucine	0.593	0.593	0.593	0.593
Digestible threonine	0.522	0.522	0.522	0.522
Digestible tryptophan	0.175	0.175	0.175	0.175
Digestible valine	0.683	0.683	0.683	0.683
Metabolizable energy (Kcal/kg)	2730	2750	2750	2730

[1]Provided per kg of the product in the diet: copper (min.) 3.33 g; iron (min) 16.65 mg; manganese (min.) 33.34 g; selenium (min.) 101 mg; zinc (min.) 33.33 g; vitamin A (min.) 4000800 IU; Vitamin D3 (min.) 1000200 IU; Vitamin E 16670 IU; Vitamin K3 (min.) 1.67 g; Vitamin B1 (min.) 0.98 g; Vitamin B2 (min.) 4g; Vitamin B6 (min.) 1.64g; Vitamin B12 (min.) 10,000 mcg; folic acid (min.) 1.03 g; pantothenic acid (min.) 4.98 g; Niacin (min.) 16.67g; Biotin (min.) 0.1 g; Hill (min.) 140.59g; Iodine (min.) 0.66 g.

Analyzed variables

From the beginning of the egg laying period (first egg), 5 daily collections of eggs were made to record the production performance. Eggs were used for data analysis only from the 23rd week onwards due to the great physiological variability observed at the beginning of the laying period.

Egg quality

On the first and last day of the production cycles (6 weeks average), the following egg quality assessments were made: egg weight (EW) - individual weighing on a 0.01g precision digital scale (Bel, model S622); 100 eggs per treatment were selected (20 eggs per repetition) using an arithmetic mean, duly identified, and sent for evaluations of internal (n=50) and shell quality (n=50). The analyzed variables for internal quality included yolk weight (YW, g), yolk index (YI), albumen weight (AW), and Haugh Unit.

The yolks were weighed individually on precision digital scales (Bel, model S622), and their diameter (mm) and height (mm) were measured with a digital pachymeter (Kingtools, model 502150Bl); the YI was then obtained by the ratio between these yolk height and diameter.

The AW was obtained by the difference between the total weight of the eggs and shell and yolk weights, as in: AW = EW - YW - eggshell weight. Thick albumen height was measured with a digital micrometer, accurate to 0.01mm (Kingtools, model 502150Bl). This measurement, together with egg weight, were used to calculate the Haugh Unit, Haugh Unit = 100 log (h + 7.57 - 1.7w0.37), where h is the thick albumen height in millimeters and w is the egg weight in grams.

For external/eggshell quality, the analyzed variables included specific gravity (SG, g/ml), eggshell percentage (ES%), eggshell thickness (ET, μm), and eggshell breaking strength (EBS, Kgf). For the evaluation of SG, the saline immersion method (1.066 to 1.102 g/ml) was used. The EBS was determined in the equatorial region of the egg with the help of a texturometer (Stable Micro Systems, model TA.XT Plus - Texture Analyser) using a 2 mm rupture probe, which registered the force necessary to break the shell and the result was expressed in kilogram-force (Kgf). Eggshells were then washed with running water and dried at room temperature for 24 hours, weighed individually on a 0.01g precision digital scale (Bel, model S622) to measure ET at the equatorial region using a digital micrometer accurate to 0.001 mm (Digimess, model IP40).

Productive performance

Analyzed production performance variables were: egg production rate (EPR), egg weight (EW) egg mass (EM), feed conversion per egg dozen (FC/dz) and feed conversion per egg mass (FC/EM). The EPR was calculated weekly and the following formula was used: EPR (%) = (EPP/NA/Y)*100, where OPP is the total number of eggs produced in the period (weeks); NA is the number of birds; and Y is the number of days in the period. Daily egg production was recorded in order to perform this calculation.

The EM indicates how many grams of egg each bird produced per day, and was calculated by the formula: EM (g) = AEW * EPR/100, where AEW is the average egg weight. The FC/EM was determined by the ratio between total feed intake (kg) and EM. Feed conversion per egg dozen (FC/dz) was determined by the ratio between total feed intake and the amount of dozen eggs produced.

