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Original Article

Effect of Dietary Zeolite Supplementation on Production, Egg Quality, Ammonia Volatilization, Organ Morphometry and Blood Parameters in Brown Laying Hens

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ABSTRACT

The objective was to examine the effects of supplementing different levels of zeolite in the diet of laying hens during the laying phase, at 54 weeks of age, on production performance, egg quality, ammonia volatilization, excreta pH, blood parameters, weight, and organ morphometry. The treatments consisted of a control diet free of zeolite and five diets including increasing levels of zeolite (0.7, 1.4, 2.1, 2.8, and 3.5%). A completely randomized design was adopted with eight replicates of five birds per plot, totaling 240 laying hens, for four 28-day periods. Results were subjected to ANOVA, Dunnett's test, and regression analysis at 5% probability. The zeolite levels had no significant effect on production performance, egg quality, and blood parameters. The zeolite levels have a significant effect on ammonia volatilization, excreta pH, liver weight, and organ morphometry specifically on the intestinal length. Ammonia and pH reached a linear plateau. Ammoniacal nitrogen levels in the excreta differed from that observed in the control group at all zeolite levels, whereas the pH was lowest at the zeolite inclusion levels of 0.7 and 1.4%. Ammonia volatilization and the pH value of the excreta decreased by 46.9% and 4.6%, when the laying hens consumed diets with 0.93% and 1.19% zeolite, respectively. There was an effect on the relative weight of the organs, with higher liver yield and intestinal length provided by zeolite inclusion. Therefore, zeolite can be included in the diet of commercial layers at up to 0.93% without affecting their production performance or egg quality.

INTRODUCTION

Egg production is an activity of global importance that has been intensified to meet the great demand for foods of high nutritional value (Dilawar *et al.*, 2021). One of the obstacles of poultry farming is the proper destination of the waste generated in the sheds. Commercial poultry farms, mainly, produce large amounts of excreta, which release gases such as ammonia (NH_3) and hydrogen sulfide, as well as volatile compounds that are detrimental to the production system. Ammonia is a health concern not only for birds but also for humans and the environment (Naseem & King, 2018).

Nutritional strategies are required to mitigate NH_3 emission (Maurer *et al.*, 2016). Zeolite is a mineral that has been studied in the diet of laying hens as a strategy to reduce the emission of NH_3 present in bird excreta through its fixation by the metallic ions present in the zeolite structure, without compromising bird performance (Gradzki *et al.*, 2020).

Several types of natural zeolites are produced worldwide, e.g., zeolite, chabazite, heulandite, modernite, phyllite, silicate, silicalite, and heroinite. However, the zeolite most used in animal research is

zeolite (Mastinu *et al.*, 2019) which can adsorb harmful substances in the gastrointestinal tract such as mycotoxins, ammonia, and heavy metals (Pavelić *et al.*, 2018). Jarosz *et al.* (2017), when used in the diet of laying hens, zeolite can improve the air quality inside the sheds without affecting production performance or immunity if it is included at low levels relative to the total diet.

Öztürk *et al.* (1998) observed a decrease in excreta moisture using 8% zeolite in the diet of laying hens in flocks with high-moisture problems. Nonetheless, further research is needed on the effectiveness of zeolite in reducing ammonia volatilization from excreta (Schneider *et al.*, 2017) as well as its effects on the production and health parameters of birds (Jarosz *et al.*, 2017).

The hypothesis is that dietary supplementation of zeolite for brown laying hens will affect egg production and quality, may act to reduce ammonia volatilization and change the pH of excreta, as well as interfere with blood parameters and organ morphometry. This is justified due to the adsorbent properties of zeolite, which can influence the absorption of nutrients, intestinal health, and the efficiency of Isa Brown laying hens, which may have a better performance.

Therefore, the present study was undertaken to evaluate the effects of including zeolite in the diet of Isa Brown brown-egg layers on egg production and quality, ammonia volatilization and pH value of excreta, blood parameters, organ weight, and organ morphometry.

MATERIAL AND METHODS

Ethical approval

All procedures were carried out in accordance with the permit of the Ethics Committee on the Use of Animals of the University Federal Rural de Pernambuco (UFRPE) approved by license number 004/2020.

Experimental location and characterization

The field experiment was carried out on a commercial farm located in the municipality of Goiana - PE, Brazil (coordinates: 07°33'38" S, 35°00'09" W).

This study proposes to examine the effect of using natural zeolite in diets for brown-egg layers on production variables, namely, egg quality, pH and ammonia of excreta, biochemical and hematological profiles, and organ morphometry.

The birds were housed in a brick shed and reared inside cages measuring 50 × 50 × 45 cm (5 birds/cage), which were equipped with trough feeders and nipple drinkers. Water was available *ad libitum*, and the amount of feed supplied was adjusted according to the requirement calculated per bird. The lighting program followed the recommendations of the line guide, consisting of 16 hours of daily light (natural + artificial). During the experimental period, the temperature and relative humidity inside the shed were recorded daily with a digital thermo-hygrometer (Incoterm 0419).

