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Original Research Article

Role of dietary gamma-aminobutyric acid in broiler chickens raised under high stocking density

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ABSTRACT

The present study was conducted to evaluate the effects of dietary gamma-aminobutyric acid (GABA) in broiler chickens raised in high stocking density (HSD) on performance and physiological responses. A total of 900 male broiler chicks (Ross 308) at 1 d old were assigned in a 2 × 2 factorial arrangement to 4 treatments (10 replicates per treatment) with stocking density, 7.5 birds/m^2 (low stocking density; LSD) or 15 birds/m^2 (HSD), and dietary GABA, 0 or 100 mg/kg. Chickens raised in HSD exhibited a decrease in body weight gain in all phases ($P < 0.05$) and feed intake in starter and whole phases ($P < 0.01$), and an increase in feed conversion ratio in the finisher phase ($P < 0.01$) compared with LSD-raised chickens. However, dietary GABA did not affect growth performance nor interacted with stocking density on production variables. The HSD vs. LSD increased relative liver weight on d 35 whereas dietary GABA increased relative liver weight and decreased relative bursa weight on d 21. Both stocking density and dietary GABA affected yield and quality of breast and leg muscles. Dietary GABA increased ($P < 0.05$) width of tibia on d 35 and interacted ($P = 0.054$) with stocking density on breaking stocking density on d 35. The HSD vs. LSD group lowered ($P < 0.05$) feather coverage scores. Significant interaction between stocking density and GABA on surface temperature of shank on d 21 was noted ($P = 0.024$). Dietary GABA exhibited an opposite effect on the concentrations of cecal short-chain fatty acids depending on stocking density leading to a moderate to significant interaction. Stocking density decreased alpha-1-acid glycoprotein whereas dietary GABA decreased heterophil-to-lymphocyte ratio and corticosterone in blood or serum samples. Serum biochemical parameters were altered by stocking density or dietary GABA. It is concluded that dietary GABA alleviated stress indices including corticosterone and heterophil-to-lymphocyte ratio, but failed to reverse stocking density-induced growth depression.

1. Introduction

In the broiler industry, one of the most critical stressful factors is to raise birds under high stocking density (HSD) in commercial production. This regimen however negatively affects the welfare, health, and productivity (i.e., growth performance or carcass quality) of commercial broilers (Simitzis et al., 2012). It is documented that increasing stocking density lowered body weight gain and feed intake (Dozier et al., 2006), downgraded poultry products (Dozier et al., 2005), induced leg problem, e.g., tibial dyschondroplasia (Sanotra et al., 2001) and foot lesion, i.e., foot pad dermatitis (Dozier et al., 2005), and in severe cases raised mortality (Imaeda, 2000). In addition, HSD led to changes in behaviors as well as increased stress indicators, e.g., heterophil-to-lymphocyte (H:L) ratio and corticosterone (Kuan et al., 1990; Shakeri et al., 2014) and susceptibility to diseases, e.g., Newcastle disease and necrotic enteritis (Mustafa et al., 2010; Tsiouris et al., 2015). It is also known that HSD vs. low stocking density

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(LSD) impairs gut functions that are linked to compromised nutrient absorption in chickens (Shakeri et al., 2014). It is thus a common practice to employ nutritional strategies to minimize stocking density-associated decrease in productivity and increase in adverse physiological responses in broiler chickens (Houshmand et al., 2012; Wang et al., 2014).

Gamma-aminobutyric acid (GABA), generally recognized as safe feed additive, is a four-carbon non-protein amino acid that acts as a primary inhibitory neurotransmitter in the central nervous system of animals (Kinnersley and Turano, 2000). Its principal role is to reduce neuronal excitability throughout the nervous system, consequently alleviating the intensity of stress (Kuffler and Edwards, 1958). In addition to neural tissues, the presence of GABA receptors in non-neural tissues including liver, pancreas and kidney indicates that GABA may exhibit biological activities in these tissues (Tillakaratne et al., 1995). Indeed, GABA has been known to possess antidiabetic, antioxidant, and immune modulating properties (Bhat et al., 2010; Kumar and Goyal, 2008; Soltani et al., 2011).

Due to the well-reported biological activities, GABA as a functional feed additive has been widely used in animal industry to improve growth performance and to prevent stress-related signs in farm animals (Zhang et al., 2012) and in heat-stressed broiler chickens (Dai et al., 2011, 2012). Tentatively, it is concluded that dietary GABA is considered an effective nutritional strategy to minimize stressor-induced factors and/or to improve gut functions of chickens. To our surprise, there is a dearth of research to overcome the negative effects of HSL on productivity and physiological responses by dietary GABA in broiler chickens. It would be expected that dietary GABA might act as a stress reliever and/or a digestion aid in broiler chickens under the overcrowding environment. Thus, the present study was aimed to test our expectation whether dietary GABA would affect growth performance, carcass and bone characteristics, short-chain fatty acids (SCFA), and physiological stress responses in broiler chickens raised in different stocking densities. In addition, 2 parameters including feather coverage score and body surface temperature commonly employed in stocking density studies (Abudabos et al., 2013; Škrbić et al., 2011; Thomas et al., 2004) were also included to see the role of GABA on behavioral and metabolic consequences.

2. Materials and methods

2.1. Animals, diets and experiment design

The experimental procedure was approved by the Institutional Animal Care and Use Committee of Konkuk University (KU182058). A total of 900 broiler chicks (Ross 308) at 1 d old were purchased from a local hatchery, weighed upon arrival, and assigned to 4 treatments with 10 replicates of 15 or 30 birds in a completely randomized design and fed experimental diets with or without GABA, thus leading to a 2 × 2 factorial arrangement. Experimental diets (Table 1) were formulated by mixing corn-soybean meal-based starter and finisher diets with or without GABA at 100 mg/kg of diet. Broiler chickens raised either at a stocking density of 7.5 or 15 birds/m^2 were fed the starter diet from d 1 to 21 and the finisher diet from d 22 to 35. Each floor pen had an area of 2 m^2, a feeder with a nipple-type waterer (52 cm diameter), and rice husk as a bedding material. Temperature of the facility was initially set at 34 °C during the first week, then gradually decreased to 24 °C on d 21 and maintained thereafter. Feed and water were provided ad libitum and light was provided 23 h/d. Body weight and feed intake per pen were weekly monitored. The incidence of mortality was recorded as it occurred during daytime and used to calculate the mortality-adjusted feed conversion ratio.

2.2. Sample collection

On d 21 and 35, one bird with its body weight close to pen average body weight was selected per pen and euthanized by carbon dioxide asphyxiation for sampling. Immediately after euthanasia, blood was taken via cardiac puncture and collected in both clot activator (BD Vacutainer, CAT, cat. Ref. 367,896) and heparinized (BD Vacutainer, LH PST, cat. Ref. 368,497) tubes. Serum samples were obtained by gentle centrifugation at 200 × *g* for 15 min and stored at −20 °C before analysis. After blood sampling, left breast and leg meats were sampled and weighed. Then, internal organs (i.e., liver, spleen, pancreas, and bursa of Fabricius) and abdominal fat were sampled and weighed. Relative organ weights were calculated and expressed as grams of organ weights per 100 g of live body weight. For measurement of secretory immunoglobulin A (sIgA) contents in ileal mucosa, a 5-cm ileal segment proximal to the Meckel's diverticulum was sampled. A pair of ceca were sampled and kept on ice before processing for SCFA and *C. perfringens* counts on the day of the sampling.

