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Technical Note

Protease Supplementation in the Diet of Light Laying Hens

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ABSTRACT

This work was developed to evaluate the impact of the addition of proteases on the performance characteristics, egg quality, relative weight of digestive organs, and intestinal morphometry of laying hens. 390 Hy Line W36® hens were allocated into five treatments and six replicates with 13 animals. The treatments were: 1) Control (standard formulation), 2) Negative control A - NCA (nutritional reduction according to protease A matrix), 3) Negative control B - NCB (nutritional reduction according to protease B matrix), 4) NCA+protease A (0.250 g/kg of feed) and 5) NCB+protease B (0.125 g/kg of feed). Hens fed the NCA, NCB, and NCA+protease A diets showed reductions in feed intake and egg mass. The addition of protease B provided better results for egg production in both percentage and per dozen as compared to the group fed with the NCA+protease A diets. The hens subjected to diets NCA and NCB showed eggs with a reduced eggshell and thickness percentage. However, supplementation with proteases A and B improved these parameters to values similar to the controls. There was no significant effect of the treatments on the relative weight of the liver, proventricle, gizzard, pancreas, and small intestine. However, the addition of protease A resulted in a decreased value for the relative weight of the large intestine. The jejunum and ileum crypt depths were, respectively, smaller in hens fed the control diet in relation to the NCB diet and the NCA and NCB diets. As it can be concluded, Protease B supplementation provided the best performance results.

INTRODUCTION

The primary protein sources in the formulation of poultry feed in Brazil are soy co-products, e.g. soybean meal, which has a high cost. The considerable increase in the price of soybean meal has raised formulation costs, leading to a constant search for other options to improve poultry diet nutritional value (Torres *et al.*, 2003). Among the alternatives, exogenous enzymes can improve protein digestibility and have been considered as a method to reduce the protein level offered through the feed without causing adverse effects on the animals' performance (Giannenas *et al.*, 2017).

In the last twenty years, researchers have extensively studied the effects of exogenous enzyme supplementation on the performance and composition of the intestinal microbiota of poultry (Bedford & Cowieson, 2012). Although the efficiency of carbohydrases, proteases, and phytases in poultry diets is well-known, there is some lack of knowledge regarding exogenous enzymes' modes of action (Giannenas *et al.*, 2017).

Studies show that proteases in poultry diets provide several benefits, such as improved digestion of protein and amino acids, and less nitrogen

loss in excreta. Supplementation with proteases can have the effect of efficiently hydrolyzing dietary proteins, cleaving peptide bonds, and making amino acids available. Proteases can also aid the hydrolysis of complexes between phytates and proteins, making available amino acids that would otherwise be excreted (Shitaneh, 2019). Besides, there is evidence that exogenous proteases could change intestinal morphology. The addition of exogenous enzymes in diets based on corn and soybeans can promote significant increases in villus height and decreases in the crypt depth of newly weaned piglets (Zuo *et al.*, 2015) and broilers (Wang *et al.*, 2008).

Although researches have studied dietary supplementation of proteases in broilers, there are few data in the literature regarding the isolated use of proteases in laying hens' diets. Moreover, the use of results obtained from other poultry species may not result in a satisfactory performance for laying hens.

Therefore, the goal of this study was to evaluate the effects of the inclusion of two proteases (considering or not its nutritional value) in the diets formulation of laying hens from 56 to 67 weeks old; analyzing its impacts on the productive performance, egg quality, relative weight of organs, and morphometry of the gastrointestinal tract.

MATERIALS E METHODS

The present study was carried out in the city of Primavera do Leste, state of Mato Grosso, Brazil, following the guidelines of the institutional committee on animal use (protocol number: 23108.099277/2015-73).

