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Effects of stocking density and dietary vitamin C on performance, meat quality, intestinal permeability, and stress indicators in broiler chickens

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Abstract

The objective of the current study was to investigate the effects of stocking density (SD) and dietary supplementation of vitamin C on growth performance, meat quality, intestinal permeability, and stress indicators in broiler chickens. The study was conducted using a completely randomized design with a 2 × 2 factorial arrangement consisting of 2 different SD and 2 supplemental levels of dietary vitamin C. A total of 1,368 Ross 308 broiler chickens of 21 days of age with similar body weights (BW) were randomly allotted to 1 of 4 treatments with 6 replicates each. Different numbers of birds per identical floor pen (2.0 m × 2.4 m) were used to create 2 different SD levels of low SD (9 birds/m^2) and high SD (18 birds/m^2). The basal diet was formulated with no supplemental vitamin C to meet or exceed nutrient recommendations of the Ross 308 manual. The other diet was prepared by supplementing 200 mg/kg vitamin C in the basal diet. The study lasted for 14 days. At the end of the study, 3 male birds per replicate were selected to analyze meat quality, intestinal permeability, and stress indicators such as blood heterophil:lymphocyte (H:L) and feather corticosterone (CORT) concentrations. Results indicated that there were no interactions between different SD and dietary supplementation of vitamin C for all measurements. For the main effects of SD, birds raised at high SD had less ($p < 0.01$) BW, BW gain, and feed intake with increasing stress responses including greater blood H:L and feather CORT concentrations ($p < 0.01$) than those raised at low SD. Transepithelial electrical resistance in the jejunal mucosa was decreased ($p < 0.05$) at high SD, indicating an increase in intestinal permeability. However, the main effects of dietary supplementation of 200 mg/kg vitamin C were insignificant for all measurements. In conclusion, high SD of broiler chickens impairs growth performance and intestinal barrier function with increasing stress responses. However, dietary supplementation of vitamin C may have little beneficial effects on broiler chickens raised at the high SD condition used in the present study.

Keywords: Broiler chicken, Dietary vitamin C, Growth performance, Intestinal permeability, Stress indicator, Stocking density

Competing interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and material
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Ethics approval and consent to participate
The protocol for the current experiment was reviewed and approved by the Institutional Animal Care and Use Committee at Chung-Ang University (IACUC No. 2019-00086).

INTRODUCTION

Stocking density (SD) is one of important concerns in broiler production because of its direct relationship with the productive performance, health, and welfare of broiler chickens [1]. It is expected that increasing SD of broiler chickens increases chicken meat production per defined space [2]. However, high SD has been frequently associated with decreased productive performance and health of broiler chickens [3]. Several studies have been performed to develop dietary interventions that alleviate the negative impacts of high SD on broiler chickens [4–6]; however, the results are not promising.

Vitamin C is a potent biological antioxidant and several animal studies have suggested that dietary supplementation of vitamin C is effective in decreasing the oxidative stress of animals raised under various stressful conditions [7,8]. However, dietary supplementation of vitamin C is not widely practiced in the poultry industry because poultry is believed to synthesize sufficient amounts of vitamin C in the body [9]. Despite the ability of chickens in producing endogenous vitamin C, the requirement or synthetic capacity of vitamin C may alter due to various factors such as individual differentiation, breeds, health status, and environment [10], which may result in possible extra requirements in poultry. One of the most-well known environmental factors increasing the demand for supplemental vitamin C in diets is heat stress. Several previous studies have reported that dietary supplementation of vitamin C ameliorated the adverse effect of heat stress on broiler performance and health [11–13]. However, although high SD is also considered a critical stressor, there are few studies on the interactive effect of high SD and dietary vitamin C on productive performance and health of broiler chickens.

Therefore, the objective of the current study was to investigate the effects of SD and dietary supplementation of vitamin C on growth performance, meat quality, intestinal permeability, and stress indicators in broiler chickens.