2.3. Meat quality

The breast and leg meats were used to measure meat quality including cooking loss, meat color, and pH. The pH values of meats were measured in duplicate with a pH meter (Testo 205, Testo AG, Lenzkirch, Germany). Meat color was measured on the central side of the breast and leg meats at 3 different points using a portable spectrophotometer (CM-2600 d, Konica Minolta, Ramsey, NJ, USA). The International Commission on Illumination (CIE) lightness (L*), redness (a*), and yellowness (b*) components were obtained from the Specular Component Excluded (SCE) mode readings. To measure the cooking loss, the breast and leg meats were packaged in a plastic bag under vacuum and chilled. Samples were cooked in a water bath at 80 °C for 30 min to an internal temperature of 70 °C as described by Huang et al. (2017). After cooking, meat samples were cooled into ice-cold water for 10 min at a room temperature and residual moisture was removed with paper towel before reweighing. Cooking loss was calculated as the percentage of weight lost by the samples.

2.4. Tibia characteristics

The left leg was excised and stored in refrigerator until analyzed. The left tibia was obtained by manually removing the attached meat, then the weight, width and length of tibia including epiphysis were measured. Bone breaking strength was measured on the fresh tibia using an Instron (Model 3342, Instron Universal Testing Machine, Instron Corp., Norwood, MA, USA) with 50-kg load rage with a crosshead speed of 50 mm/min with tibia supported on a 3.35-cm span. The graph showed the plateau curve of applied maximal force (kN) to measure the bone strength as expressed as energy stored in the bone. The sheared tibia pieces were then dried at 135 °C for 2 h and ashed in a muffle furnace at 550 °C for 6 h.

2.5. Feather coverage scoring

On d 35, a total of 5 birds per pen were randomly selected to score the feather coverage status of 6 body parts (i.e., head, dorsal neck, ventral neck, back, breast, and belly) with traditional 4-scale feather coverage scoring as described by Tauson et al. (1984). Three independent observers scored feather coverage status of the same birds by giving score 1 representing the worst feather coverage to score 4 representing the best: score 1 = a body part with heavily damaged plumage, with no or only small areas of the body covered with feathers, score 2 = a body part with clearly deteriorated

Table 1
Ingredients and nutrient composition of the basal diets (as-fed basis).

Item	Starter diet (d 1 to 21)	Grower diet (d 22 to 35)
Ingredients, g/100 g		
Corn (8.8% CP)	57.11	63.31
Soybean meal (44.8% CP)	30.50	25.00
Corn gluten meal (60% CP)	5.00	3.50
Soybean oil	2.50	4.00
Salt	0.30	0.22
Dicalcium phosphate	1.70	1.23
DL-methionine (99%)	0.34	0.26
L-Lysine (78%)	0.31	0.28
L-Threonine	0.10	0.05
Limestone	1.30	1.35
Choline	0.24	0.20
Choline chloride, 50%	0.20	0.20
Vitamin premix[1]	0.20	0.20
Mineral premix[2]	0.20	0.20
Total	100.00	100.00
Nutrient composition, g/100 g		
AMEn[3], kcal/kg	3,068	3,208
Dry matter[4]	89.20	89.30
Crude protein[4]	22.30	19.30
Lysine[3]	1.31	1.13
Total sulfur amino aicd[3]	1.04	0.95
Calcium[4]	1.00	0.90
Non phytate phosphorus[3]	0.45	0.35

[1] Vitamin premix provided following nutrients per kilogram of diet: vitamin A, 24,000 IU; vitamin D_3, 6,000 IU; vitamin E, 80 IU; vitamin K_3, 4 mg; thiamine, 4 mg; riboflavin, 10 mg; pyridoxamine, 6 mg; vitamin B_{12}, 0.04 mg; niacin, 80 mg; pantothenic acid, 20 mg; folic acid, 2 mg; biotin, 0.3 mg.
[2] Mineral premix provided following nutrients per kilogram of diet: Fe, 176 mg; Cu, 145 mg; Zn, 120 mg; Mn, 132 mg; I, 2 mg; Co, 1 mg; Se, 0.44 mg.
[3] Calculated value.
[4] Analyzed value.

feathers or larger naked areas or both, score 3 = a body part where feathers have deteriorated, but the body is still completely or almost completely covered, and score 4 = a very well feathered body part with no or few worn or otherwise deformed feathers.

2.6. Body surface temperature measurement

On d 21 and 35, a total of 6 birds per pen were randomly selected to take the images of body surface temperature using a thermal imaging camera (Model FLIR-300, FLIR Systems Inc., Wilsonville, OR) at a horizontal distance about 0.8 m away from the birds described by Zhao et al. (2013). The images were taken by the one investigator covering all the 3 body parts (i.e., the head, breast, and shank) during the time period of 10:00 to 12:00.

2.7. Ileal secretory immunoglobulin a measurement

Secretory immunoglobulin A concentrations in ileal mucosa were measured using a quantitative chicken-specific IgA enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as described by the manufacturer's recommendation. The ileal segment was kept on ice until the preparation on the day of the sampling. The ileal segment was cut longitudinally and rinsed using ice-cold phosphate buffered saline (PBS). The ileal mucosa was obtained by gentle scraping using a tissue culture scraper and homogenized with 5 mL of PBS and the mixture was centrifuged at 27,000 × *g* at 4 °C for 20 min. Supernatants were then aliquoted and stored at −20 °C until analysis. The amount of protein in supernatant samples were determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA, USA). The sIgA concentrations were expressed as nanograms of sIgA per microgram of total protein.

2.8. C. perfringens counts in cecal digesta

Approximately 1 g of cecal digesta was diluted with 9 mL of cold distilled water and the dilutions were subjected to a 10-fold serial dilution. The dilutions were then spiral-plated on tryptose-sulfite-cycloserine agar (Difco Reinforced Clostridial Medium, Difco, BD, Sparks, MD, USA) and incubated anaerobically at 37 °C for 24 h. The number of characteristic black colony was then counted and expressed as log CFU per gram of cecal digesta.

2.9. Short-chain fatty acids analysis

Approximately 1 g of cecal digesta was diluted with 9 mL of cold distilled water and mixed by using vortex mixer (C-VT Test Tube Vortex Mixer, Chang Shin Scientific Co., Incheon, Korea). The mixture was added with 0.05 mL of saturated $HgCl_2$, 1 mL of 25% H_3PO_4 and 0.2 mL of 2% pivalic acid, and centrifuged at 1,000 × *g* at 4 °C for 20 min. Then, 1 mL of supernatants was used to measure the concentrations of SCFA in cecal samples by gas chromatography (6890 Series GC System, HP, Palo Alto, CA, USA) as described by van der Wielen et al. (2000).

2.10. Heterophil-to-lymphocyte ratio

One drop of the whole blood sample from a heparinized tube was smeared on the surface of a slide glass and dyed using a Differential Quik Stain Kit (Polysciences Asia–Pacific, Inc.). Heterophils

and lymphocytes in blood samples were counted under the light microscope (Olympus BX 43, Olympus Optical Co. Ltd., Tokyo, Japan) and used to calculate H:L ratio.