390 Hy-Line W36® hens at the age of 56 weeks old were used from the commercial poultry farm where the study took place. Throughout the production phase, the animals remained in metal cages (Zucami-Poultry Equipment, Model W762) (front x bottom x front height x bottom height; 762 x 630 x 540 x 450 mm) with a wire floor, linear feeder, and nipple drinker with capacity for 13 hens. The cages were arranged in two rows, with two floors in each row. The hens were distributed in a completely randomized design with five treatments and six replicates, with thirteen hens per experimental unit. The five treatments were: 1) control: standard diet, already used in the farm for laying hens at peak production, formulated without nutritional reduction and proteases; 2) negative control A - NCA: diet formulated with nutritional reduction according to the nutritional matrix of protease A, without proteases, 3) negative control B - NCB: diet formulated with nutritional reduction according to the nutritional matrix of protease B, without proteases, 4) negative control A with the inclusion of protease A - NCA + protease A, 5) negative control B with inclusion protease B - NCB + protease B.

According to the manufacturers' recommendations, the proteases Bacillus lincheniformis - "protease A" (Cibenza®DP100, Novus International Inc, ST. Charles, MO; 0.250 g/kg of feed) and Streptomyces fradiae - "protease B" (Poultrygrow250TM - Jefo Protease, Jefo Nutrition Inc., Saint-Hyacinthe, Canada; 0.125 g/kg of feed) were added to the negative controls. The values of calcium and phosphorus from phytase were considered similar for all the treatments.

The formulation of the experimental diets (Table 1) aimed to meet the dietary requirements of laying hens according to the nutritional recommendations of Rostagno *et al.* (2011).

Hens received water and the experimental feed *ad libitum* throughout the study. Each cage represented an experimental unit.

The experimental period lasted 12 weeks and was divided at the time of statistical analysis into three cycles of 28 days.

Light phase was set at 15 hours per day, and natural and artificial light was used. An automatic timer turned the lights on and off, night and dawn, according to the poultry farm's procedure.

The variables of performance 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 59th, 63rd, and 67th weeks of the hens, all healthy eggs of the day were weighed individually, identified, and sent for determination of their specific gravity. Eggs were immersed and evaluated in saline solution (NaCl) with density ranging from 1.075 to 1.100 g/cm3, with intervals of 0.005 g/cm3.

All eggs were broken and the height of the dense albumen was then measured with a digital caliper to calculate Haugh unit, according to the following formula (Cotta, 1997): (UH=100log [h+7.57-1.7p0.37]), where "h" equals the height of the dense albumen (mm) and "p", the weight of the egg (g). Then, the yolk was separated, and a digital caliper was used to measure the height and diameter to calculate the yolk index. After that, each yolk was weighed individually. The eggshells were carefully washed under running water and left to dry at room temperature for 24 hours. Dry

Table 1 – Proximate and nutritional composition of the experimental diets.

Treatments					
Ingredients (%)	Control	Negative Control A (NCA)	Negative Control B (NCB)	NCA + Protease A	NCB + Protease B
Corn	66.467	66.508	66.898	66.508	66.898
Soybean meal 48%	10.289	9.223	11.798	9.223	11.798
Meat and bone meal 46%	4.375	4.394	4.332	4.394	4.332
Protenose®	7.926	7.715	6.000	7.715	6.000
Soybean hull meal	0.500	1.745	0.616	1.745	0.616
Vegetable oil	0.500	0.500	0.500	0.500	0.500
Limestone	8.863	8.848	8.866	8.848	8.866
Salt	0.328	0.329	0.328	0.329	0.328
Mineral and vitamin premix[1]	0.200	0.200	0.200	0.200	0.200
Inert filler	0.050	0.050	0.050	0.025	0.037
L-lysine	0.329	0.332	0.258	0.332	0.258
DL-methionine	0.108	0.106	0.108	0.106	0.108
L-threonine	0.017	-	0.007	-	0.007
L-tryptophan	0.045	0.047	0.036	0.047	0.036
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)	2.900	2.880	2.875	2.900	2.900
Crude protein (%)	17.49	16.99	16.99	17.49	17.49
Calcium (%)	4.12	4.12	4.12	4.12	4.12
Total phosphorus (%)	0.68	0.67	0.68	0.68	0.68
Available phosphorus (%)	0.48	0.48	0.48	0.48	0.48
Sodium (%)	0.17	0.17	0.17	0.17	0.17
Chlorine (%)	0.33	0.33	0.31	0.33	0.31
Digestible lysine (%)	0.82	0.79	0.78	0.82	0.82
Digestible met+cis (%)	0.64	0.62	0.61	0.64	0.64
Digestible threonine (%)	0.56	0.52	0.54	0.56	0.56
Digestible tryptophan (%)	0.17	0.17	0.17	0.17	0.17

