Dietary supplementation of methionine mitigates oxidative stress in broilers under high stocking density

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ABSTRACT We aimed to investigate whether dietary supplementation of methionine could mitigate intestinal oxidative injury in broilers under high stocking density (**HSD**). In the grower phase (d 22−42), 576 broilers with similar body weight were randomly chosen and divided into 8 groups in a 2 × 4 factorial experiment. Two different stocking densities (14 and 20 broilers per m^2) were tested with 4 different methionine levels: 0.35%, 0.4%, 0.45%, or 0.5%. Intestinal morphological and oxidative stress markers were assessed at the end of the test period. The results showed that mortality of broilers was significantly higher in the HSD group fed 0.35% methionine diet than the other groups, which was reversed by supplementation with 0.40% to 0.50% methionine. HSD significantly decreased feed intake and daily weight gain. HSD treatment significantly decreased T-AOC, activity of GPX ($P < 0.01$) and increased the level of PCO ($P < 0.01$), MDA ($P = 0.052$) of plasma. The decreased glutathione peroxidase activity in the liver and jejunum caused by HSD was alleviated by additional methionine. Supplementation of methionine increased the ration of GSH/GSSG in the plasma. The jejunum villus height and ratio of villus height to crypt depth under low stocking density conditions with 0.40% methionine diet were the highest, whereas the 0.45% methionine group was the highest under HSD conditions. Thus, additional dietary supplementation of methionine mitigates oxidative stress in broilers under HSD conditions and 0.40% to 0.45% methionine can be applied in cage rearing broiler production for amelioration of oxidative stress caused by HSD.

Key words: methionine, stocking density, mortality, oxidative stress

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INTRODUCTION

Chicken is widely considered to be a nutritious and healthy meat and is consumed in many parts of the world. In order to obtain high yields per square meter of space and more efficient production, stocking density is constantly increased. Although the application of high stocking density (**HSD**) conditions can increase meat production, HSD also poses many problems such as the fine regulation of temperature control to prevent heat stress syndrome in broilers (Feddes et al., 2002). HSD can reduce the daily weight gain of broilers, affect meat quality and leg health, induce immune stress, and increased mortality (Proudfoot et al., 1979; Pettitriley et al., 2001; Sanotra et al., 2001; Heckert et al., 2002; Sun et al., 2011; Lu et al., 2016). Crowding condition could lead to oxidative stress and a significant decrease in the bursa weight of broilers，oxidative stress could impair the health and growth performance of broilers (Simitzis et al., 2012). Many environment factors including ambient temperature and moisture which can be influenced by stocking density, also can affect the concentration of amino acid required in the diet (Martinez et al., 2017).

As a feed additives, methionine is known to promote growth, increase antioxidant activity, and improve meat quality (Chen, 2012). Previous studies have shown that supplemental methionine significantly improved the growth performance (Opoola et al., 2016; Moghadam et al., 2017; Yang et al., 2017), meat quality (Halder et al., 2007; Kobayashi et al., 2013), and intestinal antioxidant capacity in broilers (Li et al., 2014). Based on previous research, methionine can regulate

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metabolic processes, innate system and intervene in activation of endogenous antioxidant enzymes. Especially methionine can be used by the hepatocytes for the synthesis of glutathione (Martinez et al., 2017). However, there were some conflicting results related to the antioxidant function of methionine. In some studies methionine restriction stimulates the production of glutathione, mice with restricted dietary methionine showed no changes of antioxidant enzyme but reduced oxidative stress (Martinez et al., 2017). Studies have shown that supplemental methionine can effectively improve the intestinal morphology structure, intestinal barrier function and increase the number of goblet cells (Li et al., 2014; Chen et al., 2014; Shen et al., 2014).

Oxidative stress is known to occur in broilers under HSD conditions, to our knowledge, while few studies on intestinal health affected by HSD in broilers were reported. The purpose of the present study was to investigate the adverse effects of HSD on intestinal health in broilers and to determine whether dietary supplementation of methionine could mitigate these effects.

MATERIALS AND METHODS

Experimental Animals and Diets

The male Arbor Acres broilers were fed with the same starter diets from 0 to 21 d of age. At the end of d 21, a total of 576 broilers were weighed and divided into 8 groups, with 6 replicate pens in each group in term of similar body weight (**BW**), in a 2 × 4 factorial experiment. Individual cages were 100 cm long × 70 cm wide, and contained 2 nipple drinkers.

Two different stocking densities: 14 and 20 birds/m^2 were tested with 4 different methionine levels: 0.35%, 0.4%, 0.45%, or 0.5% (as a percentage of the total mass of the feed). Experimental diets were prepared by adding 0.05, 0.1, 0.15, or 0.2% supplemental DL-methionine (99%) to the basal diet. The stocking density 20 birds/m^2 is set according to actual production in multilayer cage rearing system in China and 14 birds/m^2 was set for the low level according to our previous study to achieve the optimum growth performance.

The basal diets were formulated based on reference NY/T 33-2004 standard (A standard for raising chickens in China) (Table 1) without methionine supplementation to meet the nutrient requirement of broilers. The number of broilers per replicate was determined by the stocking density: for the low stocking density (**LSD**) condition, we used 10 broilers in each replicate (14 birds/m^2), and for the HSD condition 14 broilers were raised in each replicate (20 birds/m^2). The broilers were allowed access to mashed feed and water ad libitum. Broilers were raised according to the Arbor Acres Broiler Management Guide (Aviagen Group, Beijing, China). The temperature of the rearing environment was at 34° C during 0 and 3 d of age, and was subsequently reduced until it reached 22°C by d 21. The photoperiod was set at 23:1 h light: darkness. The relative humidity was maintained at 40 to 65%.

