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Effects of feeds, supplemented with humic substances and calcium carbonate, on performance, egg quality and heart rate variability in laying hens

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Abstract

This study aimed to determine the effects of in-feed inclusion of humic substances (HS) and/or calcium carbonate ($CaCO_3$) on the performance and welfare of laying hens. A total number of 144 Hy-Line Brown laying hens (55 weeks old) were randomly assigned to four treatments, T1–T4 (36 birds per treatment). T1 hens were fed on a control diet without HS or $CaCO_3$, T2 hens were fed on the control diet + 2.00 g per bird per day $CaCO_3$, T3 hens were fed on the control diet + 0.20% HS and T4 hens were fed on the control diet + 0.20% HS + 2.00 g/bird/day $CaCO_3$. The experiment started after 15 days of adaptation and lasted 8 weeks. The parameters evaluated were percentage of hen-day egg production, food consumption, mortality, egg quality parameters and heart rate variability (welfare indicator). Hens in the T3 group showed a significantly lower feed intake than those in the other three groups, however, significantly higher daily egg production was recorded in groups T3 and T4 compared to T1 and T2. Eggshell quality characteristics were significantly improved by HS supplementation and eggs laid by hens in groups T1, and T4 presented paler yolk and shell color than those in the other groups. In conclusion, these results indicated that in-feed inclusion of HS had a beneficial effect on laying hens' productive performance including egg production and eggshells quality.

Introduction

During the past several years, probiotics, prebiotics, organic acids, phytogenics, zeolites, humates, and enzymes have all been used as feed additives for farm animals as alternatives to antibiotics due to their beneficial effect on health and growth performance combined with their lack of harmful effects on the consumers health.[1-5] Humic substances (HS) are the final and stable biotransformation products resulting from decomposition of organic matter.[6] In recent years, they have been assessed as a feed supplement in animal husbandry for their potential to improve the economic viability and ecology of animal production[5,7,8] mainly by enhancing productivity parameters, ameliorating some physiological processes, responses of the immune system[9-11] and responses to stress, which are reflected in the quality of animal products and welfare.[3] Humic substances have been documented as having beneficial effects (antibacterial, antiviral and anti-inflammatory) in animals and they have been shown to improve the immune system, aid in stress management and reducing odor in faeces.[7] More specifically humate supplementation in feed for laying hens improves performance by decreasing feed intake, improving feed utilization and feed conversion, reducing mortality as well as increasing resistance to pathogens.[2-4,12]

Egg production and egg quality are the most important economic parameters in layer farms. One of the most important factors affecting the profitability of egg production is the age-related decline in eggshell quality[13] due to reduction in mineral utilization and increase in eggshell surface as the hens age. If eggs with poor shell quality pass through the system undetected, they can constitute a risk to food safety. Therefore, control of eggshell quality is crucial.[14]

In laying hens, factors influencing calcium metabolism play a key role in the maintenance of good productive performance, especially after the peak laying period.[15] Genotype, housing system and hen age may all affect egg-shell quality.[16] Also, nutritional homeostatic maladjustment and different management activities may generate negative effects on the central nervous, endocrine and immunological

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systems of birds and negatively affect their welfare.[17,18] Moreover, immunosuppression may cause a decrease in productivity traits and changes in bird behaviour.[19]

According to recent international market trends, HS have been showing promising results as an alternative to antibiotics as a feed supplement in poultry diets, improving health and productivity. Hence, further research into their potential was felt to be justified. Previous studies in broilers have shown that dietary HS gave results comparable with that of garlic powder and herbal additives with respect to productivity parameters and internal organ weight[20,21] as well as increased phagocytosis response, respiratory burst, bacterial agglutination[9] and even mitigated the stress response.[11] Measurement of heart rate variability (HRV) is a non-invasive technique that can be used to investigate the function of the autonomic nervous system, especially the balance between sympathetic and vagal activity. Over the past years, HRV has been increasingly used in animal research to analyze changes in sympathovagal balance related to diseases, psychological and environmental stressors and individual characteristics such as temperament and coping strategies.[22] In this trial, we studied the effect of 0.20% HS as a dietary supplement, alone and in combination with a dose of calcium carbonate ($CaCO_3$) in 55-week-old Hy-Line Brown laying hens on productivity parameters and egg quality traits. Additionally, the HRV values of the laying hens were assessed as a direct welfare indicator to evaluate the effect of dietary treatments on sympathovagal balance. Heart rate variability analysis has been used in very few avian studies and, to the best of our knowledge, there are no reports concerning the effect of HS on heart rate and HRV in laying hens.

