Effect of vitamin E sources and inclusion levels in diets on growth performance, meat quality, alpha-tocopherol retention, and intestinal inflammatory cytokine expression in broiler chickens

F. M. Pitargue, J. H. Kim, D. Goo, J. B. Delos Reyes, and D. Y. Kil[1]

Department of Animal Science and Technology, Chung-Ang University, Anseong-si, Gyeonggi-do 17546, Republic of Korea

ABSTRACT Water-miscible vitamin E (WVE) has been known to be more bioavailable than conventional lipid-soluble vitamin E (LVE) in human foods because of its greater solubility in the gastrointestinal tract. However, no data regarding the comparison of the efficacy of WVE to LVE were available in poultry diets. The objective of the current experiment was to investigate the effect of vitamin E (VE) sources and inclusion levels in diets on growth performance, meat quality, alpha-tocopherol retention, and intestinal inflammatory cytokine expression in broiler chickens. A total of 420 6-d-old broiler chicks were allotted to 1 of 7 dietary treatments with 6 replicates in a completely randomized design. The corn–soybean meal-based basal diet was formulated without inclusion of supplemental VE. Additional 6 diets were prepared by supplementing the basal diet with either WVE or LVE at the inclusion levels of 33, 65, or 100 IU/kg in diets. Diets were fed to birds for 26 d. Results indicated that different VE sources and inclusion levels from 33 to 100 IU/kg in diets had no effects on growth performance in broilers. The VE sources did not affect lipid oxidation in both breast and thigh meat. However, increasing inclusion levels of VE decreased (linear and quadratic, $P < 0.05$) lipid oxidation in breast meat. Regardless of VE sources, increasing inclusion levels of VE increased (linear, $P < 0.01$) alpha-tocopherol concentrations in breast meat. Dietary WVE showed a greater reduction in the expression of both pro- and anti-inflammatory cytokine genes in the jejunum than dietary LVE. Increasing inclusion levels of both VE sources decreased (linear and quadratic, $P < 0.05$) the expression of both pro- and anti-inflammatory cytokine genes in the jejunum. The efficacy of dietary WVE for growth performance, meat quality, lipid oxidation, and alpha-tocopherol retention in broilers chickens is similar to that of dietary LVE. However, it is likely that dietary WVE is more effective in decreasing intestinal inflammatory responses than dietary LVE in broilers.

Key words: broiler chicken, inflammatory response, lipid-soluble vitamin E, lipid oxidation, water-miscible vitamin E

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INTRODUCTION

Vitamin E (**VE**) is a lipid-soluble vitamin, which is well known as an efficient chain-breaking antioxidant preventing oxidative damages to body tissues (Fellenberg and Speisky, 2006; Voljc et al., 2011). In broiler chickens, biological damages induced by oxidative stress result in several pathologies affecting growth and health (Estevez, 2015; Akbarian et al., 2016). Thus, dietary supplementation of VE is a common practice in broiler industry to counteract the deteriorative effects of oxidative stress. Moreover, it was demonstrated that the antioxidant properties of VE can improve the animal health by improving cell-mediated and humoral immunity in broiler chickens (Leshchinsky and Klasing, 2001).

The current recommendation for VE concentrations in broiler diets ranges from 10.0 IU/kg (NRC, 1994) to 80.0 IU/kg (Aviagen, 2014) depending on the stage of growth. However, recommendation levels of VE in diets are also affected by various factors including other antioxidants such as vitamin C and selenium, type and amount of lipids in diets, and environmental conditions (NRC, 1994). Therefore, the ideal inclusion levels of VE in broiler diets are still controversial (Kuttappan et al., 2012). In addition, the hydrophobic nature of VE creates a major challenge in its adequate uptake and transport as it occurs with lipid compounds (Brigelius-Flohe et al., 2002). As a feed supplement, several concerns have been raised about a conventional lipid-soluble VE (**LVE**) such as chemical instability (Gawrysiak-Witulska et al., 2009) and

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[1]Corresponding author: dongyong@cau.ac.kr

differences in bioavailability (Yang and McClements, 2013). These concerns have motivated poultry researchers to search for more efficient sources of dietary VE. For instance, water-miscible VE (**WVE**) has been reported as the therapeutic application for humans who experience with the problem of VE absorption (Harries and Muller, 1971; Soltani-Frisk et al., 2001). It was reported that dietary WVE was more efficient and bioavailable than dietary LVE because WVE was claimed to be more soluble than LVE in an aqueous environment (Muller et al., 1976). However, there have been no data regarding the comparison of the efficacy of WVE to LVE in poultry diets.

Therefore, the objective of the current experiment was to investigate the effect of different VE sources (LVE vs. WVE) and their inclusion levels in diets on growth performance, meat quality, alpha-tocopherol retention, and intestinal inflammatory cytokine expression in broiler chickens.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

All experimental procedures were reviewed and approved by the Animal Care and the Use Committee at Chung-Ang University. A total of 420 1-d-old Ross 308 broiler chickens from a local hatchery (Cheonan, Republic of Korea) were used and raised in 42 battery cages (76 cm × 78 cm × 45 cm = width × length × height for each cage) in an environmentally controlled room. At the initial 5 d of the experiment, all chicks were fed with the same commercial starter diets to prevent the early VE deficiency. At the 6 d of age, chicks were allotted to 1 of 7 dietary treatments with 6 replicates per treatment in a completely randomized design. Each replicate had 10 birds (5 male and 5 female chickens) per cage.