Table 1. Composition of basal diets for broilers.

Ingredients (%)	Starter diet	Finisher diet
Corn	52.02	58.50
Soybean meal	38.71	32.90
Soybean Oil	4.83	4.70
Dicalcium phosphate	1.90	1.31
Limestone	1.30	1.40
NaCl	0.35	0.35
Mineral premix[1]	0.20	0.20
Choline chloride (50%)	0.20	0.16
Vitamin premix[2]	0.02	0.02
Antioxidant (Ethoxyquinoline)	0.03	0.03
DL-Methionine (99%)	0.18	-
L-Lysine	0.10	0.10
Medical stone	0.16	0.33
Nutrient composition (%)		
ME (Kcal/kg)	2,989	3,050
Crude protein	21.09	19.04
Lys	1.25	1.11
Met	0.50	0.29
Met+Cys	0.70	0.65
Thr	0.89	0.80
Trp	0.28	0.25
Ca	0.99	0.89
Available phosphorus	0.45	0.35
Total phosphorus	0.69	0.58

[1]The mineral premix provided per kg diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.

[2]The vitamin premix provided per kg diet: vitamin A, 12,500 IU; vitamin D_3, 2,500 IU; vitamin K_3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_{12}, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid 12 mg; niacin, 50 mg.

The present study was approved by the Animal Care and Use Committee of China Agricultural University and performed in accordance with the Guidelines for Experimental Animal Welfare of Ministry of Science and Technology of China (Beijing, P. R. China).

Sample Collection

Growth Performance At the end of d 42, the body weight and the residual feed were recorded, after 4 h feed withdrawal. The average body weight, average daily feed intake, average daily gain, and feed conversion ratio of chicks from d 22 to 42 were calculated.

Sample Collection

After the birds were weighed, one bird per pen closest to the average body weight was selected and killed through cervical dislocation after intracardial administration of sodium pentobarbital (30 mg/kg BW). The jejunum segment was isolated immediately from the gastrointestinal tract. Spleen and bursa of *fabricius* were also removed and weighted.

After separation of the jejunum, the middle 2 cm of jejunum was placed in 4% paraformaldehyde, for histological analysis. Then the remain jejunum was immediately flushed with ice-cold phosphate-buffered saline (PBS, pH 7.4), about 5 cm sample was placed in RNase-free tubes for gene expression quantification, and another 5 cm sample was taken for superoxide dismutase (**SOD**), malondialdehyde (**MDA**), and glutathione

Table 2. Primers used for real-time PCR analysis of jejunum and liver.

Genes	Forward primer	Reverse primer	Accession number
β-actin	GCTACAGCTTCACCACCACA	TCTCCTGCTCGAAATCCAGT	L08165.1
SOD	GCGCAGGTGCTCACTTTAAT	CCTCCCTTTGCAGTCACATT	NM-205064.1
GPX	ATCGCTGATAAGGACCGAGA	GGGCCAAATATGAACACCAC	NM-001039329.1
Occludin	GCTGAGATGGACAGCATCAA	CCTCTGCCACATCCTGGTAT	NM-205128.1
Claudin-3	CCAAGATCACCATCGTCTCC	CACCAGCGGGTTGTAGAAAT	NM-204202.1
HMGCR	TGTTGTAAGGCTGCCCTCTG	TAGGCGGGCAAACCTACTTG	NM-204485
HSP70	CATGAAGCAGACGGTGGAG	GATCGAGCCAAGAGATCACC	KC706508.1

peroxidase (**GPX**) determination. Both samples were snap frozen in liquid nitrogen and stored at −80°C until analysis.

Measurement Indicators

Antioxidant Capacity Determination The tissue sample was ground in liquid nitrogen, and physiological saline was added at a ratio of 1:9. The slurry was then placed in an ice-water bath and centrifuged in an ultrasonic homogenizer at 3,000 × g for 10 min at 4°C; the supernatant obtained was used for the measurements for T-SOD, GPX enzyme activities and MDA content using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). Briefly, detecting superoxide dismutase activity by xanthine oxidase method, malondialdehyde content by thiobarbituric acid method, glutathione peroxidase activity by colorimetry. The SOD, MDA and GPX were measured absorption at 550 nm, 532 nm and 412 by using a spectrophotometer (Molecular Devices SpectraMax i3x plate reader), respectively. In the tissue homogenate, GPX and T-SOD were expressed as units per milligram of protein, MDA content as nanomoles per milligram of protein. Protein concentrations in the supernatant were assayed by BCA kits.

Jejunum Morphological Analysis The jejunum samples were embedded in paraffin and cut into 5 μm sections using a microtome. The villus height and crypt depth were determined after staining with hematoxylin and eosin, and the number of intraepithelial lymphocytes and goblet cells were determined after periodic acid-Schiff staining. The villus height (from the tip of the villus to the villus-crypt junction) and the crypt depth (from the base of the crypt to the villus-crypt junction) were measured. Three fields of view for each section were selected, and 10 villi and crypts were measured in each field and the V:C was determined. The intraepithelial lymphocytes are scattered between the intestinal villus epithelial cells. Most are located near the basement membrane of epithelial cells. Goblet cells were stained red by periodic acid-Schiff staining and scattered among epithelial cells. Five complete intestinal villi were selected for each slice. The number of intraepithelial lymphocytes and goblet cells was determined and expressed as a percentage of the total columnar cells.