Experimental design and Diets

The birds were distributed by weight and laying percentage into six treatments with eight replicates of five birds each in a completely randomized design. A total of 240 Isa Brown laying hens weighing approximately 1,750 kg, at 54 weeks of age, were used for four 28-day periods, the experimental period was 112 days. The birds started to be fed the experimental diets, which differed only in the inclusion of increasing levels of zeolite.

The treatments consisted of a control diet (0% zeolite) with 3.5% inert (washed sand) and five diets with levels of zeolite increasing (0.7, 1.4, 2.1, 2.8, and 3.5%) and decreasing inert (Table 1). All diets were isoenergetic and isoproteic, following the recommendations given in the tables by Rostagno *et al.* (2017). The proportions of other amino acids were maintained relative to the level of lysine, on an ideal protein basis.

The adsorbent-natural product evaluated was composed of zeolite of the Clinoptilolite type, which has a crystalline structure and a high Cation Exchange Capacity and was provided by the Celta Brazil Company. The chemical composition of the zeolite used in the poultry feed contained silicon dioxide SiO_2 (62 to 75%), aluminum oxide Al_2O_3 (7 to 15%), sodium oxide Na_2O (0 to 5%), potassium oxide K_2O (0.5 to 5%) and calcium oxide CaO (0.5 to 5%) and physical composition: beige to a slightly greenish color, granulometry (0.4 to 1mm), apparent density (0.5 to 2 g/cm^3), pH (6.5 to 10), humidity ≤6%, Cation Exchange Capacity (120 to 200 meq/100g).

Performance evaluation

The birds fed the diets were evaluated for the following production performance variables: initial and final body weights, feed intake (g/bird/day), feed conversion per dozen eggs (g/dz) and per egg mass (g/g), egg production per bird/week, and total production (average of the four 28-day periods). Feed intake was recorded weekly.

Table 1 – Diet formulation, nutritional composition, and experimental structure adopted with the levels of zeolite (Clinoptilolite) in diets for 54-week-old Isa Brown layers.

diet composition – Ingredients			%	Calculated nutritional composition		
Whole millet grain			50.000	Metabolizable Energy (kcal/kg)		2720.00
Soybean meal 45 %			14.138	Crude protein (%)		16.13
Ground maize			12.998	Ether extract (%)		4.050
Limestone (50% fine-50% coarse)			10.417	Crude fiber (%)		2.366
Meat and bone meal (43% CP)			4.383	Calcium (%)		4.600
Inert (Washed sand)			3.500	Available P (%)		0.360
Zeolite (Clinoptilolite)			0.000	Digestible lysine (%)		0.805
Soybean oil			2.769	Digestible Met + Cys (%)		0.789
Choline Chloride			0.500	Digestible threonine (%)		0.518
DL-Methionine 99%			0.311	Mineral matter (%)		10.97
Vitamin/Mineral supplement*			0.300	Sodium (%)		0.179
L-Lysine HCl 78.8%			0.243	Potassium (%)		0.435
Sodium bicarbonate			0.240	Chlorine (%)		0.168
Common salt			0.201	Electrolytic balance (mEq/kg)		142
Total			100.00			
Experimental structure						
Inert (Washed sand), %	3.5	2.8	2.1	1.4	0.7	0.0
Zeolite (Clinoptilolite), %	0.0	0.7	1.4	2.1	2.8	3.5

Vitamin/mineral supplement per kg of feed: copper: 8 mg; iron: 50 mg; manganese: 70 mg; zinc: 50 mg; iodine: 1.2 mg; selenium: 0.2 mg. vit. A: 7000 IU; vit. D3: 2000 IU; vit. E: 5 mg; vit. K3: 1.6 mg; vit. B2: 3 mg; vit. B12: 8 mcg; niacin: 20 mg; pantothenic acid: 5 mg; antioxidant: 15 mg.

Egg quality

Eggs were collected for analysis of egg weight (g); albumen height (mm); yolk weight (g); shell weight (g); shell thickness (mm); percentages of yolk, albumen, and shell (%), and Haugh unit.

On the last three days (26, 27 and 28d) of each 28-day cycle, three eggs were selected per experimental unit based on average weight, totaling 72 eggs per treatment in each cycle. After collection, the eggs were identified and weighed individually on a semi-analytical scale with 0.01-g precision according to the treatment and its respective replicates. Then, all eggs were broken individually and placed on a smooth and flat surface, and albumen height (mm) was measured with a 0.01-mm digital caliper. Subsequently, the yolks were separated from the albumen and individually weighed on a 0.01-g precision scale (L3102iH, Bel). The shells were carefully washed, air-dried for 48 h, and weighed, and their thickness was measured at three different points in the cross-sectional area using a precision micrometer (IGaging San Clemente, CA, USA).

