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Humic substances improve intestinal mucosal integrity in broiler chickens fed two different diets and challenged with a lipopolysaccharide

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ABSTRACT

Objective: To evaluate tibial measurements, microscopic alterations, and goblet cell (GC) counts in the jejunal villi, as well as serum liver biochemical profiles, interleukin responses, and jejunal integrity indices in broilers supplemented with humic substances (HS) derived from vermicompost, subjected to dietary formulation changes and challenged with lipopolysaccharides.

Design/Methodology/Approach: From 7 to 21 days of age, broilers were assigned to four treatments in a factorial arrangement involving two growth promoters AGP: 0.05% bacitracin methylene disalicylate (BMD) and 0.05% nicarbazin; or HS: 0.33% HS and two feeding systems: Continuous diet, consisting of Diet A (corn-soybean meal-based) offered continuously; or Alternating diets, with Diet A alternated every two days with Diet B (sorghum, soybean meal, and canola meal-based). Data were analyzed using analysis of variance.

Results: Broilers receiving HS exhibited significantly higher productivity parameters ($P<0.05$), greater tibial dimensions, more pronounced microscopic alterations, and higher GC counts in the jejunal villi compared to the AGP group, irrespective of the feeding system. Additionally, HS supplementation upregulated mucin-2 and occludin gene expression in the jejunum, whereas claudin-1 (CLDN-1) and immunoglobulin A (IgA) levels remained unaffected ($P>0.05$).

Limitations/Implications: Limitations include the small sample size, absence of replication under commercial production conditions, and assessment of only a single HS dose over a short experimental period.

Findings/Conclusions: HS supplementation enhanced the mucoprotective barrier of the jejunal epithelium.

Keywords: Broiler chickens; Humic substances; Goblet cells; Gene expression.

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INTRODUCTION

Humic substances (HS) derived from leonardite and lignite have been investigated for decades, with well-documented beneficial effects in the prevention and control of various medical conditions in both animals and humans (Peña-Méndez *et al.*, 2005; Angeles *et al.*, 2022a; EMEA, 1999). HS are primarily composed of humic acids (HA), fulvic acids (FA), and humins, formed through a complex biotransformation of organic matter (Domínguez-Negrete *et al.*, 2021; Maguey-González *et al.*, 2022). The use of HS from vermicompost produced using domestic animal manures as substrate represents a novel, environmentally friendly alternative that is gaining relevance as a potential replacement for antibiotic growth promoters (AGP) in poultry production, particularly in broiler chickens (Angeles *et al.*, 2022a; Maguey-González *et al.*, 2022; Arif *et al.*, 2019). According to the FAO (2024), more than 60 countries have banned or severely restricted AGP use in poultry, and it is estimated that approximately 55% of global poultry production is transitioning toward alternative growth enhancers, such as prebiotics, probiotics, and organic acids. Recent research has demonstrated that HS exert their effects through multiple mechanisms, including metal ion chelation, toxin adsorption, antioxidant activity, and modulation of the gut microbiota (Arif *et al.*, 2019; Xu *et al.*, 2023). These mechanisms contribute to the maintenance of intestinal homeostasis, optimization of nutrient absorption, and regulation of immune balance. Several studies have compared the physiological effects of HS with those of probiotics and prebiotics. For example, Gadde *et al.* (2017) reported that direct-fed microbials (DFM) enhanced the expression of tight junction genes (OCLN, CLDN) and reduced intestinal permeability findings consistent with those observed following HS supplementation (Tang *et al.*, 2023; Mudroňová *et al.*, 2021). Additionally, HS have been shown to modulate gut microbial communities, promoting beneficial bacteria and suppressing pathogenic populations (Wang *et al.*, 2020). It has been proposed that HS form protective layers over the digestive mucosa, limiting the translocation of harmful substances and pathogenic bacteria into systemic circulation (Angeles *et al.*, 2022a; Kühnert *et al.*, 1991). This protective action may involve interactions with epithelial glycoproteins and glycoconjugates, leading to reinforcement of the mucus layer and modulation of signaling pathways such as NF-κB, which plays a key role in intestinal inflammation (Xu *et al.*, 2023; Salvo-Romero *et al.*, 2015). Moreover, HS have been shown to upregulate the expression of mucin-secreting glycoproteins (*e.g.*, MUC-2) and tight junction-associated genes (*e.g.*, OCLN), both essential for maintaining intestinal structural integrity and functionality (Mudroňová *et al.*, 2021; Tang *et al.*, 2023). In a recent study evaluating goblet cell (GC) counts in the intestinal villi of broilers subjected to abrupt dietary changes, the inclusion of vermicompost-derived HS mitigated the reduction in GC numbers compared to AGP supplementation. Following a second dietary change, GC numbers decreased again, but the decline was less pronounced in HS-fed broilers compared to the initial dietary shift (López-García *et al.*, 2023). These findings suggest a mucoprotective role of HS in the gastrointestinal tract. Under commercial production conditions, alterations in feed ingredient composition are often associated with impaired intestinal function and inflammatory responses of the mucosa (Kuttappan *et al.*, 2015; Tellez *et al.*, 2014), particularly when incorporating ingredients with anti-nutritional factors such as sorghum and canola meal. The inclusion of these components may negatively impact poultry productivity due to reduced digestibility and nutrient assimilation (Aider & Barbana, 2011; Ambula *et al.*, 2001; Cheng *et al.*, 2022). Consequently, the present study was designed to induce a dietary challenge in order to confirm the mucosal protective effects of HS. This was achieved by alternating a sorghum-canola meal-based diet with a standard corn-soybean meal diet every two days, coupled with an intraperitoneal injection of *Escherichia coli* capsular lipopolysaccharide (LPS) to elicit a more robust immune response both locally in the gut and systemically (Chen *et al.*, 2018). Therefore, the aim of this study was to assess tibial measurements, microscopic alterations, and GC counts in the jejunal villi, alongside serum liver biochemical parameters, interleukin responses, and jejunal integrity indices in broilers supplemented with vermicompost-derived HS, subjected to alternating dietary formulations and challenged with LPS. We hypothesized that dietary inclusion of 0.33% HS would enhance villus length and upregulate tight junction gene expression (OCLN, CLDN-1), while reducing intestinal permeability (measured via FITC-d) under LPS challenge, compared to conventional AGP supplementation.