Gene Expression Determination Total RNA was extracted from the jejunum tissue using the RNAiso Plus reagent (Takara Bio, Japan), and the RNA concentration was determined by measuring absorbance at 260 nm (Spectrophotometer ND-1000, Gene Company Limited). Samples with absorbance ratios at 260/280 nm between 1.8 and 2.0 were considered to be of sufficient quality. About 1.0 μg of total RNA was reverse-transcribed into cDNA using the PrimeScript RT kit with gDNA elimination (Takara Bio). Primers information for the relative genes and β-actin were designed using Primer 3.0 Plus software (Table 2). Real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNase H Plus; Takara Bio). Gene expression was determined relative to β-actin as an internal control standard using the $2^{-\triangle\triangle CT}$ method.

Statistical Analysis

Experimental data were expressed as mean ± standard deviation. SPSS 22.0 statistical software was used to assess the main effects and interactions of methionine levels and stocking density according to the 2 × 4 experimental design. Tukey multiple comparisons were performed when the interaction had a significant difference in F test. Significant differences were defined as $P < 0.05$, while $P < 0.01$ indicates extremely significant difference.

RESULTS

Growth Performance and Relative Weights of Internal Organ

There were no interactive effects between stocking density and methionine supplementation on growth performance except mortality rate from d 21 to 42. The body weight of finishers was tended to be decreased by HSD treatment. Also daily feed intake and weight gain of broilers reared under HSD conditions was significantly lower than LSD treatment ($P < 0.05$) (Table 3). The interactive effect ($P < 0.01$) of different methionine levels and stocking density was observed on broiler mortality. HSD significantly increased the mortality rate compared with the LSD treatment, which was reversed by supplementation with the 0.40% to 0.50% methionine ($P < 0.01$) (Table 3). However, as shown in Table 4, the relative weight of spleen and bursa of *fabricius* were not significantly influenced by stocking density or addition of methionine.

Table 3. Effects of different methionine levels and stocking density on growth performance of broilers from d 21 to d 42.

Stocking density	Methionine (%)	Body weight (kg/bird)	Daily feed intake (g/bird)	Daily weight gain (g/bird)	Feed conversion ratio	Mortality rate (%)
LSD	0.35	2.38	149.63	72.48	2.07	0.00[c]
	0.40	2.35	148.68	73.85	2.01	0.00[c]
	0.45	2.30	146.43	74.23	2.05	0.00[c]
	0.50	2.30	139.11	68.60	2.05	0.00[c]
HSD	0.35	2.22	140.02	65.75	2.13	7.14[a]
	0.40	2.25	138.19	68.72	2.04	3.54[b]
	0.45	2.28	132.59	65.90	2.07	0.00[c]
	0.50	2.34	140.77	71.60	1.98	0.00[c]
SEM	0.017	1.712	1.128	0.018	0.586	
Main effect analysis						
Stocking density	LSD	2.33	145.96[a]	72.19[a]	2.05	0.00
	HSD	2.27	137.87[b]	67.99[b]	2.05	2.30
Methionine	0.35	2.30	144.82	69.11	2.10	2.68
	0.40	2.30	144.01	71.28	2.03	1.77
	0.45	2.29	139.51	69.60	2.06	0.00
	0.50	2.32	139.94	70.10	2.02	0.00
P-value						
Stocking density × Methionine		0.189	0.382	0.125	0.660	<0.01
Stocking density		0.084	0.021	0.020	0.865	<0.01
Methionine		0.915	0.602	0.851	0.418	<0.01

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is:LSD 14 broilers/m^2, HSD 20 broilers/m^2.

Table 4. Effects of different methionine levels and stocking density on internal organ index of broilers on d 42.

Stocking density	Methionine (%)	Spleen index[1] (%)	Bursa of *fabricius* index[1] (%)
LSD	0.35	0.118	0.059
	0.40	0.130	0.061
	0.45	0.141	0.116
	0.50	0.131	0.060
HSD	0.35	0.128	0.061
	0.40	0.114	0.060
	0.45	0.144	0.069
	0.50	0.149	0.058
	SEM	0.0049	0.0072
Main effect analysis			
Stocking density	LSD	0.130	0.074
	HSD	0.133	0.062
Methionine	0.35	0.123	0.060
	0.40	0.122	0.061
	0.45	0.143	0.093
	0.50	0.140	0.059
P-value			
Stocking density × Methionine		0.661	0.573
Stocking density		0.730	0.402
Methionine		0.303	0.287

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is: LSD 14 broilers/m^2, HSD 20 broilers/m^2.

[1]Spleen index= weight of spleen/BW × 100%; bursa of *fabricius* index= weight of bursa of *fabricius* /BW × 100%.

Antioxidant Status

As shown in Tables 5–7, the activities of GPX, T-SOD and the level of T-AOC, MDA, PCO, the ratio of GSH/GSSG were examined in the plasma, liver, and intestinal mucosa. In Table 5, HSD treatment significantly decreased T-AOC and GPX activity ($P < 0.01$) and increased the level of PCO ($P < 0.01$) and MDA ($P = 0.052$). The activity of T-SOD and ratio of GSH/GSSG in the plasma were not influenced by HSD compared with LSD treatment. About 0.50% methionine supplementation increased the activity of GPX ($P < 0.01$) and the ratio of GSH/GSSG in the plasma, and further enhancement of GPX was observed by 0.45% methionine treatment.

