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Original Article

Responses of Broiler Chickens Fed Low or High Non-Starch Polysaccharide Diets and the Addition of Humic Substances from a Worm Compost

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■Keywords

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ABSTRACT

The objective was to evaluate the productivity, microbiology and histopathology of the jejunum, ceca and liver in broilers fed a control or a high non-starch polysaccharide (HNSPs) diet added with an extract of humic substances (EHS). 240 broilers individually allocated, from 21-42 d of age were assigned to a factorial arrangement of 2 types of diets: 1) A corn/soybean meal diet (Control) and 2) As Control plus 7% distillers dried grain with solubles and 12% of wheat bran (HNSPs), and 3 growth promoters: 1) Antibiotic growth promoters (AGP); 2) without AGP (NAGP) and C) with 0.5% of EHS. At the end of the trial, the breast and carcass were weighed and samples of the intestine, ceca and liver were taken for microbial and histopathology analysis. Results were subjected to ANOVA. EHS-fed broilers had lower feed conversion ratio (FCR) and total aerobic bacterial (TAB) counts in the liver and higher ashes digestibility with the Control diet, but negative responses were seen with the HNSPs diet (Type of diet and growth promoter interaction, $p \leq 0.05$). The lesion scores in the jejunum were lower in EHS-fed broilers with the Control and HNSPs diet ($p \leq 0.05$). The TAB and *E. coli* were lower in the jejunum and ceca of AGP-fed broilers ($p<0.01$) compared to NAGP and EHS groups. EHS-fed broilers showed improved FCR and ileal ashes digestibility and lower TAB in the liver with the Control diet and had lower lesion score in the jejunum and similar weight gain compared to the AGP-fed broilers.

INTRODUCTION

In veterinary medicine, humic substances (HS) have been promoted as antidiarrheal agents, pain relievers, immunomodulators and antimicrobials, after the recommendations of the Veterinary Committee of the European Medicine Agency for the Evaluation of Medicinal Products (EMEA, 1999). In recent years, HS have been tested as growth promoters in animal production and are one of the promising options to face the global ban of antibiotics in feeds (Maguey-Gonzalez *et al.,* 2018a; Arif *et al.,* 2019; Dominguez-Negrete *et al.,* 2021). Humic substances are part of humus-soil organic matter, and arise from the physical, chemical and microbiological transformation (humification) of biomolecules. Approximately 80% of the total carbon in terrestrial media and 60% of the carbon dissolved in aquatic media are made up of HS; they are a complex mixture of many different acids containing carboxyl and phenolate groups (Peña-Mendez *et al.,* 2005) and their main components are fulvic (FA) and humic (HA) acids.

In broiler chickens, improvements in body weight, feed conversion and carcass weight due to the inclusion of HS in the feeds (Kocabagli *et al.,* 2002; Ozturk *et al.,* 2012; Taklimi *et al.,* 2012) or in the drinking water (Ozturk *et al.,* 2010; Rizal & Marlida, 2013) have been reported. Most

of the HS tested in poultry are commercially available or purified products mostly extracted from lignite and leonardite mines (Peña-Mendez *et al.,* 2005; Ozturk *et al.,* 2010). It has also been shown that worm composts originating from animal manures are good sources of HS which could also be used as growth promoters (Gomez-Rosales *et al.,* 2011). Broiler chickens that had a worm compost leachate added in the drinking water as a source of HS, had lower feed conversion ratio, higher energy digestibility and higher dried matter, ashes, nitrogen, and energy retention compared to the control birds (Gomez-Rosales & Angeles, 2015). The chicks that had an extract of humic acid derived from the same worm compost as the one used in the present study added in the drinking water and were subjected to feed restriction for 24 h to induce intestinal inflammation, had increased intestinal viscosity and reduced bacterial translocation to the liver compared to the control chicks (Maguey-Gonzalez *et al.,* 2018b). Furthermore, in recent studies, higher carcass yield and higher lactic acid bacteria (Dominguez-Negrete *et al.,* 2019), as well as lower feed conversion ratio (FCR) and mortality (Dominguez-Negrete *et al.,* 2021) were found in broiler chickens fed with an extract of HS (EHS) obtained from the same worm compost used in this research.

No reductions have been found on intestinal *Salmonella* or *Clostridium* spp in broilers added with HS (Dominguez-Negrete *et al.,* 2019; Maguey-Gonzalez *et al.,* 2018a) which suggest that HS may indirectly protect against pathogenic bacteria reducing their translocation from the intestinal lumen into the body by creating a protective layer over the epithelial mucosal membrane of the digestive tract (Kühnert *et al.,* 1991) due to the macrocolloidal structure of HS, ensuring the shielding on the mucous membrane. An increased digesta viscosity and reduced bacterial translocation to the liver (Maguey-Gonzalez *et al.,* 2018b), as well as higher intestinal mucin-2 gene expression (Mudroňová *et al.,* 2020), support this suggestion. This finding also agrees with the suggestion that responses to alternative additives to replace the antibiotic growth promoters (AGP) may be greater in a more challenging environment (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012) and those benefits should be demonstrated under real or simulated commercial conditions. Therefore, elucidation of the effects of HS under different stress models as inducers of intestinal inflammation deserves further clarification.

