Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2022 / v.24 / n.3 / 001-010

http://dx.doi.org/10.1590/1806-9061-2021-1450

Original Article

Growth Performance and Tibia Mineralization of Broiler Chickens Supplemented with a Liquid Extract of Humic Substances

Author(s)

Angeles ML[I] https://orcid.org/0000-0001-6399-3589
Gómez-Rosales S[I] https://orcid.org/0000-0002-0905-4959
López-Garcia YR[II] https://orcid.org/0000-0003-0154-1012
Montoya-Franco A[III] https://orcid.org/0000-0001-5912-2983

[I] National Center of Disciplinary Research in Animal Physiology and Genetics, National Institute of Research in Forestry, Agriculture and Livestock, Ajuchitlan, Queretaro, Mexico.
[II] Faculty of Higher Studies Cuautitlan, UNAM. Ajuchitlan, Queretaro, Mexico.
[III] Center for Research and Advanced Studies of the National Polytechnic Institute, CINVESTAV-IPN, Real de Juriquilla, Queretaro, Mexico.

Mail Address

Corresponding author e-mail address
María de Lourdes Angeles
National Center of Disciplinary Research in Animal Physiology and Genetics, National Institute of Research in Forestry, Agriculture and Livestock. Km 1 carretera a Ajuchitlan, Queretaro. CP 76280. Mexico.
Email: angeles.lourdes@inifap.gob.mx

Keywords

Broilers, humic substances, productive responses, tibia mineralization.

Submitted: 31/September/2021
Approved: 12/November/2021

ABSTRACT

The objective of the study was to evaluate the productive, carcass, and tibia mineralization responses in broiler chickens supplemented with a liquid extract of humic substances (HS) in the drinking water. Chicks were housed in holding cages from 8-42 days of age and were randomly assigned to one of five increasing HS levels in the drinking water (0, 161, 322, 483, and 644 µg/L). At 21 and 42 days, to obtain carcass and tibia measurements half of the broilers were slaughtered. ANOVA and linear regression were used to analyze the data. The HS chemical composition and flat structures were estimated. At 21 days, increasing levels of HS in the drinking water resulted in a cubic response on breast weight ($p<0.05$), tibia ashes percentage ($p<0.05$) and tibia Ca percentage, as well as a linear increasing response ($p<0.05$) on P percentage. HS elicited a quadratic response on the tibia DM percentage ($p<0.05$), Ca content ($p<0.01$), and P content ($p<0.05$) at 42 days. The optimal HS supplementation level to achieve the highest tibia DM percentage, Ca and P content were 345.00, 322.46, and 347.75 µg/L, respectively. Increasing HS levels also resulted in a cubic response in tibia Ca ($p<0.05$) and P percentage ($p<0.01$). In conclusions, HS supplementation in drinking water improved bone mineralization in broiler chickens at 21 and 42 days of age.

INTRODUCTION

Humic substances (HS) have been studied in poultry as an alternative to substitute the growth-promoting antibiotics in order to reduce the risk of pathogenic bacteria developing antimicrobial resistance which can spread between different bacterial populations via humans, livestock, and the entire environment (Woolhouse *et al.,* 2015). It has been demonstrated that HS improves the growth and carcass traits of broilers, as well as laying hen productivity and egg quality (Arif *et al.,* 2019). The observed benefits of HS in poultry appear to be multifaceted, including improvements in the digestive functions, increases of beneficial bacteria in the gut, improvements in the immune response and antioxidant capacity, and thus on broiler chicken health (Arif *et al.,* 2019; Domínguez-Negrete *et al.,* 2019). Because HS are the most common natural complexing ligands found in nature their primary application in environmental chemistry is in the removal of toxic metals, anthropogenic organic chemicals, and other pollutants from water (Peña-Mendez *et al.,* 2005). Because of their colloidal properties and ability to form chelates, HS have been shown to modify the toxic effects of a variety of xenobiotics and undesirable substances that enter the digestive tract in animals with feed and water (Herzig *et al.,* 2007; Livens, 1991). Several studies have been conducted to assess the ability of HS to reduce the tissue accumulation of heavy metals such as mercury, cadmium, and zinc in fish (Hammock *et al.,* 2003), lead and cadmium in rats (Rochus, 1983), and cadmium, zinc, and lead in chickens (Herzig *et al.,* 2007; Zralý *et al.,* 2008; Herzig *et al.,* 2009).

HS are recognized in plant science by their stimulating effects on mineral uptake by the roots and mineral concentrations such as Ca, P, K, Mg, Cu, Fe, Zn, and Mn in several plant tissues (Çimrin *et al.,* 2010; Denre *et al.,* 2014; Canellas *et al.,* 2015). Increases in ash and Ca content in tibia bone have also been reported in broilers fed HS (Eren *et al.,* 2000; Disetlhe *et al.,* 2017; Jad'uttová *et al.,* 2019) as have increases in Ca, Fe, and Cu concentrations in broiler meat (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012). This is consistent with the increased percentage, thickness, and hardness of eggshells reported in HS-supplemented laying hens and pheasants (Dobrzański *et al.,* 2009; Ozturk *et al.,* 2009).

In a previous study, supplementing drinking water with worm compost leachate as a source of HS in broilers aged 21-49 days, increased ash retention by 18-39% (Gómez-Rosales & Angeles, 2015). However, no differences in ash retention, tibia weight, and tibia ash content were observed in a later study in which a solid extract of HS (ESH) from a worm compost was supplemented in the feed of broilers from 14 to 35 days of age, (Domínguez-Negrete *et al.,* 2019). This disparity was most likely caused by differences in the source and form of HS supplementation, the age of the chickens and the duration of HS consumption.