MATERIALS AND METHODS

Ethical statement

This study was reviewed and approved by the Institutional Subcommittee for the Care and Use of Experimental Animals of the Universidad Nacional Autónoma de México (Protocol No. SICUAE.DC-2021/2-6).

Extraction of humic substances

The HS was extracted from vermicompost produced with sheep manure through alkaline extraction followed by acid precipitation, as described by Domínguez-Negrete *et al.* (2019). In previous studies, its composition and aromaticity were characterized. Briefly, the physicochemical analysis reported by Domínguez-Negrete *et al.* (2021) indicated a pH of 7.8, an ash content of 23.2%, and a humic acid to fulvic acid (HA:FA) ratio of 1.59 (47.1% HA and 29.6% FA). The cation exchange capacity (CEC) was 79.4 cmol(+)/kg. UV-Vis spectra exhibited a λ_max at 260 nm and an E4/E6 ratio of 4.3, while FT-IR analysis revealed characteristic absorption bands at 3400, 1715, 1600, and 1230 cm^{-1} (Peña-Méndez *et al.*, 2005; Maguey-González *et al.*, 2023a). The estimated aromaticity was 53.8% (Domínguez-Negrete *et al.*, 2019; Agregado-Palechor *et al.*, 2023).

Experimental design: Animals, treatments and management

The experimental design employed a 2×2 factorial arrangement, incorporating two growth promoters (AGP or HS) and two feeding systems (continuous or alternating). Forty male Ross 308 broiler chickens, seven days of age, with homogeneous initial body weights (mean ± SD: 130.4±2.1 g), were randomly assigned to treatments using an initial weight blocking strategy to minimize variability. Each treatment consisted of 10 birds housed individually (n=10). Figure 1 presents a flowchart detailing treatment allocation, experimental phases, and the number of birds at each stage.

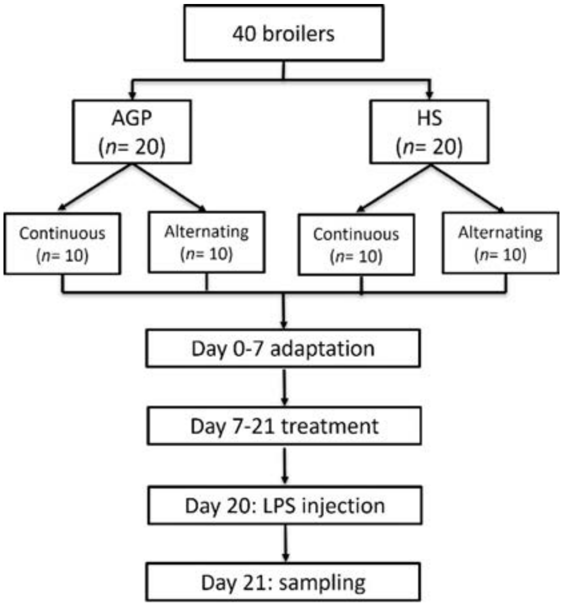

Figure 1. Flowchart of the experimental design and sampling schedule. Forty Ross 308 broiler chickens (7 days old) were assigned to a 2×2 factorial design comprising two growth promoters antibiotic growth promoter (AGP) and humic substances (HS) and two feeding systems (continuous or alternating). All birds underwent an adaptation phase (days 0-7), a treatment phase (days 7-21), a lipopolysaccharide challenge (day 20), and final sampling (day 21). The HS dosage was determined based on results from a previous study (Domínguez-Negrete *et al.*, 2021). In the alternating feeding system, diets A and B were each provided on four separate occasions.

The treatments comprised two types of growth promoters: AGP (0.05% bacitracin methylene disalicylate [BMD] and 0.05% nicarbazin) or HS (0.33% humic substances), and two feeding systems: a continuous diet (Diet A, based on corn and soybean meal, offered without interruption) or alternating diets (Diet A alternated every two days with Diet B, based on sorghum, soybean meal, and canola meal). The composition of the experimental diets is presented in Table 1. Feed and drinking water were provided *ad libitum* throughout the study.

On day 20 of age, all broilers received an intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS), serotype O55:B5 (L4005, Sigma-Aldrich Co., St. Louis, MO, USA), at a dose of 3 mg/kg body weight, a route chosen for its effectiveness in eliciting a consistent systemic inflammatory response in poultry (Chen *et al.*, 2018). On day 21, all birds were euthanized by cervical dislocation for sample collection.

Sampling and laboratory determinations

After slaughter, the left leg of each bird was sectioned, and skin, fat, and muscle were removed. Tibias were weighed before and after drying in a forced-air oven at 100 °C for 24 h to determine dry matter (DM) content. Bones were subsequently incinerated in a muffle furnace at 600 °C for 6 h to determine ash content. Both DM and ash values were expressed as percentages and in grams (Angeles *et al.*, 2022).