Under commercial conditions, changes in diet type and formulation, have been commonly associated with impaired function and inflammatory responses of the digestive mucosa (Kidd, 2004; Choct, 2009). Experimentally, different models have been used in broilers to provoke gut inflammation which led to intestinal leakage (Kuttappan *et al.,* 2015). If the epithelial barrier is injured and becomes more permeable, the innermost tissues will be continuously exposed to dietary antigens and microorganisms, causing additional inflammatory responses (Tellez *et al.,* 2014). In previous research the feeding of high non-starch polysaccharides (HNSPs) diets has been successfully used to induce the disruption of the intact barrier of the gastrointestinal tract (Tellez *et al.,* 2014; Kuttappan *et al.,* 2015). In these studies, broilers were fed high NSPs diets, based on rye, and had increased intestinal viscosity, elevated bacterial translocation to the liver and intestinal bacterial overgrowth when compared with chickens fed with corn (Tellez *et al.,* 2014; Kuttappan *et al.,* 2015).

Previous results indicate that the HS protect the epithelial barrier through the increase of the intestinal viscosity (Maguey-Gonzalez *et al.,* 2018b; Mudroňová *et al.,* 2020), but on the other hand, the increase of the intestinal viscosity due to the consumption of HNSPs diets can increase the permeability of the mucosa. Therefore, it is necessary to clarify whether the addition of HS in HNSPs diets help to protect the mucosa, or otherwise, exacerbate the negative effects of the increased viscosity on the increased leakage of the gut. Therefore, the objective of this study was to evaluate the productive parameters, carcass yield, the microbiology and histopathology of the lower jejunum, ceca and liver of broilers fed a control and HNSPs diets added with an extract of humic substances (EHS).

MATERIALS AND METHODS

The isolation and extraction of the EHS from worm compost was performed as described by (Domínguez-Negrete *et al.,* 2019). In brief, sodium hydroxide (NaOH 0.5 M) and the worm compost were mixed in a ratio of 5:1 (mL g^{-1}) in 50 mL tubes and allowed to stand for 24 h at room temperature. After this, the tubes were centrifuged for 20 min at 3000X g (5810R Eppendorf centrifuge, Hamburg, Germany), and the precipitate and supernatant were separated by decantation. The precipitate was washed twice in distilled water and centrifuged as before. The supernatants were pooled, dried in a forced air stove (Shel Lab, Cornelius, OR, USA) at 55 °C for 24 h, and ground using a Thomas Willey grinder and 1 mm sieve. The results

of functional groups, elemental analysis, crystal types and aromaticity percentage were previously reported (Domínguez-Negrete *et al.,* 2019). This research was revised and approved by the Ethical Committee of Animal Use and complied with the official Mexican norm, of the National Center of Disciplinary Research in Animal Physiology, National Institute for Research in Forestry, Agriculture and Livestock (INIFAP).

Birds, cages and treatments

A group of 240 Ross 308 male broilers were allocated in holding cages (30 cm wide x 38 cm deep x 37 cm height) providing 1140 $cm^2 bird^{-1}$ from 21 to 42 d of age. Cages were arranged in batteries and were provided with gas heaters, equipped with a plastic feeder and a cup waterer. Birds were randomly assigned to six treatments in a factorial arrangement of 2 types of diets and 3 growth promoters: 1) control diet (Control) formulated with corn and soybean meal with bacitracin methylene disalicylate and salinomycin as antibiotic growth promoters (AGP); 2) Control diet without inclusion of AGP (NAGP); 3) Control diet without inclusion of AGP and added with 0.5 % of EHS; 4) A high NSPs diet (HNSPs) formulated with corn and soybean meal plus 7 % distillers dried grain with solubles (DDGS) and 12 % of wheat bran (WB) plus AGP; 5) HNSPs diet without inclusion of AGP (NAGP); and 6) HNSPs diet without inclusion of AGP and added with 0.5 % of EHS. The concentration of HA, FA and ashes in the EHS were 47.1, 29.6, and 23.2%, respectively, on dry matter basis, with an estimated aromaticity of 53.8%. The composition of the experimental diets is presented in Table 1. The diets were mixed in a weekly basis. Feed and water were offered ad libitum throughout the experiment. The study lasted 21 days.

Data and sample collection

Broilers were weighed at the beginning and end of the trial to calculate the daily weight gain (WG, g d^{-1}).