There are also large variations in the amounts of HS used in drinking water to boost broiler productivity. Benefits in feed conversion, energy digestibility, and ash retention were reported in one study using doses ranging from 0 to 200 µg HS/L water (Gómez-Rosales & Angeles, 2015). Improvements in body weight, feed conversion, and carcass yield were observed in another study using doses ranging from 0 to 450 mg HS/L water (Ozturk *et al.,* 2010). As a result, in order to improve broiler production parameters, the dosages of HS added to the drinking water must be defined. Increasing doses of HS in broiler water, up to a maximum of 200 µg/L, caused a linear increase in tibia ash retention; however, it is unknown whether higher concentrations of HS in drinking water could cause additional improvements in bone mineralization (Gómez-Rosales & Angeles, 2015).

Because leg problems due to insufficient bone mineralization during the first three weeks of growth in broilers may be a limiting factor in achieving maximum performance in subsequent stages of production, the search for alternative options to improve the bone development is necessary (Díaz-Alonso *et al.,* 2019). Therefore, the objective was to assess the productive variables, carcass traits, and tibia mineralization responses of broiler chickens supplemented with a liquid source of HS in their drinking water.

MATERIAL AND METHODS

Animals and management

The Ethical Committee of Animal Use of the National Center of Disciplinary Research in Animal Physiology, National Institute for Research in Forestry, Agriculture, and Livestock revised and approved this study in accordance with Mexican regulations (NOM-062-ZOO, 1999). A group of Ross 308 one-day-old male broilers from a regional commercial hatchery was randomly allocated in floor pens with wood shaving and gas brooder heaters. A standard broiler starter diet was provided from 1 to 5 days after hatching. On day six, 360 chicks were randomly assigned to holding cages in groups of two (30 cm wide x 38 cm deep x 37 cm height). Cages were arranged in batteries and were provided with gas heaters, equipped with plastic feeders and cup waterers. On day eight, five increasing levels of HS in the drinking water (0, 161, 322, 483, and 644 µg/L) were used. Since the maximum ash retention in broilers added with doses ranging from 161-322 µg HS /L of water was reported in previous research, but the inflection point on the ash retention was not observed, higher concentrations of HS in the water were tested in the current study. A liquid extract of HS (LEHS) was mixed into the drinking water to achieve the increasing levels of HS. The mixture of the LEHS and water was served in plastic bottles tied to the cage. The LEHS was extracted from a worm compost using an alkaline extraction procedure described previously (Domínguez-Negrete *et al.,* 2019), and it contained 2.0% DM with 48.15 humic acid (HA) and 31.85% fulvic acid (FA). The concentrated LEHS was stored in 20 L amber plastic containers during the experiment. A starter diet from 8-14, a grower diet from 15-28, and a finisher diet from 29-42 days of age were provided. Diets were based on corn and soybean meal and were designed to meet or exceed the nutrient requirements of broilers at each stage of growth (Table 1). Diets were served *ad libitum* in mash form. Anticoccidial drugs and antibiotics used to promote growth were not included in the feeds.

Table 1 – Ingredient composition and calculated nutrient content of the experimental diets.

	Starter	Grower	Finisher
Ground corn	63.81	65.31	67.41
Soybean meal	29.40	27.00	24.70
Vegetable oil	2.10	3.30	3.80
Calcium orthophosphate	1.70	1.65	1.52
Calcium carbonate	1.60	1.50	1.43
Salt	0.32	0.30	0.28
Sodium bicarbonate	0.20	0.20	0.20
DL-Methionine	0.24	0.21	0.18
L-Lysine·HCl	0.26	0.21	0.19
L-Threonine	0.08	0.05	0.04
Premix[a]	0.20	0.20	0.20
Choline chloride	0.09	0.07	0.05
Calculated nutrient content			
Metabolizable energy, kcal/kg	3000	3100	3200
Digestible Lysine, %	1.10	1.00	0.90
Digestible Methionine, %	0.52	0.47	0.43
Digestible Threonine, %	0.71	0.65	0.60
Total Calcium, %	1.00	0.90	0.80
Available Phosphorus, %	0.50	0.45	0.40

[a] Each kg provided: 6500 IU Vit A; 2000 IU Vit D3; 15 IU Vit E; 1.5 mg Vit K; 1.5 mg thiamine; 5 mg riboflavin; 35 mg niacin; 3.5 mg pyridoxine; 10 mg pantothenic acid; 1500 mg choline; 0.6 mg folic acid; 0.15 mg biotin; 0.15 mg Vit B12; 100.0 mg Mn; 100 mg Zn; 50 mg Fe; 10 mg Cu; 1.0 mg I.

Characterization of humic substances

The concentration of HS in the LEHS was determined as previously described (Stevenson, 1982). Also, a 5 L sample was dried in a stove at 55 °C and ground using a 2 mm mesh; the dry product was used for laboratory analysis. The functional groups were determined using an infrared spectrophotometer with Fourier transformation with attenuated total reflectance (FTIR-ATR). The elemental analysis was carried out using energy dispersive X-Ray spectroscopy (EDS). The crystal types were detected with X-ray diffraction (XRD). The results were used for the calculation of aromaticity. The data of functional groups, elemental analysis, crystal types and aromaticity percentage were used to estimate the chemical properties and the flat structures of the HS molecules with aromaticity using the chemistry software ACD Lab v.12 (Advanced Chemistry Development, Toronto, Canada).