Eggs collected on the last three days of each cycle were also used to calculate the percentages of yolk and shell relative to the egg weight. Albumen weight and percentage were determined as the difference between the egg weight and the weights of the shell + yolk. In addition, with the measurements of the albumen and egg weight, the Haugh unit (HU) values of the eggs were calculated using the following formula, proposed by Card & Nesheim (1966): $HU = 100 \log (H + 7.57 - 1.7W^{0.37})$, where H is the albumen height (mm) and W is the egg weight (g). This expression of albumen height corrected to egg weight is also a means of evaluating internal egg quality.

Ammonia and pH of excreta

Fresh excreta samples were taken until 15 min after excretion, to evaluation of ammoniacal nitrogen and to perform pH analysis. Samples were weighed collecting 100 g on each replicate and placing it in a 750-mL glass flask with cap. One sample was collected per experimental unit, totaling 8 samples per treatment. Subsequently, a 50-mL beaker containing 10 mL of a 2% boric acid solution was fixed over the excreta inserted in the container to fix the ammonia released by the excreta. After a 17-hour rest period, titration was performed with standardized sulfuric acid at a concentration of 0.05. Results were expressed in milligrams of ammonia released, using the formula: , in which A: NH_3 (mg); Vt: volume of the H_2SO_4 solution used in titration (mL); N: normality of the acid used; and P: amount incubated (g) (Hernandes & Cazetta, 2001).

The analysis of pH followed the methodology of Silva & Queiroz (2002), using a digital pH meter (model Kasvi K39) previously calibrated with standard solutions of pH 4, 7, and 10.

Blood parameters

For hematological analysis, blood samples (4 mL) were collected from the vein of one of the wings of the birds at 70 weeks of age, using one hen per plot. The collection period was from 06:30 to 09:00 in the morning. To analyze the blood biochemical parameters, blood samples (4 mL), obtaining approximately 1mL of serum per hen, samples were collected from two hens per plot, also at 70 weeks of age.

The hematological analysis consisted of the analysis of erythrocytes, hemoglobin, hematocrit, platelets, total plasma proteins, leukocytes, heterophils, lymphocytes, and monocytes. Erythrocyte, leukocyte, and platelet counts were performed in a Neubauer chamber. Hematocrit was determined by the microcapillary method and by measuring the reference total plasma protein.

For biochemical analysis, the serum samples were analyzed for total protein, albumin, creatinine, calcium, urate/uric acid, urea, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase. For the analyses, commercial kits (Doles, Goiânia, GO) were used according to the manufacturer's instructions. The parameters were read by spectrophotometry.

Organ morphometry

For organ morphometry, 48 hens (eight birds per treatment) at 70 weeks of age were used. In each experimental plot, one bird was chosen per cage based on the average weight. First, the birds were euthanized by cervical dislocation, following the recommendations of CONCEA (2018). Then, the organs were weighed, including the proventriculus together with the gizzard (after removing the contents from the interior of the organs), spleen, liver, heart, and pancreas. In addition, the intestinal length of each bird was measured. Intestinal weight and length were measured to obtain the absolute weight. The percentage of each variable relative to live weight was also calculated.

Statistical analysis

The assumptions of normality and homoscedasticity errors were tested. The statistics were analyzed by analysis of variance (ANOVA), applying the F test with $\alpha = 0.05$, and using SAS software (Statistical Analysis System, version 9.4). The statistical model applied was as follows:

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

in which y_{ij} is the response variable related to the observation of treatment i; μ is the overall mean; T_i is the effect of treatment i (i = 1, 2,…., 6); on the repetition j (j = 1, 2, …., 8) and ε_{ij} is the normally distributed random experimental error with zero mean and variance $\sigma 2$ [$\varepsilon_{ij} \sim N(0, \sigma^2)$] associated with observation y_{ij}.

To compare the results obtained with each of the zeolite inclusion levels relative to the control diet, Dunnett's test was applied at 5% significance. After ANOVA, regression analyses were performed for the variables that showed a significant treatment effect, using the PROC GENMOD procedure of SAS software (Statistical Analysis System, version 9.4) to evaluate linear, quadratic, and cubic effects and the PROC NLMIXED procedure for the LP (Linear Plateau) model to identify the best level of zeolite inclusion. The linear models and the LP were compared using the Akaike Information Criterion (AIC), considering the log of maximum likelihood (-2 Log L) and the number of explanatory variables in the model.

RESULTS

The inclusion of different levels of zeolite in the diet of the laying hens did not influence (p>0.05) the performance variables of laying percentage, average egg weight, egg mass, feed intake, or feed conversion per mass and per dozen eggs (Table 2).

In terms of egg quality, the variables of egg weight, shell percentage and thickness, albumen percentage and height, yolk percentage, and Haugh unit did

Table 2 – Mean values and standard errors of the mean for production performance of Isa Brown layers evaluated for 112 days, with an initial age of 54 weeks, as a function of zeolite levels in the diet, and probability value for the F test.