A 5 cm segment was collected from the mid-jejunum of each bird. Tissues were cut longitudinally, rinsed with saline solution, and fixed onto cork boards by pinning at both

Table 1. Experimental diets.

	Diet A	**Diet B**
Ground corn	53.56	0.00
Ground sorghum	0.00	54.87
Soybean meal	37.10	31.34
Canola meal	0.00	4.00
Vegetable oil	4.69	5.06
Calcium orthophosphate	1.75	1.73
Calcium carbonate	1.60	1.53
Sodium bicarbonate	0.36	0.43
Salt	0.19	0.11
Methionine	0.23	0.27
Lysine	0.11	0.23
Threonine	0.00	0.04
Choline chloride	0.10	0.10
Vitamin and mineral premix[1]	0.20	0.20
Coccidiostat[2]	0.05	0.05
Antibiotic[3]	0.05	0.05
Calculated nutrient content		
Metabolizable energy, kcal kg^{-1}	3.05	3.05
Crude protein, %	22.67	21.81
Digestible Lys, %	1.21	1.21
Digestible Met, %	0.55	0.56
Digestible Thr, %	0.77	0.77
Ca, %	1.00	1.00
Available P, %	0.50	0.50
Na, %	0.21	0.21
Cl, %	0.17	0.17

[1] Each kg provided: 6,500 IU vitamin A; 2,000 IU vitamin D_3; 15 IU vitamin E; 1.5 mg vitamin K; 1.5 mg thiamine; 5 mg riboflavin; 35 mg niacin; 3.5 mg pyridoxine; 10 mg pantothenic acid; 1,500 mg choline; 0.6 mg folic acid; 0.15 mg biotin; 0.15 mg vitamin B_{12}; 100 mg Mn; 100 mg Zn; 50 mg Fe; 10 mg Cu; and 1.0 mg I.; [2] Nicarbazin; [3] Bacitracin methylene disalicylate.

ends, with the serosal surface in contact with the cork and the mucosal surface exposed, preserving its natural shape for subsequent villus morphometric analysis. Samples were fixed for 24 h in 10% buffered formalin, rinsed with distilled water and phosphate-buffered saline, and processed using an automated tissue processor (STP 120, Microm, Tarragona, Spain) with ethanol in ascending concentrations (75%, 90%, and 100%, 6 h each), followed by xylene (6 h), and paraffin embedding at 60 °C for 6 h. Paraffin blocks were sectioned at 5 μm using a rotary microtome (Microm HM 335 E, Germany). Sections were mounted on slides and independently stained as follows: (A) hematoxylin and eosin for villus morphometry (height, thickness) and crypt depth; (B) periodic acid-Schiff for quantification of neutral goblet cells (GC); (C) Alcian blue at pH 1.0 for strongly sulfated acidic GC; and (D) Alcian blue at pH 2.5 for non-sulfated acidic GC (López-García *et al.*, 2023). The intestinal villus area was calculated using the formula:

$$(2\pi)\times(thickness/2)\times(height)$$

The villus height-to-crypt depth ratio was also calculated. Measurements were taken from 10 villi and 10 crypts per bird for each treatment using an inverted microscope (DMi8, Leica, Germany) at 50x magnification, equipped with a digital color camera (MC170 HD, Leica, Germany). Images were analyzed with Leica LAS Interactive Measurement Software. Goblet cell (GC) quantification was performed by longitudinally sectioning each villus into three equidistant regions (basal, medial, and apical). Within each region, the number of cells reactive to the respective stain was recorded. For each staining method, GC counts were obtained for each region and for the total villus. Counts were based on 10 villi per bird, analyzed at 200x magnification using an inverted microscope (DMi8, Leica, Germany) with a digital color camera (MC170 HD, Leica, Germany). Images were processed using Leica LAS Interactive Measurement Software. Blood samples were collected and centrifuged at 4 °C for 15 min at 2,500 rpm, and the resulting serum was stored at −80 °C. Serum biochemical parameters including urea (41043, SPINREACT, S.A./S.A.U., Santa Coloma, Spain), alkaline phosphatase (ALP; 41242, SPINREACT), alanine aminotransferase (ALT; 41283, SPINREACT), and aspartate aminotransferase (AST; 41272, SPINREACT) were quantified using a UV-Vis spectrophotometer (GENESYS 10S UV-Vis, Thermo Scientific, USA) following the protocols of the respective commercial kits. Additional blood samples were collected to determine serum concentrations of proinflammatory cytokines interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) as well as mucin-2 (MUC-2). Quantification was performed by ELISA using commercial kits (IL-1: MBS261118; IL-6: MBS268769; TNF-κ MBS2509660; MUC-2: MBS017935; MyBiosource, Inc., San Diego, CA, USA) according to the manufacturers' protocols, and absorbance was read at 450 nm on a microplate reader. On day 21, fluorescein isothiocyanate-dextran (FITC-d, MW 3-5 kDa; 102239527, Sigma-Aldrich Co., St. Louis, MO, USA) was orally administered at 8.32 mg/kg body weight (1 mL/bird). One hour later, blood was collected following the recommendations of Kuttappan *et al.* (2015), centrifuged at 4 °C for 15 min at 2,500 rpm, and serum was stored at −80 °C for subsequent FITC-d analysis. Jejunal samples were collected immediately after slaughter, frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted from 20 mg of tissue using the Aurum™ Total RNA Mini Kit (7326820, Bio-Rad, Hercules, CA, USA). RNA concentration was determined using 1 μL of sample on a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA), and RNA integrity was confirmed by 2% agarose gel electrophoresis. cDNA synthesis was performed with the iScript™ Advanced cDNA Synthesis Kit (1725038, Bio-Rad) following the manufacturer's instructions, using reverse transcription at 46 °C for 20 min and enzyme inactivation at 95 °C for 1 min in a thermocycler (CFX96, Bio-Rad, USA). cDNA was quantified and stored at −20 °C. Expression of mature mRNA was measured by quantitative real-time PCR (qRT-PCR) using EvaGreen Supermix (1864034, Bio-Rad, Hercules, CA, USA). Primer sequences are listed in Table 2. Specificity was verified using the NCBI BLAST tool to ensure no cross-reactivity with unrelated sequences. Primers were synthesized, HPLC-purified, and reconstituted according to manufacturer instructions (Alpha DNA, Canada). Target gene expression was normalized to the expression level of chicken 28S ribosomal RNA (endogenous control).

