Effect of vitamin E and alpha lipoic acid on intestinal development associated with wooden breast myopathy in broilers

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ABSTRACT Intestinal development is closely associated with inflammatory wooden breast (**WB**) myopathy. Vitamin E (**VE**) and alpha lipoic acid (**ALA**) with antioxidant and anti-inflammatory effects were used independently and in combination to evaluate their effects on intestinal developmental changes in ileal morphology and expression of genes related with gut nutrient transport, structure, and inflammation in broilers during the first 3 wk posthatch. A total of 160 newly hatched Ross 708 broiler chicks were randomly assigned into a control and 3 dietary treatments with 10 replicates of 4 birds each. Supplementation of VE (160 mg/kg) and ALA (500 mg/kg) independently and in combination were fed during the first 3 wk. At 1, 2, and 3 wk of age, one chick from each pen was harvested. Plasma VE concentration and ileal morphology were determined. Gene expression was measured by real-time quantitative PCR. Broilers in VE and combination of ALA and VE group had higher plasma VE concentration than the control and ALA group at 1, 2, and 3 wk of age ($P < 0.01$). All dietary treatments increased ileal villus height at 1 wk of age ($P < 0.01$) and decreased intraepithelial lymphocytes at 3 wk of age compared to the control ($P \leq 0.05$). Combination of VE and ALA increased collagen type IV alpha 1 chain expression ($P \leq 0.05$) and improved basement membrane structure indicating increased gut basement membrane integrity at 2 and 3 wk of age compared to the control. Expression of lipopolysaccharide-induced tumor necrosis factor-alpha factor associated with inflammation was decreased in all dietary treatments at 3 wk of age compared to the control ($P < 0.01$). Ileal morphology and gene expression were closely correlated with breast muscle morphology and gene expression. These results suggest that VE and ALA especially when they were combined in the diet had positive effects on mitigating intestinal inflammation and improving nutrient transport beginning at 1 wk of age, which is likely critical in reducing the severity of WB.

Key words: alpha lipoic acid, broiler, intestine, ileal morphology, vitamin E

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INTRODUCTION

Improvement in breast meat production associated with fast-growing heavy weight broilers has resulted in breast muscle myopathies such as wooden breast (**WB**) (Brewer et al., 2012; Sihvo et al., 2014; Tijare et al., 2016; Mazzoni et al., 2020). WB has been identified globally (Kuttappan et al., 2017; Xing et al., 2019; Hasegawa et al., 2020) creating considerable economic losses because of unacceptable meat quality and product downgrades (Sihvo et al., 2014; Kuttappan et al., 2016). WB is phenotypically characterized with a rigid pectoralis major (**p. major**: breast muscle) muscle upon palpation (Sihvo et al., 2014). Histologically, WB-affected breast muscle has impaired morphological structure with moderate or severe myodegeneration such as myofiber necrosis (Sihvo et al., 2014; Clark and Velleman, 2017; Wang et al., 2020a), fibrosis (Velleman and Clark, 2015; Soglia et al., 2016), and inflammatory cell accumulation (Soglia et al., 2016; Sihvo et al., 2017). Breast muscle affected with WB has severe inflammation (Mutryn et al., 2015; Zambonelli et al., 2017), oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019), and dysregulated lipid metabolism (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020).

Gastrointestinal growth and development are closely associated with inflammatory WB myopathy because inflammation is often a systemic process affecting various physiological systems in the entire body (Bourikas and Papadakis, 2009; Chawla, 2011).

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Systemic inflammation can be produced when the gastrointestinal structure and function are negatively altered, contributing to inflammation throughout the entire body including breast muscle (Mafra et al., 2014). Wang et al. (2021) have found that the p. major muscle morphology and expression of genes related with inflammation were closely correlated with intestinal inflammation. Intestinal development during the early posthatch period affects growth performance, breast muscle morphology (Noy and Sklan, 1998; Wang et al., 2021), and inflammation (Dibner et al., 1998) in broiler chicks. In addition, the small intestinal structure and function are sensitive to nutrition early posthatch with intestinal maturation being influenced by nutritional changes (Geyra et al., 2001; Mahmoud and Edens, 2012). Therefore, early posthatch nutritional interventions to reduce intestinal inflammation and oxidative stress will likely influence intestinal development as well as WB development.

Alpha lipoic acid (**ALA**) is a short chain fatty acid with anti-inflammatory properties through inhibiting the release of proinflammatory cytokines such as tumor necrosis factor (**TNF**) alpha and interleukin 6 (Li et al., 2014; Ma et al., 2015). Moreover, ALA has antioxidant effects by scavenging the free radicals thereby reducing oxidative stress (El-Senousey et al., 2013). The antioxidant and anti-inflammatory effects will make ALA better to enhance immunity while improving the antioxidant defense system (Ma et al., 2015). Vitamin E (**VE**) is a powerful antioxidant that can prevent tissue oxidative damage (Niki et al., 1993). DL-α-tocopherol acetate is a commonly used form of VE in the poultry industry (Voljč et al., 2011; Panda and Cherian, 2014). Studies have shown that VE supplementation early posthatch not only reduced WB severity (Wang et al., 2020a,b) but also improved intestinal structure and mitigated intestinal inflammation in broilers at 58 d of age (Wang et al., 2021). Combination of VE and ALA has a synergistic function to enhance antioxidant activity (Gonzalez-Perez and Gonzalez-Castaneda, 2006).

Wang et al. (2021) found that dietary VE and ALA supplemented independently and in combination had positive effects on mitigating WB severity as early as 2 wk of age. This indicates that the beneficial changes in p. major muscle morphology as shown by Wang et al. (2020a) in broilers at market age were probably initiated early posthatch. To identify if the intestinal changes followed a similar timeline as that observed for p. major muscle development, VE and ALA independently and in combination were used to supplement commercial broiler diets immediately after hatch to determine their effects on intestinal development. The intestinal developmental effect was evaluated based on developmental changes in ileal morphological structure and expression of genes related with gut nutrient transport, structure, and inflammation at 1–3 wk of age in commercial broilers.