Statistical analyses

All data was submitted to normality (Shapiro-Wilk) and homocedasticity (Levene) analyses. Data with normal distribution were submitted to ANOVA to evaluate treatment effects and submitted to analyses of regression to evaluate treatment effects over time (weekly periods for EPR variable and every 6 weeks for other variables). The negative control treatment (with no enzyme supplementation) was considered the intercept.

When significative, means were compared by post-hoc Tukey test at 5% probability. All statistical procedures were performed using the software RStudio (RStudio Team, et al., 2015) for R Language (R Team, 2013; RStudio Team et al., 2015).

RESULTS AND DISCUSSION

Live performance

The values observed in Table 3 were used to build the following equations for each productive performance variable: EPR = 81 + 0.88(age) - 0.017(age)² + 0.000076(age)³ + treatment; EW = 30 + 1.7 (age) - 0.031 (age)² + 0.00019 (age)³ + treatment; EM = 14 + 2.6 (age) - 0.047 (age)² + 0.00026(age)³ + treatment; FC/EM = 3 - 0.063(age) + 0.0012(age)² - 0.0000067(age)³ + treatment; FC/dz = 1.5 - 0.003(age) + 0.000065(age)³² + treatment, with "age" in weeks and "treatment" defined according to the described coefficients of each treatment (Positive control , CS, OS) for each response variable and zero for the intercept (NC).

The association of phytase + xylanase improved EPR, EW, EM, FC/EM and FC/dz (P<0.001). However, the overvaluation of the nutritional matrix did not affect EW (Table 3).

Table 3. Effect of phytase and xylanase supplementation on layers' production performance variables from 23 to 88 weeks of age.

Coefficients	EPR (%)	EW (g)	EM (g)	FC/EM	FC/dz
NC (intercept)	81 ± 2.2 b	30 ± 2.5 b	14 ± 3.2 b	3 ± 0.12 b	1.5 ± 0.014 b
PC	3.1 ± 0.25 a	0.74 ± 0.31 ab	2.2 ± 0.41 a	-0.086 ± 0.015 a	-0.063 ± 0.0047 a
CS	3 ± 0.24 a	1.1 ± 0.31 a	2.6 ± 0.4 a	-0.1 ± 0.015 a	-0.06 ± 0.0046 a
OS	2.9 ± 0.25 a	0.2 ± 0.31 b	1.9 ± 0.41 a	-0.077 ± 0.015 a	-0.066 ± 0.0046 a
Age (L)	0.88 ± 0.13 ***	1.7 ± 0.16 ***	2.6 ± 0.2 ***	-0.063 ± 0.0072 ***	-0.003 ± 0.00051 ***
Age² (Q)	-0.017 ± 0.0023 ***	-0.031 ± 0.003 ***	-0.047 ± 0.0038 ***	0.0012 ± 0.00014 ***	6.5e-05 ± 4.2e-06 ***
Age³ (P)	7.6e-05 ± 1.3e-05 ***	0.00019 ± 1.8e-05 ***	0.00026 ± 2.3e-05 ***	-6.7e-06 ± 8.2e-07 ***	
R^2	0.8	0.68	0.7	0.68	0.78
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*Means within a column with different superscripts differ significantly (P < 0,05). NC: Negative control; PC: Positive control; CS: Conventional matrix with superdosing phytase; OS: Overvalued matrix with superdosing phytase; EPR: egg production rate; EW: egg weight; EM: egg mass; FC/EM: feed conversion per egg mass; FC/dz: feed conversion per dozens of eggs. L - Linear coefficient, Q - Quadratic coefficient, P – Polynomial coefficient. ***P < 0.0001. The values observed were used to build the following equations for each productive performance variable: EPR = 81 + 0.88(age) - 0.017(age)² + 0.000076(age)³ + treatment; EW = 30 + 1.7 (age) - 0.031 (age)² + 0.00019 (age)³ + treatment; EM = 14 + 2.6 (age) - 0.047 (age)² + 0.00026(age)³ + treatment; FC/EM = 3 - 0.063(age) + 0.0012(age)² - 0.0000067(age)³ + treatment; FC/dz = 1.5 - 0.003(age) + 0.000065(age)³² + treatment, with "age" in weeks and "treatment" defined according to the described coefficients of each treatment (PC , CS, OS) for each response variable and zero for the intercept (NC).