The qRT-PCR reactions were performed in a total volume of 20 μL, each containing 1,600 ng of cDNA. Amplification was carried out in a thermocycler (CFX96, Bio-Rad, USA) under the following conditions: one cycle of enzyme activation at 95 °C for 5 min; 40 denaturation cycles at 95 °C for 30 s, with annealing and extension temperatures specific to each gene ribosomal RNA gene 28S, occludin (OCLN), and mucin-2 (MUC-2) at 60 °C; claudin-1 (CLDN-1) at 65 °C; and immunoglobulin alpha heavy chain (IgA) at 66 °C followed by one cycle at 4 °C for 5 min for signal stabilization and one cycle at 90 °C for 5 min. Each assay was conducted in duplicate. Absolute mRNA quantification was achieved by generating a linear calibration curve through serial dilutions of the corresponding gene amplicons, ranging from 1×10^{11} to 1×10^{3} molecules/μL. Relative gene expression was calculated using the $2\text{^}-\Delta$Cq method.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in a completely randomized design with a 2×2 factorial arrangement, considering two growth promoters (AGP or HS) and two feeding systems (continuous or alternating diets), applying the General Linear Model procedure of the SAS software package (SAS, 1990). Each treatment included 10 replicates (one bird per cage). For morphometric measurements (villus height, thickness, and area; crypt depth; and villus height-to-crypt depth ratio) and GC counts (neutral, sulfated, and non-sulfated), 10 complete villi per bird were evaluated. Serum liver biochemical indicators, interleukins, MUC-2, and FITC-d concentrations were determined using five replicates per treatment. For IgA, MUC-2, CLDN-1, and

Table 2. Chicken (*Gallus gallus*) gene-specific primers used for RT-qPCR.

Gene name	Accession number	Oligonucleotide sequence (5′-3′)	Direction	Length pb	Author
Mucin 2	NM_001318434	ATTGAAGCCAGCAATGGTGT	F	214	(Hollemans *et al.*, 2020)
		TGACATCAGGGCACACAGAT	R		
Claudin 1	NM_001013611·2	CATACTCCTGGGTCTGGTTGGT	F	100	(Zhang *et al.*, 2017)
		GACAGCCATCCGCATCTTCT	R		
Ocludin	NM-205128·1	ACGGCAGCACCTACCTCAA	F	123	(Zhang *et al.*, 2017)
		GGGCGAAGAAGCAGATGAG	R		
Immunoglobulin alpha heavy chain	S40610	GTCACCGTCACCTGGACTACA	F	192	(Lammers *et al.*, 2010)
		ACCGATGGTCTCCTTCACATC	R		
28S ribosomal ARN gene	DQ018756	GGCGAAGCCAGAGGAAACT	F	62	(Lammers *et al.*, 2010)
		GACGACCGATTTGCACGTC	R		

OCLN gene expression analyses, jejunal samples from two birds were pooled, with five replicates per treatment. Data were tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. All variables met parametric assumptions ($P>0.05$). Significant differences among means were identified using Duncan's multiple range test at $P<0.05$. Least squares means and standard errors of the mean are presented in the results tables.

RESULTS AND DISCUSSION

Growth performance and tibia measurements

Growth performance from 7 to 21 days of age and tibia measurements at 21 days are summarized in Table 3. Final body weight, weight gain (WG), and feed intake (FI) were significantly higher ($P<0.01$) in broilers supplemented with HS compared to those receiving AGP. Additionally, FI was greater ($P<0.05$) in broilers under the alternating feeding system compared to continuous feeding; however, the remaining productive variables did not differ between feeding systems. Table 3 also presents tibia dry matter and ash content at 21 days. No interaction was observed between growth promoter type and feeding system for tibia parameters ($P>0.05$). Growth promoter supplementation influenced tibia composition, with HS-fed broilers showing higher ($P<0.01$) tibia dry matter and ash weights compared to AGP-fed birds. The feeding system had no significant effect on tibia variables.

Concentration of biochemical markers in serum

Table 4 presents the serum concentrations of biochemical markers, interleukins, MUC-2, and FITC-d. No significant effects of growth promoter type or feeding system were observed for ALT, AST, ALP, or urea levels, nor for IL-1, IL-6, or TNF-κconcentrations

Table 3. Productive performance from 7 to 21 days of age and tibia dry matter and ashes content.