In Table 6, antioxidant status of liver was examined. An interactive effect was observed on the activity of GPX. HSD significantly decreased the activity of GPX compared to the LSD treatment under the condition of 0.35% methionine level, while additional supplementation of methionine such as 0.45% and 0.50% under HSD alleviated the decrease of GPX activity caused by HSD treatment ($P < 0.001$). MDA and T-SOD were not significantly influenced by stocking density. But 0.45% methionine enhanced the activity of T-SOD in the liver ($P < 0.001$).

In Table 7, MDA and T-SOD in the jejunum tissue were not influenced by stocking density. A total of 0.35% methionine increased T-SOD in the jejunum, but also accompanied with MDA content rising. There was an interactive effect of stocking density and methionine on GPX in jejunum. Additional supplementation of methionine such as 0.40% and 0.45% alleviated the upregulation of GPX activity caused by HSD compared with LSD treatment under 0.35% methionine level.

Jejunum Morphology

In Table 8, there was an interactive effect ($P < 0.01$) of stocking density and methionine on jejunum morphology: the 0.4% methionine elevated jejunal villus height in the LSD groups which was reversed by HSD treatment. Additional supplementation of 0.45% methionine group significantly alleviated the decreased villus height caused by HSD ($P < 0.01$). Also, supplementation of different concentration methionine (0.35%–0.45%) under HSD significantly increased the crypt depth compared with the LSD treatment of the same concentration methionine. The V:C in LSD with 0.4% methionine

Table 5. Effect of different methionine levels and stocking density on plasma antioxidant status of broilers on d 42.

Stocking density	Methionine (%)	T-AOC (U/mL)	MDA (nmol/mL)	GPX (U/mL)	T-SOD (U/mL)	PCO (nmol/mgprot)	GSH/GSSG
LSD	0.35	14.30	2.36	1,870.84	85.96	0.45	1.16
	0.40	11.80	2.99	1,886.35	80.11	0.33	2.12
	0.45	8.10	2.60	2,376.65	83.80	0.46	2.14
	0.50	8.95	2.92	2,134.92	77.98	0.46	2.44
HSD	0.35	9.93	2.61	1,774.02	89.27	0.61	1.62
	0.40	7.74	3.21	1,875.37	95.10	0.54	1.98
	0.45	7.79	3.20	2,130.98	80.52	0.47	1.95
	0.50	7.86	3.17	1,892.38	85.96	0.54	2.15
	SEM	0.618	0.101	57.838	3.082	0.030	0.260
Main effect analysis							
Stocking density	LSD	10.75^{a}	2.70	$2,067.19^{a}$	81.96	0.42^{b}	2.05
	HSD	8.33^{b}	3.05	$1,918.19^{b}$	87.39	0.54^{a}	1.92
Methionine	0.35	11.92^{a}	2.48^{b}	$1,822.43^{c}$	87.62	0.53	1.41^{b}
	0.40	9.77^{ab}	3.10^{a}	$1,880.86^{c}$	86.92	0.43	2.05^{ab}
	0.45	7.93^{b}	2.90^{ab}	$2,253.81^{a}$	82.16	0.47	2.04^{ab}
	0.50	8.41^{b}	3.04^{a}	$2,013.65^{b}$	81.97	0.50	2.40^{a}
P-value							
Stocking density × Methionine		0.214	0.817	0.141	0.424	0.089	0.533
Stocking density		0.005	0.052	0.001	0.148	<0.001	0.688
Methionine		0.005	0.048	<0.001	0.573	0.152	0.029

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is: LSD 14 broilers/m^2, HSD 20 broilers/m^2.
Abbreviations: GPX, glutathione peroxidase; HSD, high stocking density; LSD, low stocking density; MDA, malondialdehyde.

Table 6. Effect of different methionine levels and stocking density on antioxidant status in liver of broilers on d 42.

Stocking density	Methionine (%)	MDA (nmol/mgprot)	GPX (U/mgprot)	T-SOD (U/mgprot)
LSD	0.35	1.07	37.01^{b}	575.38
	0.40	1.23	37.45^{b}	576.06
	0.45	1.20	42.68^{a}	744.70
	0.50	1.12	37.16^{b}	512.73
HSD	0.35	1.12	35.13^{c}	533.27
	0.40	1.29	35.88^{c}	624.50
	0.45	1.26	41.27^{a}	660.07
	0.50	1.53	44.51^{a}	589.68
	SEM	0.036	1.149	17.095
Main effect analysis				
Stocking density	LSD	1.15	38.63	602.21
	HSD	1.30	39.31	596.59
Methionine	0.35	1.09	36.07	554.33^{b}
	0.40	1.26	36.72	600.28^{b}
	0.45	1.23	41.98	710.85^{a}
	0.50	1.33	41.09	551.20^{b}
P-value				
Stocking density × Methionine		0.431	<0.001	0.071
Stocking density		0.529	0.176	0.989
Methionine		0.585	<0.001	<0.001

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is: LSD 14 broilers/m^2, HSD 20 broilers/m^2.
Abbreviations: GPX, glutathione peroxidase; HSD, high stocking density; LSD, low stocking density; MDA, malondialdehyde.

group was significantly higher than other groups ($P < 0.01$), while under HSD condition additional supplementation with 0.45% and 0.50% methionine increased the ratio of V:C compared with the 0.35% and 0.40% treatment ($P < 0.01$).