The EPR was reduced by 3% in the negative control dietary treatment (NC), without the addition of the enzyme association. However, layers fed enzyme-supplemented diets with the conventional valorization in the nutritional matrix (CS) had a higher EW (1g on average) compared to birds subjected to diets with the nutritional matrix overvalued, either with or without addition of enzymes (NC and OS). An average increase of 2.23g was observed for EM during the whole production cycle with the use of the enzymatic association, regardless of the nutritional valorization. Both FC/EM and FC/dz were respectively improved by an average of 0.088 and 0.063, evidencing a better feed efficiency for all hens fed the enzyme association. Among the parameters mentioned above, the treatment that consisted of a superdosing of phytase associated with xylanase and a overvalued matrix (OS) can be considered the most advantageous; overvaluing the matrix means to lower the inclusion of ingredients in the feed, thus reducing formulations cost without impairing the performance of the hens, seen that the results were similar to the other treatments (PC and CS) and exceeded the negative control treatment (NC).

This improvement of productive performance might be related to a combined action of the phytase/xylanase association. Kim et al. (2005) and Karimi et al. (2005) state that the containment of P within the plant cells, together with the presence of NSPs in the plant wall can reduce the availability of the content by encapsulation. Consequently, the action of phytase on P can be limited by the presence of NSPs, resulting in less phytate hydrolysis. Once xylanase has hydrolyzed the NSPs present in the plant wall, the encapsulated content is made available for phytase, so the use of xylanases (or other carbohydrases) can be regarded as a promoter of better phytase efficiency, optimizing phytate destruction and improving the release of phytic-P.

Another positive effect of phytase on the productivity of poultry was described by Ravindran (2000), who observed that saponification reactions occur when minerals complexed with phytic acid react with lipids in the digestive tract, which may impair the use of these fats as energy sources by the animal. Phytase can then improve dietary energy use through the release of minerals complexed to phytate, preventing the formation of these metal soaps and enhancing the performance and production of birds.

Similar results to the current study were observed by Abreu et al. (2018), who assessed an enzymatic association based on xylanase, B-glucanase and phytase with a caloric deficit (75 kcal of metabolizable energy) in the diet of layers at peak production (25 weeks); the authors reported that the EPR and EW were negatively affected by dietary nutritional reductions, but the inclusion of 100 g/t of enzymes led to a recovery of performance equivalent to the treatment without valorization of the nutritional matrix. Silversides et al. (2006) also observed an increase in EW with the use of phytase + xylanase in association, but not when added alone, suggesting an interaction between the two enzymes. Um and Paik (1999), in their work with Isa Brown layers from 20 to 40 weeks also found an average increase of 2.15% in EPR per bird per day

and 2.74% in EPR per bird fed a control diet with phytase but no nutritional valorization. As the control diet contained sufficient levels of available P and there was an increase in EPR, the authors concluded that phytase supplementation may not only influence the utilization of phytic P but also of other nutrients. Similarly, in a study by Ny et al. (1998), a combined enzyme supplementation (xylanase, alpha-amylase and protease) in diets with low nutrient density increased daily EM by an average of 11% when compared to diets that did not contain any enzymes and was equivalent to the results obtained when layers were fed the control diet.