Table 1 – Composition of experimental diets and calculated and analyzed dietary components.

	Control			HNSPs		
Item	AGP	NAGP	EHS	AGP	NAGP	EHS
Ground corn	57.04	57.74	57.34	38.63	39.33	39.00
Soybean meal	30.66	30.96	30.86	25.59	25.89	25.72
Wheat bran	0.00	0.00	0.00	12.00	12.00	12.00
DDGS	0.00	0.00	0.00	7.00	7.00	7.00
Vegetable oil	5.10	5.10	5.10	9.30	9.30	9.30
Calcium carbonate	1.43	1.43	1.43	1.54	1.54	1.54
Calcium phosphate	1.56	1.56	1.56	1.31	1.31	1.31
Salt	0.44	0.44	0.44	0.45	0.45	0.45
DL–methionine	0.26	0.26	0.26	0.29	0.29	0.29
L-lysine HCl	0.21	0.21	0.21	0.29	0.29	0.29
L-threonine	0.60	0.60	0.60	0.90	0.90	0.90
Vitamins and Minerals[1]	1.00	1.00	1.00	1.00	1.00	1.00
Cholinechroride	0.70	0.70	0.70	0.70	0.70	0.70
Cocciostate	0.50	0.00	0.00	0.50	0.00	0.00
Antibiotic	0.50	0.00	0.00	0.50	0.00	0.00
EHS	0.00	0.00	0.50	0.00	0.00	0.50
Calculated dietary components						
ME, kcal, kg	3160			3160		
Dig. Lys, %	1.13			1.13		
Ca, %	0.90			0.90		
Ava. P, %	0.45			0.45		
Crude fiber, %	2.43			3.64		
Analyzed dietary components						
Dried matter, %	89.51			90.07		
Ashes, %	13.62			13.54		
GE, Kcal/kg	4121.9			4362.1		
CP, %	19.13			19.99		
NDF, %	8.42			13.42		
ADF, %	3.85			5.57		

[1] 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.

Feed offered and refused was registered to calculate the daily feed intake (FI, g d^{-1}). The FCR was estimated by dividing the FI between the WG. During the last week of the experiment, 0.3% of titanium dioxide was included in the feed as an internal marker for determination of the ileal digestibility of dietary components. On the last day of the experiment, all broilers were killed by cervical dislocation and the ileal contents were collected in subgroups of five samples per replicate. Each replicate sample was stored frozen at –20°C in polyethylene bags. For measurement of microbiota, digesta content from the jejunum and ceca as well as liver samples were collected in subgroups of three samples per replicate, and immediately taken to the laboratory in sterile plastic bags placed in an insulated ice container and processed on the same day. One-cm samples from the duodenum, lower jejunum (next to the Meckel diverticulum) and liver were taken from six broilers per treatment for histopathologic evaluations. The carcass and breast were weighed and were expressed in grams and percentage relative to the final body weight.

Laboratory analyses

The ileal content samples were lyophilized and ground using a 2 mm mesh. Determinations of dry matter, ashes, nitrogen, energy and titanium were carried out in the diet and ileal digesta. The results were used to estimate the dry matter, ashes, nitrogen and energy ileal apparent digestibility, on a dry matter basis. All laboratory determinations were carried out following standard procedures according to the AOAC (). In addition, the apparent metabolizable energy corrected to zero nitrogen retention (AMEn) was estimated.

In the jejunum and ceca content and liver samples the total aerobic bacteria (TAB) counts were determined using the standard plate count method, using the Bioxon standard count agar (BX-211724, BD-Bioxon™, Becton Dickinson of Mexico). In brief, 1 g sample was homogenized in 9 mL of isotonic saline solution (ISS, 0.9% NaCl). Decimal dilutions up to 10^{-6} were prepared in ISS at 40 °C and were shaken orbitally. The plates were allowed to solidify for 10 min and inverted by incubating (Model Max Q4450, Thermo Scientific, Mexico City, Mexico) at 37 °C for 24 hours. The counts of viable *Escherichia coli* (*E. coli*) were conducted by plating serial 10-fold dilutions onto MacConkey agar plates (BD™ MacConkey II Agar, Becton Dickinson of Mexico) and were incubated for 24 h, under aerobic conditions. The lactic acid bacteria (LAB) and total fungi and yeast were also determined in the jejunum samples. The LAB counting was done in dilutions of 1:1–7 wtvol^{-1} with 0.01% peptone water. Aliquots of 100 µL were added to Petri dishes with Man Rogosa Sharpe medium (MRS) (DIBICO S.A. de C.V., Mexico City, Mexico). Plates were incubated at 35°C for 48 h in a microaerophilic atmosphere (5% O_2) using a microaerophilic container system (GasPak EZ, BD Diagnostics, Sparks MD, USA). The determination of total fungi and yeast was carried out using the selective agar plate extension technique. A1 g sample was homogenized in ISS for 1 min, and decimal dilutions from -2 to -6 were made in test tubes with 9 mL of ISS. Petri dishes were prepared with Potato Dextrose Agar (PDA)(DIBICO S.A. de C.V., Mexico City, Mexico) acidified to pH 3.4. The dishes were incubated at 25 °C during four days, and then the colonies were counted.

