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Phytase and protease supplementation for laying hens in peak egg production

Suplementação de fitase e protease para galinhas em pico de produção

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Abstract

The effects of enzyme combinations in diets for commercial laying hens need further clarification. The goal of this study was to determine if the type of protease used in diets supplemented with phytase affects performance, nutrient intake, egg quality or intestinal mucosa morphometry of laying hens during peak egg production. Seven hundred and eighty hens (25-week-old Hy-Line W36 hens) were assigned to a completely randomized design composed of five treatments/diets with 12 replicates of 13 birds each. The five treatments were: 1) positive control: diet formulated according to the Hy-Line nutritional recommendations, without proteases , 2) negative control A: positive control diet reduced in energy, protein and amino acids according to protease A matrix, without protease supplementation, 3) negative control B: positive control diet reduced in energy, protein and amino acids according to protease B matrix, without protease supplementation, 4) negative control A plus protease A, 5) negative control B plus protease B. There was no effect of the treatments ($P > 0.05$) on egg production, egg mass or feed conversion; however, the nutritional restriction imposed by the negative controls reduced egg weight (negative control A, $P=0.02$), albumen height ($P < 0.01$) and the Haugh unit ($P < 0.01$). Protease supplementation reduced the calculated intake of protein and amino acids compared to the positive control; nevertheless, protease A was able to maintain egg weight, albumen height and the Haugh unit at the same levels as that obtained with the positive control hens. The intestinal mucosa responded to treatment only at the jejunum ($P < 0.01$), but the negative controls did not modify villus height or crypt depth compared to the positive control. However, crypt depth of protease B hens was higher than that of the positive control hens. In conclusion, when included in diets supplemented with phytase, the type of protease affects performance, nutrient intake, egg quality and intestinal mucosa morphometry of laying hens during peak egg production.

Key words: Amino acid. Egg production. Egg quality. Enzyme. Nutritional restriction.

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Resumo

Os efeitos da utilização em conjunto de enzimas exógenas para aves de postura precisam ser mais explorados na literatura. No intuito de determinar se o tipo de protease, em dietas suplementadas com fitase, interfere no desempenho, qualidade do ovo, ingestão de nutrientes e morfometria da mucosa intestinal de galinhas em pico de postura, 780 galinhas Hy-Line W36 de 25 semanas foram distribuídas em um delineamento inteiramente casualizado composto por cinco tratamentos/dietas com 12 repetições de 13 aves cada. Os cinco tratamentos foram: 1) controle positivo: dieta formulada de acordo com as recomendações nutricionais da Hy-Line, sem proteases, 2) controle negativo A: dieta controle positivo reduzida em energia, proteína e aminoácidos conforme a matriz nutricional da protease A, sem proteases, 3) controle negativo B: dieta controle positivo reduzida em energia, proteína e aminoácidos conforme a matriz nutricional da protease B, sem proteases, 4) controle negativo A com inclusão da protease A, 5) controle negativo B com inclusão da protease B. Não houve efeito dos tratamentos ($P > 0,05$) sobre produção de ovos, massa de ovos e conversão alimentar; porém, a restrição nutricional imposta pelos controles negativos diminuiu o peso do ovo (controle negativo A, $P = 0,02$), a altura do albúmen ($P < 0,01$) e a unidade Haugh ($P < 0,01$). Embora a inclusão das proteases em seus respectivos controles negativos não tenha garantido o mesmo consumo de proteína e aminoácidos observado no grupo controle positivo, a adição da protease A reverteu os efeitos adversos da restrição nutricional sobre o peso do ovo, a altura do albúmen e a unidade Haugh. O efeito dos tratamentos sobre a morfometria da mucosa intestinal foi detectado somente no jejuno ($P < 0,01$), porém, o consumo dos controles negativos não alterou a altura de vilosidades e a profundidade de criptas em relação ao controle positivo. Aves suplementadas com a protease B, no entanto, apresentaram maior profundidade de criptas que o controle positivo. Em conclusão, quando utilizadas em dietas suplementadas com fitase, o tipo de protease interfere no desempenho, qualidade do ovo, ingestão de nutrientes e morfometria da mucosa intestinal de galinhas em pico de postura.