Materials and Methods

A total number of 144 Hy-Line Brown laying hens (55 weeks old) from the experimental farm of the Universidad Cooperativa de Colombia Ibagué headquarters were used. All birds were clinically healthy and their average body weight was 1,844 ± 167 g. The present study was subjected to bioethics endorsement of the scientific research and development office of the Universidad del Tolima (2.3-025/07; February 2018). Hens were located in a pyramidal module multilayer production system in individual cages. Throughout the trial, hens had 12 hr continuous natural daylight illumination per day. Temperature ranged from 19.00 - 31.00 ˚C, and average relative humidity was 74.00%. After fifteen days of adaptation, hens were randomly distributed into four dietary treatments, namely T1, T2, T3 and T4, with 12 replicates per treatment and three birds per replicate lasting for 8 weeks. The T1 hens were fed on a balanced concentrate ration without supplementation (control diet), T2 hens were fed on the control diet + 2.00 g per bird once daily $CaCO_3$ (Bm Agro, Huila, Colombia), T3 hens were fed on the control diet + 0.20% of HS Nutrihumic fino® (PBA Biotechnological Products SA, La Virginia, Colombia)[23] and T4 hens were fed on the control diet + 0.20% of HS Nutrihumic® + 2.00 g per bird once daily of $CaCO_3$. The coarse size of $CaCO_3$ granules (6.00 mm) used in this trial was chosen based on the results of a previous study.[24]

The experimental rations were prepared at a commercial feed factory and were formulated to be isonitrogenous and isocaloric. The ingredients and chemical composition of the research rations are shown in Table 1. The HS (kindly donated by PBA Biotechnological Products SA) were stabilized to 87.00% by enzymatic biotransformation of the leaves, cachaza and vinasse waste from sugar and alcohol production, having a pH of 7.50, apparent dry density of 0.70 g cm^{-3} and maximum humidity 2.00% and 54.00% ash.[23] Each kilogram of HS Nutrihumic® contained 80.00% polymeric polyhydroxy acid from a similar proportion of cachaza, leaves and vinasse from the sugar and alcohol. Feed and water were offered ad libitum.

Table 1. Ingredients and chemical composition of experimental diets T1 to T4.

Ingredients (%)	Dietary treatments			
	T1	**T2**	**T3**	**T4**
Yellow corn	53.86	53.86	53.86	53.86
Soy cake (48.00%)	18.80	18.80	18.80	18.80
Palm oil	4.00	4.00	4.00	4.00
Corn gluten	3.00	3.00	3.00	3.00
Dicalcium phosphate	1.80	1.80	1.80	1.80
Wheat bran	8.60	8.60	8.60	8.60
Limestone fine	1.26	1.26	1.26	1.26
Calcium carbonate	2.94	4.94	2.94	4.94
Premix vitamin + mineral + pigment	0.20	0.20	0.20	0.20
DL-methionine	0.18	0.18	0.18	0.18
L-lysine HCl	0.25	0.25	0.25	0.25
Common salt	0.20	0.20	0.20	0.20
Sodium bicarbonate	0.10	0.10	0.10	0.10
Choline chlorure	0.03	0.03	0.03	0.03
L-threonine	0.02	0.02	0.02	0.02
Enramycin 8.00%	0.13	0.00	0.00	0.00
Humic substances (HS)	0.00	0.00	0.20	0.20
Celite	4.63	2.76	4.56	2.56
Calculated chemical composition (%)				
Crude protein	16.00	16.20	16.00	16.30
Ether extract	6.20	5.80	6.30	5.90
Crude Fiber	2.60	2.60	2.60	2.60
Ash	13.30	12.90	13.00	13.30
Metabolizable energy (kcal kg^{-1})	2,809	2,805	2,806	2,805
Lysine	0.80	0.80	0.80	0.80
Methionine	0.40	0.40	0.40	0.40
Methionine + cystine	0.70	0.70	0.70	0.70
Available phosphorus	0.40	0.40	0.40	0.40
Calcium	4.20	6.20	4.20	6.20

T1: Control diet, T2: Control diet supplemented with 2.00 g of calcium carbonate (CaCO3), T3: Control diet supplemented with 0.20% HS, T4: Control diet supplemented with 2.00 g CaCO3 + 0.20% HS.

The parameters evaluated were productivity traits (mortality, percentage of hen-day egg production, food consumption and egg weight), egg quality characteristics (color, thickness and weight of eggshell, albumen height, Haugh Units [HU], weight and color of the yolk) and HRV (low frequency/high frequency ratio) as an indicator of sympathovagal balance.