[1]Guarantee levels per kilogram of product: Choline 67,2g; Cu 5X103mg; Fe 25g; Iodine 600mg; Mn 40g; Se 100mg; Zn 30g; Vit. A 4050X103UI; Vit. D3 1250X103UI; Vit. E 500UI; Vit. K3 1000UI; Vit. B1 500mg; Vit. B2 1750mg;Vit. B6 500mg; Vit. B12 5000mcg; Niacin 10,5g; Pantothenic Acid 3300mg; Folic acid 200mg; Biotin 7,5mg; BHT 7500mg; Zinc Bacitracin 14g.

[2]Fungal phytase (*Aspergillusniger*), 10.000 FTU/g;

[3]Bacterial protease (*Bacillus licheniformis*), 600.000 U/g;

[4]Bacterial protease (*Streptomyces fradiae*), 25.000 U/g.

eggshells were then weighted and their thickness was measured at the middle, top, and bottom of the egg with a digital caliper. The arithmetic mean of these three measurements was the average eggshell thickness. The albumen's weight was obtained by the difference between the whole egg's weight and the weight of the eggshell and yolk. All weight values from the egg components were converted into a percentage of the total egg weight. The Roché® colorimetric fan provided the results of the yolk color analysis.

At 67 weeks old, one hen from each experimental unit (6 hens/treatment) was weighed individually, according to the average replicate weight for analysis of the relative weight of digestive organs and intestinal morphometry. Hens were then slaughtered by cervical dislocation and the small and large intestines, liver with the gallbladder, proventriculus, gizzard without the adhered fat, and pancreas were dissected. 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 proportion of the body weight, and then the intestinal morphometry analysis was performed. Approximately 3 cm from the medial region of the duodenum, jejunum, and ileum were sampled for intestinal morphometry analysis. These parts were opened longitudinally, placed on a rigid paper base, washed under running water, and fixed in formaldehyde (buffered 10%) for making histological slides to measure villus height, crypt depth, and

villus:crypt ratio. After 48 hours in formaldehyde, the intestinal samples were cleaved, dehydrated in alcohol, cleared in xylol, included in paraffin, cut in a microtome, arranged in histological slides, 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 the results, the arithmetic mean of 10 villi or crypts measures was considered.

At the end of the 3rd cycle, all the hens were weighed to determine each experimental unit's average weight.

All statistical analyses were performed using the SAS® program. Upon meeting the assumptions of variance homogeneity and residue normality, the data were submitted to variance analysis. In case of significant difference between treatments, the means were compared by Tukey's test at 5% probability. The data for yolk color did not follow the principles of normality and homoscedasticity, so they were submitted to non-parametric analysis using the Kruskal-Wallis test at 5% probability. The statistical model used for variance analysis of all variables was:

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

Where:

Y_{ij} = observed result;

μ = overall mean of the experiment;

T_i = protease supplementation effect;

e_{ij}= random error associated with each observation.

RESULTS

In general, feed intake, egg production in percentage, egg production per dozen, egg mass, feed conversion per egg mass, and egg weight were decreased in NCA, NCB and NCA + protease A groups. However, there was no significant effect (p>0.05) of treatments for hen weight and conversion per dozens of eggs (Table 2).

The hens that received the NCA, NCB, and NCA + protease A diets showed lower feed intake than those fed the control treatment. However, protease B's addition promoted a similar feed intake to that of the group of birds fed the control diet.

Table 2 – Performance of Hy LineW36® laying hens from 56 to 67 weeks old.