MATERIALS AND METHODS

Birds and Experimental Diets

All bird protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University. A total of 160 newly hatched commercial Ross 708 broiler chicks were individually weighed, wing banded, and placed into pens immediately after hatch. Chicks were randomly divided into 4 groups, including a control group (corn-soybean meal basal diet), VE (160 mg/kg) supplementation group, ALA (500 mg/kg) supplementation group, and a combination of VE (160 mg/kg) and ALA (500 mg/kg) supplementation group. There were 10 pens per treatment, each pen included 4 birds. Broilers had *ad libitum* access to feed and water. Diets were formulated to meet or exceed all NRC (National Research Council, 1994) nutritional requirements and recommendations in Aviagen's Ross broiler production handbook (Aviagen, 2016). Feed ingredients and calculated nutrient composition in the starter phase and grower phase are shown in Table 1 and Table 2. At 1, 2, and 3 wk of age, one chick from each pen was harvested in accordance with humane and commercial slaughter procedures.

Plasma α-Tocopherol Measurement

Approximately 1 mL of whole blood was collected from each bird after euthanasia. To allow the blood to clot, the blood samples were then placed at room temperature for around 30 min. Plasma was isolated from the coagulated blood by centrifugation (1,500g, 15 min 4°C) and stored at –80°C for further analysis. Plasma α-tocopherol was measured as described by Traber et al. (2017). Briefly, 100 μL of plasma sample was mixed with 1% ascorbic acid and β-glucuronidase, incubated at 37°C for 1 h, and cooled to room temperature. Four milliliter diethyl ether was used to extract the plasma, and an aliquot of the ether fraction was collected and dried under nitrogen. The samples were subsequently resuspended in 1:1 (vol:vol) water:methanol for injection into the liquid chromatography–tandem mass spectrometer (API3000; Sciex, Ontario, Canada) equipped with a turbo ion-spray source that was set to negative mode. The plasma α-tocopherol concentration was calculated from the standard curve that was generated from peak areas of authentic nondeuterated compounds.

Intestinal Morphology

To evaluate intestinal morphology, a 3 cm long section of the ileum was obtained from each broiler. Tissue samples were immediately fixed in 10% (vol/vol) buffered formalin (pH 7.0) and stored at room temperature. Histological samples were dehydrated in a graded series of alcohols, cleared in Pro Par Clearant (Anatech, Battle Creek, MI), and paraffin embedded according to the procedure of (Jarrold et al., 1999). Paraffin blocks were

Table 1. Feed ingredients and calculated nutritional composition of starter diets.[1]

Item	Control	VE	ALA	VE and ALA
Ingredients, % as-fed				
Corn	51.40	51.39	51.35	51.34
Soybean meal	33.62	33.62	33.62	33.62
Poultry byproduct meal	7.50	7.50	7.50	7.50
Sodium chloride	0.22	0.22	0.22	0.22
Limestone	1.10	1.10	1.10	1.10
Dicalcium phosphate	0.46	0.46	0.46	0.46
Premix[2]	0.35	0.35	0.35	0.35
L-Lys HCL	0.15	0.15	0.15	0.15
DL-Met	0.34	0.34	0.34	0.34
L-Thr	0.11	0.11	0.11	0.11
$NaHCO_3$	0.10	0.10	0.10	0.10
Selenium	0.10	0.10	0.10	0.10
Amprolium	1.000	1.00	1.00	1.00
Dl-α-tocopherol acetate	-	0.016	-	0.016
Soy oil	3.55	3.55	3.55	3.55
ALA	-	-	0.05	0.05
Calculated nutrients and energy				
AME, kcal/kg	3,000	3,000	2,999	2,998
Protein, %	23.73	23.73	23.73	23.72
Calcium, %	0.96	0.96	0.96	0.96
Available phosphorus, %	0.48	0.48	0.48	0.48
Digestible Lys, %	1.28	1.28	1.28	1.28
Digestible Met + Cys, %	0.95	0.95	0.95	0.95

[1]Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160 mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the starter phase (0–7 d).

[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

Table 2. Feed ingredients and calculated nutritional composition of grower diets.[1]

Item	Control	VE	ALA	VE and ALA
Ingredients, % as-fed				
Corn	56.56	56.54	56.51	56.49
Soybean meal	28.06	28.06	28.06	28.06
Poultry byproduct meal	7.50	7.50	7.50	7.50
Sodium chloride	0.23	0.23	0.23	0.23
Limestone	1.02	1.02	1.02	1.02
Dicalcium phosphate	0.29	0.29	0.29	0.29
Premix[2]	0.35	0.35	0.35	0.35
L-Lys HCL	0.15	0.15	0.15	0.15
DL-Met	0.30	0.30	0.30	0.30
L-Thr	0.09	0.09	0.09	0.09
$NaHCO_3$	0.10	0.10	0.10	0.10
Selenium	0.10	0.10	0.10	0.10
Amprolium	1.00	1.00	1.00	1.00
Dl-α-tocopherol acetate	-	0.016	-	0.016
Soy oil	4.25	4.25	4.25	4.25
ALA	-	-	0.05	0.05
Calculated nutrients and energy				
AME, kcal/kg	3,102	3,102	3,100	3,100
Protein, %	21.56	21.56	21.56	21.56
Calcium, %	0.87	0.87	0.87	0.87
Available phosphorus, %	0.44	0.44	0.44	0.44
Digestible Lys, %	1.15	1.15	1.15	1.15
Digestible Met + Cys, %	0.87	0.87	0.87	0.87

[1]Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160 mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the grower phase (8–21 d).

