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Effects of different proteases on commercial laying hens at peak production

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ABSTRACT - We evaluated the effect of adding proteases in diets of laying hens at peak production on the performance, egg quality, relative weight of digestive organs, and intestinal morphometry. Hy-Line W36 layer hens (390; 28-39 weeks old) were distributed in five treatments, with 13 animals each, with six replicates. The treatments were: control (standard formulation without nutritional reduction or protease inclusion), negative control A - NCA (nutritional reduction according to protease A matrix, without protease), negative control B - NCB (nutritional reduction according to protease B matrix, without protease), NCA + protease A, and NCB + protease B. The experimental period lasted 12 weeks, divided into three cycles of 28 days. Hens subjected to treatments (NCA and NCB) showed a decrease in feed intake. However, the addition of proteases A and B promoted improvement in this trait. The diets NCA and NCB had a negative influence on the production rate of the hens, but the diet supplementation with protease B resulted in significant improvement on egg laying rate. Hens subjected to nutritional reduction presented the worst results regarding mass and conversion by egg mass. However, the addition of enzymes reversed these results. Although the diets did not affect the relative weights of yolk, albumen, eggshell, Haugh unit, and specific gravity of the eggs, a higher eggshell thickness was observed in hens that received NCA, NCB, and NCB + protease B diets. The diets did not influence the relative weights of digestive organs and the small intestine morphometry. Hens fed diets supplemented with A and B have performance recovered in relation to those that received diets with reduced levels of nutrients.

Keywords: animal nutrition, egg laying, enzyme, laying hen, morphometry

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1. Introduction

Feed costs in the egg laying industry account for about 75% of the total production costs. From this, about 85% correspond to energy and protein levels of the diet (Santana et al., 2018). For this reason, studies have been developed, seeking for alternatives to minimize the high cost of feed in poultry production.

Over the years, diets with low crude protein levels have been used to combine production rates with economic and environmental advantages (Cardinal et al., 2019). Thus, biotechnology advances have contributed to animal nutrition through the development of additives that improve production and reduce feed costs. In this context, exogenous enzymes have been used in monogastric diets to improve animal performance, since they act on the metabolism as biological catalysts (Salominski, 2011).

Supplementation of exogenous proteases can optimize the use of amino acids present in the diet, which allows the formulation of diets with lower protein levels, without compromising poultry performance and promoting higher sustainability in poultry production (Leinonen and Williams, 2015).

Cardoso et al. (2010) reported controversial results regarding the protease effectiveness. This fact may be related to different ways in which the experimental tests are conducted, the species and age of the animals, and variability of ingredients used in the diet, among other factors.

Thus, it is necessary to understand the action of exogenous protease in the metabolism to better use proteins, amino acids, and energy from macro-ingredients, as well as the protease effect on the intestinal mucosa, since the maintenance of morphofunctional integrity of the digestive system is essential, and once the digestion and absorption of nutrients depend on it to convert feed into eggs for human consumption.

Therefore, our objective was to compare the effects of two proteases in diets of light laying hens at peak production on the performance, egg quality, relative organ weight, and intestinal morphometry.

2. Material and Methods

The animal research was conducted in accordance with the institutional committee on animal use (case number 23108.099277/2015-73). The present study was carried out in Primavera do Leste, Mato Grosso State, Brazil (Latitude: 15°33'32" S, Longitude: 54°17'46" W).

We used 390 Hy-Line W36® laying hens, of 28 weeks old, taken from the commercial poultry farm where the study was conducted. They were housed during the production phase in metal cages (Zucami-Poultry Equipment, Model W762) (front × bottom × front height × bottom height; 762 × 630 × 540 × 450 mm), wire floor, provided with linear feeder, nipple drinker with capacity for 13 hens, arranged in two lines, consisting of two floors each. The hens were distributed in a completely randomized design in five treatments with six replicates, totaling thirteen hens per experimental unit. The five treatments were: control: standard diet, adopted in the farm to laying hens at peak production, formulated without nutritional reduction and without proteases; negative control A – diet formulated with nutritional reduction according to the nutritional matrix of protease A, without proteases; negative control B – diet formulated with nutritional reduction according to the nutritional matrix of protease B, without proteases; negative control A with inclusion of protease A; negative control B with inclusion protease B.