MATERIALS AND METHODS

Animals, experimental design, and diets

The experimental protocol of the current study was approved by the Institutional Animal Care and Use Committee at Chung-Ang University (IACUC approval no. 2019-00086). The study was performed using a completely randomized design with a 2 × 2 factorial arrangement consisting of 2 different SD and 2 supplemental levels of dietary vitamin C. A total of 1,368 mixed-sex Ross 308 broiler chickens (1:1 ratio of males and females) of 21 days of age with similar body weights (BW) were randomly allotted to 1 of 4 treatments with 6 replicates each. The study lasted for 14 days from 21 to 35 days of age. The experimental period was determined based on the fact that growing chickens are more sensitive to high SD than young chickens [14,15]. Different numbers of birds per floor pen were used to create 2 different SD levels, namely low SD (9 birds/m^2) and high SD (18 birds/m^2) as reported in our previous study [6]. The final floor space (4.216 m^2) was calculated by subtracting the space of immobile objects (0.584 m^2 for 2 feeders and 1 bell-shaped drinker per pen) from the floor space (2.0 m × 2.4 m). Each floor pen was covered with a 10-cm thick layer of rice hulls. The commercial-type basal diet was prepared without dietary supplementation of vitamin C as the control group (Table 1). The nutrient and energy concentrations in the basal diet were formulated to meet or exceed nutrient recommendations of the Ross 308 manual [16]. Vitamin C (> 970 g/kg coated vitamin C, Zhejiang Mingzhu Animal Health Products, Hangzhou, China) was then supplemented to the basal diet at the inclusion level of 200 mg/kg. The supplemental level of dietary vitamin C was determined based on previous studies reporting that the beneficial effect of

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Table 1. Ingredients and nutrient composition of the basal diet and experimental diet

Items	Basal diet
Ingredients (%)	
Corn	58.73
Soybean meal (46% crude protein)	24.47
Corn gluten meal	5.77
Tallow	5.55
Salt	0.20
Monodicalcium phosphate	1.48
Limestone	1.20
$_{DL}$-Methionine (85%)	0.32
$_{L}$-Lysine-HCl (50%)	0.58
$_{L}$-Threonine (98.5%)	0.10
Choline (50%)	0.10
$NaHCO_3$	0.10
Vitamin premix[1)]	0.10
Mineral premix[2)]	0.10
Celite[3)]	1.20
Total	100.00
Calculated energy and nutrient content[4)]	
AME_n (kcal/kg)	3,200
Crude protein (%)	19.54
Lysine (%)	1.16
Methionine + cysteine (%)	0.91
Threonine (%)	0.83
Tryptophan (%)	0.21
Calcium (%)	0.79
Available phosphorus (%)	0.40

[1)]Provided per kilogram of the complete diet: vitamin A, 12,000 IU; vitamin D_3, 4,000 IU; vitamin E, 80 IU; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 20 μg; biotin, 0.2 mg; vitamin B_5, 20 mg; folic acid, 2 mg; niacin, 60 mg; antioxidant, 2 mg.

[2)]Provided per kilogram of the complete diet: zinc, 100 mg; manganese, 120 mg; iron, 60 mg; copper, 16 mg; cobalt, 1 mg; iodine, 1.25 mg; selenium, 0.3 mg.

[3)]Vitamin C (Vitamin C coated 970 g/kg, Zhejiang Mingzhu Animal Health Products, Hangzhou, China) was added at the level of 200 mg/kg in replace of celite.

[4)]Calculated values from NRC and Ross 308 manual [16,17].

AME_n, nitrogen-corrected apparent metabolizable energy.

supplemental vitamin C in broiler diets was observed at 200 mg/kg [12,18].

Before the initiation of the study, all chickens were raised under the same environmental conditions and were fed the same commercial starter and grower diets for 20 days. Experimental diets and water were provided *ad libitum* for 14 days. All experimental pens were placed in an environmentally controlled room. The average room temperature was maintained at 22.3 ± 2.5℃ during overall experimental period. The study was performed under 24-hour lighting conditions. Body weight gain (BWG) and feed intake (FI) were recorded at the end of the study. Feed efficiency (FE) was calculated as BWG divided by FI after adjusting for mortality [19].

Sample collection

At the end of the study, 3 male birds with a BW close to average BW in each replicated pen were

selected. One bird was used to analyze stress indicators including blood heterophil (H):lymphocyte (L) and the concentrations of corticosterone (CORT) in the feather. The remaining 2 birds were euthanized by CO_2 asphyxiation. One bird was used for analyzing breast meat quality and liver antioxidant status, whereas the other bird was used for analyzing intestinal permeability in the jejunum by a Ussing chamber.