[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

cross sectioned at 5 μm and mounted on Starfrost Adhesive slides (Mercedes Medical, Sarasota, FL). The slides were either hematoxylin and eosin stained for measurements of villus height, crypt depth, villus width, distance between villi, and counting of intraepithelial lymphocytes (**IELs**) and epithelial cells number in villi or Periodic Acid Schiff (**PAS**; Thermo Fisher Scientific, Waltham, MA) stained for goblet cell counting and basement membrane structure evaluation. The hematoxylin and eosin staining followed the method as described by Velleman et al. (2002). After deparaffinization and rehydration, the slides were rinsed with hematoxylin for 4 min, running tap water for 10 min, and eosin Y for 2 min, dehydrated and mounted. In terms of the PAS staining, the slides were deparaffinized, rehydrated, incubated in periodic acid for 5 min, washed and incubated with Schiff's reagent for 15 min, and washed in running tap water for 10 min. After staining in hematoxylin for 1 min, slides were dehydrated and mounted. Each slide contained a minimum of 4 sections and imaged with a QImaging digital camera (QImaging, Burnaby, BC, Canada) attached to an Olympus IX 70 microscope (Olympus America, Melville, NY).

At least 4 photomicrographs from each sample were taken for measurement. In hematoxylin and eosin–stained slides, villus height was determined as the distance between the tip of the villi and the villus crypt junction. Crypt depth was measured as the distance of the invagination between 2 adjacent villi. Villus width was measured at the middle part of the villi. Distance between villi was determined as the distance between the adjacent villi at the base of the villi. Measurements were taken in 10 well-structured villi and crypts from each section of each sample using Image J 1.8.0 software (National Institutes of Health, Bethesda, MD). The ratio of villus height to crypt depth was calculated as the ratio of villus height and crypt depth. Surface area of the villi was calculated as $(2\pi) \times$ (villus width/2) $\times$ (villus height) (Solis De Los Santos et al., 2007). IELs number and epithelial cells number in villi were determined. The IELs are small round cells with nucleus centrally located and with little cytoplasm inside (Wilson et al., 1986). Epithelial cells were counted to calculate the number of IELs per 100 epithelial cells. In PAS-stained slides, basement membrane structure was observed and appears as a dense sheet underlying the epithelial cells. Goblet cells were stained purple and were counted as the number of goblet cells per 100 μm of the villi.

RNA Extraction and Real-Time Quantitative PCR

Approximately 0.5 g ileal mucosal scraping was isolated from the ileum and stored at −80°C until use. Total RNA was extracted from ileal mucosal scrapings using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Total RNA concentration was measured with a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI) from total RNA. Briefly, a total of a 13.5 μL mixture containing 1 μg RNA sample, 1 μL 50 μmol Oligo d(T) (Operon, Huntsville, AL), and nuclease-free water were used and incubated at 80°C for 5 min. In total, 0.25 μL RNasin (40U/μL), 1 μL M-MLV (200U/μL), 1 μL 10 mmol deoxynucleoside triphosphate, 5 μL 5 X M-MLV buffer (Promega), and 4.25 μL nuclease-free water were added to the mixture for further incubation at 55°C for 60 min and at 90°C for 10 min. The cDNA samples were then used for real-time quantitative PCR (**qPCR**) analysis using DyNAmo Hot Start SYBR Green qPCR kit (ThermoFisher, Waltham, MA) as described in Velleman et al. (2014). The reaction contained 1 μL of cDNA, 5 μL of DyNAmo Hot Start SYBR Green qPCR master mix, 0.5 μL of primer mixture, and 3.5 μL of RNase-DNase-free water. Solute carrier family 15 member 1 (***SLC15A1***), polypeptide N-acetylgalactosaminyltransferase 2 (***GALNT2***) associated with nutrient transport, mucin 2 (***MUC2***) and collagen type IV alpha 1 chain (***COL4A1***) associated with intestinal structure, lipopolysaccharide-induced tumor necrosis factor-alpha factor (***LITAF***), and interferon gamma (***IFNG***) associated with inflammation were selected as target sequences to be measured. Primer sequences of these genes and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***), are listed in Table 3. The qPCR was performed with the following cycling conditions: denaturation (94°C for 15 min), amplification and quantification (35 cycles of 94°C for 30 s, 53°C for *SLC15A1*, *GALNT2*, *MUC2*, and *COL4A1*; 55°C for *GAPDH*; and 60°C for *LITAF* and *IFNG* for 30 s, and 72°C for 30 s), and final extension (72°C for 5 min). The PCR products were sequenced for its amplification specificity in Molecular and Cellular Imaging Center, The Ohio State University, Wooster, OH. Gene expression was calculated as arbitrary units using the standard curve method based on Liu et al. (2006). Standard curves were constructed for each target gene and housekeeping gene using 10^{-1}–10^{-8} serial dilutions of the purified PCR products. The concentrations of the amplified cDNA samples were within the range of the standard curves to calculate the arbitrary molar concentrations based on their threshold cycle number. Target gene expression was normalized using *GAPDH* expression with each cDNA product concentration being divided by *GAPDH* concentration.

Statistical Analysis

Plasma VE, intestinal morphological attributes, and gene expression were analyzed as a completely randomized design using PROC MIXED procedure of SAS version 9.4 software (SAS Institute inc., Cary, NC). Individual pen was identified as the experimental unit. Dietary treatments were used as a fixed effect. Least square means were estimated with the LSMEANS procedure and separated with the PDIFF option. Significance was accepted at $P \leq 0.05$. To analyze correlation

Table 3. Primer sequences for real-time quantitative PCR.