Productive parameters

Broilers were weighed individually at the beginning, at 21 and 42 days of age, to calculate the daily weight gain (WG, g/d). Feed offered and refused was registered to calculate the daily feed intake (FI, g/d). The feed conversion ratio (FCR) was estimated by dividing the FI between the WG. At the beginning of the trial, there were 36 replicate cages per treatment with two birds per cage. At 21 days of age, half of the birds (18 replicate cages per treatment) were slaughtered by cervical dislocation to get the breast and carcass weight and yield. From 22-42 days, the rest of the birds were allocated individually but keeping the identification of the cage of origin and treatment. At 42 days, the other 18 replicates per treatment with two broilers per replicate were also slaughtered following the same procedures described before. In broilers killed at 21 and 42 days, the left and right tibias were removed; the bones from three chicks (six tibias in total) from the same treatment were pooled to ensure enough samples for the Ca and P analysis. All samples were frozen.

Laboratory analysis

The tibias were unfrozen, cleared of soft tissues, weighed and dried using a horizontal flow drying oven to estimate the DM percentage (Terlab S.A. de C.V., Zapopan, Jalisco, México) at 105 °C for 24 h. Then tibias were defatted in ethyl ether, and incinerated in a furnace (Furnatrol I Type 1,8200; Thermolyne, Guadalajara, Jalisco, Mexico) at 600 °C for 6 h to determine the ash percentage following the recommendations of Díaz-Alonso *et al.* (2019). The DM and ash content (g) were estimated by multiplying the DM and ash percentage by the fresh weight and dry weight of the tibia, respectively. The P percentage was determined by spectrophotometry using a GENESYS™ 10S UV Vis Spectrophotometer (Thermo Scientific, Mexico City, Mexico) and the Ca percentage was assessed using the atomic absorption methodology using an M Series AA Spectrometer (Thermo Electron Corporation, Waltham, Massachusetts, USA). The Ca and P content (mg) were estimated by multiplying the Ca and P percentage by the ash content. All laboratory determinations were carried out following standard procedures (AOAC, 2019).

Statistical analysis

Data were subjected to ANOVA using the General Linear Model of SAS (1990). Differences among means were tested by the LSD method. The growth performance and carcass traits from 8-21 and 22-42 days were analyzed using two birds per replicate and a total of 18 replicates per treatment in each period. All tibia determinations were carried out using six composite tibias from three birds for each replicate sample for a total of 12 replicates per treatment. Linear regression analysis was also used to test for linear, quadratic, and cubic responses for each response variable regarding the increasing levels of HS.

RESULTS

Characterization of humic substances

In Table 2, the chemical properties of the HA and FA molecules are presented. In Figures 1 and 2, the flat HA and FA structures with aromaticity are depicted. Higher molecular weight and higher concentration of C, H and N were estimated for HA compared to FA, while in FA a higher amount of O was observed. HA and FA had similar density.

Table 2 – Estimated chemical properties of the humic and fulvic acid molecules.[a]

	Humic acid	Fulvic acid
Formula	$C_{110}H_{105}N_7O_{50}$	$C_{25}H_{17}N_0O_{18}$
Molecular weight	2325.028	619.398
Elemental composition, %	C (56.82), H (4.55), N (4.22), O (34.41)	C (48.48), H (2.77), N (2.26), O (46.49)
Density, g/cm^3	1.870 ± 0.10	1.935 ± 0.06

[a] Using the chemistry software ACD Lab v.12.

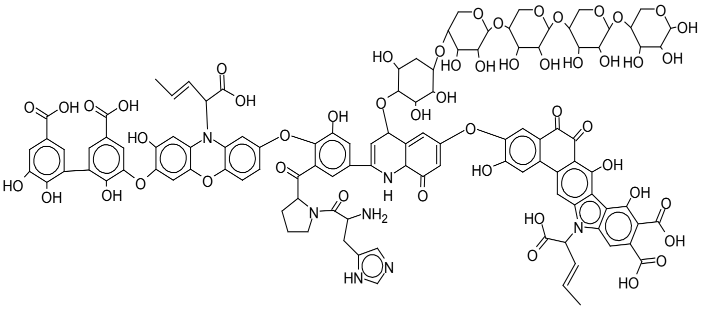

Figure 1 – Flat structure of humic acid molecule with aromaticity.

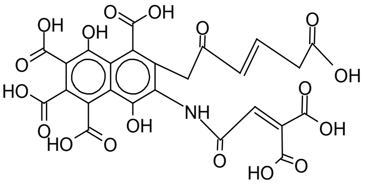

Figure 2 – Flat structure of fulvic acid molecule with aromaticity.

Productive parameters and carcass traits

The growth performance variables from 8-21 and 22-42 days of age and the carcass measurements are shown in Table 3. There was not any statistical difference on the body weight at 8, 21 and 42 days of age nor on the WG, FI and FCR on the periods from 8-21 and 22-42 days among broilers supplemented with increasing levels of HS in the drinking water. The breast weight at 21 days of age showed a cubic pattern regarding the increasing levels of HS ($y = 146.74 + 0.0734x - 0.0004x^2 - 4E\text{-}07x^3$; $R^2 = 0.99$; SEM = 3.069; $p<0.05$); the breast weight was higher in broilers supplemented with 161 and 654 µg/L of HS. The rest of the carcass measurements in 21- and 42-day old broilers were not statistically different.

Tibia measurements at 21 days of age

The tibia DM, ashes, Ca and P content in 21 days of age broilers are presented in Table 4. The increasing levels of HS in the drinking water elicited a cubic response on the tibia ashes percentage ($y = 38.1577 + 0.0209x - 7E\text{-}05x^2 + E\text{-}08x^3$; $R^2 = 0.91$; SEM = 0.539; $p<0.05$) and the tibia Ca percentage ($y = 40.02 - 0.0468x + 0.0002x2 - 3E\text{-}07x3$; $R^2 = 0.92$; SEM = 1.648; $p < 0.01$). The percentage of tibia ashes was higher in broilers supplemented with 161 and 654 µg/L of HS (similar to the response seen on the breast weight) while the Ca percentage was higher in broilers supplemented with 322 and 483 µg/L of HS. The tibia P percentage had a linear increasing response ($p<0.05$) related to the increasing levels of HS ($y = 14.029 + 0.0002x$; $R^2 = 0.80$; SEM = 0.050). The rest of the tibia measurements were similar regardless of the levels of HS.