The samples from the duodenum, lower jejunum and liver were fixed in formalin, dehydrated with an alcohol-xylene sequence, and embedded in paraffin. Three pieces of 5-µm slices were prepared and stained with hematoxylin-eosin. The histopathological changes were observed under light microscope by an experienced avian veterinarian who was blind to treatment allocations. Based upon severity of the lesions, a score of 0 (no lesions), 1 (mild lesions), 2 (moderate lesions), 3 (severe lesions) or 4 (extremely severe lesions) were recorded for each sample. The average number of lesion scores were recorded per each tissue per bird within each treatment.

Statistical analysis

Data were subjected to ANOVA using the General Linear Model of SAS (1990). Differences among means were tested by the LSD method. There were 40 replicates per treatment for the growth performance and carcass variables that were individually registered. The ileal apparent digestibility of dietary components was determined in eight replicate samples per treatment, and in each replicate, the ileal content of five broilers were mixed; the results were transformed to arcsine before analysis. The microbial counts were performed in six replicate samples per treatment; for each replicate, the jejunum or ceca content of three broilers were mixed. Results of microbial counts were expressed as $\log_{(10)}$ of colony-forming units per gram ($\log_{(10)}$CFU g^{-1}).There were six replicate measures per treatment as the lesion scores were also transformed to $\log_{10}$ before the analysis.

RESULTS AND DISCUSSION

Several modes of action have been proposed to explain the benefits observed in broiler chickens supplemented with HS; one of such mechanisms is the ability to créate protective layer sover the epitelial mucosal membrane of the digestive tract against the penetration of toxic and other bacterial contaminated substances (Kühnert *et al.,* 1991; Maguey-Gonzalez *et al.,* 2018b; Mudroňová *et al.,* 2020). In previous research (Maguey-Gonzalez *et al.,* 2018b) chicks fed with HA extracted from the same worm compost as the one used in the present study and subjected to feed restriction for 24 h, to induce intestinal inflammation, showed higher intestinal viscosity and lower bacterial liver translocation compared to control chicks. Higher intestinal viscosity, and hence, the ability of HS to créate protective layers, have been linked to the high capacity of HS to form aggregates within solutions (Peña-Mendez *et al.,* 2005). In the present experiment, the HNSPs was used as a mean to cause intestinal inflammation considering that in previous research the feeding of HNSPs diets was applied to induce the disruption of the intact barrier of the gastrointestinal tract (Kuttappan *et al.,* 2015; Tellez *et al.,* 2014). In this model, broilers were fed with rye based diets and showed increased intestinal viscosity and gut permeability, but reduced bone strength and bone mineralization. In the present experiment, it was expected that the addition of HS on broilers fed the HNSPs diet would reduce the deleterious effects of feeding higher amounts of NSPs on the integrity of the digestive mucosa.

In the present study, the interaction of type of diet and growth promoter was statistically significant in four variable responses. The first interaction of the type of diet and the growth promoter was observed on the FCR (p<0.05; Figure 1A). In broilers fed the Control diet the FCR in the EHS-fed was similar to the AGP-fed birds and was lower compared to that of the NAGP group. This result is in agreement to previous reports in which lower FCR was found in broiler chickens supplemented with HS either in the feeds (Kocabagli *et al.,* 2002; Ozturk *et al.,* 2012; Taklimi *et al.,* 2012) or in the drinking water (Ozturk *et al.,* 2010; Rizal & Marlida, 2013; Gómez-Rosales & Angeles, 2015). On the other hand, in broilersfedtheHNSPsdietthe FCR was lowest in the AGP-fed compared to the NAGP-fed group, and was intermediate on the EHS-fed birds. The FCR in EHS-fed broilers increased about 6.3% when using the HNSPs diet compared to those fed the Control diet.