The number of intraepithelial lymphocytes and goblet cells percentage in LSD and HSD with 0.4% methionine group were significantly higher than other groups under LSD or HSD treatment ($P < 0.01$).

Relative mRNA Expression of Antioxidant and Barrier Function Related Genes in Broiler Jejunum

As shown in Table 9, no significant interactive effects were found on the relative mRNA expression of SOD, GPX, claudin-3 and occludin in the stocking density treatment with different concentration of methionine ($P > 0.05$). However, 0.50% methionine significantly downregulated the gene expression of claudin-3 compared with 0.35% and 0.40% methionine ($P < 0.01$), and occludin was upregulated by 0.40% methionine ($P = 0.012$).

DISCUSSION

In the present study, daily feed intake and daily weight gain of broilers were significantly decreased by HSD compared with LSD treatment. Similar results were found in the study of Magnuson (Magnuson et al., 2020). Mortality under HSD conditions with low dietary methionine was significantly higher than that in other groups. Similarly, increased mortality was observed

Table 7. Effect of different methionine levels and stocking density on antioxidant status of jejunum in broilers on d 42.

Stocking density	Methionine (%)	MDA (nmol/mgprot)	GPX activity (U/mgprot)	T-SOD activity (U/mgprot)
LSD	0.35	1.64	32.21^{b}	1,136.88
	0.40	0.98	29.01^{c}	701.91
	0.45	0.96	30.33^{b}	1,048.32
	0.50	0.62	27.61^{c}	787.95
HSD	0.35	1.30	34.78^{a}	1,165.59
	0.40	0.87	30.92^{b}	758.36
	0.45	1.01	24.58^{d}	579.08
	0.50	1.02	35.42^{a}	755.88
	SEM	0.084	2.045	52.369
Main effect analysis				
Stocking density	LSD	1.05	29.97	918.77
	HSD	1.05	31.41	824.98
Methionine	0.35	1.48^{a}	33.24	1,151.54^{a}
	0.40	0.92^{b}	30.03	730.14^{b}
	0.45	0.98^{b}	27.46	835.03^{b}
	0.50	0.82^{b}	32.29	771.92^{b}
P-value				
Stocking density × Methionine		0.257	0.001	0.201
Stocking density		0.985	0.129	0.278
Methionine		0.005	0.003	0.011

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is: LSD 14 broilers/m^2, HSD 20 broilers/m^2.
Abbreviations: GPX, glutathione peroxidase; HSD, high stocking density; LSD, low stocking density; MDA, malondialdehyde.

Table 8. Effect of different methionine levels and stocking density on jejunum structure of broilers on d 42.

Stocking density	Methionine (%)	Villus height /μm	Crypt depth /μm	villus height/crypt depth	Number of intraepithelial lymphocytes (cell/100 absorbed cells)	Number of goblet cell (cell/100 absorbed cells)
LSD	0.35	1,780.24^{c}	264.04^{b}	7.04^{c}	19.43^{b}	17.90^{c}
	0.40	2,197.60^{a}	213.20^{c}	11.87^{a}	24.80^{a}	28.23^{a}
	0.45	1,531.64^{d}	268.00^{b}	5.55^{d}	14.40^{d}	13.91^{d}
	0.50	2,095.83^{b}	281.50^{a}	7.24^{b}	21.60^{b}	22.33^{b}
HSD	0.35	1,870.92^{c}	278.60^{a}	7.03^{c}	17.20^{c}	17.33^{c}
	0.40	1,819.89^{c}	281.67^{a}	6.34^{d}	19.80^{b}	18.00^{c}
	0.45	2,135.14^{a}	277.43^{a}	7.53^{b}	14.67^{d}	14.80^{d}
	0.50	1,967.33^{b}	283.33^{a}	7.32^{b}	15.60^{d}	15.00^{c}
	SEM	41.739	6.270	0.272	0.650	0.598
Main effect analysis						
Stocking density	LSD	1,886.86	259.73	7.71	20.97	21.41
	HSD	1,955.25	283.63	7.07	16.46	16.11
Methionine	0.35	1,854.30	272.00	7.17	18.47	18.81
	0.40	1,980.82	252.00	8.74	23.07	23.78
	0.45	1,864.32	277.08	6.61	14.66	14.33
	0.50	1,988.75	285.58	7.14	19.54	18.67
P-value						
Stocking density × Methionine		<0.01	<0.01	<0.01	<0.01	<0.01
Stocking density		<0.01	<0.01	<0.01	<0.01	<0.01
Methionine		<0.01	<0.01	<0.01	<0.01	<0.01

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is: LSD 14 broilers/m^2, HSD 20 broilers/m^2.
Abbreviations: HSD, high stocking density; LSD, low stocking density.

under HSD treatment (Pettitriley et al., 2001). Possible reason might be that HSD caused the environment deteriorate leading to higher temperature and humidity and became unconducive to broiler growth (Feddes et al., 2002). Low levels of dietary methionine did not prevent the high mortality under HSD, but additional supplementation of methionine can significantly reduce mortality. Also, in another study the total mortality was decreased by additional methionine (Acar et al., 2001).