Analysis of stress indicators

Blood samples were collected from wing veins. Blood H:L was analyzed using the method of Lentfer et al. [20] with a minor modification. Briefly, a standard microscope slide with approximately 7 μL of blood was prepared and dried at room temperature. The slides were then prefixed for 5 minutes after immersion in methanol. Afterwards, the slides were stained with 0.2 mL wright stain solution (Wright stain solution, Muto pure chemicals, Tokyo, Japan) for 2 minutes at room temperature and then rinsed with water until the edges of the slides turned pinkish red. The slides were again stained with 0.2 mL giemsa stain (Giemsa's staining solution, Duksan pure chemicals, Ansan, Korea) for 5 minutes at room temperature and then rinsed with water until the edges of the slides turned pinkish red. After air drying, the hemocyte smears were inspected under a microscope. Blood H and L were counted by the same 2 people to 100 cells per individual smear and blood H:L was calculated based on the method of Cengiz et al. [21].

Feather samples were collected from the same birds used for blood H:L analysis. The primary flight feathers were collected and stored at −80℃ before analysis. Feather CORT concentrations were analyzed using the method of Bortolotti et al. [22] with a minor modification [23]. In short, feathers without calamus were minced into pieces of < 5 mm^2 by scissors. Feather pieces were placed in 50-mL tubes with 10 mL methanol (HPLC grade, Honeywell, NC, USA). The tubes were sonicated in a water bath at room temperature for 30 minutes, and then incubated in a shaking water bath at 50℃ overnight. The methanol was then separated from the remaining feather by syringe filters (HyunDai Micro, Anseong, Korea) in a filtration funnel. The feather remnants were washed twice again with 2 mL methanol and the washes were added back to the original extract. The methanol extract was then concentrated by evaporation in a water bath at 50℃ for 7 hours. The extract residues were mixed with 1 mL phosphate-buffered saline (pH 7.4), and centrifuged at 17,900×g for 15 minutes. The resulting extracts were used for analyzing feather CORT concentrations with a CORT competitive ELISA kit according to the manufacturer's protocol (Thermo Fisher scientific, Waltham, MA, USA). The feather CORT concentrations were expressed as a function of feather length (pg/mm).

Meat quality and liver antioxidant status

Both sides of the breast muscle were excised to measure meat quality and stored at 4℃ until further analysis. The right portion was used for pH and meat color assays, whereas the left portion was used to determine water holding capacity (WHC) and thiobarbituric acid-reactive substance (TBARS) values. The detailed procedure of the meat quality analysis was reported previously [6].

Antioxidant status in the liver such as malondialdehyde (MDA) and total antioxidant capacity (TAC) was determined using a commercially available OxiSelectTM TBARS assay Kit (MDA Quantitation, STA-330, Cell Biolabs, San Diego, CA, USA) and OxiSelectTM Total Antioxidant Capacity (TAC) Assay Kit (STA-360, Cell Biolabs), respectively, according to the manufacturer's protocol. Protein concentrations were also analyzed using a commercial kit (Thermo Fisher Scientific) [24]. The relative concentrations of MDA and TAC to protein concentrations were calculated and expressed as μmol/mg and U/mg protein, respectively [25].

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Intestinal permeability

Intestinal permeability in the jejunal mucosa as a measure of intestinal barrier function was determined based on the transepithelial electrical resistance (TER) values measured in a dual-channel self-contained Ussing chamber system (U2500, Warner Instruments, Hamden, CT, USA). The detailed procedure was reported previously [6].

Statistical analysis

All data were analyzed by 2-way ANOVA as a completely randomized design using the PROC MIXED procedure (SAS Institute, Cary, NC, USA). Each replicate was considered an experimental unit. The statistical model included SD, dietary supplementation of vitamin C, and their interaction. The LSMEANS procedure was used to calculate treatment means and the PDIFF option in SAS was used to separate the means if the difference was significant. Statistical significance was considered at $p < 0.05$.