Treatment	Live weight of hens (kg)	Feed intake (kg/hen/day)	Egg production (%)	Dozen eggs production (g/dozen)	Egg mass (g/hen/day)	FC[3] egg mass (g/g)	FC[3] Dozen eggs (g/dozen)	Egg weight (g)
Control	1.581	0.096 a	79.41 ab	71.89 a	50.11 a	1.917ab	1.437	63.11a
NCA[1]	1.597	0.091 b	76.43 bc	69.17ab	44.95 b	2.028b	1.429	58.79c
NCB[2]	1.357	0.086 c	75.25 bc	68.29 ab	44.82 b	1.943ab	1.387	60.17bc
NCA+protease A	1.488	0.091 b	73.91 c	65.90 b	45.75 b	1.986ab	1.474	61.90ab
NCB+protease B	1.508	0.095 a	82.27 a	73.92 a	51.63 a	1.852a	1.394	62.74ab
Statistics								
CV(%)	12.93	2.23	4.13	4.39	5.24	4.31	3.66	2.82
p	0.15	<0.01	<0.01	<0.01	<0.01	0.02	0.07	<0.01

Means followed by the same letter, in the column, do not differ statistically from each other by the Tukey test at 5% probability;

[1]Negative control A (negative control concerning protease A);

[2]Negative control B (negative control concerning protease B);

[3]FC – Feed Conversion; CV- Coefficient of variation; *p* - *p*-value.

The inclusion of protease B provided better results for egg production in percentage and per dozen and egg mass as compared to the treatment with protease A, which were similar to those in the control group.

It was observed that hens fed with NCA, NCB, and NCA + protease A showed decreased egg mass. However, the inclusion of Protease B provided an increase in the value of this variable, reaching the levels of the controls.

The worst feed conversion per egg mass was observed in the hens that received the NCA diet and the best in the NCB + protease B treatment, but neither differed from the control treatment.

Regarding the egg quality results observed in the present study (Table 3), there was a difference (P<0.05) in the eggshell percentage and thickness, yolk index, Haugh unit, and specific gravity. In contrast, no differences were observed (p>0.05) for the yolk and albumen percentage, and yolk color.

The hens fed with nutritional restriction diets (NCA and NCB) presented eggs with decreased eggshell percentage. However, the addition of proteases A and B restored eggshell percentage to the level of controls.

It was observed that the layers submitted to diets with nutritional restriction (NCA and NCB) produced eggs with lower values for eggshell thickness than the

Table 3 – Egg quality of Hy LineW36® laying hens from 56 to 67 weeks old.

Treatment	Yolk (%)	Albumin (%)	Eggshell (%)	Eggshell thickness (mm)	Yolk index	Haugh unit	Yolk color*	Specific gravity (g/cm^3)
Control	25.69ab	65.57	8.73ab	0.36a	0.46b	107.39a	7.86	1.083ab
NCA[1]	26.09a	65.40	8.50bc	0.34b	0.48a	107.38a	7.96	1.080c
NCB[2]	25.88ab	65.86	8.25c	0.32b	0.48a	106.64ab	7.97	1.080c
NCA+protease A	25.14 b	66.00	8.86a	0.35a	0.47ab	107.33a	8.01	1.084a
NCB+protease B	25.75 ab	65.47	8.78 ab	0.35 a	0.46 b	105.36b	7.87	1.082 bc
Statistics								
CV(%)	2.05	0.87	2.26	2.81	1.98	0.74	1.23	0.13
p	0.05	0.34	<0.01	<0.01	<0.01	<0.01	0.07	<0.01

Means followed by the same letter, in the column, do not differ statistically from each other by the Tukey test at 5% probability;

[1]Negative control A (negative control concerning protease A);

[2]Negative control B (negative control concerning protease B); CV- Coefficient of variation; *p* - *p*-value.

other treatments. The supplementation of proteases in poultry diets also increased the respective values that reached the levels of controls.

Hens fed with the control, NCA, and NCA + protease A treatment showed better results for the Haugh unit index than the group that received NCB + protease B diet. However, these results did not differ from the control, NCA, and NCB treatments. Lower specific gravity values were observed for laying hens fed with nutritional restriction treatments (NCA and NCB). The addition of proteases A and B resulted in similar gravity values as compared to the group fed the control diet. Despite the statistical difference between specific gravities, they remained within the same range between 1.080 to 1.085, which is considered ideal.