Item [a]	Growth promoter				Feeding system			
	AGP	HS	SEM	P<	Continuous	Alternating	SEM	P<
Productive performance								
IBW, kg	0.13	0.13	0.001	0.6843	0.13	0.13	0.001	0.1284
FBW, kg	0.71^{b}	0.79^{c}	0.010	0.0001	0.74	0.76	0.011	0.1926
WG, kg	0.59^{b}	0.66^{c}	0.011	0.0001	0.62	0.63	0.011	0.1311
FI, kg	0.82^{b}	0.93^{c}	0.026	0.0061	0.84^{d}	0.92^{e}	0.026	0.0503
FCR	1.42	1.41	0.049	0.7474	1.38	1.46	0.049	0.3496
Tibia measurements								
DM, %	36.13	36.28	0.225	0.6423	36.36	36.05	0.222	0.3244
DM, g	2.22^{b}	2.60^{c}	0.057	0.0001	2.4	2.42	0.058	0.8063
Ash, %	40.52	41.24	0.287	0.0807	41.16	40.6	0.287	0.1698
Ash, g	0.90^{b}	1.07^{c}	0.028	0.0001	0.99	0.98	0.028	0.8762

Note: WG, weight gain; FI, feed intake; FCR, feed conversion ratio; AGP, antibiotic growth promoter; HS, humic substances; SEM, standard error of the mean; DM, dry matter [a] Least square means with 10 replication per treatment; Values within rows with different superscripts differ significantly, $^{b-c}$ $P<0.01$, $^{d-e}$ $P<0.05$.

Table 4. Serum concentrations of liver enzymes, urea, proinflammatory cytokines, mucin-secreting glycoprotein 2 (MUC-2), and fluorescein isothiocyanate-dextran (FITC-d).

Item ^{a}	**Growth promoter**				**Feeding system**			
	AGP	**HS**	**SEM**	**P<**	**Continuous**	**Alternating**	**SEM**	**P<**
ALT, U/L	8.26	8.315	1.21	0.7683	7.95	8.625	1.219	0.7684
AST, U/L	161.68	162.79	37.41	0.9835	135.84	188.63	37.271	0.3335
ALP, U/L	1181.3	773.5	240.60	0.2513	1104.8	850.0	240.974	0.4670
Urea, mmol/L	0.42	0.32	0.05	0.2113	0.39	0.35	0.052	0.5874
IL-1	243.68	304.31	34.67	0.2471	280.68	267.31	35.62	0.7923
IL-6	419.64	286	72.83	0.2152	329.42	376.23	74.83	0.6610
FNT-α	47.55	35.60	13.34	0.5608	28.46	54.69	12.99	0.1736
MUC-2	7.58	6.46	0.40	0.0606	6.88	7.17	0.41	0.6131
FITC-d	55.87^{b}	36.3^{c}	4.68	0.0001	43.47	48.7	4.68	0.4400

Note: AGP, antibiotic growth promoter; HS, humic substances; IL-1, interleukin 1; IL-6, interleukin 6; FNT-α, tumor necrosis factor alpha; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; SEM, standard error of the mean, ^{a} Least square means with five replication per treatment; $^{b\text{-}c}$ Values within rows with different superscripts differ significantly, $P<0.01$.

($P>0.05$). Serum MUC-2 levels in AGP-fed broilers showed a tendency toward higher values compared to those receiving HS ($P<0.06$). In contrast, serum FITC-d concentrations were significantly higher ($P<0.01$) in AGP-fed broilers than in those supplemented with HS.

Morphometry of the *jejunum villus* and number of goblets cells

The morphometry of the jejunal villi is presented in Figures 1-3. Villus height showed no significant differences among treatments ($P>0.05$; Figure 2A). Villus thickness (Figure 2B) exhibited a significant interaction ($P<0.01$) between growth promoter and feeding system: under a continuous feeding system with AGP supplementation, villus thickness was reduced, whereas HS supplementation under continuous feeding resulted in wider villi. Under alternating feeding, villus thickness was similar between AGP- and HS-fed broilers. Villus area (Figure 2C) was also significantly affected by the interaction between growth promoter and feeding system ($P<0.01$). Broilers receiving AGP with continuous feeding had smaller villus areas compared to the other treatments, which showed similar values. Crypt depth (Figure 3A) displayed a significant growth promoter × feeding system interaction ($P<0.01$). Birds fed AGP with continuous feeding and those fed HS with alternating feeding had shallower crypts, whereas HS-fed broilers under continuous feeding exhibited the greatest crypt depth. The villus height-to-crypt depth ratio (Figure 3B) was likewise influenced by the interaction between growth promoter and feeding system ($P<0.01$). Broilers receiving AGP with continuous feeding and those fed HS with alternating feeding had higher ratios. The numbers of neutral, strogly sulfated acidic, and non-sulfated acidic goblet cells (GC) are shown in Figure 3. In HS-supplemented broilers, the number of neutral GC was greater ($P<0.05$) compared to those receiving AGP (Figure 4A).