Tibia measurements at 42 days of age

The tibia DM, ashes, Ca and P content in 42 days of age broilers are presented in Table 5. The addition of HS resulted in a quadratic response in DM percentage ($y = 42.217 + 0.00069x - 1E\text{-}05x^2$; $R^2 = 0.91$; SEM = 0.440; $p<0.05$), Ca content ($y = 941.74 + 0.7739x - 0.0012x^2$; $R^2 = 0.57$; SEM = 45.560; $p<0.01$) and P content ($y = 391.46 + 0.1391x - 0.0002x^2$; $R^2 = 0.66$; SEM = 10.187; $p<0.05$). Based on the derivatization of the quadratic equations, the optimum supplementation level of HS in the drinking water to achieve the maximum tibia DM percentage, Ca content and P content was estimated to be 345.00, 322.46 and 347.75 µg/L, respectively. Broilers receiving the increasing levels of HS showed a cubic response on the tibia Ca percentage ($y = 34.717 - 0.0299x + 0.0002x^2 - 2E\text{-}07x^3$; $R^2 = 0.99$; SEM = 1.242; $p<0.05$) and tibia P percentage ($y = 14.022 - 0.0002x + 7E\text{-}07x^2 - 8E\text{-}10x^3$; $R^2 = 0.42$; SEM = 0.019; $p<0.01$). The Ca percentage was higher in broilers given 322 and 483 µg/L of HS, while the P percentage was higher in broilers given 322 µg/L of HS.

DISCUSSION

Functional groups, elemental analysis, crystal types, and aromaticity percentage results of HS have been previously reported (Domínguez-Negrete *et al.*, 2019). The estimated higher C and N and lower O in HA compared to FA (Table 2) agree with previous reports (Steelink, 1985; Senesi & Loffredo, 1999) that analyzed HS from soils harvested in different climate regions. Figures 1 and 2 show that the more complex

Table 3 – Growth performance variables from 8-21 and 22-42 days of age of broiler chickens added with increasing levels of humic substances in the drinking water.^{a}

	Humic substances, µg/L of water						*p* value^c		
	0	161	322	483	654	SEM^b	L	Q	C
Body weight, g									
8 days, g	128.2	130.2	129.9	130.7	129.3	1.249	0.48	0.23	0.99
21 days, g	719.8	734.1	738.4	734.8	730.9	8.013	0.37	0.14	0.70
42 days, g	2452.2	2503.6	2486.8	2496.8	2500.9	34.259	0.40	0.60	0.57
Period from 8-21 days									
Feed intake, g/d	61.3	63.1	63.7	63.1	63.4	0.797	0.10	0.16	0.42
Weight gain, g/d	42.3	43.1	43.5	43.1	43.0	0.546	0.41	0.18	0.68
FCR	1.46	1.47	1.47	1.47	1.48	0.013	0.32	0.85	0.61
Period from 22-42 days									
Feed intake, g/d	146.0	148.9	146.5	147.0	147.1	1.895	0.96	0.72	0.41
Weight gain, g/d	81.9	83.9	82.6	83.1	84.1	1.673	0.50	0.96	0.46
FCR	1.81	1.79	1.80	1.79	1.77	0.042	0.51	0.85	0.78
Carcass traits at 21 days of age									
Carcass weight, g	374	385	373	364	385	56.803	0.47	0.62	0.26
Carcass yield, %	53.8	54.2	53.7	53.3	54.6	7.357	0.45	0.59	0.19
Breast weight, g	147de	151^d	144de	141^e	150^d	3.069	0.71	0.32	0.05
Breast yield, %	21.1	21.3	20.8	20.7	21.2	0.285	0.76	0.33	0.16
Carcass traits at 42 days of age									
Carcass weight, g	1324	1341	1327	1337	1359	24.491	0.40	0.71	0.58
Carcass yield, %	54.9	54.4	54.7	54.7	55.0	0.365	0.66	0.40	0.68
Breast weight, g	574	575	581	562	572	12.232	0.65	0.86	0.51
Breast yield, %	23.8	23.3	23.9	23.0	23.2	0.275	0.10	0.72	0.99

^{a} n = 36, using two birds per replicate.

^{b} SEM = estandard error of the mean.

^{c} L, Q and C = linear, quadratic and cubic efects.

^{d,e} Means with different superscript in the same row differ significantly (p<0.05).

Table 4 – Dry matter, ashes, calcium and phosphorus content of the tibia of 21 days old broiler chickens added with increasing levels of humic substances in the drinking water.^{a}

	Humic substances, µg/L of water						*p* value^c		
	0	161	322	483	654	SEM^b	L	Q	C
Dry matter, %	35.24	36.15	35.77	35.87	36.48	0.522	0.19	0.95	0.28
Dry weight, g	2.10	1.99	2.07	2.05	2.13	0.060	0.48	0.21	0.65
Ashes, %	38.11^d	40.13^e	39.32^{de}	39.18^{de}	39.57^e	0.539	0.25	0.21	0.05
Ashes, mg	802.0	797.0	814.8	804.8	845.3	28.075	0.29	0.58	0.71
Ca, %	40.20^{fg}	36.94^f	42.04^g	42.20^g	36.70^f	1.648	0.74	0.13	0.01
Ca, mg	320.3	298.5	342.3	339.4	307.9	18.551	0.78	0.33	0.10
p, %	14.02^d	14.01^d	14.10^{de}	14.15^e	14.14^e	0.050	0.05	0.91	0.36
p, mg	112.6	111.8	114.8	113.9	119.4	4.203	0.29	0.49	0.71

^{a} n = 12, using six tibias per replicate.