Palavras-chave: Aminoácido. Enzima. Produção de ovo. Qualidade de ovo. Restrição nutricional.

Introduction

While the benefits of phytases in poultry nutrition are widely recognized, the use of proteases in commercial formulations still requires greater acceptance. Proteases alone have only started to be evaluated in recent years, as they were usually analyzed as part of multienzyme complexes, combined with xylanases, amylases, and glucanases (ADEOLA; COWIESON, 2011). Studies comparing the effects of these complexes in addition to phytases are abundant in the literature (AL-SAFFAR et al., 2013; OLUKOSI et al., 2010; TIWARI et al., 2010), but most of them were conducted with broilers. Therefore, although some improvement in performance and feed digestibility has been described in birds fed with proteases, assumptions about the combined effects of proteases and phytases that do not take into account the presence of carbohydrases, or that are related to egg production and egg quality parameters, still require further investigation.

A greater understanding of the relationship between proteases and phytases is essential for precise feed formulation and full exploitation of these additives. Different proteases and phytases can have distinct levels of affinity, and this characteristic may modify the nutritional contribution of the enzymes to the formulation. Cowieson and Adeola (2005) described the additive effect of proteases, phytases, and carbohydrases on broiler performance, but no differences in nutrient digestibility were detected when enzymes were compared alone or in combination. While information on this topic is scarce, the current practice regarding commercial formulations is to only consider the contribution of protease for nutrients that are also provided by phytase. With this approach, protease is considered to be the only enzyme providing protein, amino acids and energy to the diet, causing the possibility of excessive nutrients in the formulation.

In view of the above considerations, this experiment was designed to determine if the type

of protease used in diets supplemented with phytase affects performance, nutrient intake, egg quality or intestinal mucosa morphometry of laying hens during peak egg production

Materials and Methods

The trial was conducted in a commercial laying hen farm with Hy-Line W36 hens housed in conventional laying cages. All experimental procedures were approved by the Ethics Committee on Animal Use of Federal University of Mato Grosso (protocol nº 23108.113508/2015-69).

A total of 780 23-week-old hens were weighed individually and distributed in a completely randomized design composed of five treatments with 12 replicates of 13 birds each. The treatments consisted of five different diets, all supplemented with an *Aspergillus niger* 3-phytase (300 FTU kg^{-1} of diet), as follows: PC (positive control – formulated according to the Hy-Line nutritional recommendations, without proteases); NC_A (negative control A – positive control reduced in energy, protein and amino acids according to protease A matrix, without protease); NC_B (negative control B – positive control reduced in energy, protein and amino acids according to protease B matrix, without protease); $NC_A + PT_A$ (negative control A with protease A); and $NC_B + PT_B$ (negative control B with protease B). Protease A (*Streptomyces fradiae*, 0.125 g kg^{-1} of diet) and protease B (*Bacillus licheniformis*, 0.250 g kg^{-1} of diet) were added to the negative controls, replacing the filler, according to the manufacturers' recommendations. Furthermore, the contribution of energy, protein and amino acids from phytase was considered to be zero, in accordance with the current protocol on the combined use of feed enzymes. A complete description of diets and enzymes is given in Table 1.

Table 1. Ingredient and nutrient specifications (as-fed basis) of the experimental diets[1].

Item	Diet		
	PC	NC_A	NC_B
Ingredient (g kg^{-1})			
Corn	617.94	631.21	637.75
Soybean meal 45%	197.78	199.20	183.28
Meat and bone meal 46%	43.00	43.00	43.20
Corn gluten meal	41.15	31.20	41.20
Soybean oil	9.00	5.00	2.50
Limestone	83.50	83.48	83.50
Salt	3.20	3.20	3.20
Mineral-vitamin premix[2]	2.00	2.00	2.00
Filler (kaolin)	0.33	0.15	1.27
$_{L}$-lysine HCl 79%	0.39	0.02	0.50
$_{DL}$-methionine 99%	1.45	1.39	1.40
$_{L}$-threonine 98%	0.10	0.03	-
$_{L}$-tryptophan 99%	0.13	0.09	0.17
Phytase[3]	0.03	0.03	0.03
Calculated composition (g kg^{-1} unless indicated otherwise)[4]			
Met. energy (kcal kg^{-1})	2894	2869	2871
Crude protein	187.50	182.50	182.11
Calcium	39.65	39.65	39.65
Total phosphorus	5.78	5.78	5.78

continue

continuation

Available phosphorus	5.06	5.06	5.06
Sodium	1.69	1.68	1.69
Chlorine	2.40	2.32	2.42
Lysine	8.03	7.71	7.75
Methionine + cysteine	6.82	6.58	6.65
Threonine	5.90	5.69	5.62
Tryptophan	1.84	1.79	1.80