There was no significant effect ($p>0.05$) of treatments on the relative weight (Table 4) of the liver, proventricle, gizzard, pancreas, and small intestine (duodenum, jejunum and ileum). Nevertheless, hens submitted to diets with addition of protease A showed lower relative weight ($p<0.05$) of the large intestine than those receiving the NCB diet. The results regarding the morphometric analysis of the small intestine mucosa (Table 5) did not show any significant differences ($p>0.05$) among treatments for villus height, crypt depth, and villus:crypt ratio in the duodenum. There was no effect of treatments ($p>0.05$) on the jejunum segment regarding villus height and villus:crypt ratio. However, there was an increase ($p<0.05$) in the crypt depth in the group submitted to a nutritional restriction (NCB) compared to the control group. Although no statistical difference ($p>0.05$) in the villus:crypt ratio for the ileum segment was detected, hens submitted to diets with nutritional restriction (NCB) showed greater villus height ($p<0.05$) compared to hens of the control, NCA + protease A and NCB + protease B group. It was also observed that hens fed the NCA and NCB diets showed higher values ($p<0.05$) for crypt depth than the control group.

DISCUSSION

Studies point out that adding proteases in the diet of layers improves production parameters,

Table 4 – Relative weight of the digestive tract organs of Hy LineW36® laying hens at 67 weeks old.

Treatment	Liver[3] (%)	Proventriculus[3] (%)	Gizzard[3] (%)	Pancreas[3] (%)	Duodenum[3] (%)	Jejunum[3] (%)	ileum[3] (%)	Large intestine[3] (%)
Control	2.93	0.34	1.33	0.21	0.99	1.74	1.60	0.92 ab
NCA[1]	3.12	0.34	1.44	0.22	0.93	1.87	1.59	0.90 ab
NCB[2]	3.36	0.33	1.45	0.21	0.90	1.59	1.76	0.98 a
NCA+protease A	2.85	0.34	1.32	0.21	0.99	1.79	1.70	0.74 b
NCB+protease B	2.78	0.36	1.39	0.23	1.99	1.98	1.74	0.86 ab
Statistics								
CV (%)	12.57	18.16	13.59	11.82	17.95	16.80	19.34	14.42
p	0.87	0.94	0.58	0.64	0.39	0.25	0.83	0.04

Means followed by the same letter, in the column, do not differ statistically from each other by the Tukey test at 5% probability;

[1]Negative control A (negative control concerning protease A);

[2]Negative control B (negative control concerning protease B);

[3] Relative weight of the organ regarding the hen's live weight; CV- Coefficient of variation; *p* - *p*-Value.

Table 5 – Morphometry of the small intestine mucous membrane of Hy LineW36® laying hens at 67 weeks old.

	Duodenum			Jejunum			ileum		
Treatment	Villus[3] (µm)	Crypt[4] (µm)	Villus:crypt[5]	Villus[3] (µm)	Crypt[4] (µm)	Villus:crypt[5]	Villus[3] (µm)	Crypt[4] (µm)	Villus:crypt[5]
Control	727.54	86.85	8.42	1091.60	111.43 b	9.67	569.66 c	95.85 b	5.99
NCA[1]	839.92	101.06	8.34	1060.40	136.47 ab	7.84	792.19ab	124.35 a	6.45
NCB[2]	850.44	97.59	8.42	1091.60	148.09 a	6.76	869.46 a	131.52 a	6.68
NCA+protease A	778.35	85.37	9.00	1032.20	119.63 ab	8.66	687.77 bc	115.86 ab	5.98
NCB+protease B	940.54	100.48	9.47	1056.70	129.87 ab	8.24	748.99ab	105.65 ab	7.16
Statistics									
CV (%)	15.43	12.12	12.52	22.93	14.48	20.85	11.78	11.53	16.19
p	0.09	0.07	0.42	0.96	0.03	0.11	<0.01	<0.01	0.44

Means followed by the same letter, in the column, do not differ statistically from each other by the Tukey test at 5% probability;

[1]Negative control A (negative control concerning protease A);

[2]Negative control B (negative control concerning protease B);

[3]Villus height;

[4]Crypt depth;

[5]Villus:crypt ratio; CV- Coefficient of variation; *p* - *p*-value.

performance, intestinal mucosa morphometry, egg quality, and composition, and improves the utilization of amino acids in diets with low crude protein levels. These characteristics justify using this additive (Vieira *et al.*, 2016; Santos, 2020; Williams *et al.*, 2021).