2.11. Serum assay

The concentrations of corticosterone and alpha-1-acid glycoprotein in serum samples were determined using Corticosterone ELISIA Kit (Enzo Life Sciences Inc., NY, USA) and a chicken alpha-1-acid glycoprotein assay kit (Life Diagnostics, Inc., West Chester, PA, USA) as described by the manufacturers. The concentrations of nitric oxide in serum samples were determined as described by Lee et al. (2011). Total antioxidant capacity in serum samples was analyzed using a QuantiChrom antioxidant assay kit (BioAssay Systems, Hayward, CA, USA) and expressed as millimoles per liter Trolox equivalents. Serum samples were analyzed for glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, glucose, total cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol, total protein, albumin, globulin (calculated from total protein minus albumin), phosphorus, calcium, and uric acid using an automated dry chemistry analyzer (Film DRI CHEM 7000i, Fuji film, Tokyo, Japan).

2.12. Statistical analysis

Each pen was considered an experimental unit. Data for all variable were analyzed by a 2-way analysis of variance (ANOVA) with the model including stocking density and GABA as the main factors and their interaction using the general linear model (GLM) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was employed to determine means differences among treatments. Significant differences among treatments were determined at $P < 0.05$.

3. Results

3.1. Growth performance

Body weight gain was decreased ($P \leq 0.05$) in HSD- vs. LSD-raised chickens during starter, finisher and whole periods (Table 2). Chickens raised in HSD ate less ($P < 0.01$) compared with those raised in LSD during starter and whole periods. The HSD vs. LSD decreased feed conversion ratio during the starter period but increased it during the finisher period. Dietary GABA did not influence growth performance and no interaction between stocking density and GABA was noted.

3.2. Relative organ weight

Relative spleen, pancreas, and abdominal fat weights were not affected by stocking density or dietary GABA (Table 3). Relative liver weight was increased ($P = 0.021$) in chickens fed the GABA-supplemented diet compared with chickens fed the non-supplemented control diet on d 21 and increased ($P = 0.013$) in HSD-raised chickens compared with chickens raised in LSD on d 35. Dietary GABA decreased relative weight of bursa of Fabricius on d 21. No interaction between stocking density and GABA on organ weights was detected.

3.3. Yield and quality of breast and leg meats

Dietary GABA increased ($P = 0.016$) the absolute weight of breast meat on d 21 whereas HSD vs. LSD lowered it on d 35 (Table 4). However, none of the factors affected the relative weight of breast meat at any ages. On d 21, interaction between stocking density and GABA on cooking loss of breast meat was noted as dietary GABA increased cooking loss of breast meat in LSD-raised chickens. The b* value of breast meat on d 21 was increased ($P = 0.042$) in GABA-fed chickens compared with the non-supplemented diet-fed control groups. The pH of breast meat was decreased ($P = 0.011$) by dietary GABA on d 21 and by stocking density on d 35.

Interactions between stocking density and dietary GABA were observed in the absolute weight of leg meat ($P = 0.018$) and relative weight of leg meat ($P = 0.035$) on d 35 (Table 5). This is because leg meat weight and relative weight was increased in chickens raised in LSD by dietary GABA. Stocking density lowered the cooking loss of leg meat and dietary GABA increased it on d 21. Dietary GABA tended to increase CIE L* value of leg meat ($P = 0.055$) and to decrease the pH of the leg meat ($P = 0.050$) on d 21.

Table 2
Effect of dietary gamma-aminobutyric acid (GABA) on growth performance in broiler chickens raised in different stocking densities.[1]

Item		Body weight gain, g/d per bird			Feed intake, g/d per bird			Feed conversion ratio, g:g		
		d 1 to 21	d 22 to 35	d 1 to 35	d 1 to 21	d 22 to 35	d 1 to 35	d 1 to 21	d 22 to 35	d 1 to 35
Density[2]	GABA[3]									
Low	−	32.09	75.21	49.34	48.38	116.9	75.71	1.51	1.56	1.53
	+	33.38	76.44	50.61	48.95	117.5	76.31	1.47	1.54	1.51
High	−	31.36	71.80	47.53	43.97	115.0	72.30	1.40	1.61	1.52
	+	31.85	72.88	48.38	44.67	117.1	73.44	1.40	1.62	1.53
SEM		0.56	1.57	0.73	0.64	1.76	0.92	0.015	0.020	0.010
Main factors										
Low		32.74	75.83[a]	49.97[a]	48.66[a]	117.2	76.01[a]	1.49[a]	1.55[b]	1.52
High		31.61	72.34[b]	47.96[b]	44.32[b]	116.1	72.87[b]	1.40[b]	1.61[a]	1.52
	−	31.72	73.50	48.44	46.17	116.0	74.00	1.46	1.58	1.53
	+	32.62	74.66	49.49	46.81	117.3	74.87	1.44	1.58	1.52
P-value										
Density (D)		0.050	0.035	0.010	<0.001	0.516	0.002	<0.001	0.003	0.844
GABA (G)		0.117	0.472	0.159	0.331	0.452	0.352	0.324	0.843	0.299
D × G		0.482	0.960	0.775	0.923	0.687	0.774	0.079	0.493	0.156

SEM = pooled standard error of the means.
[a, b] Values ($n = 10$/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low stocking density, 7.5 birds/m^2; High stocking density, 15 birds/m^2.
[3] −, 0 mg/kg of diet; +, 100 mg/kg of diet.

Table 3
Effect of dietary gamma-aminobutyric acid (GABA) on relative organ weight (% BW) in broiler chickens raised in different stocking densities.[1]

Item		Liver		Spleen		Bursa of Fabricius		Pancreas		Abdominal fat	
		d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35
Density[2]	GABA[3]										
Low	−	3.46	2.78	0.119	0.141	0.255	0.237	0.394	0.317	0.779	0.991
	+	3.51	2.52	0.120	0.140	0.201	0.211	0.399	0.324	0.691	0.818
High	−	3.20	2.86	0.135	0.133	0.252	0.177	0.439	0.307	0.645	0.885
	+	3.56	3.01	0.116	0.137	0.223	0.225	0.423	0.316	0.688	0.909
SEM		0.085	0.11	0.0093	0.014	0.018	0.020	0.023	0.015	0.060	0.072
Main factors											
Low		3.48	2.65[b]	0.120	0.140	0.228	0.224	0.397	0.320	0.735	0.905
High		3.38	2.94[a]	0.126	0.135	0.237	0.201	0.431	0.311	0.666	0.897
	−	3.33[b]	2.82	0.127	0.137	0.253[a]	0.207	0.416	0.312	0.712	0.938
	+	3.54[a]	2.76	0.118	0.138	0.212[b]	0.218	0.411	0.320	0.689	0.864
P-value											
Density (D)		0.244	0.013	0.506	0.709	0.590	0.245	0.136	0.537	0.260	0.916
GABA (G)		0.021	0.608	0.348	0.926	0.029	0.575	0.824	0.572	0.710	0.309
D × G		0.075	0.077	0.299	0.863	0.483	0.069	0.641	0.968	0.280	0.183

BW = live body weight; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, 0 mg/kg of diet; +, 100 mg/kg of diet.