PC = positive control; NC_A = negative control A; NC_B = negative control B

[1] "$NC_A + PT_A$" and "$NC_B + PT_B$" diets were obtained by replacing the filler by protease A (*Streptomyces fradiae*, 3125 U kg^{-1} of diet) or protease B (*Bacillus licheniformis,* 150000 U kg^{-1} of diet) in the respective negative control.

[2] Supplied per kg of diet: 135 mg of choline (as choline chloride); 10 mg of Cu (as copper sulfate pentahydrate); 50 mg of Fe (as iron sulfate monohydrate); 1.2 mg of I (as calcium iodate); 80 mg of Mn (as manganous oxide); 0.2 mg of Se (as sodium selenite); 60 mg of Zn (as zinc oxide); 8100 IU of vitamin A (retinol); 2500 IU of vitamin D3 (cholecalciferol); 7 IU of vitamin E (dl-α-tocopherol); 2 mg of vitamin K3 (menadione); 1 mg of vitamin B1 (thiamine); 3.5 mg of vitamin B2 (riboflavin); 21 mg of vitamin B3 (niacin); 6.6 mg of vitamin B5 (pantothenic acid); 1 mg of vitamin B6 (pyridoxine); 0.15 mg of vitamin B7 (biotin); 0.4 mg of vitamin B9 (folic acid); 10 µg of vitamin B12 (cyanocobalamin); 15 mg of BHT (butyl hydroxytoluene).

[3] *Aspergillus niger* 3-phytase, 300 FTU kg^{-1} of diet

[4] Energy, protein and amino acid differences between positive and negative controls reflect the nutritional contribution of each protease to the formulation.

In order to allow the birds to completely adapt to the new diet, birds received the experimental diets for 10 consecutive days before the beginning of data collection. Data collection took place between the beginning of the 25th week and the end of the 28th week of life. Feed and water were provided *ad libitum* during this entire period.

Performance and egg quality parameters

Egg production and bird mortality were recorded daily. Feed intake was assessed weekly and multiplied by the diet composition to calculate the energy, protein and amino acid consumption. At the end of the 28th week, birds were weighed and all eggs from the final day were taken for determination of specific gravity. For this purpose, six salt solutions with different densities (varying by 0.005 g cm^{3-1}, from 1.075 to 1.100 g cm^{3-1}) were prepared and arranged in ascending order. Eggs were sequentially immersed in the solutions, and the density of the solution in which they first floated was considered to be the egg's specific gravity. Then, all eggs were cracked so the internal components could be accessed.

The height of the dense albumen was measured with a digital caliper and the Haugh unit was calculated by the following formula: $HU = 100 \log [h + 7.57 - 1.7w^{0.37}]$, where "*h*" corresponds to dense-albumen height and "*w*" to egg weight. Yolk color was determined by comparison to a commercial color fan, and subsequently the yolk was isolated and weighed. Shells were washed and set to dry at room temperature. After 24 h, shells were weighed and their thickness was measured with a digital caliper in three different regions. The mean value of the three measurements was considered as one observation. The albumen weight was calculated as the whole-egg weight minus the shell and yolk weights. The weights of the egg components were transformed to percentages of the whole egg for analysis and presentation.

Morphometry of intestinal mucosa

Six birds per treatment, each from a different plot, were weighed individually and sacrificed by cervical dislocation. Sections of approximately 3 cm from the middle part of the duodenum, jejunum and ileum were cut, opened longitudinally, extended on a cardboard base and fixed in formaldehyde solution for histological analysis. Slides of each section were stained with H&E and digital images were captured to measure the villi and crypts in ImageJ® software.