The proteases *Bacillus licheniformis* – "protease A" (Cibenza® DP100, Novus International Inc, ST. Charles, MO; 0.250 g/kg of feed) and *Streptomyces fradiae* – "protease B" (Poultrygrow 250™ - Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, Canada; 0.125 g/kg of feed) were added to the negative controls, according to recommendations of the manufacturers. Only the contribution of calcium and phosphorus from phytase was considered similar in all the treatments.

The experimental diets (Table 1) were formulated to meet the nutritional requirements of laying hens according to the nutritional recommendations of the Tabelas Brasileiras de Aves e Suínos (Rostagno et al., 2011).

Hens received water and diet *ad libitum* throughout the study. The experimental unit was represented by the cage.

The experimental period lasted 12 weeks. At the time of the statistical analysis of the results, these weeks were divided into three cycles of 28 days each.

The temperature in the poultry house was monitored twice a day, at 8:00 and 16:00 h, by maximum and minimum thermometers placed in two different spots of the house, at the height of the hens. The average maximum and minimum temperatures for cycles I, II, and III observed inside the experimental house during this test were, respectively, 28.6-23.6, 28.5-22.5, and 30.0-23.6 °C.

A light program of 15 h of daily light (natural and artificial) was adopted. For this, an automatic timer was set, turning the lights on and off, at night and dawn, according to the procedure adopted by the poultry farm where the test was carried out.

The performance variables evaluated in this study were body weight, feed intake, egg production, egg mass, feed conversion (per mass of eggs produced and per dozen eggs produced), and egg weight.

At the end of the 31st, 35th, and 39th week of age of the hens, all healthy eggs of the day were weighed individually, identified, and sent for specific gravity determination. The eggs were immersed and evaluated in saline solution (NaCl) with density ranging from 1.075 to 1.100 g/cm^3, with intervals of 0.005 g/cm^3.

All eggs were broken and, with the aid of a digital caliper, the height of the dense albumen was measured and, subsequently, the Haugh unit was calculated using the equation: $HU = 100 \log [h + 7.57 - 1.7w^{0.37}]$), in which h corresponds to the height of the dense albumen (mm) and w, to the weight of the egg (g). Then, the yolk was separated and with a digital caliper, and height and diameter were measured to calculate the yolk index. Afterwards, the yolks were weighed individually. The eggshells were carefully washed under running water and left to dry at room temperature for 24 h. Then, they were weighted, and their thickness at the central region, top, and bottom of the egg were measured with a digital caliper. The arithmetic mean of these three measurements was the average of eggshell thickness. The weight of the albumen was calculated by the difference between the weight of the whole egg and the weight of the eggshell and yolk. All weight values obtained for egg components were converted into a percentage of the total egg weight. The Roché® colorimetric fan was used to analyze the yolk color.

Table 1 - Centesimal and nutritional composition of the experimental diets

Item	Treatment				
	Control	Negative control A (NCA)	Negative control B (NCB)	NCA + protease A	NCB + protease B
Ingredient (%)					
Corn	61.916	63.660	63.381	63.660	63.381
Soybean meal 48%	16.416	15.845	16.262	15.845	16.262
Meat and bone meal 46%	4.618	4.628	4.617	4.628	4.617
Soybean hull meal	0.500	0.500	0.500	0.500	0.500
Protenose®	5.826	5.177	5.000	5.177	5.000
Vegetable oil	1.500	1.000	1.060	1.000	1.060
Limestone	8.322	8.322	8.323	8.322	8.323
Salt	0.330	0.330	0.330	0.330	0.330
Mineral and vitamin premix[1]	0.200	0.200	0.200	0.200	0.200
Inert filler (Kaolin)	0.050	0.050	0.050	0.025	0.037
L-lysine	0.132	0.121	0.103	0.121	0.103
DL-methionine	0.137	0.136	0.130	0.136	0.130
L-threonine	0.021	-	0.015	-	0.015
L-tryptophan	0.029	0.028	0.026	0.028	0.026
Phytase[2]	0.003	0.003	0.003	0.003	0.003
Protease A[3]	-	-	-	0.025	-
Protease B[4]	-	-	-	-	0.013
Nutritional composition					
Metabolizable energy (kcal/kg)	2.893	2.870	2.868	2.893	2.893
Crude protein (%)	18.75	18.19	18.25	18.75	18.75
Calcium (%)	3.96	3.96	3.96	3.96	3.96
Total phosphorus (%)	0.71	0.71	0.71	0.71	0.71
Available phosphorus (%)	0.50	0.50	0.50	0.50	0.50
Sodium (%)	0.17	0.17	0.17	0.17	0.17
Chlorine (%)	0.28	0.28	0.28	0.28	0.28
Digestible lysine (%)	0.80	0.78	0.77	0.80	0.80
Digestible Met+Cys (%)	0.68	0.66	0.66	0.68	0.68
Digestible threonine (%)	0.61	0.58	0.59	0.61	0.61
Digestible tryptophan (%)	0.18	0.18	0.18	0.18	0.18