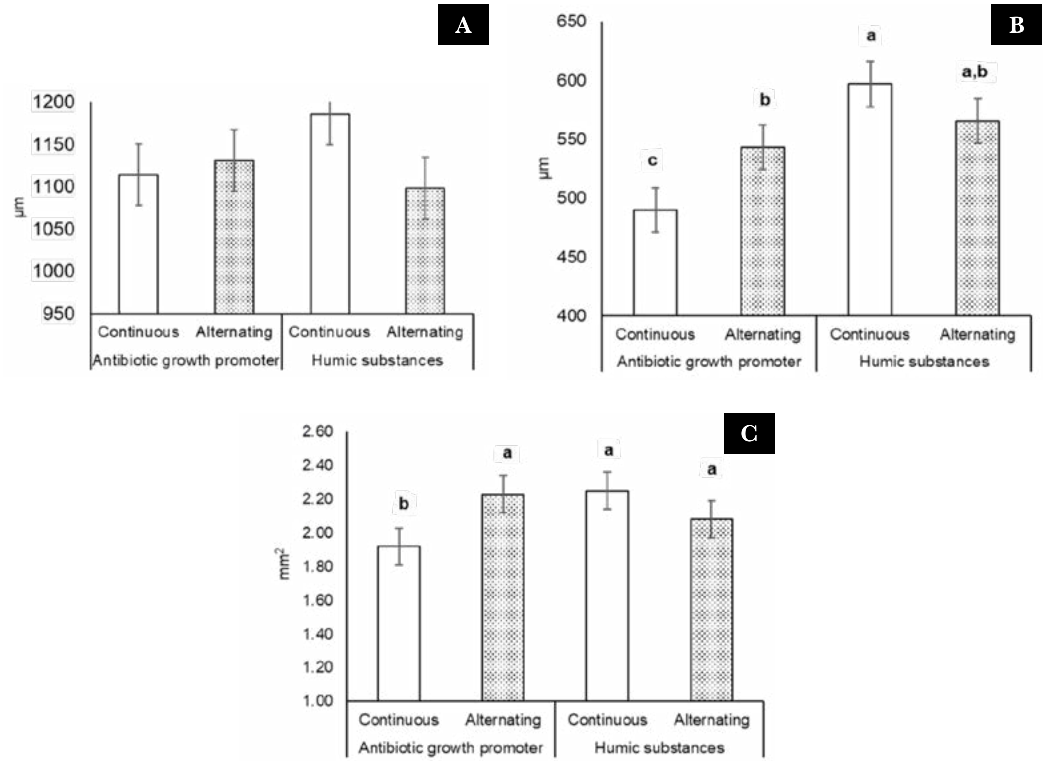

Figure 2. Height of the villi (A, $P>0.05$; SEM=36.282); Thickness of the villi (B, interaction of growth promoter and feeding system, $P<0.01$; SEM=19.175), and Area of the villi (C, interaction of growth promoter and feeding system $P<0.05$; SEM=0.111) in the jejunum. ^{a,b,c} Columns with different superscript are statistically different.

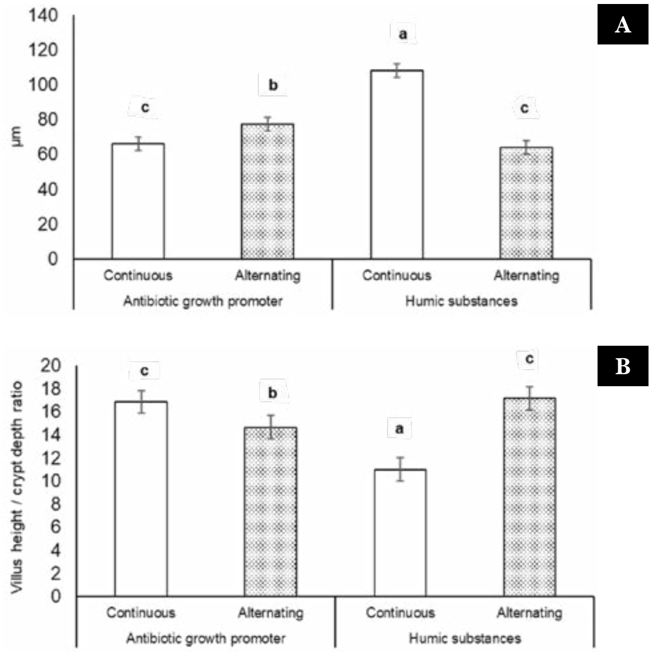

Figure 3. Depth of the crypts (A, interaction of growth promoter and feeding system, $P<0.01$; SEM=3.935) and Villus height/crypt depth ratio (B, interaction of growth promoter and feeding system, $P<0.01$; SEM=1.000). ^{a,b,c} Columns with different superscript are statistically different.