In each section, 30 measurements of villus height and crypt depth were obtained, and their arithmetic mean was considered as one observation.

Statistical analysis

Statistical procedures were performed in SAS® software. All of the variables met the assumptions of normality of residuals and homogeneity of variances before analysis of variance. One-way ANOVA was performed and, in case of differences, means were separated using Tukey's test. Body weight, egg weight and shell thickness were additionally subjected to Pearson's correlation analysis. Statistical significance was set at $P < 0.05$.

Results

All variables in this study were analyzed by a systematic comparison between treatments. First, the effect of nutrient restriction was verified by the comparison between positive and negative controls. In cases of significant effect, the ability of the proteases to overcome this effect was assessed by the comparison between diets supplemented with proteases and the positive control. When both negative controls affected a variable compared to the positive control, the efficacy of proteases was contrasted by the comparison between diets supplemented with protease A and protease B.

There was no effect (P>0.05) of treatment on body weight, egg production, egg mass or feed conversion (Table 2). Negative control A decreased (P=0.02) both feed intake and egg weight, but the addition of protease A to negative control A brought egg weight back to the same level as that obtained with the positive control. Pearson's correlation analysis showed that hen body weight was not associated with egg weight (r=0.03, P=0.86). Except for the negative control B, which did not reduce energy intake compared to the positive control, the negative controls decreased (P<0.01) daily intakes of energy, crude protein and amino acids. These responses persisted even after the addition of proteases to the diets.

Table 2. Effect of protease and phytase on performance and calculated nutrient intake of laying hens in peak egg production.

	Diet					SEM	*P*-value
	PC	NC_A	NC_B	$NC_A + PT_A$	$NC_B + PT_B$		
Performance							
Final body weight (g)	1362	1356	1390	1387	1324	12	0.51
Feed intake (g hen^{-1} day^{-1})	86.48^{a}	82.21^{b}	83.19^{ab}	81.79^{b}	82.33^{ab}	0.50	0.02
Egg production (%)	81.99	81.45	80.37	80.47	78.70	0.55	0.39
Egg weight (g)	55.85^{a}	54.20^{b}	55.20^{ab}	54.78^{ab}	54.05^{b}	0.20	0.02
Egg mass (g hen^{-1} day^{-1})	45.77	44.16	44.36	44.08	42.53	0.35	0.06
Feed conversion (g g^{-1})	1.891	1.865	1.881	1.857	1.940	0.012	0.23
Calculated nutrient intake							
Met. energy (kcal hen^{-1} day^{-1})	250.3^{a}	235.9^{b}	238.8^{ab}	236.7^{b}	238.3^{ab}	1.5	<0.01
Crude protein (g hen^{-1} day^{-1})	16.21^{a}	15.00^{b}	15.15^{b}	15.34^{b}	15.44^{b}	0.10	<0.01
Lys (mg hen^{-1} day^{-1})	694.4^{a}	633.8^{b}	644.7^{b}	656.8^{b}	661.1^{b}	4.5	<0.01
Met + Cys (mg hen^{-1} day^{-1})	589.8^{a}	540.9^{b}	553.2^{b}	557.8^{b}	561.5^{b}	3.7	<0.01
Thr (mg hen^{-1} day^{-1})	510.2^{a}	467.8^{b}	467.5^{b}	482.6^{b}	485.8^{b}	3.3	<0.01
Trp (mg hen^{-1} day^{-1})	159.1^{a}	147.1^{b}	149.7^{b}	150.5^{b}	151.5^{b}	1.0	<0.01

PC = positive control; NC_A = negative control A; NC_B = negative control B; $NC_A + PT_A$ = NC_A with protease A; $NC_B + PT_B$ = NC_B with protease B; SEM = Standard error of the mean

[a, b] Values within a row with different superscripts differ significantly at $P < 0.05$ (Tukey's test).

Egg specific gravity and relative weights of the yolk, albumen and shell were not affected by treatments (P>0.05); however, shell thickness was increased (P<0.01), and albumen height and the Haugh unit were decreased (P<0.01), by the negative controls (Table 3). The addition of protease B to negative control B had no effect (P>0.05) on these variables, but the addition of protease A to negative control A caused all of them to return to positive control standards. According to Pearson's correlation analysis, shell thickness was not associated with egg weight (r=-0.01, P=0.95).