Two sources of VE were used in the current experiment. The commercial feed-grade LVE (20,000 IU alpha-tocopherol acetate/kg) was obtained from a feed ingredient supplier (Genebiotech Co., Ltd., Seoul, Republic of Korea). The WVE was produced by mixing the commercial LVE with an emulsifier to decrease the size of LVE (DaOne Chemical Co., Ltd., Seoul, Republic of Korea). The potency of WVE (20,000 IU alpha-tocopherol acetate/kg) was validated as the same to that of LVE. A corn–soybean meal-based basal diet was formulated to meet or exceed the nutrient and energy recommendation of the Ross 308 manual (Aviagen, 2014; Table 1). However, the supplemental VE was omitted from the vitamin premix for the purpose of using no supplemental VE in the basal diet. In addition, we tried to minimize VE concentrations in the basal diet to decrease VE contributions from the basal diet in treatment diets. This is the reason why tallow was used as an energy ingredient in the basal diet. Tallow contains the least amounts of VE among conventional fat sources (Sauvant et al., 2004). The VE concentration of the basal diet calculated from tabulated values for feed ingredients (NRC, 1994) was 11.6 IU/kg, which originated mainly from natural feed ingredients and the concentration was considered marginal for meeting VE requirements of NRC (1994). Additional 6 diets were prepared by supplementing the basal diet with either LVE or WVE at the inclusion levels of 33, 65, or 100 IU/kg at the expense of celite. These inclusion levels were designed to produce treatment diets containing moderately deficient, recommended, or moderately excess VE, based on the Ross 308 manual (Aviagen, 2014). The analyzed concentrations of alpha-tocopherol in 7 dietary treatments approximated to the expected VE concentrations (Table 2).

Table 1. Composition and nutrient content of the basal diet.[1]

Items	Amounts, %
Ingredients	
Corn	41.22
Soybean meal, 46% CP	37.70
Wheat	8.00
Tallow	7.70
Monodicalcium phosphate	1.54
Limestone	1.26
Celite	1.00
DL-Methionine	0.36
L-Lysine sulfate	0.22
L-Threonine	0.08
Salt	0.20
Sodium bicarbonate	0.20
Vitamin premix[2]	0.20
Mineral premix[3]	0.12
Choline chloride	0.10
Coccidiostat	0.10
Total	100.00
Calculated energy and nutrient content[4]	
AME_n, kcal/kg	3,101
CP, %	21.49
Total lysine, %	1.30
Total methionine + cysteine, %	1.00
Total methionine, %	0.65
Total threonine, %	0.89
Total tryptophan, %	0.27
Calcium, %	0.87
Non-phytate phosphorus, %	0.44

[1]Vitamin E as an alpha-tocopherol acetate was included in the diet by replacing the same amounts of celite.

[2]Provided per kg of the complete diet: vitamin A, 10,000 IU (retinyl acetate); vitamin D_3, 4,500 IU; vitamin K_3, 3.0 mg (menadione dimethpyrimidinol); vitamin B_1, 2.50 mg; vitamin B_2, 6.50 mg; vitamin B_6, 3.20 mg; vitamin B_{12}, 18.0 μg; folic acid, 1.9 mg; biotin, 180 μg; niacin, 60 mg.

[3]Provided per kg of the complete diet: iron, 72 mg ($FeSO_4$); zinc, 120 mg ($ZnSO_4$); manganese, 144.0 mg (MnO); copper, 19.0 mg ($CuSO_4$); cobalt, 1200 μg ($CoSO_4$); selenium, 360 μg (Na_2SeO_3); iodine, 1.5 mg [$Ca(IO_3)_2$].

[4]Calculated values from NRC (1994).

Table 2. Analyzed alpha-tocopherol concentrations of experimental diets.

	Dietary treatments[1]						
	Basal	LVE, IU/kg			WVE, IU/kg		
Item	0	33	65	100	33	65	100
Alpha-tocopherol, U/kg	ND[2]	36	66	106	47	70	114

[1]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.

[2]ND, not detected.

Birds were provided with diets and water ad libitum for 26 d. The room temperature was maintained at 30°C during the first wk and then gradually decreased to 24°C at the end of the experiment. A 23-h lighting schedule was adopted throughout the experiment. The BW gain **(BWG)** and feed intake **(FI)** were recorded at the conclusion of the experiment. Mortality was recorded daily. Feed efficiency **(FE)** was calculated by dividing BWG with FI after adjusting for mortality (Kim et al., 2017).

Sample Collection

At the conclusion of the experiment (i.e., 32 d of age), 1 male broiler chicken per replicate with a BW close to the replicate mean BW (i.e., 6 birds per treatment) was euthanized by CO_2 asphyxiation, and then immediately dissected. This male bird was used for all analyses of meat quality, relative organ weight, alpha-tocopherol concentrations, and gene expressions in the jejunal tissues.

The breast and thigh muscles were excised to measure meat quality and stored at 4°C until further analysis. From the breast muscle, the right portion was used for pH and meat color assays, whereas the left portion was used to determine water holding capacity **(WHC)** and lipid oxidation as a measure of thiobarbituric acid-reactive substance **(TBARS)** values. A portion of the left breast also was collected and stored at −50°C to measure alpha-tocopherol concentrations. Tissue samples from the jejunum were collected from each chicken. These samples were immediately frozen in liquid nitrogen and stored at −85°C until the gene expression of inflammatory cytokines and alpha-tocopherol transfer protein **(TTPA)** was analyzed. Finally, the liver, spleen, thymus, and bursa of Fabricius were also collected and weighed to measure the relative organ weight as a percentage of total BW.

Meat Quality and Lipid Stability

The pH for breast meat was measured at 1-h and 24-h postmortem. The pH was measured at a 1 cm depth using a pH meter (Hanna Instruments, Nusfalau, Romania). The color of breast meat was measured only at 24-h postmortem. A colorimeter (CR-400; Minolta, Tokyo, Japan) was used to evaluate the meat color of the exterior surface of intact skinless breast muscle. The meat color was expressed as lightness (L^*), redness (a^*), and yellowness (b^*) values. The WHC of breast muscle was measured at 24-h and 48-h postmortem according to the method of Lee et al. (2017). Briefly, 1.5 g of meat samples was centrifuged at 5°C at the speed of 3000 × *g* for 15 min. The WHC was determined as: WHC (%) = [(weight before centrifugation − weight after centrifugation)/weight before centrifugation] × 100.