Table 4
Effect of dietary gamma-aminobutyric acid (GABA) on yield and quality of breast meat in broiler chickens raised in different stocking densities.[1]

Item		Fresh weight, g		Breast meat yield, % BW		Cooking loss, %		CIE L*		CIE a*		CIE b*		pH	
		d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35
Density[2]	GABA[3]														
Low	−	47.93	145.2	6.85	8.21	20.29[b]	23.37	49.96	53.28	2.23	1.05	13.69	12.06	5.93	5.75
	+	52.77	147.6	7.20	8.21	24.39[a]	22.66	51.19	53.31	2.58	0.69	14.50	12.40	5.80	5.78
High	−	47.07	140.1	7.00	8.11	22.99[a]	21.62	51.18	52.59	1.70	0.70	13.47	11.60	5.91	5.85
	+	49.17	133.1	6.81	7.69	23.02[a]	22.67	51.45	52.15	2.16	0.99	14.73	11.88	5.86	5.86
SEM		1.33	4.55	0.13	0.16	0.84	1.49	0.53	1.00	0.32	0.29	0.49	0.41	0.031	0.040
Main factors															
Low		50.35	146.4[a]	7.02	8.21	22.34	23.01	50.58	53.30	2.41	0.87	14.10	12.23	5.87	5.76[b]
High		48.12	136.6[b]	6.90	7.90	23.00	22.15	51.31	52.37	1.93	0.84	14.10	11.74	5.88	5.85[a]
	−	47.50[b]	142.7	6.92	8.16	21.64[b]	22.50	50.57	52.94	1.97	0.88	13.58[b]	11.83	5.92[a]	5.80
	+	50.97[a]	140.4	7.01	7.95	23.71[a]	22.66	51.32	52.73	2.37	0.84	14.61[a]	12.14	5.83[b]	5.82
P-value															
Density (D)		0.111	0.041	0.406	0.093	0.454	0.568	0.192	0.374	0.179	0.923	1.000	0.272	0.634	0.030
GABA (G)		0.016	0.624	0.570	0.234	0.024	0.913	0.185	0.843	0.255	0.902	0.042	0.480	0.011	0.570
D × G		0.321	0.315	0.072	0.240	0.026	0.561	0.388	0.817	0.871	0.277	0.652	0.951	0.190	0.881

BW = live body weight; CIE = International Commission on Illumination; L* = lightness; a* = redness; b* = yellowness; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, 0 mg/kg of diet; +, 100 mg/kg of diet.

3.4. Tibia characteristics

None of tibia characteristics except for relative weight and width of tibia was affected by stocking density and dietary GABA at any ages. Relative weight of fresh tibia was increased ($P = 0.023$) in chickens raised in HSD than chickens raised in LSD on d 35 (Table 6). Dietary GABA increased ($P = 0.028$) the width of tibia compared with chickens fed the non-supplemented control diet.

3.5. Feather coverage score

The HSD vs. LSD clearly lowered ($P < 0.05$) the feather coverage scores of neck, back, breast, and belly (Table 7). However, dietary GABA did not affect feather coverage scores.

3.6. Body surface temperature

Body surface temperatures at the head and breast areas were not affected by stocking density or dietary GABA at any ages (Table 8). On the other hand, the interaction between stocking density and dietary GABA on shank temperature was noted on d 21. Dietary GABA increased or decreased the shank temperature depending on the stocking density, but this effect was only seen on d 21.

3.7. Ileal secretory immunoglobulin A

Secretory immunoglobulin A concentration in ileal mucosa ranged between 8.6 and 10.1 ng per μg of protein on d 21 and increased to 16.7 to 20.4 ng per μg of protein on d 35 (data not shown). However, neither

Table 5
Effect of dietary gamma-aminobutyric acid (GABA) on yield and quality of leg meat in broiler chickens raised in different stocking densities.[1]

Item		Fresh weight, g		Leg meat yield, % BW		Cooking loss, %		CIE L*		CIE a*		CIE b*		pH	
		d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35
Density[2]	GABA[3]														
Low	−	45.09	118.7^{b}	6.45	6.74^{b}	33.25	28.97	57.14	53.14	6.78	7.05	17.88	15.74	6.46	6.31
	+	48.58	129.2^{a}	6.69	7.15^{a}	35.48	29.82	58.61	53.09	5.98	7.27	17.05	15.72	6.37	6.22
High	−	44.57	124.5^{ab}	6.40	7.20^{a}	30.60	30.23	57.03	54.75	6.43	6.22	16.89	15.61	6.51	6.26
	+	45.26	120.1^{b}	6.32	7.09^{a}	33.38	28.08	59.21	53.22	6.14	6.92	17.76	15.23	6.43	6.24
SEM		1.39	2.99	0.13	0.12	0.86	1.11	0.92	0.87	0.43	0.53	0.66	0.51	0.042	0.056
Main factors															
Low		46.84	123.9	6.57	6.95	34.36^{a}	29.40	57.88	53.12	6.38	7.16	17.46	15.73	6.42	6.27
High		44.91	122.3	6.36	7.14	31.99^{b}	29.16	58.12	53.99	6.29	6.57	17.33	15.42	6.47	6.25
	−	44.83	121.6	6.43	6.97	31.92^{b}	29.60	57.09	53.94	6.61	6.63	17.38	15.68	6.49	6.29
	+	46.92	124.6	6.51	7.12	34.43^{a}	28.95	58.91	53.16	6.06	7.09	17.41	15.48	6.40	6.23
P-value															
Density (D)		0.182	0.589	0.130	0.107	0.012	0.836	0.793	0.338	0.827	0.280	0.844	0.554	0.205	0.754
GABA (G)		0.148	0.314	0.553	0.223	0.008	0.579	0.055	0.385	0.223	0.394	0.976	0.701	0.050	0.307
D × G		0.330	0.018	0.240	0.035	0.762	0.204	0.702	0.412	0.574	0.655	0.224	0.731	0.896	0.562

BW = live body weight; CIE = International Commission on Illumination; L* = lightness; a* = redness; b* = yellowness; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, 0 mg/kg of diet; +, 100 mg/kg of diet.