Mortality and percentage of hen-day egg production was recorded daily, while feed intake was measured daily throughout the trial. Feed intake was calculated every morning by subtracting the weight of the residual feed from that supplied the previous day. The production percentage was calculated by dividing the number of eggs produced on a day by the number of hens present on that day multiplied by 100.

Egg quality traits were estimated biweekly. For this purpose, 16 eggs from each dietary treatment were randomly collected. The egg, yolk and eggshell weights were measured with a balance (Vibra AJ-4200; Shinko Denshi Co., Ltd. Tokyo, Japan). Shell color was estimated with the Hy-Line color ruler. Presence of inclusions was visually recorded. Yolk color was determined by reference to a Roche yolk color fan. Albumen height was measured using a digital micrometre (Mitutoyo, Sakado, Japan) with an accuracy of 0.001 mm). Eggshells were allowed to dry for 48 hr at room temperature before the weight of the shell was measured. Eggshell thickness at the middle and wide pole were measured with the digital micrometre to an accuracy of 0.001 mm. The percentages of yolk, albumin and eggshell were obtained based on total egg weight, and HU (%) that was calculated as Log (height albumen – 1.70 × egg weight$^{0.37}$ + 7.60) × 100.[24]

The HRV values were calculated in terms of time domain and frequency domain (low frequency/high frequency ratio) using electrocardiography at the beginning and end of the experiment. On a flat and stable table, each bird was placed in sternal recumbency, disposable electrodes were placed one on each wing (+/−) and one on each thigh (positive and pole to ground) and the data emitted in channel three were analyzed using Lab Chart Pro® Software (Power Lab 26T® (DII); ADInstruments, Bella Vista, Australia), with the HRV MLS310/8® module). Every procedure was previously standardized with trained personnel.

Statistical analysis. Using SPSS software (version 25.00; IBM Corp., Armonk, USA), the quantitative parameters of productivity, egg quality and low frequency/high frequency ratio were analyzed by analysis of variance after confirmation of statistical assumptions through a T3 Dunnett's *post hoc* test in all cases with a confidence level $\alpha = 0.05$. The normality of the data was tested with the Kolmogorov–Smirnov test and the homogeneity of variances was tested with Levene's test. The categorical variables of egg quality were evaluated by frequency distribution and Chi-squared independence test.

Results

Hens in the T3 group presented significantly lower feed intake compared to hens fed the other three diets ($p < 0.05$), whereas significantly higher percentage of hen-day egg production was recorded in T3 and T4 hens compared to T1 and T2 hens ($p < 0.05$), (Table 2). However, the dietary treatments had no effect on egg weight ($p \geq 0.05$). In addition, supplementation with HS significantly improved eggshell quality ($p < 0.05$) without having any effect on yolk and albumen percentage, yolk weight, HU and albumen height (Table 2). Shell percentage of eggs laid by hens that were fed on diets supplemented with HS was significantly higher than that of eggs laid by hens that were fed on the T1 and T2 diets ($p < 0.05$). Shell weights of eggs laid by hens in the T3 and T4 groups were higher compared to those of eggs laid by hens in the T1 and T2 groups. The observed differences were significant ($p < 0.05$) between T2 and T3 groups and between T2 and T4 groups.

Table 2. Productivity parameters and egg quality parameters of laying hens fed on experimental diets T1 to T4.

Productivity parameter	T1	T2	T3	T4	*p*-value
Hen–day egg production (%)	81.10 ± 0.24^{b}	81.60 ± 0.23^{b}	84.70 ± 0.22^{a}	84.90 ± 0.20^{a}	0.00
Feed intake (g)	108.10 ± 0.09^{b}	108.2 ± 0.09^{b}	107.50 ± 0.12^{a}	109.6 ± 0.09^{c}	0.00
Egg weight (g)	59.80 ± 3.10	58.90 ± 4.80	58.30 ± 4.80	59.10 ± 6.30	0.53
Egg quality parameters					
Albumen (mm)	8.40 ± 3.10	8.60 ± 4.70	8.30 ± 0.90	8.50 ± 1.20	0.25
Haugh Units	91.50 ± 6.90	92.60 ± 7.20	91.40 ± 5.30	92.20 ± 6.90	0.41
Yolk weight (g)	16.00 ± 1.30	15.60 ± 4.80	15.00 ± 1.80	15.40 ± 1.10	0.20
Yolk percentage (%)	26.90 ± 2.00	26.50 ± 8.70	25.80 ± 2.00	26.50 ± 4.60	0.34
Albumen percentage (%)	64.00 ± 2.50	64.40 ± 8.70	64.40 ± 2.20	63.80 ± 6.50	0.64
Shell percentage (%)	9.00 ± 0.90^{b}	8.90 ± 1.00^{b}	9.70 ± 0.70^{a}	9.50 ± 2.10^{a}	0.01
Shell weight (g)	5.30 ± 0.50^{bc}	5.20 ± 0.50^{c}	5.60 ± 0.60^{a}	5.50 ± 0.70^{ab}	0.04
Shell thickness middle (mm)	0.40 ± 0.50	0.39 ± 0.47	0.41 ± 0.03	0.40 ± 0.04	0.05
Shell thickness wide pole (mm)	0.32 ± 0.04^{b}	0.31 ± 0.04^{b}	0.33 ± 0.37^{a}	0.32 ± 0.34^{ab}	0.05