The improvement in FC/dz and FC/EM may be related to the greater availability of substrate by the synergic action of phytase and xylanase, in addition to the greater energy increase (Conte et al., 2013) from the breakdown of NSPs by xylanase. An additional beneficial effect that can be exploited by the action of exogenous xylanase in diets is the increase in retention time; the fermentation of oligosaccharides – a reaction fostered by xylanase – results in the production of short chain fatty acids; these acids stimulate the production of the hormone peptide YY by endocrine cells of the ileum and colon, and it is able to modulate the ileal brake mechanisms, thus inhibiting gastric emptying; a longer retention time eventually favors the utilization of nutrients and improves the productive performance of the animal (Sigh et al, 2012; Taylor et al., 2018).

The results obtained for FC/dz and FC/EM also corroborate with Taylor et al. (2018), who used 300 FTU/kg of phytase and 12000 BXU/kg of xylanase and observed a positive interaction between these enzymes on feed conversion, attributing this effect to the greater availability of substrate for degradation by phytase. The effect of a greater retention time was not considered by the authors as the concentration of peptide YY was not affected by the enzymes. The authors based their conclusions on the *in vitro* action of xylanase, which is shown to increase the cell permeability of the aleurone wall, increasing the availability of phytate for degradation (Parkkonen et al., 1997). Furthermore, when supplementing the superdosing of phytase (1,500 FTU/ kg), the use of xylanase had no impact on FC, suggesting that the positive effects on feed efficiency with phytase superdosing did not allow additional room for the improvements that could have been caused by xylanase. According to Lee et al. (2017), FC is the most sensitive parameter to phytase overdosage.

Egg quality

The values shown in Table 4 were used to build the following equations for each variable of external quality of eggs: SG = 1100 - 3(age) + 0.06(age)² - 0.00038 (age)³ + treatment; ES% = 13 - 0.16 (age) + 0.0031(age)² - 0.00002 (age)³ + treatment; ET = 140 + 13(age) - 0.018(age)² + 0.00066 (age)³ + treatment; EBS = 5 - 0.11(age) + 0.0018(age)² - 0.000011 (age)³ + treatment, with "age" in weeks and "treatment" defined according to the coefficients described in the table for each treatment (PC, CS, OS) in each response variable and zero for the intercept (NC).

There was no difference ($p>0.05$) between treatments for the SG, ET and EBS. The ES% was slightly lower ($p<0.0001$) when comparing layers fed the dietary treatments containing 1,500 FTU/kg with those containing 300 FTU/kg, both under the conventional nutritional matrix (Table 4).

Table 4. Effect of phytase and xylanase supplementation on external egg quality parameters of laying hens from 23 to 88 weeks of age.

Coefficient	SG	ESP (%)	EST (µm)	ESS (Kgf)
NC (intercept)	1100 ± 4.4 a	13 ± 0.39 ab	140 ± 36 a	5 ± 0.31 a
PC	-0.17 ± 0.53 a	0.036 ± 0.048 a	5.2 ± 4.8 a	-0.036 ± 0.038 a
CS	-0.82 ± 0.53 a	-0.091 ± 0.048 b	-0.65 ± 4.8 a	-0.042 ± 0.038 a
OS	-0.6 ± 0.54 a	0.012 ± 0.049 ab	-2.4 ± 4.9 a	-0.036 ± 0.038 a
Age (L)	-3 ± 0.27 ***	-0.16 ± 0.024 ***	13 ± 2.3 ***	-0.11 ± 0.019 ***
Age² (Q)	0.06 ± 0.0052 ***	0.0031 ± 0.00046 ***	-0.18 ± 0.044 ***	0.0018 ± 0.00037 ***
Age³ (P)	-0.00038 ± 3.1e-05 ***	-2e-05 ± 2.7e-06 ***	0.00066 ± 0.00026 *	-1.1e-05 ± 2.2e-06 ***
R^2	0.79	0.75	0.68	0.70
P-value	0.0972	< 0.0001	0.0793	0.0624