[1] Guaranteed levels per kilogram of product: choline, 67.2 g; Cu, 5×10^3 mg; Fe, 25 g; iodine, 600 mg; Mn, 40 g; Se, 100 mg; Zn, 30 g; vitamin A, 4050×10^3 IU; vitamin D3, 1250×10^3 IU; vitamin E, 500 IU; vitamin K3, 1000 IU; vitamin B1, 500 mg; vitamin B2, 1750 mg; vitamin B6, 500 mg; vitamin B12, 5000 mcg; niacina, 10.5 g; pantothenic acid, 3300 mg; folic acid, 200 mg; biotin, 7.5 mg; BHT, 7500 mg; zinc bacitracin, 14 g.
[2] Fungal phytase (*Aspergillus niger*), 10,000 FTU/g.
[3] Bacterial protease (*Bacillus licheniformis*), 600,000 U/g.
[4] Bacterial protease (*Streptomyces fradiae*), 25,000 U/g.

At 39 weeks old, one hen from each experimental unit (six hens/treatment) was weighed individually, according to the average repetition weight, for analysis of relative weight of the digestive organs and intestinal morphometry. Subsequently, the hens were slaughtered by cervical dislocation to proceed the extraction of intestines (small and large), liver with gallbladder, proventriculus, gizzard without adhered fat, pancreas, small intestine, and large intestine. The proventriculus and gizzard were opened and washed under running water to remove any feed content. The organs were weighed individually to calculate the relative weights as a function of live weight and subsequent analysis of intestinal morphometry. For the intestinal morphometry analysis, parts of approximately 3 cm from the median region of the duodenum, jejunum, and ileum were collected. These parts were opened longitudinally, placed on a rigid paper base, washed under running water, and fixed in formaldehyde for making histological slides to measure villus height, crypt depth, and villus:crypt ratio. After 48 h in formaldehyde, the intestinal samples were cleaved, dehydrated in alcohol, cleared in xylol, included in paraffin, cut in a microtome, arranged in histological slides, and stained in hematoxylin-eosin solution. Digitized images of the slides were analyzed using ImageJ® image editor software to measure villus height and crypt depth. For the analysis of results, the arithmetic mean of 10 villi or crypts measures was considered.

At the end of the third cycle, which was the last period of the experiment, all the hens were weighed to determine the average weight of each experimental unit.

Statistical analyses were performed using the SAS® program (Statistical Analysis System, version 9.0). After meeting the assumptions of variance homogeneity and residue normality, the data were subjected to variance analysis and, in case of significant difference between treatments, the means were compared by the Tukey's test at 5% probability. The data for yolk color violated the principles of normality and homoscedasticity, so they were subjected to non-parametric analysis using the Kruskal Wallis' test at 5% probability. The statistical model used for variance analysis of all variables was:

$$Y_{ij} = \mu + T_i + e_{ij},$$

in which Y_{ij} = observed result, μ = overall mean of the experiment, T_i = protease supplementation effect, and e_{ij} = random error associated with each observation.

3. Results

Regarding the performance results observed in the present study (Table 2), there was a difference ($P<0.05$) in feed intake, egg production, egg mass and conversion by egg mass, and egg weight. In contrast, hen weight and feed conversion by dozen eggs were not influenced ($P>0.05$) by the treatments.

The hens subjected to treatments with nutritional reduction (negative control A and B) showed decrease in feed intake. However, the addition of proteases A and B in their respective negative controls promoted an improvement infeed intake.