Stock values of 144 hens. T1: Control group, T2: Group supplemented with 2.00 g calcium carbonate ($CaCO_3$), T3: Group supplemented with 0.20% humic substances (HS), T4: Group supplemented with 2.00 g $CaCO_3$ + 0.20% HS. Values are expressed as mean ± standard deviation.
^{abc} Different superscripts letters in each row indicate significant ($p < 0.05$) differences between groups.

Moreover, the shell thickness at the broad pole of the eggshell was significantly greater ($p < 0.05$) in eggs of T3 hens than in eggs of T1 and T2 hens.

With respect to color distribution, yolk color in T1 and T4 eggs showed the highest frequency in color 11.00 of the Roche range (Fig. 1) and similarly, eggshell color in T1 and T4 eggs showed the highest frequency in color 90.00 of the Hy-Line color strip. With respect to inclusions (meat and blood spots), no significant differences were observed among the groups (data not presented).

Neither heart rate nor HRV showed significant changes in either the time or the frequency domains reported from the electrocardiograms of the birds in the trial (Table 3), thereby indicating no changes in the sympathovagal balance based on the diets provided ($p \geq 0.05$).

Discussion

The mortality rate of the hens in the present trial was in the expected range based on the manual of genetic line Hy-Line Brown[25] and was not influenced by administration of HS to their diet. Similar results have been obtained by other researchers in relevant studies carried out in laying hens.[2,15,26] In the current study, only one hen from the T4 group died and necropsy findings revealed ovoperitonitis which was not related to this investigation.

The dietary concentration of HS (0.20%) used in this research was based on results from previous studies performed on laying hens during their first production cycle and post-moulting.[11,27] As in our study, increased egg production in laying hens provided with feed or water supplemented by HS, before and after peak of laying period, has been previously demonstrated.[2,4,12,15,27,28] Other reports, however, have indicated either no dietary effect of HS on production performance (in laying hens)[3,25,29-31] or have reported a decrease in egg production in layers[32] and pheasants.[33] The exact mechanism by which HS increases egg production has not been fully understood. It has been shown that HS stabilizes the intestinal flora by forming a protective film on the mucous membrane of the gastrointestinal tract against infections and toxins, thus ensuring an improved utilization of the nutrients in the animal feed.[7] Alteration in nutrient partitioning could be associated with increased egg production in hens receiving humate.[34] Hanafy and El-Sheikh attributed the increased egg production of hens, fed on 100 or 200 mg HS kg^{-1} in their diet compared to control group, to the significantly increased relative weight of ovary and oviduct length.[27]

The results of previous investigations concerning the effect of HS dietary supplementation on feed intake and egg weight of laying hens are contradictory. As observed here, some researchers found that the addition of HS to