The bursa of *Fabricius* and spleen are essential lymphoid organs in broiler chickens. Differentiation of B lymphocytes was completed in the bursa of *Fabricius* and the mature lymphocytes migrate to the peripheral lymphoid organs like the spleen (Yanai et al., 2018). However, relative weights of the bursa of *Fabricius* and spleen were not influenced by HSD or additional supplement of methionine in our study. Additional methionine increased bursa and spleen, which might be related to the repletion of lymphoid organs and had a beneficial effect on humoral immunity (Mirzaaghatabar et al., 2011). Methionine concentration (0.90%) in broiler diet was much higher than that in our study (0.35% −0.50%), which may explain the different results related to the relative weight of bursa and spleen. Studies have shown that HSD significantly decreases chicken bursa weight and increased susceptibility to bacterial and viral infections, thus resulting in high mortality (Heckert et al., 2002; Sun et al., 2011). However, similar result was observed in another study. Absolute bursa weight and the relative weight of bursa were not significantly influenced by HSD (Buijs et al., 2009).

Table 9. Relative mRNA expression of antioxidation and tight junction related genes in broiler jejunum on d 42.

Stocking density	Methionine (%)	SOD	GPX	Claudin-3	Occludin
LSD	0.35	1.51	11.01	4.26	2.42
	0.40	1.67	12.36	3.87	3.00
	0.45	1.48	10.20	3.06	2.30
	0.50	1.50	10.76	2.15	2.28
HSD	0.35	1.43	10.68	3.71	2.17
	0.40	1.63	8.83	4.53	3.01
	0.45	1.39	10.51	3.71	2.75
	0.50	1.97	11.67	2.61	2.11
	SEM	0.096	0.838	0.359	0.120
Main effect analysis					
Stocking density	LSD	1.54	11.08	3.40	2.50
	HSD	1.60	10.51	3.64	2.46
Methionine	0.35	1.47	10.85	3.99^{a}	2.28^{b}
	0.40	1.65	10.79	4.20^{a}	3.01^{a}
	0.45	1.43	10.36	3.39^{ab}	2.52^{ab}
	0.50	1.73	11.22	2.41^{b}	2.19^{b}
P-value					
Stocking density × Methionine		0.314	0.760	0.485	0.428
Stocking density		0.605	0.672	0.353	0.929
Methionine		0.207	0.981	0.002	0.012

Means in the same column lacking a common superscript differ ($P < 0.05$). The specific stocking density is: LSD 14 broilers/m^2, HSD 20 broilers/m^2.

Abbreviations: GPX, glutathione peroxidase; HSD, high stocking density; LSD, low stocking density; MDA, malondialdehyde; SOD, superoxide dismutase.

In our study, HSD decreased T-AOC, activity of GPX and increased PCO, MDA in plasma. Decreased activity of plasm GPX caused by HSD was also shown in another study (Li et al., 2019). Methionine, an essential amino acid involved in protein, was mainly metabolized in the liver. And S-adenosylmethionine as an active form of Met was increased after feeding diets enriched with methionine. S-adenosylmethionine served as a donor of methyl group for many biological reactions, and was a precursor for glutathione thus provided protection against oxidative-stress induced liver injury (Kumar et al., 2020). The ratio of GSH/GSSG and GPX of plasma were significantly increased by 0.45% and 0.50% supplementation of methionine. Similarly, in the liver additional supplementation of methionine alleviated the decreased GPX activity caused by HSD. High level dietary methionine reversed the increased expression of oxidative stress markers in the liver such as the gene expression of *Nox2, Lox1* and *Inos*, which was produced by excessive cholesterol and sitagliptin in rats (Kumar et al., 2020).

HSD was reported to increase oxidative stress levels and induce intestinal mucosal injury (Simitzis et al., 2012; Li et al., 2019). As the first defense line against oxidative stress, intestine contains an extensive antioxidant system. The gene expression of SOD, GPX and their enzyme activity in the jejunum were determined. Supplementation of additional methionine (0.45%−0.50%) decreased the MDA content and the T-SOD in the jejunum. This is consistent with the results of Najeeb et al. (2012) that oxidative stress was enhanced when MDA was increased, and with SOD activity significantly increased.

The structural integrity of the intestine is essential for the efficient digestive function, which depends on the normal development of intestinal mucosa. The intestinal epithelial structure is composed of epithelial cells, tight junction proteins, goblet cells, and intraepithelial lymphocytes, which contribute to the integrity of intestinal mucosa (Vicente et al., 2001). The villus height and V:C values reflect the intestinal absorption capacity. In our study, the villus height and V:C under LSD conditions with 0.4% methionine diet were the highest, whereas the 0.45% methionine fed group were the highest under HSD conditions. The intestinal structure was damaged by HSD, methionine can enhance the physical barrier and immune function of the intestine and reduce oxidative damage (Pan et al., 2017). Methionine was contributed to the increase of the villus height and the number of goblet cells. Thus, the requirement of methionine was increased under HSD compared to the LSD (Li et al., 2014; Chen et al., 2014; Shen et al., 2014).

The number of intraepithelial lymphocytes and goblet cells percentage in LSD and HSD with 0.40% methionine group were significantly higher than other groups. The intestinal architecture was significantly improved by the addition of methionine to the diet which was consistent with the results of Chen et al., (2014). The jejunum structure in broilers in the HSD group fed 0.45% methionine diets was better than that in other groups, indicating that intestinal architecture can be improved under HSD conditions by supplementing basal diets with methionine.