Table 6
Effect of dietary gamma-aminobutyric acid (GABA) on tibia characteristics in broiler chickens raised in different stocking densities.[1]

Item		Fresh weight, g		Relative weight, % BW		Width, cm		Length, cm		Breaking strength, kN		Ash, % DM	
		d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35
Density[2]	GABA[3]												
Low	−	6.61	15.82	0.95	0.88	0.51	0.71	7.36	10.17	0.16	0.30	30.43	27.43
	+	6.73	16.49	0.93	0.91	0.51	0.75	7.44	10.19	0.15	0.33	30.60	27.22
High	−	6.59	16.04	0.95	0.93	0.50	0.70	7.29	10.13	0.14	0.33	30.05	27.55
	+	6.59	16.35	0.95	0.97	0.52	0.75	7.34	10.03	0.15	0.29	29.72	27.19
SEM		0.16	0.33	0.019	0.021	0.014	0.020	0.059	0.099	0.0059	0.014	0.71	0.48
Main factors													
Low		6.67	16.15	0.94	0.90^{b}	0.51	0.73	7.40	10.18	0.15	0.31	30.52	27.32
High		6.59	16.20	0.95	0.95^{a}	0.51	0.73	7.32	10.08	0.14	0.31	29.88	27.37
	−	6.60	15.93	0.95	0.91	0.51	0.71^{b}	7.32	10.15	0.15	0.32	30.24	27.49
	+	6.66	16.42	0.94	0.94	0.52	0.75^{a}	7.39	10.11	0.15	0.31	30.16	27.20
P-value													
Density (D)		0.603	0.898	0.528	0.023	1.000	0.801	0.172	0.321	0.130	0.845	0.381	0.921
GABA (G)		0.701	0.146	0.501	0.097	0.490	0.028	0.265	0.690	0.500	0.735	0.918	0.559
D × G		0.702	0.588	0.664	0.835	0.490	0.801	0.773	0.550	0.216	0.054	0.727	0.880

BW = live body weight; DM = dry matter; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, 0 mg/kg of diet; +, 100 mg/kg of diet.

stocking density nor dietary GABA affected sIgA concentrations in ileal mucosa contents at all ages. No interaction between stocking density and GABA was observed (data not shown).

3.8. *C. perfringens* counts in cecal digesta

The *C. perfringens* counts ranged from 7.09 to 7.52 log CFU per g of cecal digesta on d 35 (data not shown). However, none of the factors affected ($P > 0.05$) cecal *C. perfringens* (data not shown).

3.9. SCFA in cecal digesta

None of the factors affected SCFA in cecal digesta on d 21 or 35. On the other hand, interaction between stocking density and dietary GABA on isobutyrate ($P = 0.047$) on d 21 and total SCFA on d 35 ($P = 0.032$) in cecal digesta were observed (Tables 9 and 10).

3.10. Stress and antioxidant indicators

Dietary GABA, but not stocking density, decreased ($P = 0.037$) blood H:L ratio (Table 11). In addition, the concentration of corticosterone in serum samples tended to be decreased by 33.0% in chickens fed the GABA-added diet compared with the non-supplemented control diet-fed chickens. The HSD vs. LSD lowered ($P = 0.031$) alpha-1-acid glycoprotein in serum samples and dietary GABA increased ($P = 0.074$) it compared with the no-added control group. Nitric oxide concentration in serum samples was increased ($P = 0.028$) by dietary GABA. However, total antioxidant capacity was not affected by stocking density or dietary GABA and no interaction between two main factors was observed.

3.11. Serum biochemistry

Chickens fed the GABA-supplemented diet had more ($P < 0.05$) glutamic oxaloacetic transaminase and total cholesterol in serum

Table 7
Effect of dietary gamma-aminobutyric acid (GABA) on feather coverage score in broiler chickens raised in different stocking densities on d 35.[1]

Item		Feather coverage score[2]						
		Head	Dorsal neck	Back	Ventral neck	Breast	Belly	Average
Density[3]	GABA[4]							
Low	−	3.56	3.48	3.84	3.26	3.54	3.10	3.46
	+	3.54	3.44	3.80	3.40	3.50	3.02	3.45
High	−	3.56	3.16	3.38	3.08	2.96	2.32	3.08
	+	3.52	3.12	3.22	3.00	2.70	2.14	2.95
SEM		0.084	0.10	0.087	0.11	0.12	0.13	0.068
Main factors								
Low		3.55	3.46^{a}	3.82^{a}	3.33^{a}	3.52^{a}	3.06^{a}	3.46^{a}
High		3.54	3.14^{b}	3.30^{b}	3.04^{b}	2.83^{b}	2.23^{b}	3.01^{b}
	−	3.56	3.32	3.61	3.17	3.25	2.71	3.27
	+	3.53	3.28	3.51	3.20	3.10	2.58	3.20
P-value								
Density (D)		0.906	0.003	<0.001	0.014	<0.001	<0.001	<0.001
GABA (G)		0.724	0.698	0.256	0.790	0.232	0.327	0.313
D × G		0.906	1.000	0.493	0.332	0.378	0.704	0.413

SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Score 1 = a body part with heavily damaged plumage, with no or only small areas of the body covered with feathers; score 2 = a body part with clearly deteriorated feathers or larger naked areas or both; score 3 = a body part where feathers have deteriorated, but the body is still completely or almost completely covered; score 4 = a very well feathered body part with no or few worn or otherwise deformed feathers.
[3] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[4] −, GABA at 0 mg/kg of diet; +, GABA at 100 mg/kg of diet.

Table 8
Effect of dietary gamma-aminobutyric acid (GABA) on body surface temperature (°C) in broiler chickens raised in different stocking densities.[1]

Item		Head		Breast		Shank	
		d 21	d 35	d 21	d 35	d 21	d 35
Density[2]	GABA[3]						
Low	−	35.52	34.87	36.08	35.06	35.69^{b}	35.51
	+	35.59	34.79	36.39	34.94	36.09^{ab}	35.62
High	−	35.57	34.99	36.47	35.19	36.42^{a}	35.54
	+	35.52	34.92	36.21	35.15	36.12^{ab}	35.71
SEM		0.17	0.15	0.16	0.25	0.15	0.26
Main factors							
Low		35.55	34.83	36.23	35.00	35.89^{b}	35.57
High		35.54	34.96	36.34	35.17	36.27^{a}	35.62
	−	35.55	34.93	36.27	35.12	36.06	35.52
	+	35.55	34.85	36.30	35.05	36.10	35.67
P-value							
Density (D)		0.961	0.400	0.507	0.494	0.017	0.842
GABA (G)		0.961	0.626	0.879	0.757	0.753	0.596
D × G		0.701	0.965	0.077	0.882	0.024	0.906

SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, GABA at 0 mg/kg of diet; +, GABA at 100 mg/kg of diet.

samples than chickens fed the non-supplemented control diet (Table 12). The HSD vs. LSD increased ($P < 0.05$) total cholesterol, HDL cholesterol, total protein, albumin, and globulin in serum samples. The albumin-to-globulin ratio in serum samples showed the interaction ($P = 0.026$) between stocking density and dietary GABA. However, glutamic pyruvic transaminase, calcium, phosphorus, and uric acid in serum samples were not affected by stocking density or dietary GABA. Marginal interaction between stocking density and GABA for concentration of calcium in serum was observed ($P = 0.051$).

4. Discussion

4.1. Growth performance

The negative effects of HSD on productivity have been well documented (Dozier et al., 2006) which corroborates our findings being substantial reduction of body weight gain and feed intake in HSD- vs. LSD-raised birds. In contrast to our expectation, dietary GABA failed to affect growth performance of chickens raised in different stocking densities. Initially, it was expected that dietary GABA would mitigate the stocking density-induced growth depression as it promoted the secretion of growth hormone (Willoughby et al., 1986), enhanced the digestive enzyme activities in heat-stressed Roman hens (Zhang et al., 2012) and ameliorated performance loss by heat stress in broilers (Dai et al., 2011). The lack of effect of dietary GABA on growth performance cannot be attributed to the addition levels in diets as dietary GABA at the level of 25 to 100 mg per kg of diet increased growth performance of broiler chickens exposed to heat stress (Chand et al., 2016). It may be likely that dietary GABA would be more effective in mitigating growth depression induced by heat stress rather than by stocking density.