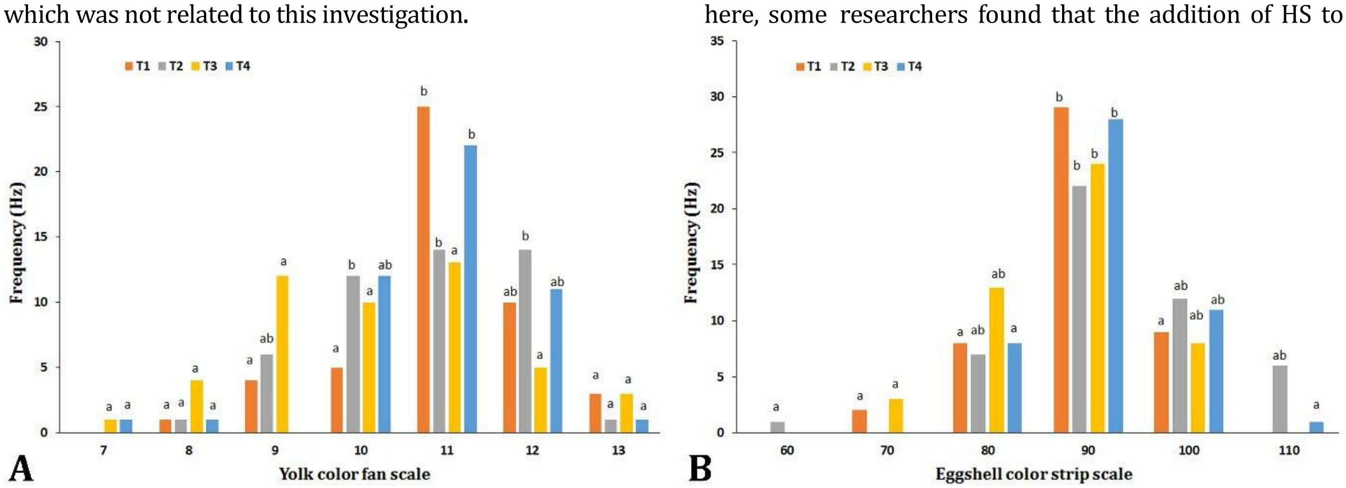

Fig. 1. Distribution of **A)** yolk color and **B)** eggshell color frequencies (Roche fan and strip color) in the eggs of hens supplemented with humic substances and calcium carbonate. Different letters of the same color denote significant difference.

Table 3. Heart rate variability in the time and frequency domain in hens supplemented with humic substances and calcium carbonate.

Heart rate variability		T1	T2	T3	T4	*p*- value
	X S-S (ms)	198.30 ± 3.10	207.90 ± 5.80	198.70 ± 2.80	207.50 ± 5.00	0.17
	SDSS (ms)	34.60 ± 4.80	33.30 ± 4.80	31.80± 4.00	37.00 ± 4.40	0.47
Time domain	HR (bpm)	311.50 ± 3.80	298.5 ± 8.30	309.70 ± 3.50	300.20 ± 7.30	0.18
	SD SD (ms)	50.50 ± 6.60	46.80 ± 6.80	48.70 ± 5.20	53.10 ± 6.70	0.53
	RMSSD (ms)	50.40 ± 6.60	46.70 ± 6.80	48.60 ± 5.20	52.80 ± 6.70	0.54
Frequency domain	LF/HF ratio	0.10 ± 0.00	0.30 ± 0.10	0.00 ± 0.00	0.10 ± 0.00	0.05

T1: Control group, T2: Group supplemented with 2.00 g CaCO3, T3: group supplemented with 0.20% HS, T4: Group supplemented with 2.00 g CaCO3 + 0.20% HS. Values are expressed as mean ± standard deviation.

X S-S: Average S-S interval; SDSS: Standard deviation of S-S intervals; HR: Heart rate; SD SD: Standard deviation of the difference between consecutive S-S intervals; RMSSD: Square root of the mean of the squared differences of the successive S-S intervals; LF/HF ratio: Low frequency/high frequency ratio. No significant differences were detected among groups $p > 0.05$.

the diet decreased feed intake[3,12] and had no effect on egg weight,[2-4,12,26,29,31] while others observed improved egg weight[27] and no effect on feed intake.[2,25,27,29,30] More recently, the addition of HS supplied in drinking water was associated with positive results with respect to egg weight but no effect on feed intake when offered to 50 week old laying hens.[15] Additionally, Sopoliga *et al.* suggested that supplementing the diet of pheasants with 0.50% HS significantly decreased egg weight compared to the control group.[33]