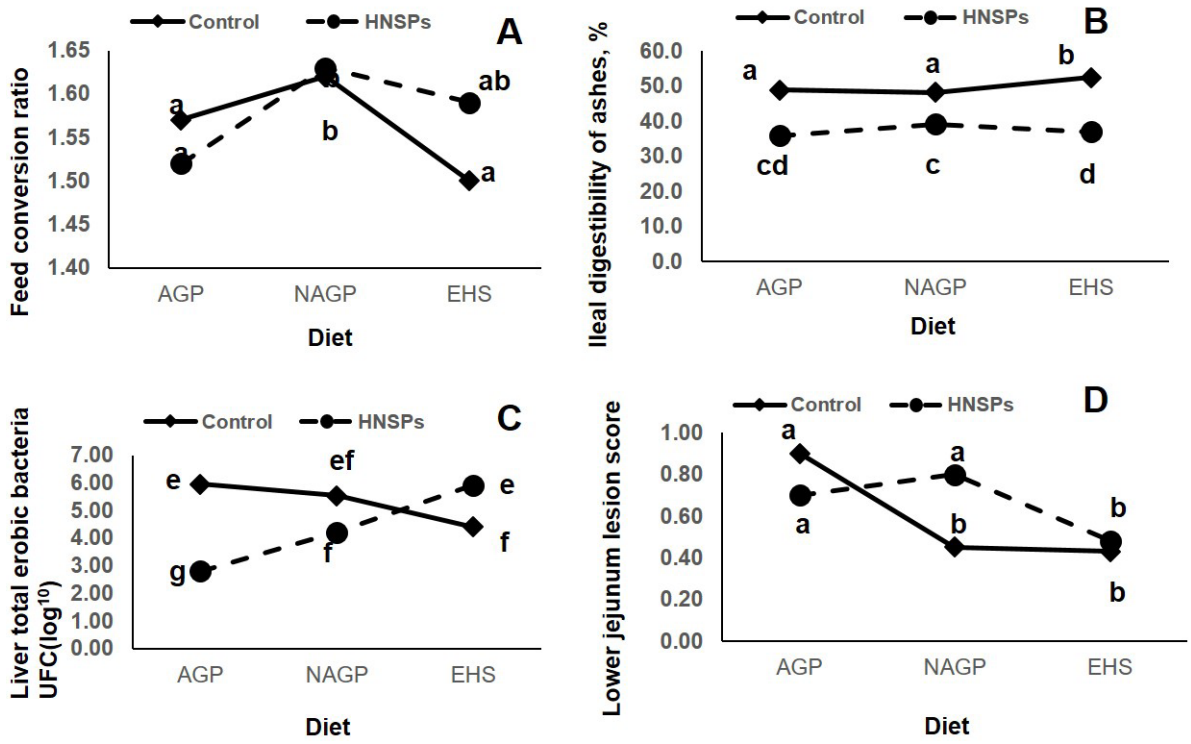

Figure 1 – Effect of the two way interaction of A) Type of diet and Growth promoter on the feed conversion ratio (SEM = 0.036, p<0.05), B) Ileal aparente digestibility of ashes (SEM = 1.259, p<0.05), C) liver total aerobic bacteria (SEM = 0.576, p<0.01), and D) Lower jejunum lesión score (SEM = 0.103, p<0.05). The treatments consisted of the combination of two types of diets: Control = formulated with corn and soybean meal as main feed ingredients, and HNSPs diet (high non-starch polysaccharides) formulated with corn and soybean meal plus 7 % distillers dried grain with solubles and 12 % of wheat bran, and three Growth promoters: AGP = diet added with bacitracin methylene disalicylate and salinomycin as antibiotic growth promoters. [a-d]Means lacking a common superscript are significantly different at p<0.05. [e-g]Means lacking a common superscript are significantly different at p<0.01.

The second interaction of the type of diet and growth promoter was found on the ashes ileal digestibility (p<0.05; Figure 1B). In broilers fed the Control diet, the ashes digestibility was higher in EHS-fed compared to the AGP and NAGP-fed birds. This result also agrees to previous research in which increases in the retention of ashes and the tibia content of ash and Ca have also been reported in broilers supplemented with HS (Eren *et al.,* 2000; Gómez-Rosales & Angeles, 2015; Disetlhe et al, 2017; Jad'uttová *et al.,* 2019). However, in broilers fed the HNSPs diet the ashes digestibility was higher in the NAGP compared to AGP control birds, and was intermediate on the EHS-fed bids. The ashes digestibility in EHS-fed broilers dropped dramatically by about 30 % when using the HNSPs compared to the Control diet.

The third interaction of the type of diet and growth promoter (p<0.01) was found on the TAB counts on the liver (p<0.01; Figure 1C). In broilers fed the Control diet the TAB were lower in EHS-fed compared to the AGP and NAGP-fed birds. This finding agrees with a previous research (Maguey-Gonzalez *et al.,* 2018b) in which chicks fed HA extracted from the same worm compost as the one used in the present study and subjected to feed restriction for 24 h, to induce intestinal inflammation, showed higher intestinal viscosity and lower bacterial liver translocation compared to control chicks. The reduction of the bacterial liver translocation was associated to a reduction on the mucosal permeability by the HA (Maguey-Gonzalez *et al.,* 2018b). Opposite to this, in broilers fed the HNSPs diet, the TAB counts in the liver were highest in the EHS-fed, were lowest in the AGP and intermediate on the NAGP-fed birds.