*Means within a column with different superscripts differ significantly ($P < 0,05$). NC: Negative control; PC: Positive control; CS: Conventional matrix with superdosing phytase; OS: Overvalued matrix with superdosing phytase; SG: Specific gravity; EST: Eggshell thickness; ESP: Eggshell percentage; ESS: Eggshell strength. L - Linear coefficient, Q - Quadratic coefficient, P – Polynomial coefficient. ***$P < 0.0001$ and *$P < 0.05$. The values observed were used to build the following equations for each variable of external quality of eggs: : SG = 1100 - 3(age) + 0.06(age)² - 0.00038 (age)³ + treatment; ES% = 13 - 0.16 (age) + 0.0031(age)² - 0.00002 (age)³ + treatment; ET = 140 + 13(age) - 0.018(age)² + 0.00066 (age)³ + treatment; EBS = 5 - 0.11(age) + 0.0018(age)² - 0.000011 (age)³ + treatment, with "age" in weeks and "treatment" defined according to the coefficients described in the table for each treatment (PC, CS, OS) in each response variable and zero for the intercept (NC).

This difference can be explained by the increased amounts of internal components of the egg, as observed by the increased EW for the CS diet. Despite the higher EW and lower ES%, eggshell quality was not negatively affected overall, as ET and EBS values remained unaltered. It was not possible to affirm if this was due to the beneficial effects of the enzymes or if the dietary levels of Ca were sufficient to meet the requirements for good eggshell formation, even in larger eggs, indicating that mineral requirements could be above the real needs of the layers.

Araújo et al. (2008) and Oba et al. (2013) also observed no effect of the addition of enzyme combinations on egg SG and ET, stating that the control diets met the specific needs of the birds for these quality parameters. However, previous studies (Lim et al. 2003; Englmaeirová et al. 2017) reported that the addition of phytase improved egg quality parameters regarding ET and EBS due to an increased Ca and P availability.

The findings of the current study for ES% differ from the results obtained by Silva et al. (2012), who assessed the ES% in eggs from commercial Isa Brown layers fed diets containing phytase and carbohydrases with or without nutritional valorization (30 kcal MS, 0.24% CP, 0.15% Ca and 0.11% P) but found no significant effects on ES%. The ES% was also not affected by the factors evaluated in the work of Vieira et al., 2011 and Lichovnicova (2007). The authors stated that there was no effect of phytase on the Ca balance, and that the deposition of Ca in the eggshell was similar between diets, although the efficiency of Ca deposition in the eggshell decreased when increasing dietary Ca. Lichovnicova (2007) and Chandramoni & Sinha (1998) found an important effect on dietary Ca retention, as lower levels of Ca are better absorbed by the bird (or less excreted), and that eggshell Ca deposition can also be maintained at the expense of bone Ca, resulting in a negative body Ca balance. This may help explain the results of the present study, in which eggshell quality may have been maintained even with the non-supplemented diets due to lower concentrations of dietary Ca, and/or due to the mobilization of bone Ca. In the case of Ca being removed from the bone, despite the long period of experimentation (88 weeks), no pathology or injury to the animals' health was observed.

The values shown in Table 5 were used to build the following equations for each variable of internal quality of eggs: YW = -3.5 + 1.1(age) -0.019(age)² + 0.00011(age)³ + treatment (PC, CS or OS); AW = 29 + 0.62(age) - 0.012 (age)² + 0.000077(age)³+ treatment; GI = 48 - 0.42(age) + 0.0036 (age)² + treatment; Haugh Unit = 150 - 3.8 (age) + 0.067(age)² - 0.00035(age)³ + treatment, with "age" in weeks and "treatment" defined according to the coefficients described in the table for each treatment (PC, CS, OS) in each response variable and zero for the intercept (NC).

The results for internal quality of eggs showed no difference ($p>0.05$) between treatments for the parameters YW, YI and Haugh Unit (Table 5). The AW was higher ($p<0.0001$) when using the enzyme association with phytase superdosing and a conventional valorization of the nutritional matrix (CS), and the increment in EW and EM previously described can be attributed to the increase of this component.