The results of the present study revealed that the addition of HS to the diet of laying hens significantly improved eggshell quality without having any adverse effect on yolk and albumen percentage, yolk weight, HU and albumen height. Previous studies showed inconsistent results for egg quality traits with dietary supplementation of HS. Similar to our results, increased eggshell thickness, weight and percentage of eggshell have been documented by some researchers.[12,27] Other investigators, however, found no difference in shell quality of eggs laid by hens that were fed on diets supplemented with HS.[2-4,26,29] The increased eggshell quality observed in T3 and T4 groups could be related to a beneficial effect of HS on absorption of nutrients such as calcium and phosphorous and/or changes in metabolic profile of those nutrients. It has been shown that HS improve protein digestion as well as calcium and trace element utilization.[7] Hayirli *et al*. suggested that changes in metabolic profile due to humate supplementation might be related to alteration in partitioning of nutrient metabolism.[34] Furthermore, due to the ion capture characteristics of HS, some trace elements may act as co-factors and, consequently, increase the activity of several enzymes for digestion and utilization of some nutrients. According to Hanafy and El-Sheikh the increase in plasma calcium concentration found in hens, fed on 100 or 200 mg HS kg^{-1} diet, could be responsible for the increase in eggshell percentage and eggshell thickness observed in the eggs laid by those hens compared to controls.[27] Similarly, Avci *et al*. found that serum calcium concentrations were increased in Japanese quails fed on diets supplemented with 600 mg kg^{-1} HS.[1] Studies in broilers have shown that inclusion of HS in their diet increased the height and width of the intestinal villi and depth of intestinal crypts, positively influenced digestion and assimilation dynamics that were consequential to the improvement in bone and immune development of the birds.[10] Similar to the results obtained here, Ergin *et al*. reported that yolk weight was not affected by the inclusion of 30.00 and 90.00 ppm of humic acid in liquid form in the diet of Isa Brown layers from 51 - 61 weeks of age.[12] Trials carried out in laying hens in an early phase of the production cycle (17 weeks old) presented similar findings for yolk weight.[31] As in the current study, in a significant number of reports it has been documented that the addition of HS to the diet or water of laying hens had no effect on HU,[2,3,15,25,26] albumen height,[15,26,27] percentage of albumen[26,27] and percentage of yolk.[26] Other researchers, however, found that HS exerted a positive effect on albumen index and HU[4,31] or caused a decrease in egg yolk percentage.[27] The variability in results of experiments involving dietary HS on egg quality observed in different studies may be attributable principally to the differences in the composition of HS preparations, the inclusion rate and differences in the poultry species and ages.[15,35]

It has been demonstrated that larger particles of calcium provide greater retention in the upper part of the digestive tract, making calcium available, slowly and evenly, during the period of eggshell formation.[24,36] From this perspective, the findings of the current study regarding eggshell thickness imply that if diet is supplemented with HS then it is not necessary to increase the percentage of $CaCO_3$ to achieve increased eggshell thickness during the phase after the peak of production.

In the current study, eggs laid by hens in groups T1 and T4 presented paler yolk and shell color than those in the other groups. It is known that the color of eggshell is determined primarily by the genetics of the hen.[37] However, stress, age of bird, chemotherapeutic agents and disease are also considered to be responsible for decreasing the intensity of brown shell color in eggs.[38] Research evidence suggests that the color of eggshells becomes paler with age of the flock.[39] Odabaşi *et al*. observed that older hens lay lighter colored eggs due to an increase in egg size associated with no proportionate change in the quantity of pigment deposited over the shell surface.[40] On this basis, the paler shell color of eggs laid by hens in groups T1 and T4 could be attributed to the numerically higher egg weight documented in those groups compared to that observed in groups T2 and T3.

Yolk color is affected by the diet of the laying hens.[16] Previous investigators have shown that the addition of HS in feed or water improved yolk colour[4,15] while others found no significant effect.[2,32] In the current research the eggs laid by hens in groups T2 and T3 had significantly darker yolk color than in eggs laid by hens in groups T1 and T4 ($p < 0.05$). Our findings implied that dietary treatments with either $CaCO_3$ or HS alone improved the bioavailability of the nutrient components such as carotenoids that were responsible for yolk coloration by increasing either their absorption or their supply to the oviduct.

Measurement of HRV is a particularly good technique for the non-invasive assessment of autonomic nervous system activity in response to psychophysiological stress.[22] Dietary supplementation with HS alone or in combination with $CaCO_3$ had no significant effect on heart rate and HRV in the hens. This result indicated that the sympathovagal balance was not affected by the dietary treatments in this investigation with data values remaining at baseline.[41] Previous studies reported changes in heart rate in birds due to ageing, health, management and other aspects.[22,41,42] Other results showed that the supplementation of poultry diets with HS improved the response to stress as measured by indirect welfare indicators, increased mean corpuscular volume, decreased heterophile/lymphocyte ratio and tonic immobility.[11,43] However, it is important to bear in mind that, in this trial, birds were not undergone stressful challenge as in the publications mentioned above.[42] Results from our study show that the addition of HS to the diet of laying hens had a beneficial effect on production performance by decreasing feed intake and increasing hen-day egg production. Additionally, dietary HS supplementation with or without $CaCO_3$ improved eggshell quality and had no adverse effect on the other egg quality traits evaluated in this study or on HRV.