RESULTS

Growth performance

During 14 days of the study (from 21 to 35 days of age), no interaction between different SD and dietary supplementation of 200 mg/kg vitamin C was observed for growth performance of broiler chickens (Table 2). For the main effects, birds raised at high SD had less ($p < 0.01$) BW, BWG, and FI than those raised at low SD, whereas dietary supplementation of vitamin C had no effects on growth performance of broiler chickens, regardless of SD.

Table 2. Effects of stocking density (SD) and dietary supplementation of vitamin C (Vit C) on growth performance of broiler chickens

Treatment		Growth performance				
SD (birds/m^2)	Vit C (mg/kg)	IBW (g)	FBW (g)	BWG (g)	FI (g)	FE (g/kg)
9	0	614	1,586	972	1,633	596
	200	613	1,555	942	1,633	577
18	0	614	1,481	867	1,476	587
	200	613	1,465	852	1,470	580
SEM (n = 6)		1.5	22.4	22.4	31.4	8.3
Main effect						
SD (birds/m^2)						
9		613	1,570	957	1,633	586
18		613	1,473	860	1,473	583
SEM (n = 12)		1.1	15.8	15.8	22.2	5.9
Vit C (mg/kg)						
0		613	1,533	920	1,554	592
200		613	1,510	897	1,551	578
SEM (n = 12)		1.1	15.8	15.8	22.2	5.9
Effect (*p*-value)	*df*					
SD	1	0.98	< 0.01	< 0.01	< 0.01	0.73
Vit C	1	0.52	0.31	0.33	0.93	0.13
SD × Vit C	1	0.71	0.74	0.76	0.93	0.53

IBW, initial body weight; FBW, final body weight; BWG, body weight gain; FI, feed intake; FE, feed efficiency.

Stress indicators

There was no interaction between SD and dietary supplementation of 200 mg/kg vitamin C for blood H:L and feather CORT concentrations (Table 3). However, birds raised at high SD had greater ($p < 0.01$) blood H:L and feather CORT concentrations than those raised at low SD. Dietary supplementation of 200 mg/kg vitamin C had no effects on blood H:L and feather CORT concentrations, regardless of SD.

Meat quality and liver antioxidant status

No interaction between SD and dietary supplementation of 200 mg/kg vitamin C was observed for breast meat quality (Table 4) and antioxidant status such as MDA and TAC concentrations in the liver (Table 5). In addition, there were no main effects of SD and dietary supplementation of vitamin C on meat quality and antioxidant status in the liver.

Intestinal permeability

No interaction between SD and dietary supplementation of 200 mg/kg vitamin C was observed for TER values in the jejunal mucosa as a measure of intestinal permeability (Table 6). However, birds raised at high SD had less ($p < 0.05$) TER values than those raised in low SD. However, TER values were not affected by dietary supplementation of vitamin C.

DISCUSSION

Many previous studies have reported that high SD decreased BWG, FI, and FE of broiler chickens compared with low SD, which is in agreement with the current observation [4,21,26]. The reason

Table 3. Effects of stocking density (SD) and dietary supplementation of vitamin C (Vit C) on stress indicators of broiler chickens

Treatment		Stress indicators	
SD (birds/m^2)	Vit C (mg/kg)	Blood H:L	Feather CORT concentrations (pg/mm)
9	0	0.40	9.2
	200	0.40	10.3
18	0	0.83	18.5
	200	0.75	17.6
SEM (n = 6)		0.038	0.96
Main effect			
SD (birds/m^2)			
9		0.40	9.8
18		0.79	18.0
SEM (n = 12)		0.027	0.68
Vit C (mg/kg)			
0		0.61	13.8
200		0.57	14.0
SEM (n = 12)		0.027	0.68
Effect (*p*-value)	*df*		
SD	1	< 0.01	< 0.01
Vit C	1	0.30	0.91
SD × Vit C	1	0.29	0.31

CORT, corticosterone; H:L, heterophil:lymphocyte.