The breast and thigh samples (without skin) were analyzed for lipid oxidation by measuring TBARS values during 7 d of the storage at 4°C. The TBARS value was determined as described by Lee et al. (2017). In short, 5 g of meat sample were weighed and placed into a 50 mL test tube and homogenized with 15 mL of deionized distilled water for 10 s at the highest speed. Afterwards, 1 mL of homogenate was transferred into a disposable test tube (13 mm × 100 mm), to which 50 μL of 100 mL/L butylated hydroxyanisole and 2 mL of TBA/trichloroacetic acid were added. The mixture was then vortexed and incubated in a boiling water bath for 15 min for the color development and cooled in an ice bath for 10 min. The samples were vortexed again and centrifuged for 15 min at 2000 × *g*. The absorbance of the resulting supernatant solution was determined at 531 nm using a spectrophotometer (SpectraMax® 190; Molecular Devices LLC; San Jose, CA, USA).

Determination of Alpha-tocopherol Concentration

Alpha-tocopherol was extracted from diets and breast meat samples by the simplified procedure described by Liu et al. (1996). In short, 1 g of fresh sample was weighed and placed into a test tube. Then, 250 mg of ascorbic acid and 7.3 mL of 11% KOH digestion solution were added. The test tubes were incubated for 15 min at 80°C in a shaking water bath, cooled, and then 4 mL of isooctane was added. The test tubes were then vortexed for 2 min. After the phases separate, an aliquot of the upper layer was transferred to an autosampler vial. All the samples were prepared in duplicates and filtered.

Alpha-tocopherol concentrations in the samples were measured using Ultra Performance Liquid Chromatography system (Milford, MA, USA) as demonstrated by Wong et al. (2014). Briefly, chromatographic separation was conducted at 42°C on a Kinetex PFP column (150 × 2.1 mm, 2.6 μm; Phenomenex, CA, USA). Maximal separation was induced by a binary mobile phase containing methanol and water with gradient conditions at a constant flow rate of 0.38 mL/min. The injection volume was 2 μL. All solvents, standards, and sample solutions were filtered through 0.22 μm nylon filter membranes (Agilent Technologies, Waldbronn, Germany). Fluorescence was detected at the excitation and emission at the wavelengths of 297 and 328 nm, respectively, with a sampling rate of 10 points per second. The wavelength of UV was set at 292 nm with a sampling rate of 20 points per second.

Analyses of Gene Expression of Inflammatory Cytokines and Alpha-tocopherol Transfer Protein

The expression of TTPA gene and inflammation-related cytokine genes in the jejunum was analyzed. The total RNA was extracted from the jejunal tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA,

Table 3. Primers used for quantitative RT-PCR.

Primer name[1]	Primer sequence[2] 5′–3′	Ta[3], °C	Product size, bp	GenBank accession number
GAPDH	F: GGTGGTGCTAAGCGTGTTAT R: ACCTCTGTCATCTCTCCACA	50 to 65	264	K01458
TTPA	F: TCCAGCAGTGGCCAAGAAAA R: GCGAAGACTGGGTGGAAGAA	60	108	XM_0,012,34375
IFN-γ	F: AGCTGACGGTGGACCTATTATT R: GGCTTTGCGCTGGATTC	58	259	Y07922
IL-1β	F: TGGGCATCAAGGGCTACA R: TCGGGTTGGTTGGTGATG	60	244	Y15006
IL-6	F: CAAGGTGACGGAGGAGGAC R: TGGCGAGGAGGGATTTCT	60	254	AJ30954
IL-4	F: ACCCAGGGCATCCAGAAG R: CAGTGCCGGCAAGAAGTT	60	258	AJ621735
IL-10	F: CGGGAGCTGAGGGTGAA R: GTGAAGAAGCGGTGACAGC	58	272	AJ621614
TGF-β$_4$	F: CGGGACGGATGAGAAGAAC R: CGGCCCACGTAGTAAATGAT	60	258	M31160

[1]*GAPDH*, glyceraldehyde-3-phosphate; *TTPA*, alpha-tocopherol transfer protein; *IFN-γ*, interferon gamma; *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-4*, interleukin 4; *IL-10*, interleukin 10; *TGF-β$_4$*, transforming growth factor beta 4.
[2]F, forward; R, reverse.
[3]Ta, annealing temperature

USA). Following extraction, the RNA was diluted with 20 μL of RNase-free water. Total RNA concentration was determined at an optical density **(OD)** 260 nm (NanoDrop-1000, Thermo Fisher Scientific, Watham, MA) and RNA purity was verified by evaluating the ratio of OD 260 nm to OD 280 nm. Afterwards, 2 μg of total RNA was reverse transcribed to cDNA by using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA), according to manufacturer's protocol, and the cDNA was stored at −20°C.