^{b} SEM = estandard error of the mean.

^{c} L, Q and C = linear, quadratic and cubic efects.

^{d,e} Means with different superscript in the same row differ significantly (p<0.05).

^{f,g} Means with different superscript in the same row differ significantly (p<0.01).

structure of HA, due to higher molecular weight and a greater percentage of aromaticity, compared to FA, is consistent with previous reports (Varanini & Pinton, 1995; Calace *et al.,* 2000). While the chemical properties of composite HS (mixtures of HA and FA) from organic sources such as manure and compost have previously been reported (Ayuso *et al.,* 1997; Rupiashi & Vidyasagar, 2009; Campitelli *et al.,* 2012) as far as we are aware, this is the first study to publish the chemical properties and flat structures of HA and FA extracted from a worm compost. Figures 1 and 2 depict structures that correspond to other HA and FA depicted in previous reports (Buffle, 1977; Stevenson, 1982; Mirza *et al.,* 2011). However, it

Table 5 – Dry matter, ashes, calcium and phosphorus content of the tibia in 42 days old broiler chickens added with increasing levels of humic substances in the drinking water.[a]

	Humic substances, µg/L of water						*p* value[c]		
	0	161	322	483	654	SEM[b]	L	Q	C
Dry matter, %	42.25^{d}	43.06^{de}	43.16^{de}	43.35^{e}	42.17^{d}	0.440	0.95	0.05	0.72
Dry weight, g	7.79	8.32	8.26	8.39	8.21	0.218	0.19	0.14	0.67
Ashes, %	35.84	35.64	35.11	36.34	35.34	0.596	0.88	0.94	0.32
Ashes, mg	2785.5	2966.1	2894.4	3046.8	2891.0	73.941	0.22	0.11	0.81
Ca, %	34.73^{d}	33.17^{de}	35.81^{d}	35.98^{d}	31.11^{e}	1.242	0.26	0.06	0.05
Ca, mg	969.0^{fg}	984.0^{fg}	1039.2^{f}	1102.5^{f}	891.9^{g}	45.542	0.82	0.01	0.05
p, %	14.02^{fg}	13.98^{f}	14.04^{g}	13.97^{f}	13.97^{f}	0.019	0.13	0.38	0.01
p, mg	390.6^{d}	414.5^{de}	409.9^{de}	425.9^{e}	402.0^{d}	10.187	0.21	0.05	0.83

[a] n = 12, using six tibias per replicate.

[b] SEM = estandard error of the mean.

[c] L, Q and C = linear, quadratic and cubic efects.

[d,e] Means with different superscript in the same row differ significantly ($p<0.05$).

[f,g] Means with different superscript in the same row differ significantly ($p<0.01$).

has been suggested that the elemental and chemical composition of HA and FA are affected by a variety of factors, including, geographical, climatic, physical and biological conditions, as well as the chemical composition of the raw materials from which they are derived (Peña-Mendez *et al.,* 2005).

One issue confronting animal production today is the risk of the pathogenic bacteria developing antimicrobial resistance, which can spread between different bacterial populations via humans, livestock, and the entire environment (Woolhouse *et al.,* 2015). To deal with antimicrobial resistance, various alternatives are in place around the world, such as the ban or reduction of the use of growth promoter antibiotics in poultry, as well as an intensive search for products that can replace them, while maintaining optimal levels of growth and health of the flocks (Gómez-Rosales & Angeles, 2015; Domínguez-Negrete *et al.,* 2019). Several products have been tested in poultry, and according to recent reports, HS appear to be a promising choice for improving broiler growth and carcass traits, as well as laying hens' productivity and egg quality (Arif *et al.,* 2019).

Higher lactic acid bacteria counts in the ileal content were found, but there was no effect on the total bacteria, *Salmonella* and *Escherichia coli* counts in broiler guts supplemented with HS extracted from worm compost (Maguey-González *et al.,* 2018a; Maguey González *et al.,* 2018b; Domínguez-Negrete *et al.,* 2019). Previous research has found conflicting results in gut microbiology in chickens supplemented with SH. However, one of the suggested growth-promoting effects of HS is related to their ability to form protective barriers over the digestive tract´s epithelial mucosal membrane against the penetration of pathogenic bacteria or toxic substances from bacteria (Kühnert *et al.,* 1991; Maguey-Gonzalez *et al.,* 2018b). This effect has been linked to the macro colloidal structure of HS, which provides good shielding on stomach and gut mucous membranes (Mudronová *et al.,* 2020). Additionally, the colloidal properties of HS and their ability to form chelates (Livens, 1991; Herzig *et al.,* 2007) have been linked to the improved mineral uptake in plants and bone mineralization in broilers (Eren *et al.,* 2000; Disetlhe *et al.,* 2017; Jad'uttová *et al.,* 2019). However, the use of an alkaline solution of HS extracted from worm compost as a means to improve bone mineralization in broilers has never been tested. That was the focus of the current research.

The lack of effects of HS on FI (Table 3) in the present research agrees with some studies (Taklimi *et al.,* 2002; Gómez-Rosales & Angeles, 2015; Domínguez-Negrete *et al.,* 2019) but disagrees with reports in which FI was depressed in broilers supplemented with HS (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012). The lack of an effect of HS on the FCR contradicts previous studies in which FCR was improved in broilers fed HS (Taklimi *et al.,* 2002; Ozturk *et al.,* 2010; Ozturk *et al.,* 2012). In a previous study from our laboratory, broilers given increasing amounts of worm compost leachate as the source of HS in their drinking water had lower FCR than the control birds (Gómez-Rosales & Angeles, 2015).