Table 2 - Performance of Hy-Line W36 laying hens between 28 and 39 weeks old

Treatment	Hen weight (kg)	Feed intake (kg/hen/day)	Egg production (%)	Egg mass (g/hen/day)	FC egg mass (g/g)	FC dozens of eggs (g/dozen)	Egg weight (g)
Control	1.336	0.087AB	79.60AB	45.42B	1.91AB	1.31	57.09A
NCA[1]	1.256	0.082C	76.91B	42.79C	1.91AB	1.28	55.66AB
NCB[2]	1.254	0.084BC	77.47B	42.80C	1.96B	1.30	55.26B
NCA + protease A[3]	1.278	0.087AB	82.51AB	46.84AB	1.85A	1.26	56.77AB
NCB + protease B[4]	1.313	0.089A	83.91A	48.46A	1.83A	1.27	56.40AB
				Statistics			
CV (%)	8.22	2.51	4.21	2.97	3.09	4.50	1.80
P-value	0.66	<0.01	<0.01	<0.01	<0.01	0.56	<0.01

FC - feed conversion; CV - coefficient of variation.
[1] Negative control A = negative control for protease A.
[2] Negative control B = negative control for protease B.
[3] Protease A = *Bacillus licheniformis* (Cibenza® DP100, Novus International Inc, ST. Charles, MO; 0.250 kg/ton of feed).
[4] Protease B = *Streptomyces fradiae* (Poultrygrow 250™ - Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, Canada; 0.125 kg/ton of feed).
Means followed by the same letter in the column do not differ statistically by the Tukey Test at 5% probability.

The groups of hens that were fed the treatments negative control A and B showed reduction in egg production rate in relation to hens that received the treatment negative control B + protease B. However, supplementing the diets with protease B resulted in significant improvement in the egg laying rate.

Egg mass was negatively influenced in the groups of hens fed diets with nutritional reduction (negative control A and B). However, there was an increase in the egg mass from hens that received diets with proteases A and B.

Feed conversion by egg mass presented the worst indexes in the group of hens that received the control diets and diets with nutritional reduction (negative control A and B). However, an improvement in the value of this variable was observed after the addition of proteases A and B in their respective negative controls negative control A + protease A and negative control B + protease B.

It was observed that hens that received the negative control B treatment had lower egg weight compared with the ones subjected to the control treatment.

There was no significant treatment effect (P>0.05) on the relative weights of yolk, albumen, and eggshell, Haugh unit, and specific gravity of the eggs (Table 3). However, a higher eggshell thickness (P<0.05) was observed in the negative control A, negative control B, and negative control B + protease B treatments. In addition, treatments negative control A and B showed the best results for yolk index and color (P<0.05) in relation to the other treatments.

There was no significant effect (P>0.05) of treatments on the relative weights of liver, proventriculus, gizzard, pancreas, and small and large intestines (Table 4).

No treatment effect (P>0.05) was found in the morphometric analysis of the small intestine mucosa (Table 5).

4. Discussion

The use of exogenous proteases in Hy-Line laying diets at peak production improves the productive performance of birds. Proteases increase the digestibility of proteins and amino acids, especially when the ingredients have low quality or low bioavailability (Kocher et al., 2002). Thus, besides providing nutritional benefits, the use of enzymes can contribute to the maintenance of the normal balance of the animal's organism, reflecting in better conditions for the performance and egg production (Vieira Filho et al., 2015).

According to Ferreira (2005), the ambient temperature indicated for the production phase may oscillate between 15 and 28 °C. We can infer that during the experimental period, the birds were in a condition of thermal comfort, since the averages of the maximum and minimum temperature registered inside the experimental house were 29.0 and 23.2 °C, respectively.

Table 3 - Egg quality from Hy-Line W36 laying hens between 28 and 39 weeks old

Treatment	Yolk (%)	Albumen (%)	Eggshell (%)	Eggshell thickness (mm)	Yolk index	Haugh unit	Yolk color	Specific gravity (g/cm³)
Control	24.85	66.22	8.93	0.34B	0.52AB	105.81	7.60B	1.085
NCA[1]	25.25	66.21	8.76	0.36A	0.53A	106.87	7.77A	1.085
NCB[2]	24.75	66.50	8.81	0.36A	0.53A	105.92	7.80A	1.084
NCA + protease A[3]	25.32	65.57	9.11	0.31C	0.52AB	105.53	7.69AB	1.086
NCB + protease B[4]	25.74	65.22	9.04	0.36A	0.51B	105.03	7.75AB	1.085
				Statistics				
CV (%)	2.99	1.19	2.45	2.93	1.93	1.07	1.08	0.18
P-value	0.19	0.06	0.06	<0.01	<0.01	0.11	<0.01	0.78