In this study, there was a decrease in feed intake of hens fed diets with nutritional restriction (NCA and NCB) and NCA + protease A. These results are in agreement with those found by Vasconcellos *et al.* (2012), who observed a linear decrease in the intake of broilers between 1 and 21days old fed with diets with reduced protein levels. Novak *et al.* (2006) also found that Hy Line W98 laying hens showed reduced feed intake when receiveing diets with reduced protein levels. According to these authors, the decrease in feed intake may be caused by the sudden change in circulating amino acids in the blood. In free form, these amino acids are quickly absorbed throughout the gastrointestinal tract, reflecting in the increase in amino acids in the bloodstream. In response to these amino acids' high plasma concentrations, the animal decreases feed intake once its nutritional requirements are met. In the present study, the protein and amino acid content showed a more significant decrease than the energy content in nutritional compositions of the NCA and NCB diets compared to the control diet. In the NCA diet, there was a more significant decrease in the amino acid threonine (6.17%); and in the NCB diet, a more significant decrease in energy, lysine, methionine + cystine, and tryptophan (0.86%, 3.93%, 3.77%, and 2.98%, respectively) was observed. It can be inferred that the decrease in the amino acid level in the hens' diet may have caused the decrease in the feed intake of hens submitted to nutritional restriction treatments.

In contrast, protease B's inclusion restored the decrease in the diet's nutritional value, keeping the feed intake similar to the control treatment. It possibly happened due to the enzymes increasing the digestibility of nutrients. The same compensation was not observed when adding protease A, which is possibly due to its lower enzymatic efficiency compared to protease B. As Barbosa *et al.* (2014) reported, exogenous enzymes improve the utilization of amino acids in diets, reducing formulation costs by allowing the reduction of levels of metabolizable energy and certain nutrients, such as amino acids and minerals. Thus, the present study results endorse the addition of protease B in hens' diets, since they improve the utilization of protein and, consequently, the performance of laying hens. Moreover, their effects stand out in diets with low levels of essential amino acids or total protein.

Some improvement in the hens' performance was observed in this study, especially regarding the production, mass, and conversion into egg mass with supplementation of protease B, which promoted a result similar to that of the control diet. Resende *et al.* (2017) also found similar results, since they observed that the inclusion of an enzyme complex (β-glucanases, β-xylanases, cellulases, and phytases) at the dose of 50g/t in diets of Hy-Line Brown hens from 28 to 40 weeks old, provided improved rates for egg production, egg mass, conversion by dozen, and egg mass compared to hens fed diets with low nutritional density. Vieira Filho *et al.* (2015) observed an increase in the laying rate by adding 500 g ton-1 of protease (100 Ug-1) to diets of Isa Brown commercial layers at 44 weeks old with reduced nutritional levels. Similarly,

Silva *et al.* (2012) observed a similar effect, reporting that birds fed diets with nutritional reduction and without supplementation of enzymes presented worse rates of feed conversion, confirming the positive effect of the inclusion of enzymes in diets of laying hens. From this study, it can be concluded that the inclusion of protease B improved the digestibility of nutrients and, consequently, increased the availability of amino acids for absorption, thus improving protein synthesis.

As indicated in the present study, percentage and thickness of eggshell, yolk index, Haugh unit, and the specific gravity of the eggs had different values in the experimental groups. However, the addition of proteases generated the same values for percentage and thickness of the eggshell and specific gravity of eggs as compared to hens fed the control diet. These results differ from those found by Resende *et al.* (2017), who did not find any significant differences in the percentage and thickness of eggshell, Haugh unit, and specific gravity of eggs from Hy-Line Brown hens from 28 to 40 weeks old fed diets with or without the enzyme complex (β- glucanases, β-xylanases, cellulases, and phytases) in the proportion of 50g/t.