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Table 4. Effects of stocking density (SD) and dietary supplementation of vitamin C (Vit C) on breast meat quality of broiler chickens

Treatment		Breast meat quality							
SD (birds/m^2)	Vit C (mg/kg)	Yield (%)	pH (1 h)	pH (24 h)	WHC (%)	CIE L*	CIE a*	CIE b*	TBARS
9	0	16.78	6.38	5.69	69.41	44.72	3.48	12.68	0.45
	200	16.69	6.29	5.75	69.38	42.98	4.27	12.68	0.43
18	0	15.84	6.28	5.72	73.24	43.02	4.11	12.00	0.43
	200	16.33	6.40	5.74	68.51	43.63	3.38	11.84	0.44
SEM (n = 6)		0.466	0.066	0.042	1.458	0.888	0.467	0.427	0.010
Main effect									
SD (birds/m^2)									
9		16.73	6.33	5.72	69.39	43.85	3.87	12.68	0.44
18		16.09	6.34	5.73	70.88	43.33	3.74	11.92	0.44
SEM (n = 12)		0.329	0.046	0.030	1.031	0.628	0.330	0.302	0.007
Vit C (mg/kg)									
0		16.31	6.33	5.70	71.32	43.87	3.79	12.34	0.44
200		16.51	6.34	5.75	68.94	43.30	3.82	12.26	0.44
SEM (n = 12)		0.329	0.046	0.030	1.031	0.628	0.330	0.302	0.007
Effect (*p*-value)	*df*								
SD	1	0.18	0.96	0.82	0.32	0.56	0.79	0.09	0.85
Vit C	1	0.67	0.79	0.33	0.12	0.53	0.95	0.86	0.59
SD × Vit C	1	0.55	0.13	0.67	0.12	0.20	0.12	0.86	0.20

Yield, relative breast weight; WHC, water holding capacity; TBARS, thiobarbituric acid-reactive substances (malondialdehyde equivalents per g of meat sample); CIE, Commission Internationale de l'Eclairage.

Table 5. Effects of stocking density (SD) and dietary supplementation of vitamin C (Vit C) on liver antioxidant status of broiler chickens

Treatment		Antioxidant status	
SD (birds/m^2)	Vit C (mg/kg)	MDA (μm/mg protein)	TAC (U/mg protein)
9	0	1.71	713
	200	1.84	702
18	0	1.69	681
	200	1.66	694
SEM (n = 6)		0.178	10.3
Main effect			
SD (birds/m^2)			
9		1.78	707
18		1.67	688
SEM (n = 12)		0.126	7.3
Vit C (mg/kg)			
0		1.70	697
200		1.75	698
SEM (n = 12)		0.126	7.3
Effect (*p*-value)	*df*		
SD	1	0.57	0.07
Vit C	1	0.77	0.92
SD × Vit C	1	0.66	0.27

MDA, malondialdehyde; TAC, total antioxidant capacity.

Table 6. Effects of stocking density (SD) and dietary supplementation of vitamin C (Vit C) on intestinal permeability in the jejunal mucosa of broiler chickens

Treatment		Intestinal permeability		
SD (birds/m^2)	Vit C (mg/kg)	TER (Ω/cm^2)	PD (mV)	ISC (μa/cm^2)
9	0	336	300	0.90
	200	297	173	5.69
18	0	218	125	1.73
	200	244	258	2.24
SEM (n = 6)		36.3	99.4	2.758
Main effect				
SD (birds/m^2)				
9		316	236	3.30
18		231	192	1.99
SEM (n = 12)		25.6	70.3	1.950
Vit C (mg/kg)				
0		277	213	1.32
200		271	215	3.97
SEM (n = 12)		25.6	70.3	1.950
Effect (*p*-value)	*df*			
SD	1	0.03	0.66	0.64
Vit C	1	0.86	0.98	0.35
SD × Vit C	1	0.38	0.21	0.45

TER, trans-epithelial electrical resistance; PD, trans-epithelial voltage; ISC, short circuit current.

for these negative outcomes from high SD has been associated with various environmental and behavioral factors. First, high SD restrains the movement of birds in a floor space, which leads birds to have a difficulty in accessing feeders and drinkers [21]. Moreover, high SD may create an undesirable environment for birds owing to decreased air and floor quality, promoting health problems [6]. It is also suggested that birds raised at high SD may undergo moderate heat stress because of a reduction in heat dissipation by crowding [27]. These environmental and behavior problems caused by high SD are known to increase stress responses, which was confirmed by our observation for increasing stress responses with increasing blood H:L and feather CORT concentrations [28,29].