In conclusion, the results of the current study provided evidence that feeding laying hens with HS beneficially affected their performance by decreasing feed intake and increasing egg production. Additionally, it ameliorated eggshell quality without having any adverse effect on the rest of the quality traits evaluated or on birds' welfare.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Avci M, Denek N, Kaplan O. Effects of humic acid at different levels on growth performance, carcass yields and some biochemical parameters of quails. J Anim Vet Adv 2007; 6(1): 1-4.
2. Yörük MA, Gül M, Hayirli A, et al. The effects of supplementation of humate and probiotic on egg production and quality parameters during the late laying period in hens. Poult Sci 2004; 83(1): 84-88.
3. Kucukersan S, Kucukersan K, Colpan I, et al. The effects of humic acid on egg production and egg traits of laying hen. Vet Med-Czech 2005; 50(9): 406-410.
4. Macit M, Çelebi Ş, Esenbuga N, et al. Effects of dietary humate supplementation on performance, egg quality and egg yolk fatty acid composition in layers. J Sci Food Agric 2009; 89(2): 315-319.
5. Papatsiros V, Katsoulos PD, Koutoulis K, et al. Alternatives to antibiotics for farm animals. CAB Rev: Perspect Agric Vet Sci Nutr Nat Resour 2013; 8(32): doi: 10.1079/PAVSNNR20138032.
6. Vlcová Z. Chemical and physical transformations of humic acids. PhD Thesis. Institute of Physical and Applied Chemistry. BRNO University of Technology. Brno, Czechia 2009.
7. Islam KM, Schuhmacher A, Gropp JM. Humic acid substances in animal agriculture. Pak J Nutr 2005; 4(3): 126-134.
8. Sanmiguel-Plazas RA, Aguirre Pedreros WJ, Rondón Barragán IS. Perspectives on the use of humic substances in poultry production. [Spanish]. Ces Med Vet Zootec 2014; 9(1): 104-113.
9. Sanmiguel RA, Rondon IS. Supplementation with humic substances affects the innate immunity in layer hens in posfasting phase. Rev MVZ Córdoba 2016; 21(1): 5198-5210.
10. Disetlhe AR, Marume U, Mlambo V, et al. Humic acid and enzymes in canola-based broiler diets: Effects on bone development, intestinal histomorphology and immune development. S Afr J Anim Sci 2017; 47(6): 914-922.
11. Arif M, Alagawany M, Abd El-Hack ME, et al. Humic acid as a feed additive in poultry diets: a review. Iran J Vet Res 2019; 20(3): 167-172.
12. Ergin O, Isa C, Nuh O, et al. Effects of dietary humic substances on egg production and egg shell quality of hens after peak laying period. Afr J Biotechnol 2009; 8(6): 1155-1159.
13. Nys Y. Nutritional factors affecting eggshell quality. Czech J Anim Sci 1999; 44(3): 135-143.
14. Bain MM. Recent advances in the assessment of eggshell quality and their future applications. Worlds Poult Sci J 2005; 61(2): 268-276.
15. Arafat RY, Hassan Khan S, Abbas G, et al. Effect of dietary humic acid via drinking water on the performance and egg quality of commercial layers. Am J Biol Life Sci 2015; 3(2): 26-30.
16. Ahmadi F, Rahimi F. Factors affecting quality and quantity of egg production in laying hens: A Review. World Appl Sci J 2011; 12(3): 372-384.
17. Feng Y, Yang XJ, Wang YB, et al. Effects of immune stress on performance parameters, intestinal enzyme activity and mRNA expression of intestinal transporters in broiler chickens. Asian-Australas J Anim Sci 2012; 25(5): 701-707.
18. Sordillo LM, Aitken SL. Impact of oxidative stress on the health and immune function of dairy cattle. Vet Immunol Immunopathol 2009; 128(1-3): 104-109.
19. Broom DM. Welfare in relations to feelings, stress and health. Rev Electrón Vet 2007; 8(12B): 1695-1704.
20. Pistová V, Arpášová, H, Hrnčar C, et al. The effect of the humic acid and herbal additive supplement on production parameters of broiler chicken. Sci P Anim Sci Biotechnol 2016; 49(2): 166-169.