HS have been shown to increase broiler growth and carcass features, as well as laying hen productivity and egg quality (Arif *et al.,* 2019). The lack of an effect of HS on carcass, breast weight, and yield in 21 and 42-day old broilers (Table 3) contradicts the increased carcass and breast weight reported in other studies in which broilers were supplemented with several sources of HS (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012; Disetlhe *et al.,* 2019). Furthermore, broilers fed a solid HS source derived from the same worm compost as the LEHS used in this study had higher carcass yield (Domínguez-Negrete *et al.,* 2019).

The differences in growth and carcass characteristics observed in this study versus previous results of broiler chickens treated with HS could be attributed to a number of factors. Differences in HA and FA content, inclusion level, and form (liquid or solid) of previously used HS in broilers, as well as chain length, side-chain composition, and origin (plant, soil, peat, and coal-derived) are all important factors to consider (Pea-Mendez *et al.,* 2005; Arif *et al.,* 2019). In a previous study, broilers given worm compost leachate in their drinking water had higher body weight, WG, and FCR than the control birds (Gómez-Rosales & Angeles, 2015); However, in that study, the HS were collected in an aqueous solution, whereas in this study, the HS were extracted using an alkaline solution, and the HS concentration was approximately 2.6 times higher than in the first study. In more recent studies, broiler chickens treated with a solid source of HS from a worm compost had higher carcass yield (Domínguez-Negrete *et al.,* 2019), despite the fact that the HS were added to the feed rather than the drinking water. Two additional parameters that may influence broiler responses to HS addition are the age of the birds when HS were first supplemented and the length of the HS consumption period.

Another specific issue confronting poultry production is the intensive selection of modern broilers for the optimization of growth traits, which has resulted in high incidence of leg problems due to inadequate mineralization of bones during the first three weeks of growth (Dinev, 2012; Disetlhe *et al.,* 2017), which could be a limiting factor for achieving maximum performance in subsequent stages of production; thus, the search for alternative options to improve performance is ongoing (Díaz-Alonso *et al.,* 2019). Supplementation of HS in broiler feed or water is another option because they are among the most common natural complexing ligands found in nature (Peña-Mendez *et al.,* 2005) and can chelate macro- and microminerals inside the intestine improving their assimilation (Ozturk *et al.,* 2012; Disetlhe *et al.,* 2017; Jauttová *et al.,* 2019). Macrominerals, such as Ca and P, are natural and essential components of tissues and body fluids, that serve a body-building function. They are thought to be the most important elements required for adequate bone development and mineralization (Díaz-Alonso *et al.,* 2019).

It has also been proposed that HS can cause intestinal morphophysiological changes, stimulate changes in intracellular divalent calcium levels, and act as dilators, increasing mucosal and cellular permeability (Pizzari & Birkhead, 2000; Johnsson *et al.,* 2015). Increased gut permeability, and thus cell membrane permeability in the body, may facilitate mineral assimilation and transfer from blood to bone and cells, resulting in better skeletal development (Dinev, 2012; Jad'uttová *et al.,* 2019; Rybalka *et al.,* 2020). These suggestions are supported by improved digestive capabilities such as increased intestinal villus height and width (Taklimi *et al.,* 2012; Disetlhe *et al.,* 2017; Lala *et al.,* 2017), improved tibia ash and Ca content (Eren *et al.,* 2000; Jad'uttová *et al.,* 2019), and higher meat mineral concentrations, such as Ca, Fe and Cu (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012) of broilers supplemented with HS.

The increased tibia Ca percentage at 21 and 42 days in broilers supplemented with 322 and 483 µg/L of HS, as well as the estimated higher tibia Ca content at a dose of 322.46 µg/L of HS (Tables 4 and 5) agree with the previously reported increases on tibia ash and Ca content (Eren *et al.,* 2000; Disetlhe *et al.,* 2017; Jad'uttová *et al.,* 2019) and the increases in the meat concentration of Ca, Fe and Cu (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012) in broilers supplemented with HS. These findings are consistent with the increased percentage, thickness and hardness of eggshell reported in HS-supplemented laying hens and pheasants (Hanafy & El-Sheikh, 2008; Dobrzański *et al.,* 2009; Ozturk *et al.,* 2009).

The current study is the first to show linear increases in P percentage in 21-day old broilers supplemented with increasing levels of HS, as well as higher P percentage and P content in 42-day old broilers supplemented with HS (322 and 347.75 µg/L of HS, respectively; Tables 4 and 5). In line with these findings, an increase in tibia P content was observed in broilers fed a diet added with canola meal and potassium humate (Disetlhe *et al.,* 2017). However, contrary to our findings, reductions in tibia P content were observed in broilers fed HS in another study (Jad'uttová *et al.,* 2019). This reduction could be attributed to the addition of much higher dietary amount of HS compared to the levels of HS provided in the drinking water in the current study.

HS are recognized in plant science for their stimulating effects on the growth, mineral uptake, and increases in mineral concentration of several plant tissues (Çimrin *et al.,* 2010; Denre *et al.,* 2014;

EL-Sayed *et al.,* 2014; Canellas *et al.,* 2015), but it is clear that the effects of HS on the mineral use and accumulation by plant tissues vary depending on the dosage and form of application. These findings published in Plant Science, suggest that future studies with broiler chickens should define the optimum level of supplementation and the best form (liquid or solid) to supply the HS extracted from a worm compost in order to improve the efficiency of use of Ca and P and the bone mineralization responses.