Quantitative real-time PCR was performed using the CFX Connect™ Real-time PCR Detection System (Bio Rad Laboratories, Hercules, CA, USA) in a 20-μL reaction mixture containing 1 μL cDNA, 10 μL of 2x AMPIGENE qPCR Green Mix Lo-ROX (Enzo Life Sciences Inc., Farmingdale, NY, USA), and 10 pmol each of forward and reverse primers of the selected genes. The primers of the selected genes for pro-inflammatory cytokines (IFN-γ, IL-1β, and IL-6) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β_4; Hong et al., 2006), and TTPA were designed based on sequences available from public databases and were synthesized by Genotech Co. Ltd. (Daejeon, Republic of Korea). Details for primers are presented in Table 3. Thermal conditions for performing qPCR are as follows: initial incubation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and termination by final incubation at dissociation temperatures 95°C (10 s), 65°C (60 s), 97°C (1 s), and 37°C (30 s). Relative quantification of gene expression was performed using the chicken glyceraldehyde-3-phosphate dehydrogenase gene **(GAPDH)** as an internal control gene to normalize for RNA abundance. Each reaction was run in duplicate. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with the GAPDH (Livak and Schmittgen, 2001).

Statistical Analysis

All data were analyzed by ANOVA as a completely randomized design using the PROC MIXED procedure (SAS Institute Inc., Cary, NC). Each replicate was considered an experimental unit. The LSMEANS procedure was used to calculate treatment means and the PDIFF option of SAS was used to separate the means if the difference was significant. The statistical model included VE sources, inclusion levels of VE, and their interaction. In addition, preplanned orthogonal polynomial contrast test was performed to verify linear and quadratic effects of increasing VE concentrations in diets, regardless of VE sources (Seo et al., 2018). Finally, an additional simple contrast test for basal diets vs. all VE treatment groups was conducted. Significance for statistical tests was set at $P < 0.05$.

RESULTS

The concentrations of α-tocopherol in diets were close to their expected concentrations (Table 2), which indicated that the potency of each VE source as claimed by the provider was verified and the diet mixing was appropriate in the present experiment.

Growth Performance

The main effects of VE sources and inclusion levels and their interaction were not significant for BWG, FI, and FE (Table 4). There were no linear and quadratic effects of increasing inclusion levels of VE in diets on growth performance of broiler chickens. However, the simple contrast test revealed that birds fed basal diets had less ($P < 0.05$) FI than those fed diets containing supplemental VE.

Table 4. Effects of vitamin E (VE) sources and inclusion levels in diets on growth performance of broiler chickens.[1]

Items[2]	Added VE, IU/kg	Growth performance[3]		
		BWG, g	FI, g	FE, g/kg
Basal	0	1,589	2,199[c]	723
LVE	33	1,624	2,281[a,b]	711
	65	1,642	2,292[a,b]	716
	100	1,661	2,306[a]	720
WVE	33	1,657	2,312[a]	717
	65	1,672	2,302[a]	726
	100	1,578	2,217[b,c]	712
Pooled SEM (n = 6)		27.1	30.4	6.3
Main effect				
VE source				
LVE		1,642	2,293	716
WVE		1,636	2,277	718
Pooled SEM (n = 18)		17.5	18.0	3.5
VE level				
33		1,640	2,296	714
65		1,657	2,297	721
100		1,620	2,262	716
Pooled SEM (n = 12)		21.1	21.8	4.2
P-values				
1-way ANOVA		0.15	0.04	0.54
2-way ANOVA				
VE source		0.79	0.52	0.61
VE level		0.45	0.41	0.47
Source × level		0.09	0.12	0.27
Contrast				
VE level (linear)		0.21	0.26	0.74
VE level (quadratic)		0.94	0.50	0.24
Basal vs. VE groups		0.10	0.01	0.38

[a-c]Means with different superscripts within a column differ ($P < 0.05$).
[1]Data are least squares means of 6 replicates per treatment. Each replicate had 10 birds (5 male and 5 female birds).
[2]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.
[3]BWG, body weight gain; FI, feed intake; FE, feed efficiency (BWG: FI, g/kg).

Table 5. Effects of vitamin E (VE) sources and inclusion levels in diets on relative organ weight of broiler chickens.[1]

Items[2]	Added VE, IU/kg	Relative organ weight[3], %			
		Liver	Spleen	Thymus	BF
Basal	0	2.94[a]	0.13	0.25	0.13
LVE	33	2.63[b]	0.11	0.25	0.17
	65	2.54[b]	0.11	0.25	0.18
	100	2.63[b]	0.12	0.38	0.18
WVE	33	2.80[a,b]	0.11	0.31	0.16
	65	2.53[b]	0.13	0.34	0.16
	100	2.50[b]	0.12	0.27	0.19
Pooled SEM (n = 6)		0.116	0.012	0.042	0.017
Main effect					
VE source					
LVE		2.60	0.11	0.29	0.18
WVE		2.61	0.12	0.30	0.17
Pooled SEM (n = 18)		0.063	0.007	0.024	0.010
VE level					
33		2.71	0.11	0.28	0.16
65		2.53	0.12	0.29	0.17
100		2.57	0.12	0.32	0.18
Pooled SEM (n = 12)		0.078	0.009	0.029	0.012
P-values					
1-way ANOVA		0.01	0.69	0.21	0.19
2-way ANOVA					
VE source		0.88	0.48	0.72	0.61
VE level		0.21	0.74	0.57	0.44
Source × level		0.37	0.67	0.05	0.87
Contrast					
VE level (linear)		0.19	0.46	0.30	0.20
VE level (quadratic)		0.25	0.85	0.86	0.93
Basal vs. VE groups		0.01	0.32	0.30	0.02

[a,b]Means with different superscripts within a column differ ($P < 0.05$).
[1]Data are least squares means of 6 replicates per treatment (i.e., 1 male bird per replicate).
[2]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.
[3]Relative organ weight was expressed as a percentage of BW (BF, bursa of Fabricius).

Relative Organ Weights

As important immune organs, the relative weights of the liver, spleen, thymus, and bursa of Fabricius were measured (Table 5). Similar to the results for growth performance, the main effects of VE sources, inclusion levels, and their interaction were not significant for all relative organ weights. However, birds fed basal diets had greater ($P < 0.05$) relative weights of the liver, but less ($P < 0.05$) relative weights of bursa of Fabricius compared with those fed diets containing supplemental VE.