Vitamin C is a well-known antioxidant for animals but dietary supplementation of vitamin C is considered unnecessary for poultry because poultry can endogenously synthesize vitamin C in the kidney and liver [30,31]. However, under some conditions when poultry cannot synthesize enough vitamin C to satisfy its requirements such as stressful environment or aging, dietary supplementation of vitamin C may be required [12]. Likewise, several previous studies have reported that dietary supplementation of vitamin C exerted beneficial effects on productive performance of broiler chickens exposed to heat stress [11–13]. However, there is limited information regarding the effects of dietary supplementation of vitamin C on broiler chickens raised at high SD. Therefore, we hypothesized that dietary supplementation of vitamin C may ameliorate the negative effect of high SD on broiler chickens. However, our results indicated that dietary supplementation of 200 mg/kg vitamin C had no beneficial effect on growth performance in broiler chickens, regardless of SD. The reason is not clear; however, it may be related to the fact that the extent of the high SD used in the present study may be insufficient to impart a significant stress on broiler chickens because previous studies suggested that the beneficial effect of dietary vitamin C may be insignificant when

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birds were raised under the normal or low stressful condition [12]. However, it should be noted that growth performance of birds raised at high SD was decreased in the current study, which makes it difficult to conclude that our experimental condition was not significantly stressful. One possible reason for little benefit of dietary supplementation of vitamin C may be that birds raised at high SD may have increased requirement of vitamin C, but this increase may be compensated by increased endogenous synthesis of vitamin C, which likely blunts the effect of feeding additional vitamin C to broiler chickens. To our knowledge, however, there are no previous studies regarding the interactive effect of dietary vitamin C and different SD on endogenous synthesis of vitamin C in broiler chickens. Thus, it is suggested that more studies are required to reveal the relationship between dietary supplementation of vitamin C and different levels of SD in broiler chickens.

High SD and dietary supplementation of vitamin C did not influence meat quality in the current study. It has been reported that high SD impairs broiler meat quality because high SD promotes oxidative stress, which is one of the main causes of decreased meat quality [32,33]. However, previous results have been equivocal. Our previous study [15] and several other studies [14,34] reported little association between high SD and broiler meat quality. The reason for these inconsistent results has been related to the variation in animals and the differing extent of high SD among studies. Likewise, the extent of the high SD used in the present study may also be too small to induce oxidative stress that affects meat quality in broiler chickens, which may be supported by our results that high SD had no effects on MDA and TAC concentrations in the liver. Accordingly, it appears that dietary supplementation of 200 mg/kg vitamin C as an antioxidant may exert a limited effect on meat quality of broiler chickens raised at our high SD conditions. Moreover, most values for meat quality in the current study were placed in the normal quantitative range for broiler breast quality, indicating that the current high SD conditions and dietary supplementation of vitamin C may not affect meat quality of broiler chickens.

The damage in intestinal barrier function has been associated with decreased productive performance and increased health problems in animals by increasing inflammatory responses and oxidative stress [35,36]. In the current study, TER values as a measure of intestinal barrier function were decreased by high SD, which agrees with our previous findings that increasing SD linearly decreased TER values by increasing intestinal permeability (i.e., decreasing intestinal barrier function) [15]. This decreased intestinal barrier function may be one of the reasons why broiler chickens raised at high SD had decreased performance in the current study. In previous studies, increased oxidative damage in intestinal cells was reported to decrease intestinal barrier function [37,38]. Therefore, it was hypothesized that dietary supplementation of vitamin C may ameliorate decreased intestinal barrier function of broiler chickens by a high SD. However, our hypothesis was not verified in the current study. The reason may be related to the fact that decreased intestinal barrier function by various stressors may not be caused only by increased oxidative stress in intestinal cells [35]. In addition, absorbed vitamin C may be rapidly transferred to blood circulation instead of being retained in the enterocyte [39], which may be the reason why vitamin C had little benefit on intestinal barrier function.

CONCLUSION

High SD of broiler chickens decreases productive performance and intestinal barrier function but increases stress responses without affecting meat quality and antioxidant status in the liver. However, no clear beneficial effects of supplemental vitamin C in diets at the level of 200 mg/kg are observed for broiler chickens raised at the high SD condition used in this study.

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