Zeolite level, %	Laying percentage, %	Average egg weight, g	Egg mass, g/day	Feed intake, g/bird/day	Conversion, g feed/g egg	Conversion, kg feed/dz
0.0	90.51±1.27	59.33±0.77	53.67±0.80	107.0±0.4	2.001±0.031	1.183±0.011
0.7	87.79±1.61	59.34±0.73	52.10±1.11	106.3±0.3	2.051±0.042	1.211±0.014
1.4	87.28±1.44	60.33±0.71	52.65±0.87	106.7±0.4	2.037±0.038	1.222±0.016
2.1	87.22±1.46	59.15±0.65	51.60±1.24	105.9±0.4	2.066±0.042	1.214±0.013
2.8	87.47±1.36	59.37±0.59	51.98±0.55	106.6±0.3	2.063±0.020	1.219±0.015
3.5	86.56±1.12	59.14± 0.40	51.21±0.82	106.0±0.2	2.078±0.035	1.224±0.018
Mean	87.80±0.53	59.44± 0.26	52.20±0.38	106.4±0.2	2.049±0.014	1.212±0.006
p-value	0.3762	0.8244	0.5608	0.1508	0.335	0.2759

not show any statistical difference (p>0.05) between treatments (Table 3).

However, there was an effect (p=0.05) on ammonia nitrogen values, with a reduction in ammonia emission caused by increasing the levels of zeolite included in the diet, when compared with the control treatment (without zeolite). As for the pH values of the excreta (p=0.05), the lowest values occurred at the zeolite inclusion levels of 0.7 and 1.4%, which caused it to drop 3.86% and 4.82%, respectively (Table 4).

Table 3 – Mean values and standard errors of weight and composition of eggs from Isa Brown layers with an initial age of 54 weeks as a function of zeolite levels in the diet, and probability values for the F test.

Variable	Zeolite level, %						Overall mean	*p*-value
	0.0	0.7	1.4	2.1	2.8	3.5		
Egg weight (g)	60.29±0.39	59.99±0.31	60.69±0.44	59.69±0.45	60.28±0.47	60.53±0.28	60.24±0.16	0.5213
Shell weight (g)	5.890±0.07	5.876±0.052	5.983±0.074	5.858±0.064	5.842±0.087	5.895±0.064	5.890±0.027	0.7683
Shell (%)	9.79±0.13	9.81±0.09	9.86±0.10	9.82±0.07	9.70±0.10	9.75±0.12	9.79±0.04	0.9069
Shell thickness (mm)	0.391±0.005	0.390±0.004	0.395±0.004	0.393±0.003	0.389±0.003	0.393±0.004	0.392±0.001	0.8849
Albumen weight (g)	39.28±0.34	39.17±0.30	39.65±0.31	38.99±0.36	39.56±0.38	39.82±0.33	39.41±0.14	0.4779
Albumen (%)	65.11±0.30	65.26±0.29	65.30±0.21	65.29±0.24	65.58±0.29	65.76±0.31	65.38±0.11	0.5762
Albumen height (mm)	7.523±0.144	7.259±0.053	7.483±0.119	7.235±0.093	7.808±0.148	7.399±0.161	7.451±0.056	0.0534
Haugh Unit	86.09±0.85	84.61±0.33	85.91±0.66	84.54±0.61	87.86±0.84	85.32±0.98	85.72±0.33	0.0521
Yolk weight (g)	15.12±0.18	14.94±0.16	15.06±0.17	14.84±0.16	14.88±0.13	14.82±0.15	14.94±0.06	0.7159
Yolk (%)	25.10±0.24	24.93±0.25	24.83±0.23	24.89±0.25	24.72±0.21	24.49±0.27	24.83±0.10	0.5760

Table 4 – Mean values and standard errors of the mean of ammonia nitrogen content released from the excreta and pH of the excreta of 70-week-old Isa Brown layers as a function of zeolite levels in the diet, and probability values for the F test.

Zeolite level, %	Excreta Ammonia-N release, mg/100 g	Excreta pH
0.0	1.11±0.20	7.26±0.14
0.7	0.72±0.16*	6.98±0.08*
1.4	0.58±0.13*	6.91±0.07*
2.1	0.53±0.09*	7.07±0.09
2.8	0.64±0.11*	7.14±0.10
3.5	0.60±0.13*	7.22±0.10
Mean	0.70±0.06	7.10± 0.04
p-value	0.050	0.049

Means in the column followed by an asterisk differ from the control diet by Dunnett's test ($p \leq 0.05$).

Based on Linear Plateau regression analysis (p=0.0033), there was an estimated 46.9% reduction in ammonia release from the excreta (Figure 1). This value was estimated from the equation from an estimated value of 1.105 mg of released ammonia/100 g of excreta in the zeolite-free diet, against an estimated constant value of 0.587 mg/100 g at the level of 0.93% of the additive.