Gene	Accession number	Forward primer	Reverse primer	Amplicon (bp) size
Nutrient transport				
SLC15A	NM_204365.1	5′-TCCCATGGAGTCAACAGGCT-3′	5′-GCTAGAAACAATGCCGGCTG-3′	160
GALNT2	XM_015284386.2	5′-GCAGGAAGGAGGACCCAAAC-3′	5′-GATCCTGACCAGATCGCACC-3′	171
Gut structural component				
MUC2	NM_001318434.1	5′-CTGGAAGGTTGCTACCCCAG-3′	5′-CTCAATGGATCCTGAGGGGC-3′	183
COL4A1	NM_001162399.3	5′-CTAGGGCCTCCAGGTGTT-3′	5′-AAGGCCCTGTTACTCCTTGC-3′	247
Inflammation				
LITAF	NM_204267.1	5′-TGTGGGGCGTGCAGTG-3′	5′-ATGAAGGTGGTGCAGATGGG-3′	194
IFNG	NM_205149.1	5′-ATGTAGCTGACGGTGGACCT-3′	5′-TCAAGTCGTTCATCGGGAGC -3′	246
Housekeeping gene				
GAPDH	U94327.1	5′-GAG GGT AGT GAA GGC TGC TG-3′	5′-CCACAACACGGTTGCTGTAT-3′	175

Abbreviations: *COL4A1*, Collagen type IV alpha 1 chain; *GALNT2*, Polypeptide N-acetylgalactosaminyltransferase 2; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *IFNG*, Interferon gamma; *LITAF*, Lipopolysaccharide-induced tumor necrosis factor-alpha factor; *MUC2*, Mucin 2; *SLC15A1*, Solute carrier family 15 member 1.

between gut development and WB development, body weight, p. major muscle weight, and p. major muscle morphology and gene expression from Wang et al. (2021; Supplemental table 1) were used for correlation coefficients analysis with ileal morphology and gene expression. Pearson correlation coefficients were determined with the CORR procedure of SAS. The $P \leq 0.05$ was considered as a significant difference.

RESULTS

Plasma α-Tocopherol Concentration

Plasma α-tocopherol concentration in the broilers used in this study is shown in Table 4. Broilers in the VE group and combination of ALA and VE group had higher plasma α-tocopherol concentration than the control and ALA group at 1, 2, and 3 wk of age ($P < 0.01$). Broilers supplemented with a combination of VE and ALA had 20.33% increased concentration of plasma α-tocopherol compared to those supplemented with VE independently at 3 wk of age ($P = 0.04$).

Ileal Morphology

Broiler ileal morphology at 1–3 wk of age is shown in Table 5. Villus height was increased by 4.74, 12.62, and 8.13% in VE, ALA, and combination of VE and ALA groups compared to the control group at 1 wk of age, respectively ($P < 0.01$). There was a 2.64% increase of villus height in the combination of VE and ALA group compared to the control group at 2 wk of age ($P = 0.02$), and a 3.94 and 3.74% increase of villus height in the ALA ($P < 0.01$) and combination of VE and ALA groups ($P = 0.01$) compared to the control group at 3 wk of age. Broilers in the ALA group and combination of VE and ALA group had a 16.08 and 12.04% decrease in the number of IELs compared to the control group at 2 wk of age ($P < 0.01$). Supplementation of VE ($P = 0.03$), ALA ($P < 0.01$), and combination of VE and ALA ($P < 0.01$) decreased the number of IELs by 9.40, 14.25, and 15.50% compared to the control group at 3 wk of age. There was no significant difference in crypt depth, the ratio of villus height to crypt depth, villus width, surface area, distance between villi, or goblet cell number among the treatments within each age ($P > 0.05$). The integrity of the basement membrane was increased in the VE, ALA, and combination of VE and ALA groups, especially in the combination of VE and ALA group, at 2 wk of age compared to the control group as shown in Figure 1. A well-defined basement membrane structure has a robust, uninterrupted structure underneath the epithelium as shown in Figure 1D. The basement membrane structure in the control group was less well defined than the other dietary treatments as shown in Figure 1A.

Ileal Gene Expression

In terms of genes associated with nutrient transport, broilers fed dietary ALA ($P = 0.01$) and combination of VE and ALA ($P < 0.01$) had 6.89 and

Table 4. Effect of vitamin E and alpha lipoic acid on broiler plasma α-tocopherol concentration.

		Treatments[1]					
Item (μM)	Age (wk)	Control	VE	AlA	VE and ALA	SEM	*P*-value
Plasma α-tocopherol	1	26.12^{b}	92.18^{a}	36.28^{b}	90.42^{a}	7.54	<0.01
	2	21.24^{b}	86.24^{a}	31.87^{b}	89.29^{a}	7.36	<0.01
	3	20.02^{c}	57.70^{b}	23.74^{c}	69.43^{a}	3.02	<0.01

$^{a–c}$Means within a row without a common letter are significantly different ($P \leq 0.05$).

[1]Values are means of 10 pens per treatment, each pen included 3 birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160 mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (0–21 d). At 1, 2, and 3 wk of age, one chick from each pen was harvested.

Table 5. Effect of vitamin E and alpha lipoic acid on broiler ileal morphology.

Item	Age (wk)	Treatments[1]				SEM	*P*-value
		Control	VE	AlA	VE and ALA		
Villus height (μm)	1	400.66^{d}	419.66^{c}	451.23^{a}	433.25^{b}	3.47	<0.01
	2	533.19^{b}	524.61^{b}	525.27^{b}	547.27^{a}	3.15	<0.01
	3	623.88^{b}	624.99^{b}	648.52^{a}	647.2^{a}	14.66	<0.01
Crypt depth (μm)	1	76.63	74.75	73.16	80.81	3.51	0.46
	2	89.41	84.33	84.13	86.35	3.59	0.69
	3	92.60	93.07	99.14	87.78	4.95	0.38
Villus/crypt	1	5.33	5.71	6.27	5.47	0.29	0.14
	2	6.03	6.27	6.36	6.39	0.25	0.74
	3	6.76	6.82	6.57	7.51	0.28	0.13
Villus width (mm^2)	1	128.19	112.49	114.25	128.94	6.28	0.14
	2	121.06	124.18	125.77	114.79	4.86	0.4
	3	132.42	139.09	130.99	125.13	8.33	0.74
Surface area (mm^2)	1	0.16	0.15	0.16	0.18	0.01	0.55
	2	0.20	0.21	0.21	0.19	0.01	0.96
	3	0.26	0.27	0.26	0.24	0.02	0.48
Distance between villi (μm)	1	11.43	11.17	10.25	10.81	0.88	0.8
	2	13.84	13.07	12.83	11.31	0.79	0.20
	3	13.19	14.47	14.35	14.2	0.88	0.75
IELs	1	11.99	12.17	12.67	11.67	2.11	0.78
	2	16.11^{a}	16.00^{a}	13.52^{b}	14.17^{b}	0.45	<0.01
	3	13.61^{a}	12.33^{b}	11.67^{b}	11.50^{b}	0.41	<0.01
Goblet cell	1	10.07	10.39	10.95	10.92	0.48	0.51
	2	9.26	9.95	9.47	9.63	0.42	0.73
	3	9.89	10.25	10.35	10.54	0.47	0.79

[a–d]Means within a row without a common letter are significantly different ($P \leq 0.05$).