The results of these three interactions indicate that in EHS-fed broilers receiving the HNSPs diet had negative effects on the FCR, ashes digestibility and TAB counts in the liver compared to broilers supplemented with ESH but fed the Control diet. These findings suggest that the combination of ESH to the HNSPs diet probably had an additive effect on intestinal viscosity which caused a reduction on the efficiency of nutrient digestion and absorption, and also worsen the damage due to the increased viscosity of the epithelial integrity which lead to an increased permeability of the TAB through the mucosal barrier. This topic deserves further clarification in future studies.

The last interaction between the type of diet and growth promoter was observed on the lesion scores in the lower jejunum (p<0.05; Figure 1D). In broilers fed the Control diet the lesions were lower in the NAGP and EHS-fed broilers compared to the AGP birds; whilst in broilers fed the HNSPs diet, the lesion scores were lowest in the EHS-fed compared to the AGP and NAGP birds. All the lesions scored were below 1 (mild lesions) which means that there were only minor damages on the mucosa. The lower lesion score on the EHS-fed broilers when both Control and HNSPs diets were offered is in accordance to the suggestion that HS has the ability to créate protective layers over the epitelial mucosal membrane of the digestive tract against the penetration of toxic and other bacterial contaminated substances (Gomez-Rosales & Angeles, 2015; Maguey-Gonzalez *et al.,* 2018b; Mudroňová *et al.,* 2020). However, the lower lesion score in EHS-fed broilers receiving the HNSPs diet is in opposition to the higher FCR, reduction on the ashes ileal digestibility and higher TAB counts on the liver found on the same broilers. The explanation for these discrepancias is unknown, but it is posible that the measurement of the lesion scores at the end of the jejunum did not allow to associate the degree of these lesions with the negative effects observed on the FCR, ashes digestibility and TAB counts on the liver. It is posible that the measurements of the lesion in the middle part of the jejunum, in which most of the digestion and absorption of nutrients takes place, may better correlate with performance, digestibility and microbiological response variables.

In Table 2, the results of the growth performance and carcass traits of broilers are depicted. In broilers fed the HNSPs diet, the final bodyweight (2961.68 vs 3023.81 g; SEM = 18.834; p< 0.05), FI (171.39 vs 178.76 g d^{-1}; SEM = 0.912; p< 0.01), WG (110.00 vs 114.37 g d^{-1}; SEM = 1.152; p<0.05), breast weight (731.95 vs 762.41 g; SEM = 9.651; p<0.05) and carcass weight (1246.37 vs 1291.82 g; SEM = 12.511; p<0.01). When chickens are fed high dietary levels of ingredients with high NSPs content such as DDGS and WB, and hence, bulky diets, poor performance is expected due tonegative effects on the FI and the growth performance of broilers. These results are in agreement with previous reports (González-Alvarado *et al.,* 2007; Jiménez-Moreno *et al.,* 2009).

In Table 3, the results of the ileal digestibility of dietary components of broilers chickens are presented. The apparent ileal digestibility of dry matter (73.53 vs 79.61 %; SEM = 0.266; p<0.01), nitrogen (75.88 vs 78.65 %; SEM = 0.587; p<0.01), energy (73.70 vs 79.79%; SEM = 0.297; p<0.01), FDN (15.35 vs 21.20%; SEM = 1.397; p<0.01) and FDA (7.36 vs 12.29%; SEM = 1.649; p< 0.05) were also lower when using the HNSPs diet compared to the responses of

broilers fed the Control diet (Table 3). When chickens are fed high dietary levels of ingredients with high NSPs content such as DDGS and WB, poor performance and lower ileal digestibility of dietary components (Liu *et al.,* 2011; Wickramasuriya *et al.,* 2019; Szambelan et al., 2020; Al-Qahtani *et al.,* 2021), higher intestinal and cecal microbiota abundance as well as greatermicrobial fermentations have been reported (Abudabos *et al.,* 2017; Pérez *et al.,* 2011). For these reasons, high dietary inclusion of feed ingredients with HNSPs is not recommended in broiler chickens due to the negative effects caused on the growth performance. On the other hand, in broilers fed the HNSPs diet, the LAB counts in the jejunum (8.34 vs 7.72 $\log_{(10)}$CFU g^{-1} of content; SEM = 0.207; p<0.050) were higher compared to broilers fed the Control diet. This result is in agreement with the report of Mateos *et al.* (2012) and Tellez *et al.* (2014) in which higher LAB counts were found in the intestine of broilers fed diets with high or moderate levels of NSPs.

Table 2 – Effect of the type of diet (TD) and growth promoter (GP) on the growth performance and carcass traits of broilers chickens.