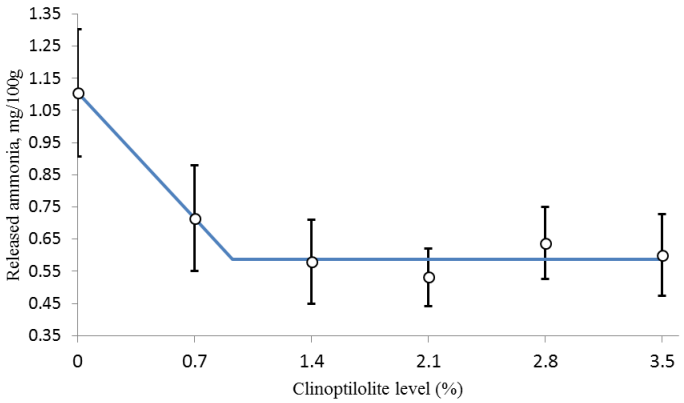

Figure 1 – Effect of zeolite level in Isa Brown laying hens on the ammonia released in excreta.

The zeolite levels did not influence (p>0.05) any of the hematological (Table 5) or serum biochemical (Table 6) variables evaluated.

In organ morphometry, the diets with zeolite also had no effect on the relative weights of proventriculus, gizzard, spleen, heart, or pancreas, but influenced the relative weight of the liver (p=0.002) and intestinal length (p=0.048) (Table 7). Regression analysis revealed a quadratic effect on relative liver weight (p=0.0043, Figure 2) and intestinal length (p=0.0047, Figure 3), which increased up to the levels of 2.16% and 2.03%, respectively.

DISCUSSION

In this study, zeolite showed to be able to reduce the ammonia and increase the relative weight of the liver and intestinal length of laying hens.

The presence of zeolite in the diet did not affect production performance or egg quality, which were maintained in the period from 54 to 70 weeks of age. The lack of effect of zeolite inclusion on these variables can be explained by the intrinsic characteristics of this additive, as it is an inorganic compound that is not absorbed by the organism of birds. Therefore, it does not replace the nutrients present in the diet, despite providing elements that participate in reactions in the animal organism (Prasai *et al.* 2017), having ion

Table 5 – Mean values and standard errors of the mean of hematological variables of 70-week-old Isa Brown layers as a function of zeolite levels in the diet, and probability values for the F test.

Parameter	Zeolite level, %						Overall mean	*p*-value
	0.0	0.7	1.4	2.1	2.8	3.5		
Heterophils, %	50.83± 6.23	52.17± 3.64	59.67± 5.25	54.17± 2.04	46.67± 5.05	45.33± 6.09	51.47± 2.03	0.4677
Lymphocytes, %	45.83± 5.87	46.33± 3.74	38.17± 5.16	39.83± 2.98	50.33± 5.20	52.00± 5.43	45.42± 2.02	0.3548
Monocytes, %	2.333±0.955	1.500±0.342	1.500±0.224	4.167±1.740	2.500±1.118	2.333±0.843	2.389±0.409	0.5700
Total platelets, /mm³	5500±885	7000±2000	5667±615	4500±428	5000±816	5833±703	5583±372	0.5868
Leukocytes, /mm³	1970±200	2213±294	2047±206	2200±493	2535±342	3117±335	2347±140	0.0815
Erythrocytes, /mm³	2337±161	2247±203	2150±141	2435±251	2393±166	2228±143	2298± 71	0.8027
Heterophils: Lymphocytes	1.079±0.268	1.193±0.168	1.317±0.284	1.408±0.134	1.151±0.191	0.981±0.213	1.185±0.081	0.6874
Hematocrit, %	26.33± 0.92	27.83± 1.40	27.00± 0.26	26.17± 0.65	26.33± 1.12	25.67± 0.88	26.56± 0.37	0.4592
Hemoglobin, g/dL	8.750±0.335	9.350±0.527	8.817±0.111	8.800±0.254	8.850±0.419	8.433±0.410	8.833±0.146	0.5479
Medium corpuscular hemoglobin, pg	38.41± 3.03	42.55± 2.77	41.95± 2.95	37.48± 2.86	37.50± 2.06	38.45± 2.48	39.39± 1.08	0.7371
Mean corpuscular hemoglobin concentration, g/dL	33.21± 0.29	33.54± 0.34	32.65± 0.16	33.62± 0.33	33.60± 0.69	32.84± 0.97	33.24± 0.21	0.7935
Mean corpuscular volume, fL	115.5± 8.6	126.9± 8.3	128.4± 8.6	111.7± 8.9	111.3± 4.3	116.9± 6.0	118.4± 3.1	0.5959
Plasma total protein, g/dL	7.533±0.813	8.240±0.695	7.333±0.169	7.250±0.280	7.800±0.529	7.400±0.556	7.574±0.213	0.6091
Hemoglobin×3: Hematocrit	0.996±0.009	1.006±0.010	0.980±0.005	1.009±0.010	1.008±0.021	0.985±0.029	0.997±0.006	0.7935
Erythrocytes×3: Hemoglobin	0.806±0.065	0.719±0.044	0.733±0.051	0.828±0.072	0.812±0.045	0.796±0.050	0.782±0.022	0.7322

Table 6 – Mean values and standard errors of the mean of serum biochemistry variables of 70-week-old Isa Brown layers as a function of zeolite levels in the diet, and probability values for the F test.