4.2. Relative organ weight

The immune organs (i.e., bursa, thymus, and spleen) have been measured as indicators of physiological or immunological stresses as regressed lymphoid organs have been shown to occur in response to stress hormones (Puvadolpirod and Thaxton, 2000). In this study, stocking density did not affect the relative bursal weight at any ages, but dietary GABA decreased it on d 21. It is not clearly understood how dietary GABA affected bursa weight on d 21 but not on d 35. In any event, whether GABA-induced reduction in bursa weight is related to impaired immune function needs to be addressed. However, the anti-inflammatory effect by GABA (Duthey et al., 2010) and lack of effect of dietary GABA on relative bursa weight in the stressed chickens (Xie et al., 2013) have been reported.

Relative liver weight was increased in chickens fed the GABA-supplemented diet on d 21 and increased in HSD- vs. LSD-raised chickens on d 35. Stocking density-induced increase in liver weight can be attributed to an increase in liver fat caused by increased liver lipids in stressed chickens (Puvadolpirod and Thaxton, 2000). In line with our finding, it is reported that increasing stocking density increased relative liver weight in chickens (Simitzis et al., 2012). Dietary GABA did increase relative liver weight, but this effect was only seen on d 21. It is known that GABA receptors are present in

Table 9
Effect of GABA on concentration of SCFA in cecal digesta in broiler chickens raised in different stocking densities on d 21.[1]

Item		SCFA, mmol/L							
		Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	BCFA[2]	Total SCFA[3]
Density[4]	GABA[5]								
Low	−	40.06	5.38	0.43	11.35	0.46	0.72	1.60	58.92
	+	34.62	4.77	0.33	10.93	0.35	0.65	1.46	51.62
High	−	37.22	5.68	0.37	10.66	0.39	0.63	1.53	54.68
	+	36.94	5.57	0.48	9.73	0.49	0.69	1.89	54.18
SEM		2.96	0.42	0.050	1.46	0.069	0.083	0.18	4.45
Main factors									
Low		37.34	5.08	0.38	11.14	0.41	0.68	1.53	55.27
High		37.08	5.63	0.42	10.19	0.44	0.66	1.71	54.43
	−	38.64	5.53	0.40	11.00	0.42	0.67	1.57	56.80
	+	35.78	5.17	0.41	10.33	0.42	0.67	1.67	52.90
P-value									
Density (D)		0.932	0.229	0.388	0.526	0.619	0.772	0.398	0.856
GABA (G)		0.356	0.424	0.875	0.651	0.988	0.959	0.603	0.401
D × G		0.402	0.578	0.047	0.862	0.154	0.411	0.236	0.464

GABA = gamma-aminobutyric acid; SCFA = short-chain fatty acids; BCFA = branched chain fatty acid; SEM = pooled standard error of the means.
[1] Values are least-square means of 10 replicates per treatment.
[2] Branched-chain fatty acids were isobutyrate, isovalerate, and valerate.
[3] Total SCFA were acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate.
[4] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[5] −, GABA at 0 mg/kg of diet; +, GABA at 100 mg/kg of diet.

Table 10
Effect of dietary GABA on concentration of SCFA in cecal digesta in broiler chickens raised in different stocking densities on d 35.[1]

Item		SCFA, mmol/L							
		Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	BCFA[2]	Total SCFA[3]
Density[4]	GABA[5]								
Low	−	70.86	9.95	1.31	26.39	1.53	1.88	4.72	123.2[a]
	+	46.97	8.00	1.03	14.25	1.25	1.38	3.66	72.9[b]
High	−	62.77	9.34	1.34	19.45	1.57	1.92	4.83	96.4[ab]
	+	74.82	9.68	1.52	20.36	1.62	1.99	5.11	114.9[ab]
SEM		10.49	1.79	0.25	3.66	0.32	0.33	0.86	15.00
Main factors									
Low		58.92	8.98	1.17	20.32	1.39	1.63	4.19	98.0
High		68.79	9.51	1.43	19.91	1.59	1.95	4.97	105.7
	−	66.82	9.65	1.33	22.92	1.55	1.90	4.77	109.8
	+	60.90	8.84	1.27	17.31	1.43	1.69	4.39	93.9
P-value									
Density (D)		0.356	0.770	0.299	0.911	0.536	0.344	0.393	0.623
GABA (G)		0.579	0.660	0.827	0.134	0.727	0.531	0.671	0.311
D × G		0.098	0.533	0.375	0.083	0.622	0.393	0.463	0.032

GABA = gamma-aminobutyric acid; SCFA = short-chain fatty acids; BCFA = branched chain fatty acid; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Branched-chain fatty acids were isobutyrate, isovalerate, and valerate.
[3] Total SCFA were acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate.
[4] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[5] −, GABA at 0 mg/kg of diet; +, GABA at 100 mg/kg of diet.

neuronal tissues and non-neuronal tissues including the small intestine, pancreas, and liver (Tillakaratne et al., 1995) and GABA is also acting as a modulator in proliferation of the tissues (Watanabe et al., 2006). Thus, it is likely that dietary GABA upon ingestion might be absorbed via the small intestine and then reach to the liver via the portal system to be metabolized. As GABA is also considered being protective to liver injury (Wang et al., 2016), future studies are warranted to reveal the modulatory role of GABA in hepatocyte functions of chickens.

4.3. Yields and quality of breast and leg meats

Stocking density is known to affect the yields and qualities of breast meats of chicken especially at later days (Dozier et al., 2006). As expected, HSD vs. LSD decreased the yield of breast meat but increased the pH of breast meat on d 35. It is well reported that low pH of the meat is associated with altered meat qualities (e.g., pale meat color and high water hold capacity) due to denaturation of protein (Wilhelm et al., 2010). However, other than pH of the meat, meat qualities including colors and cooking loss of the meats were not affected by stocking density.

Of interest, dietary GABA increased the meat yield and CIE b* values but lowered the pH of the meat and these effects were only seen on d 21. It is not clear how dietary GABA lowered the pH of the meats. On the other hand, dietary GABA increased the absolute weight of breast meats, which was linked to an increase of body weight gain by approximately 2.8% on d 21. Thus, our study is in line with Dai et al. (2011) who reported GABA-induced increase in breast meat yield but is against Dai et al. (2012) who demonstrated that dietary GABA affected meat qualities including meat colors and meat

Table 11
Effect of dietary gamma-aminobutyric acid (GABA) on blood or serum parameters relating to stress indicators in broiler chickens raised in different stocking densities on d 35.[1]

Item		Stress indicators in blood or serum				
		H:L ratio	CORT, ng/mL	AGP, mg/mL	NO, μmol/L	TAC, mmol/L
Density[2]	GABA[3]					
Low	−	0.30	0.55	1.36	10.57	0.94
	+	0.22	0.39	1.47	12.85	0.91
High	−	0.35	0.48	1.21	9.36	0.90
	+	0.21	0.28	1.33	13.70	0.99
SEM		0.046	0.098	0.064	1.40	0.052
Main factors						
Low		0.26	0.47	1.42[a]	11.71	0.92
High		0.28	0.38	1.27[b]	11.53	0.94
	−	0.33[a]	0.51	1.28	9.97[b]	0.92
	+	0.22[b]	0.34	1.40	13.28[a]	0.95
P-value						
Density (D)		0.705	0.363	0.031	0.901	0.701
GABA (G)		0.037	0.088	0.074	0.028	0.569
D × G		0.585	0.843	0.939	0.479	0.309

H:L ratio = heterophil-to-lymphocyte ratio; CORT = corticosterone; AGP = alpha-1-acid glycoprotein; NO = nitric oxide; TAC = total antioxidant capacity; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, GABA at 0 mg/kg of diet; +, GABA at 100 mg/kg of diet.