CV - coefficient of variation.
[1] Negative control A = negative control for protease A.
[2] Negative control B = negative control for protease B.
[3] Protease A = *Bacillus licheniformis* (Cibenza® DP100, Novus International Inc, ST. Charles, MO; 0.250 kg/ton of feed).
[4] Protease B = *Streptomyces fradiae* (Poultrygrow 250™ - Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, Canada; 0.125 kg/ton of feed).
A-C - Means followed by the same letter in the column do not differ statistically by the Tukey Test at 5% probability.
A-B - Means followed by the same letter, for yolk color, do not differ statistically by the KrusKal Wallis Test at 5% probability.

Table 4 - Relative weight of the digestive system organs of Hy-Line W36 laying hens at 39 weeks old

Treatment	Liver (%)	Proventriculus (%)	Gizzard (%)	Pancreas (%)	Duodenum (%)	Jejunum (%)	Ileum (%)	Large intestine (%)
Control	2.83	0.42	1.37	0.22	1.13	1.93	1.59	1.10
NCA[1]	3.26	0.44	1.48	0.23	1.09	1.89	1.48	1.08
NCB[2]	3.04	0.41	1.46	0.22	1.06	1.55	1.39	1.13
NCA + protease A[3]	2.82	0.43	1.58	0.21	1.21	1.89	1.42	1.12
NCB + protease B[4]	2.86	0.40	1.43	0.22	1.10	1.94	1.68	1.09
				Statistics				
CV (%)	10.84	13.64	10.00	12.93	13.44	21.61	15.09	18.26
P-value	0.12	0.66	0.18	0.73	0.48	0.43	0.18	0.99

CV - coefficient of variation.
[1] Negative control A = negative control for protease A.
[2] Negative control B = negative control for protease B.
[3] Protease A = *Bacillus licheniformis* (Cibenza® DP100, Novus International Inc, ST. Charles, MO; 0.250 kg/ton of feed).
[4] Protease B = *Streptomyces fradiae* (Poultrygrow 250™ - Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, Canada; 0.125 kg/ton of feed).

Table 5 - Morphometry of the small intestine mucosa of Hy-Line W36 laying hens at 39 weeks old

Treatment	Duodenum			Jejunum			Ileum		
	Villus height (µm)	Crypt depth (µm)	Villus:crypt ratio	Villus height (µm)	Crypt depth (µm)	Villus:crypt ratio	Villus height (µm)	Crypt depth (µm)	Villus:crypt ratio
Control	1670.17	306.70	5.77	1032.95	200.39	5.22	749.74	146.42	5.68
NCA[1]	1619.86	337.10	4.94	990.08	214.62	4.67	655.85	159.03	4.21
NCB[2]	1516.53	332.91	4.60	1071.36	209.86	5.17	659.26	150.94	4.49
NCA + protease A[3]	1655.33	322.04	5.43	1138.62	232.12	5.10	728.38	145.36	5.05
NCB + protease B[4]	1703.33	291.31	6.14	1011.57	196.58	5.21	719.86	145.70	4.98
				Statistics					
CV (%)	17.72	18.92	27.65	14.83	16.17	19.41	13.44	24.07	23.79
P-value	0.87	0.79	0.55	0.52	0.45	0.85	0.33	0.96	0.25

CV - coefficient of variation.
[1] Negative control A = negative control for protease A.
[2] Negative control B = negative control for protease B.
[3] Protease A = *Bacillus licheniformis* (Cibenza® DP100, Novus International Inc, ST. Charles, MO; 0.250 kg/ton of feed).
[4] Protease B = *Streptomyces fradiae* (Poultrygrow 250™ - Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, Canada; 0.125 kg/ton of feed).

In this study, we observed that groups of hens fed diets with reduced nutrients (negative control A and B) had lower feed intake. However, the addition of proteases A and B improved this variable. These data are in agreement with those presented by Viana et al. (2009), who also found positive effect of protease supplementation on the performance and metabolism of Bovans Goldline® laying hens fed anenzymatic complex composed of glucanases, xylanases, pectinases, proteases, and phytase between 24 and 36 weeks old.