Parameter	Zeolite level, %						Overall mean	*p*-value
	0.0	0.7	1.4	2.1	2.8	3.5		
Total protein (g/dL)	8.94±0.40	9.53±0.40	9.651±0.321	9.837±0.333	9.708±0.309	9.618±0.265	9.548±0.137	0.5452
Albumin (g/dL)	1.65±0.13	1.728±0.122	1.744±0.231	1.805±0.094	1.918±0.137	1.967±0.149	1.802±0.060	0.7138
Globulin (g/L)	7.29±0.33	7.806±0.292	7.907±0.323	8.032±0.303	7.790±0.276	7.651±0.315	7.746±0.123	0.6118
Glob : Alb ratio	4.64±0.48	4.622±0.235	5.179±0.759	4.528±0.290	4.238±0.402	4.082±0.381	4.548±0.184	0.6496
Creatinine (mg/dL)	0.30±0.03	0.31±0.039	0.31±0.020	0.30±0.023	0.29±0.034	0.28±0.039	0.30±0.013	0.9585
Phosphatase (U/L)	539.30±74.50	558.5±64.5	560.5±46.4	527.3±30.5	475.2±45.9	422.2±48.7	515.8±22.0	0.5527
AST-TGO (U/L)	171.60±6.70	166.1±5.0	164.1±7.8	162.9±6.7	167.0±11.0	176.0±8.5	168.0±3.1	0.8661
GGT (U/L)	295.10±65.50	378.1±69.5	399.8±19.4	398.4±35.7	393.5±68.0	390.2±70.2	375.5±22.9	0.6198
TGP-ALT (U/L)	21.66±2.70	16.96±3.74	15.96±3.92	18.05±3.62	20.35±6.15	23.74±5.57	19.45±1.76	0.7694
Calcium (mg/dL)	32.52±1.48	33.05±0.82	32.94±1.56	32.55±0.79	32.00±1.23	30.82±0.68	32.31±0.46	0.7786
Urate (mg/dL)	3.68±0.38	3.537±0.435	3.316±0.429	3.173±0.511	3.051±0.570	2.966±0.424	3.293±0.183	0.8733
Urea (mg/dL)	1.92±0.22	1.990±0.209	2.032±0.164	2.033±0.161	1.947±0.217	1.727±0.125	1.941±0.074	0.7819

AST-TGO - oxaloacetic transaminase or aspartate aminotransferase; TGP-ALT - pyruvic transaminase or alanine aminotransferase; GGT - gamma-glutamyl transferase.

Table 7 – Mean values and standard errors of the mean of organ morphometry of brown-egg layers fed different levels of zeolite, and probability values for the F test.

Variable	Zeolite level, %						Overall mean	*p*-value
	0.0	0.7	1.4	2.1	2.8	3.5		
Slaughter weight (g)	1928±18	1908±13	1879±12	1887±15	1894±19	1914±13	1901± 6.4	0.268
Intestine (%)	4.43±0.30	4.59±0.09	4.77±0.27	4.99±0.17	5.11±0.26	4.51±0.21	4.73±0.10	0.2535
Liver (%)	1.95±0.10	2.07±0.07	2.09±0.09	2.43±0.08*	2.26±0.07*	2.09±0.06	2.15±0.04	0.002
Gizzard (%)	1.26±0.07	1.27±0.04	1.33±0.02	1.30±0.07	1.42±0.04	1.28±0.05	1.31±0.02	0.303
Cecum (%)	0.91±0.02	0.89±0.03	1.01±0.06	0.95±0.05	0.93±0.03	0.86±0.04	0.93±0.02	0.153
Heart (%)	0.38±0.03	0.37±0.03	0.38±0.03	0.39±0.01	0.40±0.02	0.40±0.03	0.39±0.01	0.908
Proventriculus (%)	0.32±0.02	0.31±0.02	0.35±0.012	0.33±0.02	0.35±0.02	0.32±0.01	0.33±0.01	0.367
Pancreas (%)	0.17±0.02	0.16±0.01	0.17±0.01	0.18±0.01	0.19±0.01	0.16±0.01	0.17±0.01	0.698
Spleen (%)	0.07±0.01	0.09±0.01	0.08±0.01	0.10±0.01	0.08±0.01	0.08±0.01	0.09±0.001	0.439
Intestinal length (cm)	140.5± 6.6	150.1± 3.6	156.3± 4.9*	161.6± 4.1*	159.3± 3.1*	148.0± 5.0	152.6± 2.1	0.048

Means followed by an asterisk differ by Dunnett's test ($p \leq 0.05$).