Table 3. Effect of protease and phytase on egg quality of laying hens in peak egg production.

	Diet					SEM	*P*-value
	PC	NC_A	NC_B	$NC_A + PT_A$	$NC_B + PT_B$		
Relative weight (% of egg)							
Yolk	23.81	23.70	23.87	24.28	23.87	0.11	0.07
Albumen	66.26	66.27	66.10	65.64	66.10	0.18	0.15
Shell	9.93	10.03	10.03	10.08	10.03	0.13	0.96
Shell thickness (mm)	0.353[b]	0.406[a]	0.405[a]	0.353[b]	0.401[a]	0.010	<0.01
Albumen height (mm)	11.589[a]	10.930[bc]	10.550[c]	11.434[ab]	10.929[bc]	0.074	<0.01
Haugh unit	106.54[a]	104.25[b]	102.66[b]	106.05[a]	104.03[b]	0.28	<0.01
Specific gravity (g cm^{3-1})	1.086	1.086	1.085	1.086	1.086	0.010	0.38

PC = positive control; NC_A = negative control A; NC_B = negative control B; $NC_A + PT_A$ = NC_A with protease A; $NC_B + PT_B$ = NC_B with protease B; SEM = Standard error of the mean

[a-c] Values within a row with different superscripts differ significantly at $P < 0.05$ (Tukey's test).

Intestinal mucosa morphometry was affected by treatments only at the jejunum (P<0.01), but negative controls did not modify villus height or crypt depth compared to the positive control (Table 4). However, crypt depth of protease B hens was higher than that of the positive control.

Table 4. Effect of protease and phytase on intestinal mucosa morphometry of laying hens in peak egg production.

	Diet					SEM	*P*-value
	PC	NC_A	NC_B	$NC_A + PT_A$	$NC_B + PT_B$		
Villus height (μm)							
Duodenum	1456	1444	1724	1616	1616	36	0.05
Jejunum	604[ab]	534[b]	573[ab]	534[b]	648[a]	12	<0.01
Ileum	713	656	753	761	793	21	0.30
Crypt depth (μm)							
Duodenum	387.1	402.9	402.0	382.3	430.7	10.5	0.65
Jejunum	134.0[b]	141.9[b]	152.5[ab]	126.7[b]	174.0[a]	4.3	<0.01
Ileum	150.1	122.1	141.8	128.7	137.6	3.8	0.15

PC = positive control; NC_A = negative control A; NC_B = negative control B; $NC_A + PT_A$ = NC_A with protease A; $NC_B + PT_B$ = NC_B with protease B; SEM = Standard error of the mean

[a, b] Values within a row with different superscripts differ significantly at $P < 0.05$ (Tukey's test).

Discussion

The results of this study indicate that the reduced daily intake of protein and amino acids was the reason for the adverse effects of the negative control diets on egg weight, albumen height and the Haugh unit. The addition of proteases to the negative controls did not restore the intake of nutrients to the same amount as that consumed by positive-control hens; nevertheless, protease A brought egg weight, albumen height and the Haugh unit back to the same level as that observed in the positive control. While different assumptions could be made to explain the better performance by protease A, the results of all treatments led to one big question: does protease A alone have a more suitable nutritional matrix than protease B alone, or is the actual nutritional contribution of the combination of protease A and phytase greater than the nutritional contribution of protease alone? Because different levels of interaction between phytases and multienzyme complexes with proteases have been described for broilers and hens (AL-SAFFAR et al., 2013; OLUKOSI et al., 2010; TIWARI et al., 2010), it seems reasonable to consider the second option as a valid statement. In this case, increased or decreased affinity between enzymes under study may be responsible for the different responses observed for each protease.