Considering that in this present work the experimental diets negative control A and B were formulated with reduced levels of proteins and amino acids in relation to the control diet and that birds tend to have less feed intake in diets with imbalance of amino acids (Swatson et al., 2002), it can be inferred that the reduction in amino acid levels may have caused the low feed intake of birds. When adding proteases A and B, there was an improvement in intake, which can be explained by the fact that the addition of exogenous proteases effectively increases the apparent digestibility of amino acids and proteins in poultry diets with reduced levels of protein (Angel et al., 2011; Law et al., 2015).

Regarding egg production, we observed that the supplementation of diets with protease B provided an improvement in the indexes of this variable. Likewise, Vieira Filho et al. (2015) conducted an experiment with laying hens of the commercial lineage Isa Brown at 44 weeks old fed diets with reduced nutritional levels containing 500 g ton^{-1} protease (100 U g^{-1}) and observed an increase in egg laying

rate. The authors stated that the protease supplementation was able to match the nutrient availability, allowing them to be directed towards keeping the productive performance, which guaranteed the maximum productive efficiency of the animals. Thus, considering that exogenous enzymes act increasing the nutrient digestibility, it can be inferred that the addition of protease B in its respective negative control (negative control B) may have promoted an improvement in the use of energy and amino acids in diets with nutritional reduction, which increased egg production.

Regarding egg mass, it was found that this variable was influenced by diets with nutritional reduction. However, Lima et al. (2012) did not observe any significant treatment effect on the egg mass when providing diets with low levels of crude protein (0.0 and 4.0 g/kg) with or without supplementation of enzymatic complex for light laying hens at 30 weeks old. Similar results were also found by Vieira et al. (2016), who did not observe any significant differences in egg production of light laying hens at peak production fed diets with reduced energy, proteins, and amino acids, with or without the inclusion of proteases (*Streptomyces fradiae*, 0.125 g kg^{-1} in the diet and *Bacillus licheniformis*, 0.250 g kg^{-1} in the diet) in their composition. Considering that the reduction in the crude protein level of the diet can cause reduction in egg mass values of light laying hens (Silva et al., 2010; Mousavi et al., 2013), it can be inferred that the nutritional reduction of diets negative control A and B promoted reduction in the egg mass values found in the present research, since they had lower levels of crude protein and amino acids in their compositions. The addition of proteases A and B in the diets, possibly promoted a better use of energy and amino acids of the diets, which resulted in an increase in the value of this variable in relation to the negative control.

The feed conversion by egg mass decreased in the group of hens that received diets with nutritional reduction. These data corroborate those found by Rosário et al. (2018), who also observed significant differences in feed conversion by egg mass from light laying hens fed diets with nutritional reduction, supplemented or not with exogenous enzymes (*Bacillus licheniformis*, 0.250 kg/ton of feed). These authors found that after adding protease to the diet, in addition to improving feed conversion by egg mass, there was also an increase in egg production and egg mass, suggesting that the inclusion of the enzyme increased the availability of amino acids for absorption and, consequently, for higher protein synthesis. In this study, we observed that the addition of exogenous enzymes in diets with nutritional reduction also improved feed intake, egg mass, and conversion by egg mass, since they increased the digestibility of proteins, amino acids, and energy.

In this study, hens subjected to negative control B treatment showed reduction in egg weight when compared with the control diet. However, the addition of protease B in the diet provided an increase in this variable. These results are similar to those presented by Vieira et al. (2016), who also observed a reduction in egg weight values of light laying hens at peak production fed a diet with reduced energy, protein, and amino acids. The authors found that the addition of the enzyme (*Streptomyces fradiae*, 0.125 g kg^{-1} in the feed) to the hens' diet promoted an increase in egg weight.

In the present study, the protease A matrix (*Bacillus licheniformis*, 0.250 kg/ton of feed) shows a 5.85% reduction in the amino acid threonine compared with protease B matrix (*Streptomyces fradiae*, 0.125 kg/ton of feed), which corresponds to 3.53%. According to Nogueira (2006), among other functions, this amino acid optimizes the use of methionine and lysine in the diet. Thus, threonine deficiency reduces the efficiency of utilization of methionine + cystine and lysine, which are considered the first and second limiting amino acidsin poultry diets, respectively (Atencio et al., 2004). Based on these reports, we expected lower protein deposition and egg weight from hens that received the negative control A diet in relation to those that received the negative control B diet. However, the hens that received the negative control B diet were the only ones that reduced the egg weight compared with the control. It is suggested that this result was a consequence of the amino acids imbalance caused by the reduction of lysine, methionine + cystine, and tryptophan in the diet. Thus, formulating diets for laying hens that meet the requirement of lysine, methionine + cystine, and tryptophan at minimum cost is essential to express their maximum genetic potential and reduce the imbalance between amino acids.