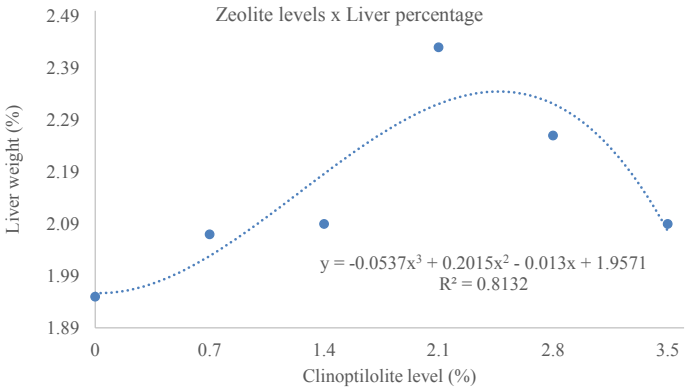

Figure 2 – Effect of zeolite level in Isa Brown laying hens on relative liver weight.

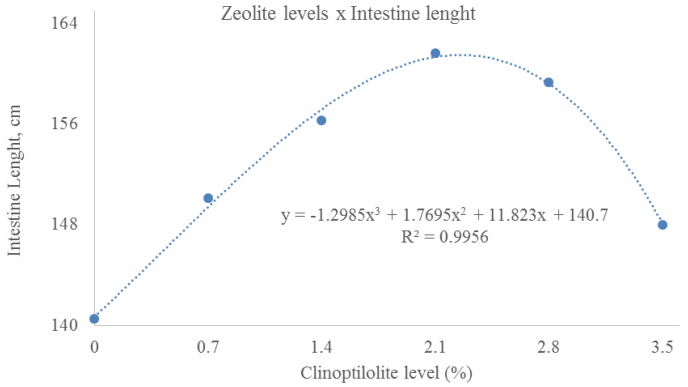

Figure 3 – Effect of zeolite level in Isa Brown laying hens on intestine length.

adsorption and cation-exchange capacities and thus not compromising the production performance of birds.

Kermanshahi *et al.* (2011) found no differences in laying percentage or egg weight after adding zeolite to the diet of laying hens. On the other hand, Macháček *et al.* (2010) studied levels of inclusion of zeolite (2 and 4%) - the same type of zeolite used in this experiment, but in Bovans Goldline hybrid layers - and found an increase in average egg weight with the inclusion of up to 2% zeolite in the diet of laying hens compared with the control diet, and a reduction in feed intake. Emam *et al.* (2019) only found a positive effect on laying percentage and egg quality after including 4% zeolite in the diet of laying hens. Fendri *et al.* (2012) observed an effect of zeolite at the level of 1% addition to the diet on the variables of egg weight and albumen weight, both of which increased. Romero *et al.* (2012) also observed satisfactory results in laying percentage and egg quality in an experiment with diets containing zeolite for laying hens.

Nonetheless, the results reported in the literature on the amount of zeolite included in the diet of laying hens and its effects on the productive and qualitative aspects of eggs are still very inconsistent, as they can vary according to several factors such as type and origin of the zeolite, bird line, technological level used in the system, among others. Thus, in the present study conditions, in which Isa Brown layers weighing approximately 1,750 kg were subjected to isonutritive diets with inclusion levels of clinoptilolite (Table 1), which refers to the zeolite tested in this experiment, for 112 days, there were no effects on production parameters or egg quality. These results, however, were satisfactory, considering that the primary purpose was including zeolite to mitigate the ammonia release present in the excreta and thus probably improve the welfare and health of the hens.

The decrease in the pH of the excreta observed in this study was also described in other works, such as those of Romero *et al.* (2012) and Schneider *et al.* (2017). The evaluation of excreta pH is an important factor in poultry production, as it is decisive in the process of ammonia emission in the sheds, since the acidification (pH<7.0) of the excreta can minimize the release and the impacts caused by ammonia (Schneider *et al.*, 2017). According to Romero *et al.* (2012), diets thus formulated allow a reduction of excreta pH due to the protonation of volatile ammonia (NH_3) to the less volatile ammonium ion (NH_4^+). Due to its structural arrangement, zeolite can adsorb ammonia, reducing the potential for volatilization of ammonia compounds (Pavelić *et al.*, 2018).