Despite the above-mentioned facts, we expected more pronounced effects of nutritional restriction imposed by the negative controls on performance and egg quality parameters. As discussed by Lei et al. (2011), the phytase already present in the negative controls alone could be enough to compensate for a small restriction in energy, protein and amino acids. Furthermore, this weak response could indicate that the nutritional levels of the positive control diet were above the requirement of hens for most of the evaluated variables. While the positive control diet was formulated according to Hy-Line recommendations for the age and feed intake pattern of the birds, both body weight and egg production of the flock were lower than expected. Accordingly, it seems reasonable to assume that the positive control diet exceeded the nutritional requirements of the birds. Moreover, hens were fed the experimental diets for 38 consecutive days, considering the entire experimental period and the first 10 days of adaptation. There might have been more evident signs of nutrient restriction had the hens consumed the diets for a longer period of time.

Proteases A and B contributed relatively more crude protein (2.67 and 2.87%, respectively) and amino acids (3.99 and 3.49% for Lys, respectively; 3.52 and 2.49% for Met + Cys; 3.56 and 4.75% for Thr; and 2.72 and 2.17% for Trp) than metabolizable energy (0.86% and 0.79%, respectively) in the formulation. Thus, negative controls showed greater reduction of protein and amino acids than energy when compared with the positive control. According to Antar et al. (2004), laying hens regulate their feed intake primarily to meet the energy requirements for maximum egg production, and then the resulting amount of amino acids consumed determines the average weight of the eggs. Our findings support this assumption since only egg weight, but not egg production, was affected by the treatments. Nevertheless, because no difference in nutrient intake was observed between hens fed the proteases, the reason why only protease A restored egg weight back to the positive control value remains unclear. One could suggest that the lower egg weight of hens fed protease B was associated with their body weight; however, correlation analysis showed that this was not the case (r=0.03, P=0.86).

With regard to egg quality, the negative controls reduced both albumen height and the Haugh unit, and these responses were reversed only by protease A. Because the weight of the eggs from the hens fed protease B was lower than that of the eggs from positive-control hens, we can assume that only the lower albumen height of the protease-B hens was responsible for its lower Haugh unit value, since egg weight is negatively associated with the Haugh unit while albumen height is positively associated. Contrary to our results, Shim et al. (2013) described

an increase in the Haugh unit after reduction of dietary crude protein. This response was probably a consequence of the lower egg weight; nevertheless, because the albumen height was not provided by the authors, our comparison remains limited.

Although shell thickness was greater in both negative controls, this effect was not followed by changes in shell weight or egg specific gravity. Correlation analysis showed that egg weight (and indirectly, egg size) was not associated with this increase in shell thickness (r=-0.01, P=0.95). These findings indicate that shell structure, but not shell composition, was affected by nutrient restriction. Jiang et al. (2013) concluded that dietary energy levels do not interfere with eggshell thickness. However, because eggshell calcium crystals begin their formation attached to a protein matrix (MAZZUCO; BERTECHINI, 2014), it is speculated that low levels of dietary protein and amino acids could change this matrix and affect crystal organization and the thickness of the shell. In fact, Novak et al. (2006) stated that providing proper amounts of amino acids, especially the sulfur amino acids, is essential for eggshell improvement. More recently, Khajali et al. (2008) and Ghasemi et al. (2014) demonstrated that dietary reduction of crude protein does not influence shell thickness as long as the levels of lysine and sulfur amino acids are kept constant.

The effect of the treatments on the intestinal mucosa morphometry was minimal, whereas none of the negative controls affected villus height or crypt depth compared to the positive control. Moreover, there was no difference in villus height between the treatments with proteases and the positive control. The intestinal mucosa was affected by treatments only in the jejunum, with birds fed protease B showing greater ($P < 0.01$) villus height and crypt depth than those treated with protease A. Although the larger crypt depth of protease-B hens suggests a higher rate of cell turnover in the villus (WONG; WRIGHT, 1999), this was probably a compensatory response associated with high levels of extrusion in the apex of the villus (CARULLI et al., 2014) and does not reflect any trophic effect of protease B on the intestinal epithelium. However, the reason for this higher rate of cell turnover was not clear.

Conclusions

The addition of proteases to laying hen diets supplemented with phytase reduces the calculated intake of protein and amino acids, and modifies the jejunal mucosa morphometry. In this situation, egg weight, albumen height and the Haugh unit are kept constant only with protease A supplementation. These data indicate that, when included in diets supplemented with phytase, the type of protease affects performance, nutrient intake, egg quality and intestinal mucosa morphometry of laying hens during peak egg production.

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