Meat Quality, Lipid Oxidation, and Alpha-tocopherol Retention

No main effects of VE sources, inclusion levels, and their interaction were observed for breast meat quality, except for meat redness (a^*) and WHC at 48-h postmortem (Table 6). Increasing inclusion levels of VE in diets increased (linear, $P < 0.05$) meat redness (a^*). There was a quadratic association ($P < 0.05$) between inclusion levels of VE in diets and WHC at 48-h postmortem. Birds fed basal diets had greater ($P < 0.05$) meat yellowness (b^*) than birds fed diets containing VE; however, there were no differences in other meat quality measurements between birds fed basal diets and birds fed diets containing supplemental VE. The extent of lipid oxidation of breast and thigh meat was determined based on TBARS values that were measured during 7 d of storage at 4°C (Table 7). For breast meat, birds fed diets containing 65 or 100 IU/kg of LVE had less ($P < 0.05$) TBARS values than those fed diets containing 33 IU/kg of LVE, whereas no differences in TBARS values were observed for WVE treatment groups, which indicated an interaction ($P < 0.05$). Different VE sources did not influence TBARS values. Increasing inclusion levels of VE in diets decreased (linear and quadratic, $P < 0.05$) TBARS values. Birds fed basal diets had greater ($P < 0.01$) TBARS values than those fed diets containing supplemental VE. For thigh meat, however, no main effects of VE sources, inclusion levels, and their interaction were identified. However, birds fed diets containing supplemental VE had less ($P < 0.05$) TBARS values than those fed basal diets. No interaction between VE sources and inclusion levels was observed for alpha-tocopherol concentrations in breast meat (Table 8). Different VE sources had no effect on alpha-tocopherol concentrations in breast meat; however, increasing inclusion levels of VE in diets increased (linear, $P < 0.01$) alpha-tocopherol concentrations in breast meat, regardless of VE sources.

Table 6. Effects of vitamin E (VE) sources and inclusion levels in diets on breast meat quality of broiler chickens.[1]

		pH values		Meat color values[3]			WHC, %[4]	
Items[2]	Added VE, IU/kg	1 h	24 h	L*	a*	b*	24 h	48 h
Basal	0	6.40	5.94	51.2	7.7[a]	11.1	80.2	82.8
LVE	33	6.49	5.96	51.9	8.0[a]	10.0	80.4	82.1
	65	6.42	5.92	51.3	7.0[a,b]	8.6	78.8	80.4
	100	6.45	5.89	51.1	8.3[a]	10.5	81.0	82.4
WVE	33	6.28	5.99	50.5	5.5[b]	8.9	81.8	81.7
	65	6.38	5.97	51.0	5.4[b]	9.1	81.0	80.9
	100	6.50	5.97	51.7	8.4[a]	8.6	80.7	85.3
Pooled SEM (n = 6)		0.075	0.053	0.86	0.94	0.70	1.28	1.15
Main effect								
VE source								
LVE		6.45	5.92	51.5	7.9	9.7	80.1	81.6
WVE		6.39	5.97	51.1	6.5	8.9	81.2	82.6
Pooled SEM (n = 18)		0.041	0.026	0.71	0.55	0.35	0.76	0.67
VE level								
33		6.39	5.97	51.2	6.8[a,b]	9.5	81.1	81.9[a,b]
65		6.40	5.94	51.2	6.2[b]	8.9	79.9	80.6[b]
100		6.47	5.93	51.4	8.4[a]	9.6	80.9	83.8[a]
Pooled SEM (n = 12)		0.051	0.032	0.87	0.67	0.44	0.93	0.83
P-values								
1-way ANOVA		0.38	0.82	0.95	0.01	0.11	0.79	0.10
2-way ANOVA								
VE source		0.28	0.16	0.81	0.23	0.11	0.31	0.29
VE level		0.47	0.62	0.43	0.02	0.48	0.64	0.03
Source × level		0.21	0.91	0.43	0.18	0.15	0.62	0.37
Contrast								
VE level (linear)		0.25	0.34	0.41	0.01	0.27	0.89	0.10
VE level (quadratic)		0.65	0.88	0.32	0.77	0.64	0.35	0.03
Basal vs. VE groups		0.81	0.94	0.98	0.46	0.02	0.78	0.58

[a,b]Means with different superscripts within a column differ ($P < 0.05$).
[1]Data are least squares means of 6 replicates per treatment (i.e., 1 male bird per replicate).
[2]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.
[3]L*, lightness; a*, redness; b*, yellowness.
[4]WHC, water holding capacity.

All VE treatment groups had greater ($P < 0.01$) alpha-tocopherol concentrations in breast meat than the basal treatment group.

Expression of Alpha-tocopherol Transfer Protein and Inflammatory Cytokine Genes

No interactions between VE sources and inclusion levels in diets were observed for all expression of TTPA gene and inflammatory cytokine genes in the jejunum of broiler chickens (Table 9). However, there were significant main effects of VE sources and inclusion levels for some gene expressions. The expression of TTPA gene was not affected by VE sources, but was decreased (linear and quadratic, $P < 0.05$) as inclusion levels of VE in diets were increased. The expression of TTPA gene for birds fed diets containing supplemental VE was less ($P < 0.01$) than for those fed basal diets. For the expression of pro-inflammatory cytokine genes (i.e., IFN-γ, IL-1β, and IL-6) in the jejunum, birds fed diets containing LVE had greater ($P < 0.01$) expression of IFN-γ and IL-1β than those fed diets containing WVE. However, VE sources did not affect the expression of IL-6. Increasing inclusion levels of VE in diets decreased (linear and quadratic, $P < 0.01$) the expression of IFN-γ, IL-1β, and IL-6. The expression of IFN-γ, IL-1β, and IL-6 was less ($P < 0.01$) for birds fed diets containing supplemental VE than for those fed basal diets. Similar results were observed for the expression of anti-inflammatory cytokine genes (i.e., IL-4, IL-10, and TGF-β_4). Birds fed diets containing LVE had greater ($P < 0.05$) expression of IL-4 and IL-10 than those fed diets containing WVE. However, the expression of TGF-β_4 was not influenced by VE sources. Increasing inclusion levels of VE in diets decreased the expression of IL-4 and IL-10 (linear and quadratic, $P < 0.01$), and TGF-β_4 (quadratic, $P < 0.05$). The expression of IL-4, IL-10, and TGF-β_4 was less ($P < 0.01$) for birds fed diets containing supplemental VE than for those fed basal diets.