In a research in which reduced-ammonia-emission diets were compared, an impressive decrease of 48% of ammonia release to the environment has been reported (Romero *et al.*, 2012). In the present experiment, the mean ammonia release reduction value calculated for the five diets with zeolite inclusion was 46.9%, and in the estimation of the best-fitting regression equation, the corresponding estimated value would be achieved at the zeolite inclusion level of 0.93%. This characteristic effect on ammonia release is associated with an estimated maximum reduction of up to 4.6% in excreta pH at the optimum dietary zeolite level of 1.18%. With the two levels of zeolite (0.7 and 1.4%) at which the excreta pH differed significantly from that of the control group, on average, a reduction can be observed. The magnitude of the reduction of excreta pH does not depend only on the additives used, but also on the composition of the diets. For this reason, it is not possible to make a comparison between different studies. Notwithstanding this, Romero *et al.* (2012) reported a 9.94% decline in pH with the use of a low-ammonia-emission, maize- and soybean meal-based diet containing gypsum and zeolite.

The formulated diets differed only in the relative change between inert and zeolite for the different levels, with the other ingredients being kept constant (Table 1). This reinforces the exclusive effect (when present) of the evaluated additive.

Blood profile parameters are indicators of the physiological, pathological, and nutritional status of animals. In this respect, changes in hematological variables are indicators to be used to elucidate the impact of nutritional factors, which are present in the diet fed to the birds (Kim *et al.*, 2017). B and T lymphocytes are considered the defense mechanisms, and, together with antibodies and lymphokines, they form the immune response. T lymphocytes stimulate the immune system in response to infectious or stress-inducing agents (Zekarias *et al.*, 2002). Jarosz *et al.* (2017) used zeolite as an additive in the diet of broilers and found that the metabolic energy acquired from the diet is used in the proliferation of lymphocytes, which are part of the immune response to antigens and act in the immune defense of birds. However, the modulating or suppressive effect of zeolite on the defense mechanism of the body remains unknown.

The hematology and biochemistry values observed in this study were within the standards for the studied variables. Hemoglobin (8.750 g/dL) values were close to those found by Macháček *et al.* (2010), who added 2% zeolite to the diet and detected hemoglobin values around 7.940 g/dL. In other words, the zeolite levels used in this study did not change the blood parameters of the laying hens.

However, in the present experiment, there was an increase in the relative weight of the liver at the zeolite levels of 2.1 and 2.8%, which likely reflects the dynamics of the metabolism of nitrogen compounds and other nutrients. As an adsorbent, zeolites eliminate a series of toxic substances (salts, heavy metals, nitrates, mycotoxins, radionuclides, and metabolism products such as ammonia), and also adhere to pathogenic bacteria (Andronikashvili *et al.*, 2009).

Furthermore, the ability of zeolite to fix ammonia in the intestine and excrete it influences the nitrogen load in the plasma, benefiting metabolic processes that occur in the liver (Berto *et al.*, 2013). The different levels of zeolite inclusion did not influence organ weight, except for the liver. The present findings concerning these variables were like those described by Safameher (2008), who supplied 2% zeolite to broilers and did not obtain significant results.

The increase in intestinal length, whose maximum estimated value was at the zeolite inclusion level of 2.25%, may be related to the digestibility of the feed in the gastrointestinal tract. This increase is in line with Berto *et al.* (2013), who stated that the fixation of excess ammonia produced by zeolite in the processes of intestinal digestion and fermentation can benefit intestinal integrity and consequently, positively affect the absorption and utilization of diet nutrients. The authors also contextualized that in addition to the benefits for intestinal integrity, ammonia adsorption can also benefit certain metabolic processes. However, higher levels of zeolite induced a reduction in intestinal length. This finding agrees with Macháček *et al.* (2010), who declared that other effects should be considered in the long-term administration of zeolite since high rates of this mineral can affect biologically active substances such as vitamins, trace elements, and other substances with a specific action, which can influence nutrient metabolism.

Wu *et al.* (2013) evaluated the inclusion of 2% of a natural zeolite in broiler diets and observed beneficial changes in intestinal morphology, as evidenced by the increased intestinal villi, despite finding no significant difference in production data, as in the present study. Also, according to these authors, the addition of zeolite to the diet can also have selective actions on the intestinal microbiota in the digestive tract by changing the pH of the intestine and cecum of broiler chickens.

Oral application of zeolite in humans suggests that it has a positive impact on the intestinal tract, due to its influence on the integrity of the intestinal wall (Mastinu *et al.*, 2019).

In general, the literature reports the beneficial effects of the action of zeolite at dietary levels of 2 to 4%. However, it is important to note that in the present study, the zeolite level of 0.93% already exerted considerable effects on the emission and circulation of ammonia present in hen excreta to the environment. According to Swelum *et al.* (2021), this contributes to improving animal welfare, considering that high levels (>25 ppm) of this gas inside sheds can be toxic to birds and cause health damage and economic losses.

In the present experimental conditions, zeolite is recommended for inclusion in the diet of brown-egg layers at the level of 0.93%, which would result in less ammonia emission to the environment, lower excreta pH, and improved intestinal morphology and liver metabolism, thus not compromising egg production or quality.

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