The formation of tight junctions in poultry intestines is thought to mainly rely on the expression of occludin, claudins, and ZO-1, ZO-2, and ZO-3 (Gonzalez-Mariscal et al., 1998; Ozeden et al., 2010; Suzki et al., 2013). The intestinal epithelial cells are linked together by tight junction proteins to form a mechanical barrier to the intestinal tract (Vicente et al., 2001). However, 0.50% downregulated the gene expression of occludin and claudin-3. High level dietary methionine (1.95%) decreased the intestinal transmembrane proteins such as claudin 8, claudin 9, and claudin 10 in the proximal jejunum of mice (Miousse et al., 2017). And methionine supplementation increased the occludin in the jejunum of intrauterine growth retardation piglets (Su et al., 2018). In another study in broilers, gene expression of claudin-1 in jejunum was decreased by addition of methionine under heat stress (Del Vesco et al., 2020). Thus, the increased gene expression of claudin-3 may contribute to the integrity of jejunum.

In conclusion, HSD adversely affected the growth performance and lead to oxidative damages and increased mortality. Additional dietary methionine alleviated the increased morbidity induced by HSD by enhancement of inherent antioxidant defenses. During the oxidative stress caused by HSD, 0.45% supplementation of methionine reversed the decreased GPX in the liver and plasma. Therefore, additional methionine was needed under crowding stress and 0.45% methionine can be applied in broiler production for amelioration of

oxidative stress caused by HSD in multilayer cage rearing system.

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DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

Acar, N., G. F. Barbato, and P. H. Patterson. 2001. The effect of feeding excess methionine on live performance, carcass traits, and ascitic mortality. Poult. Sci. 80:1585–1589.

Buijs, S., L. Keeling, S. Rettenbacher, E. Van Poucke, and F. A. Tuyttens. 2009. Stocking density effects on broiler welfare: identifying sensitive ranges for different indicators. Poult. Sci. 88:1536–1543.

Chen, Y., D. F. Li, Z. L. Dai, X. S. Piao, Z. L. Wu, B. Wang, Y. H. Zhu, and Z. K. Zeng. 2014. L-methionine supplementation maintains the integrity and barrier function of the small-intestinal mucosa in post-weaning piglets. Amino Acids. 46:1131–1142.

Del Vesco, A. P., A. D. S. Khatlab, T. P. Santana, P. C. Pozza, M. A. Menck Soares, C. O. Brito, L. T. Barbosa, and E. Gasparino. 2020. Heat stress effect on the intestinal epithelial function of broilers fed methionine supplementation. Livest. Sci. 240 104152.

Feddes, J. J., E. J. Emmanuel, and M. J. Zuidhoft. 2002. Broiler performance, body weight variance, feed and water intake, and carcass quality at different stocking densities. Poult. Sci. 81:774–779.

Chen, Y. P. 2012. Effect of incubation weight and dietary methionine level on growth, immunity and antioxidant activity in broilers. Diss. Nanjing Agricultural University.

Gonzalez-Mariscal, L., A. Betanzos, and P. Nava. 1998. Tight junction proteins [Review][J]. Progr. Biophys. Mol. Biol. 1:1–44.

Halder, G., and B Roy. 2007. Effect of herbal or synthetic methionine on performance, cost benefit ratio, meat and feather quality of broiler chicken. Int. J. Agric. Res. 2:987–996.

Heckert, R. A., I. Estevez, E. Russekcohen, and R. Pettitet. 2002. Effects of density and perch availability on the immune status of broilers. Poult. Sci. 81:451–457.

Kobayashi, H., K. Nakashima, A. Ishida, A. Ashihara, and M. Katsumata. 2013. Effects of low protein diet and low protein diet supplemented with synthetic essential amino acids on meat quality of broiler chickens. Anim. Sci. J. 84:489–495.

Kumar, A., R. Pathak, H. A. Palfrey, K. P. Stone, T. W. Gettys, and S. N. Murthy. 2020. High levels of dietary methionine improves sitagliptin-induced hepatotoxicity by attenuating oxidative stress in hypercholesterolemic rats. Nutr. Metab. 17:2, doi:10.1186/s12986-019-0422-z.

Li, H., J. F. Wang, M. Yves, X. Zhang, C. Wu, X. Wu, L. Tang, L. Che, Y. Lin, S. Xu, G. Tian, D. Wu, and Z. Fang. 2014. Changes in plasma amino acid profiles, growth performance and intestinal antioxidant capacity of piglets following increased consumption of methionine as its hydroxy analogue. Brit. J. Nutr. 112:855–867.

Li, W., F. Wei, B. Xu, Q. Sun, W. Deng, H. Ma, J. Bai, and S. Li. 2019. Effect of stocking density and alpha-lipoic acid on the growth performance, physiological and oxidative stress and immune response of broilers. Asian Austral J. Anim. 32:1914–1922.

Lu, Y. J., Z. Q. Miao, J. H. Li, Y. Yang, and Y Yang. 2016. Effects of stocking density and activity space on immune and stress indexes in broilers. J. Anim. Nutr. 28:2927–2935.

Magnuson, A. D., G. Liu, T. Sun, S. A. Tolba, L. Xi, R. Whelan, and X. G. Lei. 2020. Supplemental methionine and stocking density affect antioxidant status, fatty acid profiles, and growth performance of broiler chickens. J. Anim. Sci. 98:skaa092, doi:10.1093/jas/skaa092.