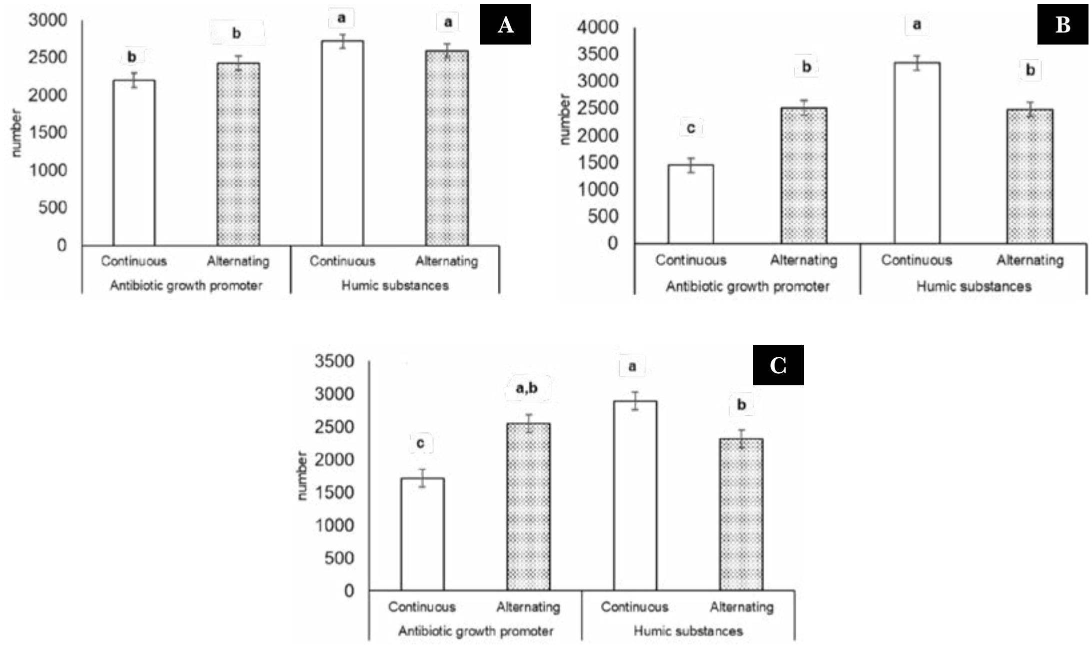

Figure 4. Number of neutral cells (A, effect of growth promoter, $P<0.05$; SEM=93.846); Number of sulfated acid cells (B, interaction of growth promoter and feeding system, $P<0.05$; SEM=135.628); and Number of non-sulfated acid cells (C, interaction of growth promoter and feeding system, $P<0.05$; SEM=133.203) in the jejunum. ^{a,b,c} Columns with different superscript are statistically different.

In the number of sulfated acidic GC (Figure 4B) and non-sulfated acidic GC (Figure 4C), the interaction between growth promoter and feeding system was significant ($P<0.05$). Under the continuous feeding system, broilers receiving AGP had the lowest GC counts, whereas those supplemented with HS had the highest counts; under the alternating feeding system, birds exhibited intermediate values.

Relative quantification of gene expression

The results of the relative quantification of IgA, OCLN, CLDN-1, and MUC-2 gene expression in the jejunal mucosa, determined by real-time PCR, are presented in Table 5. No significant differences in IgA gene expression were observed between AGP- and HS-supplemented broilers, nor between continuous and alternating feeding systems. Growth promoter type influenced OCLN, CLDN-1, and MUC-2 expression: OCLN and MUC-2 expression levels were higher in HS-supplemented birds compared to those receiving AGP ($P<0.01$), whereas CLDN-1 expression was higher in AGP-fed broilers than in those supplemented with HS ($P<0.05$).

Humic substances (HS) derived from vermicompost have been reported to enhance the productive performance of broilers (Maguey-González *et al.*, 2022). In the present study, dietary HS supplementation increased final body weight, weight gain (WG), and feed intake (FI) from 7 to 21 days compared with AGP-fed birds, regardless of feeding system. The absence of differences in feed conversion ratio (FCR) between HS- and AGP-supplemented groups aligns with previous findings, where broilers fed HS from vermicompost showed

Table 5. Relative quantification of immunoglobulin A (IgA), ocludin (OCLN), claudine-1 (CLDN-1) and mucin-secreting glycoprotein 2 (MUC-2) genes expression in jejunum *,†.

	Growth promoter				**Feeding system**			
Genes	**AGP**	**HS**	**SEM**	**P<**	**Continuous**	**Alternating**	**SEM**	**P<**
IgA	0.518	0.507	0.008	0.3598	0.518	0.508	0.008	0.4119
Ocludin	0.224^{b}	0.289^{c}	0.015	0.0007	0.241	0.272	0.015	0.1573
Claudin	0.634^{d}	0.544^{e}	0.025	0.0236	0.618	0.560	0.025	0.1263
MUC-2	0.482^{b}	0.569^{c}	0.016	0.0013	0.518	0.533	0.016	0.5195

* Normalized units of the transcript with housekeeping 28S gene; † Data are means of five replicates in pulls of two chicks/treatment.
Note: AGP=Antibiotic growth promoter, HS=Humic substances; SEM=Standard error of the mean; Values within rows with different superscripts differ significantly, $^{b\text{-}c}$ $P<0.01$, $^{d\text{-}e}$ $P<0.05$.

similar FCR to control groups (Angeles *et al.*, 2022a; Domínguez-Negrete *et al.*, 2019). The inclusion of 0.33% HS in this study did not significantly improve FCR despite positive trends in FI and WG. This outcome could reflect a suboptimal inclusion level, as recent meta-analyses (Gao *et al.*, 2023) suggest that HS effects on productive parameters may be dose-dependent and influenced by their origin and extraction method. Similarly, in previous work where broilers underwent steady digestive conditions interspersed with two abrupt diet changes, no differences in body weight, WG, FI, or FCR were found between HS- and AGP-fed birds (López-García *et al.*, 2023). To increase digestive and productive challenge compared with the previous study, the current experiment incorporated: (a) diet alternation every two days, (b) inclusion of sorghum and canola meal in diet B, and (c) induction of systemic and digestive disturbances via LPS injection. Remarkably, HS-fed broilers under alternating feeding, despite undergoing eight dietary changes over 15 days and consuming sorghum- and canola-based diets four times, maintained growth performance comparable to those under continuous feeding. This pattern was also observed in AGP-fed birds, although their overall growth rate was lower than that of HS-fed birds.