The present study also showed that hens subjected to diets with nutritional reduction showed worse egg external quality. Eggshell production is directly related to collagen synthesis, which is crucial for calcium ions to be appropriately deposited on the organic matrix to form the eggshell. Collagen is a fibrous protein containing peptide chains of glycine, proline, lysine, hydroxylysine, hydroxyproline, and alanine. These chains are organized parallel to the axis, forming collagen fibers, which provide strength and elasticity to the structure (Campbell, 2000). According to Novak *et al.* (2006), adequate amounts of amino acids in laying hens' diets, especially sulfur, are essential to improve eggshell thickness. Mazzuco & Bertechini (2014) reported that low levels of protein and dietary amino acids could alter this matrix and affect the eggshell's crystal structure and thickness, since calcium crystals in the eggshell begin their formation linked to a protein matrix. According to these reports, it can be inferred that diets with nutritional reduction NCA and NCB promoted changes in the eggshell composition. However, the addition of proteases possibly increased the digestibility of amino acids, especially sulfur ones, thus promoting better results for the percentage and thickness of eggshells as compared to NCA and NCB groups. Although no changes were observed in the relative weight of the digestive system organs, hens submitted to the NCA + protease A diet showed a decrease in the large intestine's relative weight when compared to the NCB diet. Ndazigaruye *et al.* (2019) observed a decrease in the relative weight of the pancreas of Ross 308 broilers at 35 days old that were fed diets supplemented with protease (Bacillus clausii), with either normal or reduced levels of crude protein. Similarly, Yuan *et al.* (2008) reported that adding exogenous enzymes in broiler diets promoted a decrease in the pancreas' relative weight and the relative length and weight of the duodenum, jejunum, and ileum. According to these authors, supplementation of exogenous proteases may have caused a decrease in the pancreas' relative weight, which indicates that the concentration of enzymes may have influenced the secretion of pancreatic enzymes, substrates, or products of their hydrolysis in the small intestine lumen. Zanella *et al.* (1999) also observed a 40% decrease in enzyme secretions (trypsin, chymotrypsin, lipase, and α-amylase) by the duodenal mucosa in diets supplemented with exogenous enzymes. It is known that adding proteases in the diet of laying hens can complement the action of endogenous enzymes (Wiryawan & Dingle, 1999). Nevertheless, according to the data presented in this study, it can be inferred that the amount of enzyme supplemented in the diets of the layers was not capable of reducing the secretion of enzymes from digestive organs, consequently reducing in the relative weight of the digestive tract's viscera.

In this research, the treatments possibly influenced the jejunum and ileum segments of the laying hens. The groups that received diets with a nutritional reduction (NCA and NCB) presented greater crypt depths (jejunum and ileum) and villus heights (ileum) compared to the controls. However, the addition of proteases promoted similar results compared to the group fed the standard diet; except for protease B, which provided greater villus height in the ileum's mucous membrane. A similar effect was found by Gomides *et al.* (2019). They evaluated the intestinal histomorphometry of the small intestine of broilers at 21 days and found that the addition of exogenous enzymes promoted statistical differences in the villus height of the jejunum and ileum; however, the inclusion of the protease did not induce superior values compared to that of the control treatment. Likewise, Vieira *et al.* (2016) found that laying hens fed diets with the addition of protease B (*Bacillus licheniformis*, 0.250 g kg^{-1} in the diet) presented greater villus height, and crypt depth in the jejunal mucous membrane compared to the group that received diets with protease A (*Streptomyces fradiae*, 0.125 g kg^{-1} in the diet).

In recent years, there has been an interest in the "extra-proteinaceous" effects of protease, such as influences on enteric resilience and interactions with the digestibility of non-protein nutrients. The origin of the beneficial effect of protease on intestinal health is unclear, but it may be a combination of several interacting factors (Cowieson & Roos, 2016). Such factors may include a rotting decrease in the distal digestive tract (Windey *et al.*, 2012), hydrolysis of protein anti-nutrients and antigenic proteins (Rooke *et al.*, 1998; Ghazi *et al.*, 2002; Cowieson *et al.*, 2015), greater availability of amino acids for mucin synthesis (Cowieson & Roos, 2014), and enterocyte turnover (Cowieson *et al.*, 2015). Given these reports, it appears that the addition of exogenous enzymes in this study possibly promoted the growth of epithelial cells (enterocytes, goblet, and enteroendocrine cells) that were essential for the repair of the intestine.

CONCLUSION

Adding protease B in diets with reduced nutritional levels provides better performance results, as the proteases significantly influence nutrient utilization by Hy Line W36 laying hens at 56 weeks old.

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