Martinez, Y., X. Li, G. Liu, P. Bin, W. Yan, D. Mas, M. Valdivie, C. A. Hu, W. Ren, and Y. Yin. 2017. The role of methionine on metabolism, oxidative stress, and diseases. Amino Acids 49:2091–2098.

Miousse, I. R., R. Pathak, S. Garg, C. M. Skinner, S. Melnyk, O. Pavliv, H. Hendrickson, R. D. Landes, A. Lumen, A. J. Tackett, N. Deutz, M. Hauer-Jensen, and I. Koturbash. 2017. Short-term dietary methionine supplementation affects one-carbon metabolism and DNA methylation in the mouse gut and leads to altered microbiome profiles, barrier function, gene expression and histomorphology. Genes Nutr. 12:22.

Mirzaaghatabar, F., A. A. Saki, P. Zamani, H. Aliarabi, and H. R. Hemati Matin. 2011. Effect of different levels of diet methionine and metabolisable energy on broiler performance and immune system. Food Agr. Immunol. 22:93–103.

Moghadam, M. H. B., A. Shehab, and G. Cherian. 2017. Methionine supplementation augments n-3 fatty acid and tocopherol content in broiler birds fed flaxseed. Anim. Feed Sci. Technol. 228:149–158.

Najeeb, Q., N. Bhasker, I. Masood, S. Wadhwa, H. Kaur, and S. Ishaq. 2012. Malondialdehyde (MDA) Superoxide dismutase (SOD) levels-distinguishing parameters betweenbenign malignant pleural effusions. Free Radic. Antioxid. 2:8–11.

Opoola, E., S. O. Ogundipe, G. S. Bawa, O. J. Makinde, and O Abejide. 2016. Determination of optimum methionine requirements of broiler chickens reared during the hot season under tropical environment. Anim. Res. Int. 13:2421–2427.

Ozeden, O., B. L. Black, and C. M. Ashwell. 2010. De-velopm ental profile of claudin-3, -5, and -16 proteinsin the epithelium of chick intestine. Anat. Record Adv. Integr. Anat. Evolut. Biol. 293:1175–1183.

Pan, F. Y., P. Wu, L. Feng, W. D. Jiang, S. Y. Kuang, L. Tang, W. N. Tang, Y. A. Zhang, X. Q. Zhou, and Y. Liu. 2017. Methionine hydroxy analogue improves intestinal immunological and physical barrier function in young grass carp (Ctenopharyngodonidella). Fish Shellfish Immunol. 64:122–136.

Pettitriley, R., and I. Estevez. 2001. Effects of density on perching behavior of broiler chickens. Appl. Anim. Behav. Sci. 71:127–140.

Proudfoot, F. G., H. W. Hulan, and D. R. Ramey. 1979. The effect of four stocking densities on broiler carcass grate, the incidence of breast blisters, and other performance traits. Poult. Sci. 58:791–793.

Sanotra, G. S., J. D. Lund, and A. K. Ersball. 2001. Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. World. Poult. Sci. J. 57:55–69.

Shen, Y. B., A. C. Weaver, and S. W. Kim. 2014. Effect of feed grade L-methionine on growth performance and gut health in nursery pigs compared with conventional DL-methionine. Am. Soc. Anim. Sci. 92:5530–5539.

Simitzis, P. E., E. Kalogeraki, M. Goliomytis, M. A. Charismiadou, K. Triantaphyllopoulos, A. Ayoutanti, K. Niforou, A. L. Hager-Theodorides, and S. G. Deligeorgis. 2012. Impact of stocking density on broiler growth performance, meat characteristics, behavioural components and indicators of physiological and oxidative stress. Br. Poult. Sci. 53:721–730.

Su, W., H. Zhang, Z. Ying, Y. Li, L. Zhou, F. Wang, L. Zhang, and T. Wang. 2018. Effects of dietary l-methionine supplementation on intestinal integrity and oxidative status in intrauterine growth-retarded weanling piglets. Eur. J. Nutr. 57:2735–2745.

Sun, Z., L. Lv, and H. Lin. 2011. Effects of stocking density and dietary lysine level on growth performance , carcass composition and health status of sepatated-sex reared broilers. Aquaculture 23:578–588.

Suzki, T. 2013. Regulation of intestinal epithelial permea-bility by tight junctions. Cell. Mol. Life Sci. 70:631–659.

Vicente, Y., C. D. Rocha, J. K. Yu, G. Hernandez-Peredo, L. Martinez, B. Perez-mies, and J. A. Tovar. 2001. Architecture and function of the gastroesophageal barrier in the piglet. Digest. Dis. Sci. 46:1899–1908.

Yanai, T., M. I. Abo-Samaha, S. E. El-Kazaz, and H. G. Tohamy. 2018. Effect of stocking density on productive performance, behaviour, and histopathology of the lymphoid organs in broiler chickens. Eur. Poult. Sci. 82:247, doi:10.1399/eps.2018.247.

Yang, Z., Z. Y. Wang, H. M. Yang, L. Xu, and D. Q. Gong. 2017. Effects of dietary methionine and betaine on slaughter performance, biochemical and enzymatic parameters in goose liver and hepatic composition. Anim. Feed Sci. Tech. 8401:30269–30304.