DISCUSSION

The beneficial effects of dietary VE on growth performance, meat quality, and immune responses of broiler chickens have been widely reported (Fellenberg and Speisky, 2006; Khan et al., 2012). However, as a feed supplement in the vitamin premix, several concerns regarding lipid soluble property of VE have been raised owing to chemical instability (Gawrysiak-Witulska et al., 2009) and variations in bioavailability (Yang and McClements, 2013). Water-miscible preparations of alpha-tocopherol have been reported as the therapeutic application for humans with VE malabsorption (Harries and Muller, 1971; Soltani-Frisk

Table 7. Effects of vitamin E (VE) sources and inclusion levels in diets on TBA-reactive substance (TBARS) values in breast and thigh meat of broiler chickens.[1]

Items[2]	Added VE, IU/kg	TBARS[3]	
		Breast meat	Thigh meat
Basal	0	0.63[a]	0.60[a]
LVE	33	0.57[a]	0.51[a,b]
	65	0.42[b]	0.47[b]
	100	0.39[b]	0.44[b]
WVE	33	0.44[b]	0.50[a,b]
	65	0.39[b]	0.57[a]
	100	0.42[b]	0.51[a,b]
Pooled SEM (n = 6)		0.029	0.035
Main effect			
VE source			
LVE		0.46	0.47
WVE		0.42	0.53
Pooled SEM (n = 18)		0.017	0.019
VE level			
33		0.51[a]	0.51
65		0.40[b]	0.52
100		0.41[b]	0.48
Pooled SEM (n = 12)		0.021	0.024
P-values			
1-way ANOVA		<0.01	<0.01
2-way ANOVA			
VE source		0.08	0.05
VE level		<0.01	0.38
Source × level		0.04	0.29
Contrast			
VE level (linear)		<0.01	0.39
VE level (quadratic)		0.04	0.27
Basal vs. VE groups		<0.01	0.01

[a,b]Means with different superscripts within a column differ ($P < 0.05$).
[1]Data are least squares means of 6 replicates per treatment (i.e., 1 male bird per replicate).
[2]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.
[3]TBARS values were quantified as malondialdehyde (MDA) equivalents per g of meat sample at 7 d of storage at 4°C.

Table 8. Effects of vitamin E (VE) sources and inclusion levels in diets on alpha-tocopherol concentrations in breast meat of broiler chickens.[1]

Items[2]	Added VE, IU/kg	Alpha-tocopherol, IU/kg
Basal	0	8.1[e]
LVE	33	13.3[c,d]
	65	15.3[b,c]
	100	23.0[a]
WVE	33	12.0[d]
	65	17.1[b]
	100	23.1[a]
Pooled SEM (n = 6)		1.04
Main effect		
VE source		
LVE		17.2
WVE		17.4
Pooled SEM (n = 18)		0.61
VE level		
33		12.6[c]
65		16.2[b]
100		23.0[a]
Pooled SEM (n = 12)		0.77
P-values		
1-way ANOVA		<0.01
2-way ANOVA		
VE source		0.86
VE level		<0.01
Source × level		0.36
Contrast		
VE level (linear)		<0.01
VE level (quadratic)		0.08
Basal vs. VE groups		<0.01

[a–e]Means with different superscripts within a column differ ($P < 0.05$).
[1]Data are least squares means of 6 replicates per treatment (i.e., 1 male bird per replicate).
[2]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.

et al., 2001). The WVE was reported to be putatively less dependent on the solubilization, suggesting that dietary WVE is likely to be more efficient and bioavailable than the conventional LVE (Muller et al., 1976). However, there has been a lack of information regarding the comparison of the efficacy of WVE to LVE in poultry nutrition.

In the current experiment, VE sources and their inclusion levels in diets did not affect growth performance of broiler chickens. Birds fed the basal diet exhibited less FI than those fed diets containing supplemental VE. In general, animals fed diets deficient in a specific nutrient exhibited a compensatory increase in FI (Leeson et al., 1996). However, the compensatory increase in FI by feeding diets with no VE supplementation was not observed in the current experiment. The reason for this observation may be related to the fact that the basal diet was not sufficiently deficient in VE. That it, the calculated concentration of VE in the basal diet was 11.6 IU/kg, which was close to the VE requirement suggested by NRC (1994). In addition, Pompeu et al. (2018) suggested that the effect of dietary VE on growth performance of broiler chickens may be affected by the type and composition of various feed ingredients in diets. Moreover, the positive effects of increasing inclusion of VE may be more apparent when animals are raised under stressful conditions (Sahin et al., 2001; Sahin et al., 2002; Khan et al., 2011); however, it is postulated that our experimental conditions were not severe enough to increase the stress of broiler chickens.