HS possess one of the highest complexation capacities among natural ligands, allowing strong chelation with various ions and potentially enhancing mineral utilization in both plants and animals (Peña-Méndez *et al.*, 2005). Tibia measurements are reliable indicators of mineral assimilation and mucosal integrity in broilers. Increased tibia ash content, Ca and P percentages at 21 days, and higher dry matter and mineral content at 42 days have been reported in broilers receiving HS from vermicompost in drinking water (Angeles *et al.*, 2022; Gómez-Rosales & Angeles, 2015). In this study, HS supplementation increased tibia dry matter and ash weights by 15.38% and 15.89%, respectively, compared to AGP-fed broilers, irrespective of feeding system. Other studies have also reported higher Ca, P, Fe, and Cu in broiler meat (Ozturk *et al.*, 2010; 2012; 2014) and increased Ca, Mg, Zn, and Fe in breast and thigh tissues (Skalická *et al.*, 2019). Higher serum Ca and P concentrations in HS-fed birds have been linked to improved bone strength and reduced locomotor problems (Disetlhe *et al.*, 2017), consistent with the present findings. HS from vermicompost also exerted a trophic effect on the intestinal mucosa, increasing villus thickness (Figure 2B) and villus area (Figure 2C) regardless of feeding system. This enhancement in absorptive surface may contribute to the improved tibia mineralization observed, as nutrient absorption is maximized in the jejunum. Similar effects greater villus height, thickness, and surface area have been documented in HS- or FA-supplemented broilers (Disetlhe *et al.*, 2017; Lala *et al.*, 2017; Taklimi *et al.*, 2012; Mao Y, 2019; Tang *et al.*, 2023). In this study, villus architecture in HS-fed birds was maintained across feeding systems, unlike in AGP-fed broilers, which showed structural variation. The protective role of HS has been attributed to their colloidal properties and stimulation of mucin production, forming a barrier over the gastrointestinal mucosa (Angeles *et al.*, 2022a; EMEA, 1999; Kühnert *et al.*, 1991). Previous research has shown that HA supplementation increased intestinal viscosity, reduced bacterial translocation, and lowered permeability (FITC-d) in feed-restricted broilers (Maguey-González *et al.*, 2018a), and upregulated MUC-2 expression in laying hens (Mudroňová *et al.*, 2021). In the present trial, HS supplementation increased neutral GC numbers by 15% (Figure 4A) compared to AGP-fed birds, regardless of feeding system. Under continuous feeding, HS-fed birds had 56.7% and 40.6% more sulfated acidic GC (Figure 4B) and non-sulfated acidic GC (Figure 4C), respectively, than AGP-fed birds. This suggests that HS protect the mucosa by enhancing mucin secretion, thereby limiting pathogen and toxin contact with enterocytes. Despite the LPS challenge and frequent diet changes designed to induce both intestinal and systemic inflammation, liver enzyme and urea levels were unaffected by treatment, consistent with López-García *et al.* (2023). Similarly, no differences in serum IL-1, IL-6, or TNF-α were found, echoing findings in FA-supplemented broilers (Tang *et al.*, 2023). However, reductions in these cytokines have been observed in HS-fed turkey poults challenged with aflatoxin Bκ(Maguey-González *et al.*, 2023b) and in sodium humate-supplemented pigs and calves (Wang *et al.*, 2020; 2022), as well as in HS-fed mice (Xu *et al.*, 2023). A key difference is that those studies compared HS against unsupplemented controls, whereas this trial compared HS to AGP. No significant differences in jejunal IgA gene expression were detected between HS and AGP treatments, regardless of feeding system, paralleling findings in HS-fed broilers (Mudroňová *et al.*, 2020) but contrasting with increased cecal IgA expression in laying hens (Mudroňová *et al.*, 2021). Similar variable effects have been reported for serum IgA in livestock (Wang *et al.*, 2022; Tang *et al.*, 2023; Maguey-González *et al.*, 2018). This study is the first to assess the effects of vermicompost-derived HS on CLDN-1 and OCLN expression in broiler jejunum. HS increased OCLN expression compared to AGP, whereas CLDN-1 expression was higher in AGP-fed birds. Since OCLN and CLDN-1 share localization and function in tight junctions and are co-regulated through phosphorylation (Salvo-Romero *et al.*, 2015), these opposite effects may reflect a compensatory regulatory mechanism. HS supplementation also upregulated MUC-2 expression compared to AGP, consistent with the hypothesis that HS reinforce the mucus layer. Lower serum FITC-d levels in HS-fed birds indicate improved intestinal barrier integrity, aligning with previous HA studies (Maguey-González *et al.*, 2018a) and with other barrier-enhancing additives such as sodium butyrate (Hu *et al.*, 2021) and essential oils (Mohiti-Asli *et al.*, 2020). The concurrent trend toward lower serum MUC-2 in HS-fed birds may indicate reduced leakage of this glycoprotein into circulation, whereas higher FITC-d and MUC-2 levels in AGP-fed birds suggest epithelial barrier disruption. Limitations of this study include the relatively small sample size (n=10 per group), controlled experimental conditions without commercial-scale replication, a single HS inclusion level (0.33%), and a short evaluation period (21 days post-challenge). Future research should investigate larger-scale production settings, multiple inclusion rates, and longer trial durations to clarify the long-term potential of HS as AGP alternatives in poultry production.

CONCLUSIONS

Supplementation with 0.33% vermicompost-derived humic substances reduced intestinal permeability by 23% (FITC-d, P=0.034), increased villus height by 18% (P=0.021), and enhanced tibial bone strength (P=0.016) in LPS-challenged broilers. Furthermore, increases in neutral goblet cell counts and the expression of MUC-2 and OCLN genes were observed, suggesting an early epithelial repair response. These findings indicate that HS could serve as a partial alternative to AGPs for preserving intestinal integrity, particularly in sorghum-canola-based diets. Further research should evaluate dose-response effects, microbiota modulation, and economic viability under commercial production conditions.

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