CONCLUSION

The addition of increasing levels of HS in the drinking water improved the ashes, Ca and P percentages in 21-day-old broilers but the results did not allow for the calculation of an optimum supplementation dosage. In 42-day-old broilers, there were also quadratic responses in tibia DM percentage and Ca and P content with estimated optimum levels of HS supplementation of 345.0, 322.5 and 347.8 µg/L of water, respectively. The addition of HS also improved the tibia Ca and P percentages in 42-day-old broilers. The results indicate that adding a liquid source of HS to broiler chickens drinking water improved bone mineralization at 21 and 42 days of age.

REFERENCES

AOAC. International official methods of analysis. 21st ed. Rockville; 2019. Available from: https://www.aoac. Official-methods-of-analysis-21st-edition.

Arif M, Alagawany M, Abd El-Hack ME, Saeed M, Arain MA, Elnesr SS. Humic acid as a feed additive in poultry diets: A review. Iranian Journal of Veterinarian Research 2019;20:167–172.

Ayuso M, Moreno JL, Hernández T, García C. Characterisation and evaluation of humic acids extracted from urban waste as liquid fertilisers. Journal of the Science of Food and Agriculture 1997;75:481-488.

Buffle J. Les substances humiques et leurs interactions avec les ions mineraus. Proceedings of Conference de la Commission d'Hydrologie Appliquee de l'A.G.H.T.M; 1977. Ile de France: L'University d'Orsay; 1977. p.3–10.

Calace N, Furlani G, Petronio BM, Pietroltti M. Sedimentary humic and fulvic acids: structure, molecular weight distribution and complexing capacity. Annaly di Chimica 2000;90;25-34.

Campitelli P, Velasco M, Ceppi S. Characterization of humic acids derived from rabbit manure treated by composting-vermicomposting process. Journal of Soil Science and Plant Nutrition 2012;12:875-891.

Canellas LP, Olivares FL, Aguiar NO, Jones DL, Nebbioso A, Mazzeic P, et al. Humic and fulvic acids as biostimulants in horticulture. Scientia Horticulturae 2015;196:15-27.

Çimrin KM, Onder T, Turan M, Burcu T. Phosphorus and humic acid application alleviate salinity stress of pepper seedling. Journal of African Biotechnology 2010;9:5845–5851.

Denre M, Ghanti G, Sarkar K. Effect of humic acids application on accumulation of mineral nutrition and pungency in garlic (Allium sativum L.). International Journal for Biotechnology & Molecular Biology Research 2014;5:7–12.

Díaz-Alonso JA, Gómez-Rosales S, Angeles ML, Ávila-González E, López-Coello C. Effects of the level and relationship of calcium and available phosphorus on the growth and tibia mineralization of broiler starter chickens. Journal of Applied Poultry Research 2019;28:339–349.

Dinev I. Clinical and morphological investigations on the incidence of forms of rickets and their association with other pathological states in broiler chickens. Research in Veterinary Science 2012;2:273-277.

Disetlhe ARP, Marume U, Mlambo V, Dinev I. Humic acid and enzymes in canola-based broiler diets: effects on bone development, intestinal histomorphology and immune development. South African Journal of Animal Science 2017;47:914-922.

Disetlhe ARP, Marume U, Mlambo V, Hugo A. Effects of dietary humic acid and enzymes on meat quality and fatty acid profiles of broiler chickens fed canola-based diets. Asian-Australasian Journal of Animal Science 2019;32:711–720.

Dobrzański Z, Trziszka T, Herbut E, Krawczyk J, Tronina P. Effect of humic preparations on productivity and quality traits of eggs from Greenleg Partridge hens. Annals of Animal Science 2009;9:165-174.

Domínguez-Negrete A, Gómez-Rosales S, Angeles ML, López-Hernández LH, Reis-de Souza TC, López-García Y, et al. Effect of the addition of humic substances as growth promoter in broiler chickens under two feeding regimens. Animals 2019;9:1101.

EL-Sayed SAA, Hellal FA, Mohamed KAS. Effect of humic acid and phosphate sources on nutrient composition and yield of radish grown in calcareous soil. European International Journal of Science and Technology 2014;3:168-177.

EMEA. Humic acids and their sodium salts, summary report. Committee for Veterinary Medicinal Products. Amsterdam: The European Agency for the Evaluation of Medical Products; 1999.

Eren M, Deniz G, Gezen SS, Turkmen II. Effects of humates supplemented to the broiler feeds on fattening performance, serum mineral concentration and bone ash. Ankara Universitesi Veteriner Fakultesi Dergisi 2000;47:255-263.

Gomez-Rosales S, Angeles ML. Addition of a worm leachate as source of humic substances in the drinking water of broiler chickens. Asian-Australasian Journal of Animal Science 2015;28:215–222.

Hammock D, Huang CC, Mort G, Swinehart JH. The effect of humic acid on the uptake of mercury(II), cadmium(II), and zinc(II) by Chinook salmon (Oncorhynchus tshawytscha) eggs. Archives of Environmental Contamination and Toxicology 2003;44:83-88.

Hanafy MM, El-Sheikh AMH. The effect of dietry humic acid supplementation on some productive and physiological traits of laying hens. Egyptian Poultry Science 2008;28:1043-1058.

Herzig I, Navrátilová M, Suchý P, Totušek J. Model trial investigating retentionin selected tissues using broiler chicken fed cadmium and humic acid. Veterinarni Medicina 2007;52;162-168.

Herzig I, Navrátilová M, Totušek J, Suchý P, Večerek V, Blahová J, et al. The effect of humic acid on zinc accumulation in chicken broiler tissues. Czech Journal of Animal Science 2009;54:121–127.