	Type of diet (TD)			Growth promoter (GP)				*p* -value		
	Control	HNSPs	SEM[1]	AGP	NAGP	EHS	SEM	TD	GP	TD*GP
Initialweight, g	1422.6	1421.7	9.715	1422.6	1428.1	1415.8	11.924	0.751	0.575	0.095
Final weight, g	3023.8[a]	2961.7[b]	19.007	2988.6	2967.0	3022.6	23.328	0.028	0.182	0.786
Feedintake, g/d	178.76[c]	171.39[d]	0.921	173.09	175.74	176.40	1.130	0.001	0.066	0.617
Weightgain, g/d	114.37[a]	110.00[b]	1.163	112.86[fg]	109.93[f]	113.78[g]	1.427	0.018	0.026	0.081
Feedconversionratio[h]	1.57	1.58	0.021	1.56	1.63	1.54	0.026	0.743	0.055	0.045
Breast, g	762.9[a]	732.7[b]	9.120	733.3	752.4	757.6	11.038	0.017	0.256	0.787
Breast, %	24.9	24.6	0.221	24.3	24.9	25.0	0.268	0.335	0.155	0.697
Carcass, g	1292.3[c]	1247.0[d]	12.630	1255.2	1273.8	1279.9	15.287	0.010	0.477	0.388
Carcass, %	42.1	41.8	0.244	41.6	42.1	42.2	0.296	0.295	0.254	0.981

[1] Standard error of the mean.

[a-b]Effect of the Type of diet, p<0.05.

[c-d]Effect of the Type of diet, p<0.01.

[f-g]Effect of the Growth promoter, p<0.05.

[h]Effect of the interaction of Type of diet and Growth promoter, p<0.05.

In broilers fed the EHS diet, the WG (Table 2) was similar compared to AGP but was higher compared to NAGP-fed birds (AGP = 112.86, NAGP = 109.92 and EHS = 113.78 g d^{-1}; SEM = 1.427; p<0.05). In previous reports, higher WG was also reported in broilers supplemented with HS (Ozturk *et al.,* 2010; Ozturk *et al.,* 2012; Taklimi *et al.,* 2012). However, the energy digestibility (Table 3) was lowest in broilers fed the EHS diet, intermediate in NAGP and highest in AGP birds (PC= 77.46, NC = 76.65 and EHS = 76.12%; SEM = 0.364; p<0.05). Higher ileal energy digestibility in broiler chickens and higher ileal digestibility of crude protein and fat in pigs supplemented with HS have been reported (Gómez-Rosales & Angeles, 2015;

Table 3 – Effect of the type of diet (TD) and growth promoter (GP) on the ileal digestibility of dietary components of broilers chickens.

	Type of diet (TD)			Growth promoter (GP)				*p* -value		
	Control	HNSPs	SEM[1]	AGP	NAGP	EHS	SEM	TD	GP	TD*GP
Drymatter, %	79.61[a]	73.53[b]	0.266	77.08	76.55	76.08	0.326	0.001	0.106	0.128
Ashes, %	45.99	37.36	0.727	40.41	41.66	42.95	0.890	0.001	0.145	0.038
Nitrogen, %	78.65[a]	75.88[b]	0.588	78.11	77.07	76.62	0.719	0.002	0.337	0.314
Energy, %[c]	79.79[a]	73.70[b]	0.298	77.46[c]	76.65[d]	76.12[d]	0.364	0.001	0.042	0.262
FDN, %	21.20[a]	15.35[b]	1.397	18.18	20.57	16.07	1.711	0.005	0.190	0.317
FDA, %	12.29[e]	7.36[f]	1.649	10.66	11.29	7.51	2.019	0.041	0.374	0.378

[1] Standard error of the mean.

[a-b]Effect of the Type of diet, p<0.01.

[c]Effect of the interaction of Type of diet and Growth promoter, p<0.05.

[d-e]Effect of the Growth promoter, p<0.05.

[f-g]Effect of the Type of diet, p<0.05.

Písaříková *et al.,* 2010) which do not agree with the lower energy digestibility in the present study. The reason for this contrasting result is unknown, but it seems that the energy digestibility in EHS-fed broilers fell 0.6 and 2.9 % with the Control and HNSPs diet respectively, which indicates that the overall mean of the energy digestibility was strongly influenced by the higher drop in the HNSPs diet.