Abbreviations: Goblet cell, Number of goblet cell per 100 μm of the villi; IELs, Number of intraepithelial lymphocyte per 100 epithelial cells; Villus/crypt, Ratio of villus height to crypt depth.

[1]Values are means of 10 pens per treatment, each pen included 3 birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160 mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (0–21 d). At 1, 2, and 3 wk of age, one chick from each pen was harvested.

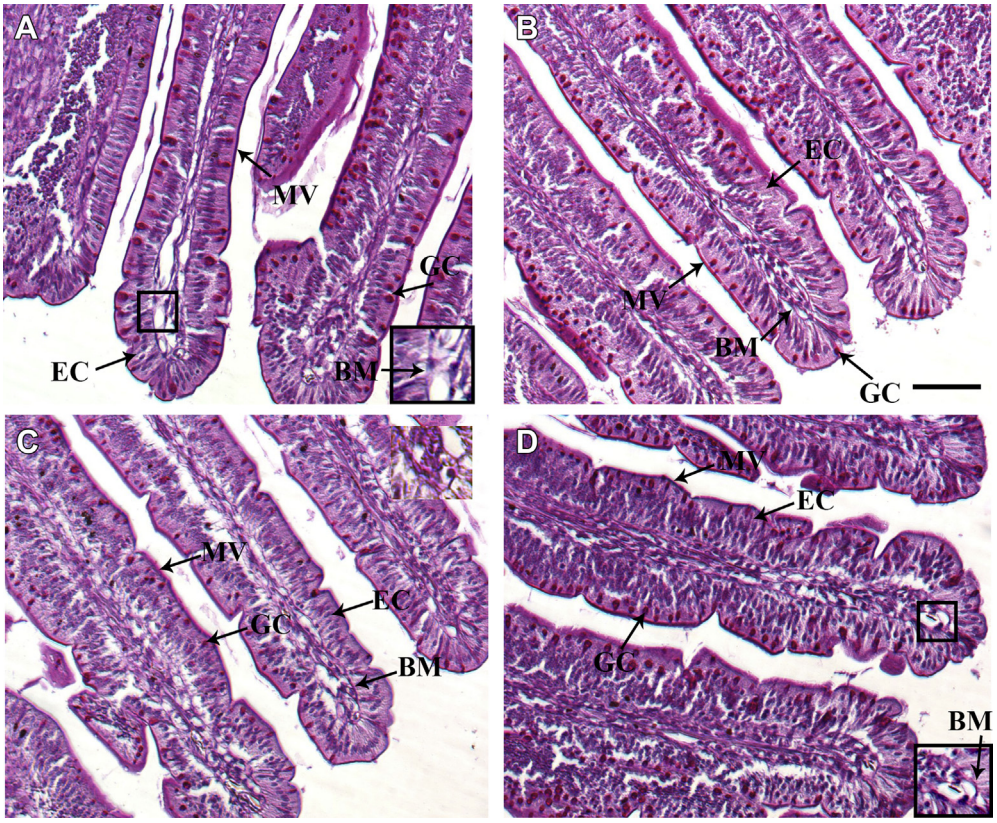

Figure 1. Representative photomicrographs of the broiler ileum at 2 wk of age in the 4 treatments. Broilers in the control group (A) were fed corn-soybean meal basal diet. Vitamin E (VE; 160 mg/kg) was supplemented in the VE group (B) and alpha lipoic acid (ALA; 500 mg/kg) was supplemented in the ALA group (C). Combination of VE and ALA was supplemented in the VE and ALA group (D). The boxes contain enlargements of the basement membrane. Scale bar = 50 μm. Abbreviations: BM, Basement membrane; EC, Epithelial cell; GC, Goblet cell; MV, Microvilli.

8.14% increased *SLC15A1* expression at 2 wk of age compared to the control group (Table 6). Supplementation of VE ($P = 0.04$), ALA ($P = 0.02$), and combination of VE and ALA ($P = 0.01$) had 5.05, 5.53, and 6.00% increase in *SLC15A1* expression at 3 wk of age compared to the control group, respectively. The VE ($P < 0.01$) and combination of VE and ALA ($P = 0.02$) supplementation increased 21.63 and 17.87% *GALNT2* expression at 2 wk of age compared to the control group. Supplementation of ALA ($P < 0.01$) and combination of VE and ALA ($P = 0.02$) increased 13.33 and 10.86% *GALNT2* expression at 3 wk of age compared to the control group. With regard to genes associated with gut structure, *MUC2* expression was increased by 16.19 and 9.46% when broilers were supplemented with VE ($P < 0.01$) and combination of VE and ALA ($P = 0.03$) at 3 wk of age compared to the control group. Dietary VE, ALA, and combination of VE and ALA increased 6.80, 5.77, and 11.50% expression of *COL4A1* associated with intestinal basement membrane integrity at 2 wk compared to the control group ($P < 0.01$). Combination of VE and ALA supplementation increased 4.55% *COL4A1* expression at 3 wk of age compared to the control group ($P = 0.01$). In terms of genes related with inflammation, expression of *LITAF* was decreased by 13.25, 27.56, and 32.19% in VE, ALA, and combination of VE and ALA groups at 3 wk of age compared to the control group ($P < 0.01$). There was no significant difference in *IFNG* expression among the treatments within each age ($P > 0.05$).