We observed that the treatments did not influence the percentage of yolk, albumen, eggshell, Haugh unit, and specific gravity. Similarly, Lima et al. (2012) did not find any influence of the enzyme complex supplementation (Allzyme SSF) on the internal and external characteristics of the eggs. In this study, since there is no significant difference in the percentage of yolk and albumen, we suggest that the nutritional reduction was not enough to influence the weight of these egg constituents.

Although the eggshell thickness showed higher values in the group of hens that were fed diets with nutritional reduction, there was no change in the eggshell percentage and in the specific gravity of eggs from hens subjected to these treatments. Similar results were found by Vieira et al. (2016), who observed thicker eggshells in diets with nutritional reduction. Mazzuco and Bertechini (2014) reported that the calcium crystals in the eggshell start their formation linked to a matrix protein. Therefore, it is inferred that low levels of protein and amino acids in the diet can alter this matrix and, consequently, affect the organization of calcium crystals and the eggshell thickness. In this research, we found that the protease addition improved the eggshell thickness in relation to the group of hens fed the control diet. According to Novak et al. (2006), adequate amounts of amino acids, especially those sulfurized in diets for laying hens, are essential to promote improvement in eggshell thickness. Thus, it is suggested that protease B added to its respective negative control B increased the amino acid digestibility, especially sulfur acids, promoting higher eggshell thickness compared with the control group.

It was found that the relative weights of the digestive tract organs were not influenced by the treatments. These findings are in agreement with those found by Lima et al. (2012), who did not observe any reduction in the weight of liver and the lengths and weights of large and small intestines of light laying hens that received diets containing an enzyme complex. However, the authors found significant differences in the pancreas weight. According to them, the supplementation with exogenous enzymes possibly caused a lower enzymatic activity in the pancreas, promoting a reduction in the size and weight of this organ.

Reports in literature indicate that the inclusion of exogenous enzymes in poultry diets can lead to a reduction in the synthesis of endogenous enzymes, which consequently would lead to a reduction in the activity of the organs and, consequently, reduction of weight. Zanella et al. (1999) observed that trypsin, chymotrypsin, lipase, and α-amylase had a reduction of 40% in duodenal secretion when diets were supplemented with these exogenous enzymes. In addition, these authors found that amylase and protease supplementation in the diet based on corn and soybean for broilers reduced the synthesis of these endogenous enzymes by 23.4 and 35.5%, respectively. In the present study, it can be inferred that the amount of enzyme added to the diets was not enough to reduce the enzyme activity of the digestive organs and influence their size.

The small intestine mucosa of laying hens was not influenced by the treatments. These results differ from those found by Vieira et al. (2016), who found that hens fed diets with the addition of protease B (*Bacillus licheniformis*, 0.250 g kg^{-1} in the diet) presented higher values of villus height and crypt depth in the jejunum mucosa in relation to the group that received diets containing protease A (*Streptomyces fradiae*, 0.125 g kg^{-1} in the diet). Cardinal et al. (2019) also observed that protease supplementation (*Streptomyces griseus*, 1.25 g kg^{-1}) in the diet of broilers, with or without reduction in crude protein, promoted an improvement in the indexes related to the intestinal health (thickness of the lamina propria and epithelial surface, and enterocyte proliferation). These authors suggested that the use of exogenous protease may be associated with increased availability of protein-bound amino acids, since beneficial effects of the enzyme are associated with reduction in the amount of undigested nutrients in the ileum, such as proteins and starch (Romero and Plumstead, 2013; Romero et al., 2011), which, when in excess, can result in proteolytic fermentation with synthesis of toxic components that can compromise the morphology of the broilers' intestines, and negatively affect their performance and growth.

Based on the results found in this study, it can be inferred that the level of nutritional restriction in the diets was not sufficient to alter the small intestine morphometry of Hy-Line W36 laying hens.

5. Conclusions

Protease inclusion recovers the performance of hens. These enzymes significantly influence the utilization of nutrients by Hy-Line W36 laying hens at peak production.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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