There were no main effects of VE sources, inclusion levels, and their interaction on the relative organ weight of broiler chickens, which indicates that the development of immune organs of broiler chickens are unaffected by different VE sources and inclusion levels in diets. However, birds fed the basal diet had a greater relative liver weight than those fed diets containing supplemental VE in the present experiment. The reason for this increase in liver weight by feeding the basal diet is likely due to its relatively higher sensitivity to the oxidative stress. Oxidative stress and potential induction of inflammatory responses may be increased by diets containing a small amount of antioxidants in the basal diet, which could lead to an enlarged liver, given that the liver is the most sensitive organ in the body to oxidative stress (Lu et al., 2014). Lymphoid organs such as bursa of Fabricius, thymus, and spleen are important parts of the avian immune system (Schat et al., 2014). The development and differentiation of B-lymphocytes and T-lymphocytes occur in the bursa of Fabricius and thymus, respectively (Schat et al., 2014). The relative weight of immune tissues reflects the

Table 9. Effects of vitamin E (VE) sources and inclusion levels in diets on the expression of alpha-tocopherol transfer protein (TTPA) gene and inflammatory cytokine genes in the jejunum of broiler chickens.[1]

			Pro-inflammatory cytokines			Anti-inflammatory cytokines		
Items[2]	Added VE, IU/kg	TTPA	IFN-γ	IL-1β	IL-6	IL-4	IL-10	TGF-β_4
Basal	0	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{a}
LVE	33	0.69^{b}	0.56^{b}	0.72^{b}	0.46^{b}	0.71^{b}	0.54^{b}	0.63^{b,c}
	65	0.10^{c}	0.10^{d,e}	0.15^{c}	0.15^{c}	0.21^{d,e}	0.15^{c,d}	0.54^{c,d}
	100	0.57^{b}	0.23^{c,d}	0.28^{c}	0.20^{c}	0.39^{c,d}	0.25^{c}	0.55^{c,d}
WVE	33	0.64^{b}	0.31^{c}	0.25^{c}	0.50^{b}	0.45^{c}	0.49^{b}	0.96^{a,b}
	65	0.15^{c}	0.02^{e}	0.02^{c}	0.04^{c}	0.04^{e}	0.04^{d}	0.27^{d}
	100	0.10^{c}	0.04^{e}	0.04^{c}	0.07^{c}	0.02^{e}	0.04^{d}	0.58^{c,d}
Pooled SEM (n = 6)		0.114	0.070	0.096	0.068	0.085	0.069	0.126
Main effect								
VE source								
LVE		0.45	0.30	0.38	0.27	0.44	0.31	0.57
WVE		0.30	0.12	0.10	0.20	0.17	0.19	0.60
Pooled SEM (n = 18)		0.068	0.041	0.058	0.042	0.053	0.043	0.077
VE level								
33		0.66^{a}	0.43^{a}	0.49^{a}	0.48^{a}	0.58^{a}	0.51^{a}	0.80^{a}
65		0.12^{b}	0.06^{b}	0.09^{b}	0.10^{b}	0.12^{b}	0.10^{b}	0.40^{b}
100		0.33^{b}	0.13^{b}	0.16^{b}	0.13^{b}	0.20^{b}	0.15^{b}	0.57^{a,b}
Pooled SEM (n = 12)		0.084	0.052	0.071	0.052	0.067	0.053	0.093
P-values								
1-way ANOVA		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
2-way ANOVA								
VE source		0.11	<0.01	<0.01	0.26	<0.01	0.05	0.78
VE level		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02
Source × level		0.07	0.52	0.24	0.45	0.56	0.51	0.09
Contrast								
VE level (linear)		0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.09
VE level (quadratic)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02
Basal vs. VE groups		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a-e]Means with different superscripts within a column differ ($P < 0.05$).
[1]Data are least squares means of 6 replicates per treatment (i.e., 1 male bird per replicate).
[2]LVE, lipid-soluble vitamin E; WVE, water-miscible vitamin E.

immune functionality in broiler chickens (Al-Khalifa et al., 2012). In agreement with our results, Konieczka et al. (2017) showed a greater relative weight of bursa Fabricius in chickens fed diets containing 300 IU/kg VE as compared to those fed diets containing 50 IU/kg. Likewise, it was reported that VE deficiency decreased the size of immune organs such as the bursa of Fabricius and thymus (Marsh et al., 1986). Thus, our results suggested that the proper development of immune organs, especially for bursa of Fabricius, in broiler chickens requires sufficient amounts of VE in diets (Konieczka et al., 2017).

The results of the current experiment showed that dietary VE supplementation affected meat redness. The high redness of the meat is perceived by consumers as an indicative of a greater freshness (Morrissey et al., 1994). Moreover, the rate of meat discoloration is likely related to the effectiveness of oxidative processes and enzymatic reducing systems in controlling metmyoglobin levels (Faustman et al., 1989). In beef, a sufficient amount of alpha-tocopherol in diets was reported to effectively control loss of desirable color and decrease the accumulation of metmyoglobin (Faustman et al., 1989; Arnold et al., 1993). In pork meat, similar results were presented by Monahan et al. (1994) who reported that pigs fed diets containing 100 or 200 mg/kg of alpha-tocopherol had a greater redness for the pork as compared to those fed diets containing 10 mg/kg of alpha-tocopherol.