Correlation Coefficient of Morphology and Gene Expression

Correlation coefficients between ileal morphology and gene expression and broiler body weight, p. major muscle weight, morphology, and gene expression are shown in Table 7. Broiler body weight and p. major muscle weight were positively correlated with ileal villus height, crypt depth, the ratio of villus height to crypt depth, surface area, and expression of *MUC2*, *SLC15A1*, and *GALNT2* ($P \leq 0.05$). The p. major muscle morphology score, with a higher score representing more well-structured muscle fiber, was positively correlated with ileal villus height, crypt depth, surface area, ileal expression of *MUC2* and *GALNT2* ($P \leq 0.05$). The p. major muscle fiber width was positively correlated with ileal villus height, crypt depth, the ratio of villus height to crypt depth, surface area, and expression of *MUC2* ($P < 0.01$). Expression of the lipid metabolism genes *PPARγ* and *SELE*, which are associated with inflammation in the p. major muscle, was negatively correlated with ileal villus height and the ratio of villus height to crypt depth ($P \leq 0.05$).

DISCUSSION

WB-affected breast muscle is characterized by severe inflammation (Mutryn et al., 2015; Zambonelli et al., 2017) and oxidative stress (Mutryn et al., 2015;

Table 6. Effect of vitamin E and alpha lipoic acid on broiler ileal gene expression.

Item	Age (wk)	Treatments[1]				SEM	*P*-value
		Control	VE	ALA	VE and ALA		
Nutrient transport							
SLC15A1	1	54.90	53.74	55.41	55.52	1.05	0.58
	2	71.41^{c}	73.49^{b,c}	76.33^{a,b}	77.22^{a}	1.34	0.01
	3	70.85^{b}	74.43^{a}	74.77^{a}	75.10^{a}	1.19	0.03
GALNT2	1	2.72	2.81	2.75	2.66	0.10	0.42
	2	3.19^{b}	3.88^{a}	3.45^{a,b}	3.76^{a}	0.16	0.02
	3	4.05^{b}	4.30^{a,b}	4.59^{a}	4.49^{a}	0.15	0.02
Gut structural component							
MUC2	1	30.46	33.92	30.73	34.43	1.69	0.22
	2	62.07	65.43	64.50	63.35	2.21	0.72
	3	141.79^{c}	164.75^{a}	148.37^{b,c}	155.21^{a,b}	4.31	0.01
COL4A1	1	77.31	78.76	80.19	80.23	1.23	0.28
	2	195.79^{c}	209.12^{b}	207.09^{b}	218.30^{a}	3.87	<0.01
	3	132.22^{b}	133.56^{b}	134.97^{a,b}	138.24^{a}	2.51	0.04
Inflammation							
LITAF	1	1.18	1.19	1.12	1.16	0.05	0.81
	2	8.16	8.15	8.07	8.14	0.44	0.86
	3	17.74^{a}	15.39^{b}	12.85^{c}	12.03^{d}	0.81	<0.01
IFNG	1	1.20	1.18	1.18	1.14	0.04	0.59
	2	4.26	4.30	4.13	4.18	0.18	0.82
	3	4.11	4.10	4.09	3.97	0.13	0.84

[a–c]Means within a row without a common letter are significantly different ($P \leq 0.05$).

Abbreviations: *COL4A1*, Collagen type IV alpha 1 chain; *GALNT2*, Polypeptide N-acetylgalactosaminyltransferase 2; *IFNG*, Interferon gamma; *LITAF*, Lipopolysaccharide-induced tumor necrosis factor-alpha factor; *MUC2*, Mucin 2; *SLC15A1*, Solute carrier family 15 member 1.

[1]Values are means of 10 pens per treatment, each pen included 3 birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160 mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (0–21 d). At 1, 2, and 3 wk of age, one chick from each pen was harvested.

Table 7. Correlation coefficients for ileal and breast muscle morphology and gene expression.[1]

P. major muscle characteristics	Villus height	Crypt depth	Villus/crypt[2]	Surface area	*SLC15A1*	*GALNT2*	*MUC2*	*COL4A1*	*LITAF*	*IFNG*
Body weight										
Pearson	0.96	0.53	0.44	0.67	0.23	0.21	0.53	0.18	−0.18	−0.17
P-value[3]	<0.01	<0.01	<0.01	<0.01	0.02	0.02	<0.01	0.06	0.10	0.12
P. major muscle weight										
Pearson	0.93	0.51	0.45	0.64	0.20	0.18	0.51	0.11	−0.15	−0.11
P-value	<0.01	<0.01	<0.01	<0.01	0.04	0.05	<0.01	0.26	0.18	0.30
Morphology score[4]										
Pearson	0.40	0.19	0.15	0.20	0.04	0.19	0.25	0.13	−0.13	−0.17
P-value	<0.01	0.05	0.11	0.03	0.69	0.04	0.01	0.16	0.18	0.08
Fiber width										
Pearson	0.84	0.43	0.40	0.56	0.15	0.16	0.39	0.15	−0.04	−0.04
P-value	<0.01	<0.01	<0.01	<0.01	0.14	0.09	<0.01	0.11	0.71	0.74
PPARγ										
Pearson	−0.38	−0.12	−0.30	−0.15	−0.05	−0.01	−0.18	0.03	0.11	0.13
P-value	<0.01	0.28	0.01	0.18	0.66	0.97	0.10	0.77	0.27	0.21
SELE										
Pearson	−0.25	0.03	−0.21	0.01	0.01	0.12	−0.10	<0.01	0.02	0.01
P-value	0.02	0.79	0.05	0.91	0.98	0.24	0.35	0.97	0.87	0.91

Abbreviations: *COL4A1*, Collagen type IV alpha 1 chain; *GALNT2*, Polypeptide N-acetylgalactosaminyltransferase 2; *IFNG*, Interferon gamma; *LITAF*, Lipopolysaccharide-induced tumor necrosis factor-alpha factor; *MUC2*, Mucin 2; *PPARγ*, Peroxisome proliferator-activated receptor gamma; *SELE*, Selectin E; *SLC15A1*, Solute carrier family 15 member 1.