Table 12
Effect of dietary gamma-aminobutyric acid (GABA) on serum biochemistry in broiler chickens raised in different stocking densities on d 35.[1]

Item		Serum biochemistry												
		GPT, U/L	GOT, U/L	GLU, mg/dL	TCHO, mg/dL	TG, mg/dL	HDL, mg/dL	TP, g/dL	ALB, g/dL	GLB, g/dL	ALB: GLB	P, mg/dL	Ca, mg/dL	UA, mg/dL
Density[2]	GABA[3]													
Low	−	4.00	162.4	458.9	73.44	136.0	29.22	2.58	0.92	1.75	0.52[b]	12.13	11.78	9.70
	+	3.90	186.6	474.2	84.10	147.4	41.20	2.59	0.96	1.63	0.59[a]	11.79	12.27	10.74
High	−	4.00	153.5	423.2	89.90	160.0	42.63	3.00	1.08	1.92	0.56[a]	13.31	12.49	11.66
	+	4.00	173.5	416.1	89.00	138.8	45.89	3.26	1.19	2.07	0.58[a]	12.47	12.04	11.03
SEM		0.30	7.91	26.46	3.76	14.20	3.31	0.12	0.047	0.083	0.012	0.63	0.22	0.90
Main factors														
Low		3.95	167.8	466.6	78.77[b]	141.7	35.21[b]	2.58[b]	0.94[b]	1.69[b]	0.56	11.96	12.02	10.22
High		4.00	161.9	419.7	89.45[a]	149.4	44.26[a]	3.13[a]	1.13[a]	1.99[a]	0.57	12.89	12.26	11.35
	−	4.00	152.9[b]	441.1	81.67[b]	148.0	35.92	2.79	1.00	1.84	0.54[b]	12.72	12.13	10.68
	+	3.95	176.8[a]	445.2	86.55[a]	143.1	43.54	2.92	1.08	1.85	0.58[a]	12.13	12.15	10.89
P-value														
Density (D)		0.872	0.495	0.094	0.009	0.613	0.015	<0.001	<0.001	0.001	0.232	0.148	0.304	0.221
GABA (G)		0.872	0.009	0.881	0.037	0.747	0.134	0.289	0.135	0.875	0.002	0.355	0.933	0.822
D × G		0.872	0.116	0.682	0.223	0.285	0.411	0.334	0.507	0.123	0.026	0.694	0.051	0.361

GPT = glutamic pyruvic transaminase; GOT = glutamic oxaloacetic transaminase; GLU = glucose; TCHO = total cholesterol; TG = triglyceride; HDL = high density lipoprotein; TP = total protein; ALB = albumin; GLB = globulin; P = phosphorus; Ca = calcium; UA = uric acid; SEM = pooled standard error of the means.
[a, b] Values (n = 10/treatment) without a common superscript letter within a column differ ($P < 0.05$).
[1] Values are least-square means of 10 replicates per treatment.
[2] Low, 7.5 birds/m^2; High, 15 birds/m^2.
[3] −, GABA at 0 mg/kg of diet; +, GABA at 100 mg/kg of diet.

nutrient composition in broiler chickens. In addition, there was a significant interaction between stocking density and dietary GABA on cooking loss on d 21 and the interaction was detected due to an increase of cooking loss by dietary GABA in LSD-raised chickens. Castellini et al. (2002) postulated that low pH and water holding capacity of the meat in organic broiler chickens might be associated with glycogen accumulation in meats due to improved welfare status. If the latter status holds true, then our current findings can be explained by GABA-induced reduction in stress and/or increase in glycogen accumulation. In any event, all values for meat qualities are within the range of average chicken meats.

Both stocking density and dietary GABA failed to affect the absolute and relative weights of leg meats at any ages and meat qualities on d 35. However, significant interaction between main factors on the absolute and relative weights of leg meats were detected on d 35. This interaction was found as dietary GABA increased yields of leg meats in LSD-raised chickens but decreased them in HSD-raised chickens. In addition, dietary GABA lowered the pH of the leg meats on d 21 and this was associated with increased cooking loss and CIE L* values. However, all values seen in this study were acceptable and within the standard quality of leg meats. Thus, further studies are warranted to address how dietary GABA affect digestion and absorption processes of the nutrients at the gut level and metabolic fates of the observed nutrients at the cellular levels which would help understand the role of dietary GABA in meat qualities of broiler chickens.

4.4. Tibia characteristics

It is known that HSD vs. LSD deteriorated the bone quality of chickens due to the reduced activity (Kestin et al., 1992) and impaired mineral metabolism (Simsek et al., 2011) in addition to

decreased feed intake. It is expected that dietary GABA might mitigate stocking density-induced decrease in bone quality as it plays a role in health and function of the small intestine (Chen et al., 2014) which is linked to improved mineral utilization (Ortiz et al., 2009). In this study, HSD vs. LSD failed to affect tibia characteristics at all ages except for relative tibia weight on d 35. The latter finding is related to lower live body weight in HSD- vs. LSD-raised chickens as fresh tibia weight did not differ in chickens raised under different stocking density. In line with our findings, no clear effect of stocking density on tibia characteristics was reported elsewhere (Tablante et al., 2003).

Dietary GABA tended to increase the absolute and relative weight of fresh tibia and significantly increased the width of tibia on d 35. However, in contrast to our expectation, dietary GABA failed to affect the breaking strength and ash content of tibia. In studies with laying hens, it was reported that dietary GABA at 50 mg per kg of diet increased the breaking strength and thickness of eggshell (Park and Kim, 2015; Zhang et al., 2012) and it was postulated that dietary GABA could increase the utilization of dietary calcium and phosphorus (Zhu et al., 2015). Finally, although not significant, moderate interaction between stocking density and dietary GABA on tibia breaking strength on d 35 was noted. This interaction was found as dietary GABA increased the tibia strength in LSD-raised chickens but decreased it in chickens raised in HSD. It is thus tempting to conclude that dietary GABA may exhibit a regulatory role in absorption and metabolism of macro-minerals including calcium and phosphorus. Our conclusion is in part supported by earlier studies (Chen et al., 2014; Zhang et al., 2012) that dietary GABA increased activities of digestive enzymes and nutrients digestibility in chickens.