Table 5. Effect of phytase and xylanase supplementation on internal egg quality variables of laying hens from 23 to 88 weeks of age.

Coefficients	YW (g)	AW (g)	YI (%)	Haugh Unit
NC (intercept)	-3.5 ± 1 a	29 ± 2.4 b	48 ± 1.4 a	150 ± 8.2 a
PC	0.12 ± 0.13 a	0.37 ± 0.3 ab	0.068 ± 0.47 a	-0.008 ± 1 a
CS	0.079 ± 0.13 a	0.92 ± 0.3 a	0.53 ± 0.48 a	-0.66 ± 1 a
OS	-0.16 ± 0.13 a	0.091 ± 0.31 b	0.49 ± 0.48 a	0.091 ± 1 a
Age (L)	1.1 ± 0.065 ***	0.62 ± 0.15 ***	-0.42 ± 0.051 ***	-3.8 ± 0.51 ***
Age² (Q)	-0.019 ± 0.0012 ***	-0.012 ± 0.0028 ***	0,0036 ± 0,00045 ***	0.067 ± 0.0098 ***
Age³ (P)	0.00011 ± 7.3e-06 ***	7.7e-05 ± 1.7e-05 ***		-0.00035 ± 5.8e-05 ***
R^2	0.79	0.68	0.59	0.57
P-value	0.2657	< 0.0001	0.1298	0.1136

*Means within a column with different superscripts differ significantly (P < 0,05). NC: Negative control; PC: Positive control; CS: Conventional matrix with superdosing phytase; OS: Overvalued matrix with superdosing phytase; YW: yolk weight; AW: albumen weight; YI: Yolk index. L - Linear coefficient, Q - Quadratic coefficient, P – Polynomial coefficient. ***P < 0.0001. . The values observed were used to build the following equations for each variable of internal quality of eggs: YW = -3.5 + 1.1(age) -0.019(age)² + 0.00011(age)³ + treatment (PC, CS or OS); AW = 29 + 0.62(age) - 0.012 (age)² + 0.000077(age)³+ treatment; GI = 48 - 0.42(age) + 0.0036 (age)² + treatment; Haugh Unit = 150 - 3.8 (age) + 0.067(age)² - 0.00035(age)³ + treatment, with "age" in weeks and "treatment" defined according to the coefficients described in the table for each treatment (PC, CS, OS) in each response variable and zero for the intercept (NC).

Because the control of egg size can be influenced by different energy and/or fat and/or linoleic acid levels or by adjustments in protein and/or methionine and/or methionine + cystine levels (Leeson & Summers, 2005), the results found in the present study may come from the superdosing of phytase. Phytase can release more energy, amino acids and other nutrients when supplemented in higher doses (Cowieson et al. 2011), and in this case the albumen component was the most affected.

The results agree with Silversides et al. (2006), who studied the interaction between phytase and xylanase in wheat-based diets for laying hens and found an increase in EW when supplementing both enzymes in association; this increase was mainly related to the increase in AW rather than eggshell weight.

The Haugh Unit can be considered a standard universally accepted measure in the poultry industry and is used an indication of the internal quality of the egg (Vargas et al., 2016); The higher its numerical value, the better the quality of the egg (Alleoni and Antunes 2001). In the current study was possible to verify that Haugh Unit was not affected by treatments regardless of the dietary supplementation with the enzyme complex or the matrix valorization used throughout the production cycle. Oba et al. (2013) and Vargas et al., (2016) also found no effects on Haugh Unit, possibly because the control diet was sufficient to meet this quality parameter.

CONCLUSIONS

The supplementation of xylanase in association with phytase to the diet of commercial layers improves the productive performance during the entire production, increasing the amount of albumen and egg weight. Even nutritional matrices with overvalued requirements can be used alongside the enzyme association when phytase is added in overdoses. Egg quality is not affected by the associated enzymatic action of phytase and xylanase.

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