21. Pistová V, Arpášová H, Hrnčar C. The effect of the humic acid and garlic (*Allium sativum* L.) on performance parameters and carcass characteristic of broiler chicken. J Cent Eur Agric 2016; 17(4): 1168-1178.
22. von Borell E, Langbein J, Després G, et al. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals - a review. Physiol Behav 2007;92(3): 293-316.
23. Nutri-Humic. Technical data sheet. Code 963. 2018. Available at: https://www.pba.com.co/Productos/ Accessed Oct 08, 2022.
24. Sanmiguel-Plazas RA, Mejía-Rojas GR, Lozano-Covaleda LM, et al. Evaluation of different calcium grain sizes in laying hens diet. [Spanish]). Ciencia Agricultura 2016; 13(2): 67-72.
25. Hy-Line. Brown Commercial layers-Australia. Management guide (2018); available at: https://www.hyline.com/ filesimages/ BrownAccessed Oct 08, 2022.
26. Yalcin S, Ergun A, Ozsoy B, et al. The effects of dietary supplementation of L-carnitine and humic substances on performance, egg traits and blood parameters in laying hens. Asian- australas J Anim Sci 2006; 19(10): 1478-1483.
27. Hanafy M, El-Sheikh AMH. The effect of dietry humic acid supplementation on some productive and physiological traits of laying hens. Egypt Poult Sci 2008; 28: 1043-1058.
28. Abo-EglaES, EL-Samra H, Ismail FS, et al. Effect of humic acid and BIO-MOS supplementation on egg production and quality parameters in local hens. J Anim Poult Prod 2011; 2(4): 55-63.
29. Hakan KB, Gultekin Y, Ozge S. Effects of boric acid and humate supplementation on performance and egg quality parameters of laying hens. Braz J Poult Sci 2012; 14(4): 283-289.
30. Sanmiguel Plazas RA, Rondón I. Supplementation with humic substances in laying hens during molt. [Spanish]. CES Med Vet Zootec 2014; 9(2): 169-178.
31. Arpášová H, Kacaniová M, Pistová V, et al. Effect of probiotics and humic acid on egg production and quality parameters of laying hens eggs [Abstract]. Sci Pap Anim Sci Biotechnol 2016; 49(2): 1-9.
32. Wang Q, Yoo JS, Chen YJ, et al. Effects of supplemental humic substances on egg production and quality in laying hens. Korean J Poult Sci 2006; 33(4): 317-321.
33. Sopoliga IA, Hreško-Šamudovská A, Demeterová M, et al. Effect of humic substances on the production parameters of pheasant hens. Acta Fytotechn Zootech 2016; 19(1): 11-14.
34. Hayirli A, Esenbuğa N, Macit M, et al. Nutrition practice to alleviate the adverse effects of stress on laying performance, metabolic profile, and egg quality in peak producing hen: II. The probiotic supplementation. Asian-Aust J Anim Sci 2005; 18(12): 1752-1760.
35. Monira KN, Salahuddin M, Miah G. Effect of breed and holding period on egg quality characteristics of chicken. Int J Poult Sci 2003; 2(4): 261-263.
36. Świątkiewicz S, Arczewska-Włosek A, Krawczyk J, et al. Effects on performance and eggshell quality of particle size of calcium sources in laying hens' diets with different Ca concentrations. Arch Anim Breed 2015; 58(2): 301-307.
37. Goger H, Demirtas SE, Yurtogullari S. A selection study for improving eggshell colour in two parent lines of laying hens and their hybrids. Ital J Animal Sci 2016; 15(3): 390-395.
38. Butcher GD, Miles R. Factors causing poor pigmentation of brown shelled eggs. University of Florida 2003. Available at: http://edis.ifas.ufl.edu/pdffiles/VM/VM04700.pdf. Accessed Oct 08, 2022.
39. Lang MR, Wells JW. A review of eggshell pigmentation. Worlds Poult Sci J 1987; 43(3): 238-246.
40. Odabaşi AZ, Miles RD, Balaban MO, et al. Changes in brown eggshell color as the hen ages. Poult Sci 2007; 86(2): 356-363.
41. Kjaer JB, Jørgensen H. Heart rate variability in domestic chicken lines genetically selected on feather pecking behavior. Genes Brain Behav 2011; 10(7): 747–755.
42. Müller MS, Vyssotski AL, Yamamoto M, et al. Heart rate variability reveals that a decrease in parasympathetic (‘rest-and-digest’) activity dominates autonomic stress responses in a free-living seabird. Comp Biochem Physiol A Mol Integr Physiol 2017; 212: 117-126.
43. Rath NC, Huff WE, Huff GR. Effects of humic acid on broiler chickens. Poult Sci 2006; 85(3): 410-414.