4.5. Feather coverage

Feather coverage, as an indicator of welfare status, has been used in laying hens (Tactacan et al., 2009) and broiler chickens exposed to various stress condition such as heat stress or stocking density. Indeed, the lowered feather coverage in HSD-raised broiler chickens has been reported (Škrbić et al., 2011) that is presumably linked to the increased frequency of direct bird-to-bird contact (Arnould and Faure, 2003) and/or elevated secretion of hormones related to feathering such as thyroxine and estrogen (Leeson and Walsh, 2004). In this study, we confirmed the impact of stocking density on feather loss in broiler chickens. On the other hand, it is clear from this study that dietary GABA did not affect feather coverage score nor interacted with stocking density, indicating that feathers were molted due to mainly physical contact rather than an increase in hormone secretion induced by stress per se. If the latter plays a significant role in feather loss via environmental stress triggering to secrete more hormone, then dietary GABA might be expected to intervene feather loss as it is known to mitigate stresses in chickens (Kuffler and Edwards, 1958).

4.6. Body surface temperature

It is reported that body surface temperature is elevated in chickens raised in HSD vs. LSD and is considered a reliable indicator to assess welfare status of chickens (Abudabos et al., 2013). Dietary GABA is known to reduce heat stress-induced body temperature in broilers (Al Wakeel et al., 2017) that might be linked to GABA's role in lowering heat production in a hot environment (Miyazawa et al., 2012). It is clear from this study that both stocking density and dietary GABA did not affect the surface temperatures of the head and breast at all ages. Of interest, dietary GABA increased the surface temperature of shank in LSD-raised chickens but decreased it in HSD-raised counterparts, leading to significant interaction on d 21. However, this interaction was not maintained to d 35. In a study with rats, GABA is known to affect cold and warm sensitive neurons in an opposite fashion modulating body temperature (Jha et al., 2001). If the latter hold true in chickens, it is hypothesized that dietary GABA can act as a modulator regulating body temperature depending on the internal- and/or external environments leading to an increase or a decrease in body temperature in chickens. This hypothesis can be easily tested by raising chickens in hot and cold chambers and fed them diets containing with or without GABA.

4.7. C. perfringens counts and SCFA in cecal digesta

HSD vs. LSD is known to increase *C. perfringens* in intestinal contents of broiler chickens (Tsiouris et al., 2015) whereas GABA exhibits an augmentation of host local immune response (Kim et al., 2018) and lowers pH of cecal digesta associated with increased production of SCFA, thus exhibiting antibacterial activity (Xie et al., 2017). In contrast to our expectation, neither stocking density nor dietary GABA affected *C. perfringens* counts in cecal digesta. Clear explanation is not readily available at this stage.

Neither stocking density nor dietary GABA affected SCFA in cecal digesta on d 21 and 35. On the other hand, significant interaction between stocking density and GABA on isobutyrate on d 21 and acetate, butyrate, and total SCFA on d 35 was noted. These interactions were detected due to the opposite effect of dietary GABA depending on the stocking density: it lowered SCFA in the LSD-raised chickens but increased it in the HSD-raised chickens. It is well-known that SCFA are the major end byproducts of undigested nutrients by gut microbiota (Peng et al., 2016) and gut microbiota interact with various environments including host immunity, disease status, diet, stress, and raising environment (Tsiouris et al., 2015). As dietary GABA affected SCFA depending on the stocking density, further studies are warranted to explore the functional analysis of gut microbiome of chickens which will increase our understanding on the role of GABA in gut function and physiology.

4.8. Blood and serum parameters relating to stress indicator

In order to assess chickens exposed to stressors such as stocking density, high environmental temperature or pathogen exposure, a great array of physiological parameters relating to metabolism, immunity, antioxidant response, and stress response in blood or serum samples is monitored (Puvadolpirod and Thaxton, 2000). For example, H:L ratio is accepted as one of the best recognized stress indicators in poultry. If chickens are exposed to stressors, heterophil numbers increase but lymphocyte numbers decrease leading to an increase of the H:L ratio (Siegel, 1995). In addition, the commonly measured stress hormone is corticosterone in chickens (Mcfarlane and Curtis, 1989), which is the major avian adrenal glucocorticoid (Siegel, 1995). As expected, the increases in plasma corticosterone was noted in chickens raised in HSD vs. LSD (Jahanian and Mirfendereski, 2015).

In this study, HSD vs. LSD significantly decreased the concentration of alpha-1-acid glycoprotein in serum samples. Alpha-1-acid glycoprotein is one of dominant acute phase proteins in chickens and considered as an indicator of innate immune response (Holt and Gast, 2002). Thus, our study corroborates earlier study (Mustafa et al., 2010) showing that HSD vs. LSD impaired immune response in broiler chickens. In contrast to earlier studies (Kuan et al., 1990; Shakeri et al., 2014), we failed to observe stocking density-induced increase in H:L ratio in blood samples and corticosterone in serum samples. No clear explanation is apparent at this stage.

On the other hand, dietary GABA lowered H:L ratio and corticosterone but increased alpha-1-acid glycoprotein and nitric oxide. Our study clearly provided evidence that dietary GABA mitigates stress responses and augment innate immune response as reported elsewhere (Dai et al., 2011; Zhang et al., 2012). On the other hand, GABA-induced regulation of stress and immune responses was noted independent to the stocking density.

4.9. Serum biochemistry

Serum biochemical profile is often used to identify and assess poultry health (Lumeij and De Bruijne, 1985) and serum metabolites reflect the immediate nutritional status of chickens. In this study, HSD vs. LSD increased total cholesterol, HDL cholesterol, total protein, albumin and globulin but decreased glucose levels in serum samples. In general, it is reported that chickens exposed to stress conditions such as stocking density or heat stress exhibit metabolic alterations in blood biochemical profiles including glucose, total protein, albumin, total cholesterol, and HDL cholesterol (Onbaşilar et al., 2008). For example, heat stress decreased concentrations of total protein and glucose but increased triglyceride in serum samples (Zhang et al., 2012). It is reported that increased concentration of total cholesterol and HDL cholesterol are expected as HDL cholesterol transports cholesterol to liver from body tissues in excess needs (Tall, 1998). In addition, an increase in blood glucose and total cholesterol was detected in HSD-raised chickens (Houshmand et al., 2012; Onbaşilar et al., 2008). Although dietary GABA did not interact with stocking density on the serum biochemical profiles, it increased glutamic oxaloacetic transaminase and total cholesterol in serum samples. Serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase are used to assess liver function and increased values indicate the pathophysiological changes of the liver and kidney (Gao et al., 2014). Our finding is in contrast with an earlier report (Wang et al., 2016) on hepatoprotective property by GABA. Nonetheless, it should be kept in mind that the observed glutamic oxaloacetic transaminase values are within normal physiological ranges from about 140 to 190 IU/L (An et al., 2018).

5. Conclusion

In conclusion, the present study demonstrated that increasing stocking density impaired growth performance and feather coverage status. Dietary GABA did not affect growth performance but lowered H:L ratio and corticosterone in blood or serum samples regardless of stocking densities. Dietary GABA interacted with stocking density on variables including organ weight, meat quality, tibia breaking strength, shank surface temperature, and SCFA concentration in cecal digesta. Our finding suggests that dietary GABA is effective in mitigating stress responses, but the effect is independent to stocking density.

Author contributions

Su-Been Jeong: investigation, writing - original draft. Yoo Bhin Kim: methodology. Jeong-Woo Lee: methodology. Da-Hye Kim: formal analysis. Byung-Hern Moon: resources. Hong-Hee Chang: conceptualization. Yang-Ho Choi: conceptualization. Kyung-Woo Lee: supervision, writing - review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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