[1]Pearson correlation coefficient for ileal morphology and gene expression (in columns) and pectoralis major muscle (p. major muscle; breast muscle) morphology and differentially expression genes (in rows).

[2]Villus/crypt = Ratio of villus height to crypt depth.

[3]*P*-value for each Pearson correlation coefficient.

[4]Morphology scoring scale of 1–5 was used for p. major muscle overall morphology evaluation. Samples with limited or no perimysial or endomysial connective tissue space and excessive myofiber degradation were given a score of 1. Samples with morphology score of 5 have ample perimysial and endomysial connective tissue spacing and well-structured muscle fibers. Score of 2–4 are intermediate.

Abasht et al., 2016; Brothers et al., 2019). The development of WB is closely associated with intestinal structure and intestinal inflammatory state (Wang et al., 2021). Previous studies have identified that supplementation with the antioxidant VE early posthatch reduced WB severity (Wang et al., 2020a,b) and reduced intestinal inflammation at market age in broilers (Pitargue et al., 2019; Wang et al., 2021). The beneficial effects of VE and ALA with antioxidant and anti-inflammatory activities on mitigating the WB severity were initiated as early as 2 wk of age of the broilers (Wang et al., 2021). Therefore, effects of VE (160 IU/kg) and ALA (500 mg/kg) independently and in combination on developmental changes in intestinal morphology and expression of genes related with nutrient transport, intestinal structure, and inflammation at 1–3 wk of age in commercial broilers were further investigated in the current study to identify the relationship between intestinal development and the onset of WB in the p. major muscle.

Intestinal structure is an essential attribute reflective of gut nutrient absorption (Celi et al., 2017). Villus height is an important morphological attribute for nutrient absorption and transport in the gastrointestinal system. It was increased in the VE and ALA dietary treatments both independently and in combination beginning at 1 wk of age compared to the control group, with the combination of VE and ALA group having the highest villus height. This is consistent with Yoo et al. (2016) that combination of VE and ALA increased villus height in broilers. The digestive and absorptive capacity could be enhanced with an increased villus height through higher surface area of nutrient absorption and brush border enzymes expression (Yamauchi et al., 1996). The increased villus height in the dietary treatments is suggestive of an improvement in ileal morphology, nutrient uptake, and absorptive efficiency beginning at 1 wk of age from the VE and ALA supplementation both independently and in combination. The combination of VE and ALA had a maximal beneficial effect.

Genes associated with gut nutrient transport were differentially expressed in the dietary treatments as well. The *SLC15A1* expression was increased in ALA and combination of VE and ALA groups at 2 wk of age and in VE, ALA, and combination of VE and ALA groups at 3 wk of age compared to the control group. This is in agreement with Wang et al. (2021) that VE supplementation from 0 to 10 d posthatch increased ileal *SLC15A1* expression compared to the control at 58 d of age in broilers, which suggests that the effect of VE on *SLC15A1* expression in broilers at market age could be initiated as early as 2 wk of age. The SLC15A1, also called peptide transporter 1, belongs to the superfamily proton oligopeptide transporters (Ingersoll et al., 2012). It is responsible for transportation of dipeptides and tripeptides in the enterocytes (Osmanyan et al., 2018), a major regulatory process of protein degradation and utilization (Gaildrat et al., 2005). The higher *SLC15A1* expression is suggestive of greater protein digestion and absorption in the broilers supplemented with VE and ALA independently and in combination compared to the control. Another gene related with nutrient transport, *GALNT2*, had higher expression in

VE and combination of VE and ALA groups at 2 wk of age, and ALA and combination of VE and ALA groups at 3 wk of age compared to the control. The *GALNT2* encodes polypeptide N-acetylgalactosaminyltransferase 2, which is a member of glycosyltransferase 2 protein family (Wu et al., 2011). The polypeptide N-acetylgalactosaminyltransferase 2 then activates mucin type O-glycosylation of peptides with N-acetyl galactosamine transferring to the hydroxyl group of a serine or threonine residue (Ten Hagen et al., 2003). Triglyceride levels are modulated by GALNT2 as well through its regulatory effects on high-density lipoprotein cholesterol (Roman et al., 2015). Increased *GALNT2* expression in the dietary groups supplemented with VE, ALA, and combination of VE and ALA will positively regulate nutrient transport by influencing lipid metabolism. Interestingly, WB development has been previously hypothesized to be associated with the dysregulation fatty acid metabolism (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020). Wang et al. (2021) identified that supplementation of VE and ALA decreased the incidence and severity of WB in broilers at 2 and 3 wk of age. The higher WB severity along with a higher ileal *GALNT2* expression in the control group in the current study suggests that WB severity may be associated with dysregulated intestinal lipid metabolism.

As for intestinal structural components, the intestinal mucus layer is primarily composed of mucin glycoproteins and serves as a barrier protecting the intestinal epithelium from the pathogens (Deplancke and Gaskins, 2001; Velcich et al., 2002). Mucin 2 plays a key role in maintaining the thickness of the intestinal mucus layer contributing to the barrier structure and nutrient absorption (Uni et al., 2003; Horn et al., 2009). Supplementation of VE and combination of VE and ALA had higher expression of *MUC2* at 3 wk of age compared to the control suggestive of improved intestinal barrier integrity and function. Another gene, *COL4A1,* is involved in intestinal basement membrane integrity. Type IV collagen is a triple helix composed of 2 α1 chains and one α2 chain (Qian and Glanville, 1984). The repetitive tripeptide sequence of the triple helix is consisted with amino acids Gly-X-Y repeats, in which X and Y can be any amino acids (Trüeb et al., 1982). Type IV collagen is the primary component of the basement membrane, which supports the epithelial cells in the gut (Brazel et al., 1987). It forms a network interacting with other extracellular matrix molecules such as proteoglycans and non-collagenous glycoproteins (Vercellotti et al., 1985; Basson, 2003). Through these complex interactions, type IV collagen is involved in various cellular activities such as cell adhesion and cell migration (Herbst et al., 1988; Chelberg et al., 1989). Increased *COL4A1* expression in the broilers supplemented with VE, ALA, and combination of VE and ALA at 2 wk of age is a potential indicator of improved basement membrane integrity. Combination of VE and ALA with the highest expression of *COL4A1* suggested the most improvement on the basement membrane structure. This is consistent with ileal morphology that basement membrane structure was better defined in the dietary treatments especially in the combination of VE and ALA group than the control group. Additionally, the increased expression of *COL4A1* along with the increased villus height is consistent with Brautigan et al. (2017) that enhanced intestinal collagen expression was associated with an increase in villus height. With improved basement membrane and villus structure, the epithelium will be adhered more strongly resulting in increased nutrient absorption and transport function. Interestingly, it was found that VE and ALA supplementation reduced the WB severity from 1 to 3 wk of age with combination of VE and ALA having the most significant effect (Wang et al., 2021). This is similar to the current study in which a similar trend was observed with ileal *COL4A1* expression and the basement membrane structure, suggesting that ileal basement membrane integrity may be closely related with WB development.