In Table 4, the results of the microbiology and histopathology of broilers chickens are shown. In the jejunum of broilers fed the AGP diet the counts of TAB (AGP = 6.09, NAGP = 8.38 and EHS = 8.62 $\log_{(10)}$CFU g^{-1} of content; SEM = 0.215) and *E. coli* (AGP = 6.00, NAGP = 7.95 and EHS = 8.08 $\log_{(10)}$CFU g^{-1} of content) were lower (p<0.01) compared to those fed the NAGP and the EHS treatments. In the ceca of broilers fed the AGP diet the counts of TAB (AGP= 5.88, NAGP = 8.76 and EHS = 8.61 $\log_{(10)}$CFU $g^{-1}$of content; SEM = 0.124) and *E. coli* (AGP = 5.09, NAGP = 8.08 and EHS = 8.61 $\log_{(10)}$CFU g^{-1} of content;) were also lower (p<0.01) compared to those fed the NAGP control and the EHS-diet. In line with our results, broiler chickens fed with BMD as growth promoter showed reductions of the population of *E. coli* in the small intestinal contents compared to control bids without BMD (Park *et al.,* 2016). Furthermore, broiler chickens added with BMD and challenged with an oral or intramuscular inoculation with *E. coli* showed reductions the population of *E. coli* in the ceca and improved WG, FI, FCR and villi height and width compared to the control bird (Manafi *et al.,* 2017; Daneshmand *et al.,* 2019).

In opposition to BMD, in *in vitro* and *in vivo* studies in which the effect of HS on the growth of *E. coli* have been evaluated, contrasting results have been found. In *in vitro* trials, the use of natural HS from peat and lignite had insignificant growth inhibition of *E. coli* (Yarkova, 2011) and the use of 87 sources of HS, mainly from soils, did not show any antimicrobial activity against *E. coli* (Ansorg & Rochus, 1978). However, inan *in vitro* study in which a modified FA by wet oxidation (oxifulvic acid) was used the growth of *E. coli* was effectively inhibited. In *in vivo* studies in which broiler chickens were fed diets added with HS, the use of a commercial product containing HS caused lower *E. coli* counts in the digesta content from the small intestine and ceca (Aksu & Bozkurt, 2009), but in broilers added with a mined humate compound the *E. coli* populations in the ceca were between 10-100 times greater compared to the control group (Shermer *et al.,* 1998). The last report agrees with our findings and also two of the in vitro studies (Ansorg & Rochus, 1978; Yarkova, 2011).

CONCLUSION

In EHS-fed broilers, improved FCR and ileal ashes digestibility and reduced liver TAB when fed a Control diet were found, but in EHS-fed broilers receiving the

Table 4 – Effect of the type of diet (TD) and growth promoter (GP) on the microbiology and histopathology of broilers chickens.

	Type of diet (TD)			Growth promoter (GP)				*p*-value		
	Control	HNSPs	SEM[1]	AGP	NAGP	EHS	SEM	TD	GP	TD*GP
Lower jejunum										
Total aerobic bacteria	7.36[a]	8.03[b]	0.176	6.09[c]	8.38[d]	8.62[d]	0.215	0.014	0.001	0.131
E. coli	7.15	7.53	0.206	6.00[c]	7.95[d]	8.08[d]	0.253	0.207	0.001	0.656
Lactic acid bacteria	7.72[a]	8.34[b]	0.207	8.31	8.27	8.38	0.191	0.050	0.063	0.512
Yeast	3.02	3.32	0.289	3.33	2.84	3.34	0.354	0.473	0.535	0.092
Fungi	1.76	1.39	0.602	1.25	2.50	0.98	0.611	0.602	0.202	0.279
Ceca										
Total aerobic bacteria	7.80	7.70	0.101	5.88[c]	8.76[d]	8.61[d]	0.124	0.495	0.001	0.252
E. coli	6.96	7.00	0.097	5.09[c]	8.08[d]	7.77[d]	0.132	0.792	0.001	0.344
Liver										
Total aerobic bacteria[e]	5.29	4.30	0.390	4.37	4.88	5.15	0.478	0.088	0.514	0.010
E. coli	2.64	2.43	0.322	2.11	2.35	3.17	0.394	0.651	0.167	0.132
Lesion score										
Liver[f]	0.85	1.06	0.087	0.91	1.07	0.89	0.107	0.439	0.012	0.049
Lowerjejunum	0.59	0.66	0.059	0.80	0.63	0.45	0.073	0.099	0.472	0.677

[1] Standard error of the mean.

[a-b]Effect of the Type of diet, p<0.01.

[c-d]Effect of the Growth promoter, p<0.05.

[e]Effect of the interaction of Type of diet and Growth promoter, p<0.01.

[f]Effect of the interaction of Type of diet and Growth promoter, p<0.05.

HNSPs diet these three variable responses were negatively affected. The findings suggest that the combination of ESH and the HNSPs diet probably increased the intestinal viscosity damaging the epithelial integrity leading to increased permeability, which explains the higher FCR and liver TAB. The lesion scores in the lower jejunum were reduced and the WG was similar in EHS-fed broilers compared to the AGP-fed birds.

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