Jad'uttová I, Marcinčáková D, Bartkovský M, Semjon B, Harčárová M, Nagyová A, et al. The effect of dietary humic substances on the fattening performance, carcass yield, blood biochemistry parameters and bone mineral profile of broiler chickens. Acta Veterinaria Brno 2019;88:307–313.

Johnsson M, Johnsson KB, Andersson L, Jensen P, Wright D. Genetic regulation of bone metabolism in the chicken: similarities and differences to mammalian systems. PLOS Genetics 2015;11:5.

Kühnert VM, Bartels KP, Kröll S, Lange N. Huminsäurehaltige tierarzneimittel in therapie and prophylaxe bei gastrointestinalen erkrankungen von hund und katze. Monatshefte für Veterinärmedizin 1991;46:4-8.

Lala AO, Okwelum N, Oso AO, Ajao AO, Adegbenjo AA. Response of broiler chickens to varying dosage of humic acid in drinking water. Journal of Animal Production Research 2017;29:288-294.

Livens FR. Chemical-reactions of metals with humic material. Environmental Pollution 1991;70:183-208.

Maguey-Gonzalez JA, Michel MA, Baxter MF, Solis-Cruz B, Hernandez-Patlan D, Merino-Guzman R, et al. Effects of humic acids on recovery of *Salmonella enterica* serovar *Enteritidis*. Annals of Animal Science 2018a;18:387–399.

Maguey-Gonzalez JA, Michel MA, Baxter MF, Tellez Jr G, Moore Jr PA, Solis-Cruz B, et al. Effect of humic acids on intestinal viscosity, leaky gut and ammonia excretion in a 24 hr feed restriction model to induce intestinal permeability in broiler chickens. Animal Science Journal 2018b;89:1002–1010.

Mirza MA, Agarwal SP, Rahman MA, Rauf A, Ahmad N, Alam A, et al. Role of humic acid on oral drug delivery. Drug Development and Industrial Pharmacy 2011;37:310–319.

Mudrovnová D, Karaffová V, Pevsulová T, Kovsvcová J, Maruvsvcáková IC, Bartkovsk`y M, Marcinvcáková D. The effect of humic substances on gut microbiota and immune response of broilers. Food and Agriculture Immunology 2020;31:137–149.

Ozturk E, Coskun I, Ocak N, Erener G. Effects of dietary humic substances on egg production and egg shell quality of hens after peak laying period. African Journal of Biotechnology 2009;8:1155-1159.

Ozturk E, Ocak N, Coskun I, Turhan S, Erener G. Effects of humic substances supplementation provided through drinking water on performance, carcass traits and meat quality of broilers. Journal of Animal Physiology and Animal Nutrition 2010;94:78–85.

Ozturk E, Ocak N, Turan A, Erener G, Altop A, Cankaya S. Performance, carcass, gastrointestinal tract and meat quality traits, and selected blood parameters of broilers fed diets supplemented with humic substances. Journal of the Science of Food and Agriculture 2012;92:59–65.

Peña-Méndez EM, Havel J, Patôcka J. Humic substances compounds of still unknown structure: Applications in agriculture, industry, environment, and biomedicine. Journal of Applied Biomedicine 2005;3:13–24.

Pizzari T, Birkhead TR. Female feral fowl eject sperm of subordinate males. Nature 2000;405:787-789.

Rochus W. The effect of peat humic acids on the intake, shedding and distribution of lead and cadmium in the rat organism. Zeitschrift fur Physiotherapie 1983;35:25–30.

Rupiasih NN, Vidyasagar PB. Analytical study of humic acid from various sources commonly used as fertilizer: emphasis on heavy metal content. International Journal of Design & Nature and Ecodynamics 2009;4:32–46.

Rybalka MA, Stepchenko LM, Shuleshko OO, Zhorina LV. The impact of humic acid additives on mineral metabolism of rabbits in the postnatal period of ontogenesis. Regulatory Mechanisms in Biosystems 2020;11;289–293.

SAS Institute. SAS user's guide: statistics. 64th de. Cary; 1990.

Secretaría de Gobernación. [NOM-062-ZOO] Norma Oficial Mexicana. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Ciudad de México: Diario Oficial de la Federación; 1999.

Senesi N, Loffredo E. The chemistry of soil organic matter. In: Sparks, D.L. (Ed.), Soil Physical Chemistry, second Ed. Boca Raton: CRC Press; 1999.

Steelink C. Implications of elemental characteristics of humic substances. In: Aiken GR, Mcnight DM, Wershaw RL, MacCarthy P, editors. Humic substances in soil, sediment and water geochemistry. Isolation and characterization. New York: Wiley-Interscience Publication; 1985.

Stevenson FJ. Humus Chemistry; genesis, composition, reactions. New York: Wiley-Inter-Science; 1982.

Taklimi SM, Ghahri H, Isakan MA. Influence of different levels of humic acid and esterified glucomannan on growth performance and intestinal morphology of broiler chickens. Agricultural Science 2012;3:663-668.

Varanini Z, Pinton R. Humic substances and plant nutrition. In: Behnke HD, Lüttge U, Esser K, Kadereit JW, Runge M, editors. Progress in botany. Berlin: Springer; 1995.

Woolhouse M, Ward M, van Bunnik B, Farrar J. Antimicrobial resistance in humans, livestock and the wider environment. Philosophical Transactions of the Royal Society B: Biological Sciences 2015;370:20140083.

Zralý Z, Písaříková B, Trčková M, Navrátilová M. Effect of humic acids on lead accumulation in chicken organs and muscles. Acta Veterinaria Brno 2008;77:439–445.