In terms of inflammation-related attributes, IELs were significantly decreased in ALA and combination of VE and ALA groups at 2 wk of age and in all of the dietary treatments at 3 wk of age compared to the control group. This is in agreement with previous study that VE and polyunsaturated fatty acids supplementation decreased IELs in the broilers at 58 d (Wang et al., 2021). The IELs are interspersed in intestinal epithelial cell layer providing various regulatory functions including cytokine production for the mucosal immune system (Yamamoto et al., 1998; Kakar et al., 2003). The intestinal IELs and their released cytokines activate the subsequent protective immune functions (Kakar et al., 2003; Rieger et al., 2015). The IELs are the site where *LITAF* is expressed (Ateya et al., 2019). As a proinflammatory cytokine, LITAF can be upregulated mediating the host immunity against pathogens (Hong et al., 2006). Same with the IELs, *LITAF* was decreased expressed in VE, ALA, and combination of VE and ALA groups at 3 wk of age compared to the control group. The decreased IELs and *LITAF* expression in the dietary treatments suggest that VE and ALA both independently and in combination reduced intestinal inflammation at 2 and 3 wk of age. The combination of VE and ALA showed the most significant effect on reducing ileal inflammation because of its lowest expression of *LITAF* at 3 wk of age.

The ileal morphological attributes were consistent with the expression of genes associated with gut nutrient transport, structure, and inflammation indicating positive effects of VE and ALA supplementation on improving gut health and nutrient absorption, with the combination of VE and ALA showing a better performance than supplementation of VE or ALA independently. This is consistent with plasma α-tocopherol concentration in the current study. Plasma VE concentration in the combination of VE and ALA group was higher than in the VE group at 3 wk of age, which suggests that plasma VE was more efficiently accumulated in the broilers when ALA was combined supplemented with VE. After being absorbed into the gastrointestinal

tract in the broilers, ALA is rapidly reduced to dihydrolipoic acid, which can react with reactive oxygen species and enhance antioxidative enzymes (Bjørklund et al., 2019). In addition, dihydrolipoic acid can recycle VE from its oxidized form by reducing glutathione disulphide, dehydroascorbate, and semidehydroascorbyl radical, and ubiquinone (Sohaib et al., 2018). This results in the synergistic function of VE and ALA on preventing oxidative stress. As oxidative stress can trigger multiple inflammatory pathways, combination of VE and ALA showed synergistic effects on reducing inflammation as well.

Ileal morphology and gene expression were correlated with broiler body weight, p. major muscle weight, fiber width, and gene expression. Broiler body weight, p. major muscle weight, morphology score with a higher score representing more well-structured muscle fiber, and fiber width were positively correlated with ileal morphology and expression of genes associated with nutrient transport. The positive correlations between body weight, p. major muscle weight, morphology score, fiber width, and ileal morphology and gene expression were also found in broilers at market age (Wang et al., 2021). The positive correlations suggest that improved ileal structure, nutrient absorption, and transport function have a beneficial influence on total growth performance and breast muscle development. Expression of *PPARγ* and *SELE* in the p. major muscle was negatively correlated with ileal villus height and the ratio of villus height to crypt depth. The *PPARγ* is an adipogenic gene regulating lipid metabolism (Hu et al., 1995; Kliewer et al., 1997; Rosen et al., 1999). Expression of *PPARγ* is an important maker for the development of WB as WB has been linked with dysregulation of lipid deposition (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020). The negative correlations between p. major muscle *PPARγ* expression and the ileal morphological attributes indicate that the dysregulated lipid metabolism of the p. major muscle is related with the impaired ileal morphology. This suggests that the WB development may be closely associated with intestinal development. Selectin E is involved in chronic and acute inflammation (Lundberg, 2000; Ley, 2003). The negative correlations between p. major muscle *SELE* expression and the ileal villus height and the ratio of villus height to crypt depth indicate that reduced inflammatory level in p. major muscle was related with improved ileal morphology. This is suggestive that inflammation in p. major muscle has a negative influence on intestinal morphology associated with nutrient absorption. The close correlations between p. major muscle morphology and expression of adipogenic and proinflammatory genes and ileal morphology and expression of genes related with nutrient transport strongly suggest that WB development is influenced by gut health.

In conclusion, supplementation of VE and ALA independently and in combination showed a beneficial effect on improving intestinal morphology at 1, 2, and 3 wk of age. Genes involved in gut nutrient transport, structure, and inflammation were differentially expressed in VE, ALA, and combination of VE and ALA groups at 2 and 3 wk of age compared to the control group. Combination of VE and ALA supplementation showed a more beneficial influence on ileal morphology and expression of genes associated with intestinal structure and inflammation than VE and ALA supplementation independently at 1, 2, and 3 wk of age. Ileal morphology and gene expression were closely correlated with broiler body weight, p. major muscle weight, morphology, and gene expression. Research focused on the mechanism of intestinal development targeting lipid metabolism and responses to inflammation identified in the current study in WB-affected and unaffected broilers will be addressed in future studies.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.12.049.

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