Lipid oxidation is the primary cause of decreased meat quality, which is associated with the production of off-flavors, odors, and even toxic compounds such as peroxides and aldehydes (Morrissey et al., 1994). The VE supplementation in broiler diets has been reported to decrease lipid oxidation of meat, which is highly related to improved meat quality and increased shelf life (Barroeta, 2007). Similar results for breast and thigh meat in broiler chickens fed diets containing supplemental VE as compared to those fed the basal diet were observed in the current experiment. Interestingly, there was a significant interaction for TBARS values in breast meat. The reason for the interaction may be related to the fact that LVE at the level of 33 IU/kg in diets had no effects on lipid oxidation in breast meat, but WVE at the same level decreased lipid oxidation. This result indicates that WVE may be more efficient than LVE in preventing lipid oxidation at the low level in diets. However, there were no main effects of VE sources, inclusion levels, and their interaction on TBARS values in thigh meat. This result was not expected because lipid oxidation may occur more rapidly in thigh meat than in breast meat due to the greater total lipid concentrations in thigh meat than in breast meat (Kanner, 1994). It was reported, however, that lipids in breast meat were more unsaturated than in thigh meat (Gong et al., 2010). It is well-known that unsaturated lipids are more susceptible to lipid oxidation due to the presence of double bonds in fatty acids, which are prone to

be oxidized by free radicals (Parkin and Damodaran, 2003). Thus, the different effects of VE supplementation between breast and thigh meat may be related to the differences in their fatty acid compositions. In addition, no effects of increasing inclusion levels of VE in diets on TBARS in thigh meat indicate that a greater than 33 IU/kg VE, regardless of VE sources had no further benefits on the prevention of lipid oxidation.

One of the main objectives of the present experiment was to compare the efficacy of different VE sources (LVE vs. WVE) on the fortification of VE in poultry meat. As we expected, increasing inclusion levels of both VE sources linearly increased alpha-tocopherol concentrations in breast meat. This linear increase in VE concentrations was also reported in other animal products (Flachowsky et al., 2002; Chae et al., 2006; Voljc et al., 2011). However, there were no effects of VE sources and interaction between VE sources and inclusion levels for alpha-tocopherol concentration in breast meat. No significant differences in TBARS values in breast meat between LVE and WVE also support the fact that alpha-tocopherol concentrations in breast meat were not affected by different VE sources.

The transport of alpha-tocopherol occurs within the liver by TTPA that specifically binds with alpha-tocopherol among all VE isoforms, thus enhances its transport between membranes (Sato et al., 1993). The TTPA was initially identified as a liver-specific protein that is necessary for the transfer of alpha-tocopherol to the peripheral tissues in association with very-low-density lipoprotein (Bjorneboe et al., 1990; Sato et al., 1993). However, very limited information for TTPA expression in the intestine is available. In the current experiment, VE sources did not affect the expression of TTPA gene in the jejunum. This result was expected because both VE sources are absorbed via the same absorptive mechanism in the small intestine. However, TTPA expression was quadratically related to increasing inclusion levels of LVE or WVE with TTPA expression being the least at the level of 65 IU/kg among 3 different inclusion levels. The moderate VE concentrations in diets showed the least TTPA expression in the intestine as compared to low or high amounts of VE in diets for broiler chickens. Similar quadratic association between hepatic TTPA expressions and increasing VE concentrations in diets was observed for broiler chickens fed diets containing different VE concentrations (Rengaraj et al., 2019). The clear reason is not identified; however, it is speculated that deficient or excessive intake of VE may accelerate TTPA expression in the intestine and other tissues to control or regulate VE utilization (Rengaraj et al., 2019). This notion is partly supported by our results for a greater expression of TTPA for birds fed the basal diet than for birds fed diets containing supplemental VE.

The VE is a well-known antioxidant that scavenges free radicals produced from lipid peroxidation during normal metabolic state and inflammation (Khan et al., 2012). Inflammatory cytokines are essential for the innate and adaptive immune functions, and the balance of pro-inflammatory cytokines and anti-inflammatory cytokines is important for the prevention of the development of a pathological state (Kidd, 2003; Sultani et al., 2012). The current experiment revealed that VE sources and inclusion levels had considerable effects on intestinal inflammatory responses as measured by relative mRNA expression levels of pro-inflammatory (IFN-γ, IL-1β, and IL-6) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β_4).

The expression of both pro-inflammatory and anti-inflammatory cytokines in the jejunum was decreased by dietary VE supplementation in the current experiment. This result agreed with Zhang et al. (2010) who reported that dietary supplementation of alpha-tocopherol reduced plasma protein levels of both pro-inflammatory cytokines (IFN-γ, IL-1β, IL-2, and IL-6) and anti-inflammatory cytokines (IL-4 and IL-10). These results appear to be associated with maintaining the proper balance of the inflammatory cytokines during inflammatory responses. It has been suggested that the relative balance within the cytokine network can be maintained by both pro- and anti-inflammatory cytokine responses (Kaiser et al., 2012). Thus, our observation for the decrease in both inflammatory cytokines with increasing inclusion levels of VE may indicate that increasing inclusion levels of VE lead to an increase in the balance of the inflammatory responses. In comparison of VE sources, dietary WVE had a greater reduction in both types of inflammatory cytokines than dietary LVE although significance was only observed for IFN-γ, IL-1β, and IL-4. The clear reason is not known for this observation; however, we speculated that WVE may be more easily absorbed, and thus, WVE may accumulate temporarily at a larger amount than LVE in the intestinal cells, which may improve the balance of inflammatory cytokines. More researches are required to elucidate the reason why dietary WVE is more effective in the balance of intestinal inflammatory cytokines than dietary LVE.

CONCLUSION

Different VE sources (LVE and WVE) and their inclusion levels from 33 to 100 IU/kg in diets have little effects on growth performance and relative organ weights of broiler chickens. Lipid oxidation of breast and thigh meat is not affected by VE sources. However, regardless of VE sources, increasing inclusion levels of VE in diets decrease lipid oxidation of breast meat, but it is not the case for thigh meat. Increasing inclusion levels of VE linearly increase alpha-tocopherol concentrations in breast meat, but the extent of the increase is similar between VE sources. Increasing inclusion levels of both VE sources in diets decrease the expression of pro- and anti-inflammatory cytokines. Dietary WVE exhibits a greater reduction in the expression of both pro- and anti-inflammatory cytokines than dietary LVE.

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