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Differential Effects of Dietary Methionine Isomers on Broilers Challenged with Acute Heat Stress

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In this study, we investigated the effect of methionine isomers (D- and L-methionine) on growth performance, blood metabolite levels, nutrient digestibility, intestinal morphology, and foot pad dermatitis in broilers challenged with acute heat stress. In total, 240 broilers were randomly allocated in a 2×2 factorial arrangement consisting of two dietary treatments (D- vs. L-methionine) and two thermal environmental conditions (thermo-neutral vs. acute heat stress). Methionine isomers were added to the diet as an ingredient according to the diet formulation. The broilers were exposed to acute heat stress at 33℃ for 5 h on day 14. The average daily gain and feed conversion ratio of birds fed L-methionine were higher than those fed D-methionine ($P<0.05$) from the time of hatching till 21 days. Induced acute heat stress impaired ($P<0.05$) the daily gain and feed intake of the broilers on day 21. Furthermore, the blood urea nitrogen levels of birds subjected to acute heat stress on days 14 and 21 were higher ($P<0.05$) than those of their counterparts. Longer villi ($P<0.05$) were observed in broilers fed L-methionine-supplemented diet than in those fed D-methionine-supplemented diet on day 14, irrespective of thermal environmental conditions. Heat stress reduced ($P<0.01$) nutrient digestibility of the broilers on days 14 and 21. Higher incidence and severity of foot pad dermatitis were observed ($P<0.05$) in broilers fed diet containing D-methionine than in those fed L-methionine-supplemented diet. In conclusion, L-methionine-supplemented diet improved growth performance, overcame growth depression, and reduced the incidence of foot pad dermatitis when broilers were exposed to acute heat stress in the starter period.

Key words: acute heat stress, broiler, digestibility, growth performance, methionine isomers

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Introduction

Methionine is the first limiting essential amino acid for broilers consuming diet formulated with corn and soybean meal and is known to directly affect its production (Goulart *et al*., 2011; Farkhoy *et al*., 2012). Several studies have demonstrated the importance of L-methionine in broilers with respect to growth performance indices such as meat and feather development and immune responses (Xie *et al*., 2004; Eriksson *et al*., 2007; Zeng *et al*., 2015).

Synthetic methionine is frequently added to poultry diets as either DL-methionine (i.e., 50% L- and 50% D-methionine) or an aqueous solution of 2-hydroxy-4-(methylthio)-butanoic acid (Shen *et al*., 2015; Zhang *et al*., 2015). Poultry have the ability to utilize both methionine isomers and analogs for protein synthesis via a unique enzymatic pathway that converts D-methionine to L-methionine in the liver and kidneys (Baker, 2006; Thwaites and Anderson, 2007, Shen *et al*., 2015). D-amino acid oxidase, which converts the D-isomer or D-analog to the L-isomer, is abundant in the liver and kidney (Bauriedel, 1963). However, the expression of D-amino acid oxidase is significantly low in young animals (D'Aniello *et al*., 1993). Therefore, growth performance and gut integrity of broilers fed L-methionine-supplemented diet should be better than those fed D-methionine-supplemented diet. Previous studies have compared the growth performance of poultry fed diet containing DL- methionine, L- methionine, and a methionine hydroxy analogue (Shen *et al*., 2015; Esteve-Garcia and Khan, 2018). However, none of these studies separately compared the effects of D- and L- methionine on growth performance and the intestinal responses of broilers.

Both methionine isomers follow the sodium-dependent and sodium-independent pathways to traverse the gut wall in

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animals (Knight *et al*., 1994; Soriano and García *et al*., 1998). Absorption via the sodium-independent pathway is altered during heat stress, resulting in lower uptake of D-methionine than that of L-methionine. Consequently, conversion of D-methionine to L-methionine results in energy loss in heat-stressed broilers (Knight *et al*., 1994). Thus, L-methionine has a competitive advantage in broilers during heat stress.

Dietary methionine shows wound healing property (Perez-Tamayo and Ihnen, 1953) and a relationship with foot pad dermatitis in poultry (Murillo and Jensen, 1976; Shepherd and Fairchild, 2010). However, evidence regarding the effect of methionine isomers on healing of foot pad dermatitis lesions in broilers is limited.

Therefore, the objective of this study was to investigate the differential responses of L-methionine and D-methionine on growth performance, blood metabolite levels, intestinal health, nutrient digestibility, and foot pad dermatitis in broilers challenged with acute heat stress.

Materials and Methods

The experimental protocol for the current study was reviewed and approved by the Animal Ethics Committee of the Chungnam National University (Protocol No. CNU - 00779).

Experimental Design and Management

In total, 240 one-day-old Ross broilers were obtained from a commercial hatchery and randomly allocated in 2×2 factorial arrangements with six replications. The corresponding factors were two methionine isomers (D- vs. L- methionine) and two thermo-environmental conditions (thermo-neutral control vs. acute heat stress). Ten broilers with a mean body weight of 47.50±0.20 g (mean±SEM) were housed in raised wire-floor pens (0.85 m×0.55 m×0.35 m). Owing to space limitation in the research facility, the experiment was conducted in two consecutive periods to create two thermo-environmental conditions in the same facility using identical procedures (120 broilers per period).

The experimental diets were offered on an *ad-libitum* basis for 5 weeks using a metal trough. The broilers had free access to fresh clean drinking water via nipple drinkers throughout the experiment. All management practices were in accordance with the guidelines of the Ross broiler management handbook (Avigen, 2014a).

Experimental Diets

A basal diet was formulated based on corn, soybean meal, and barley (Table 1) to meet or exceed the nutrient requirements specified in the Ross 308 Nutrition Specification (Avigen, 2014b). The formulated diet was mixed without adding crystalline methionine to obtain a methionine-deficient basal diet.

Two experimental diets were constituted by separately adding crystalline D- and L- methionine isomers to the methionine-deficient basal diet per the diet formulation. The diets did not contain any antimicrobial growth promoters or alternatives. At the time of mixing the treatment diets, 0.3% chromium oxide (Cr_2O_3: >99.9%, Sigma-Aldrich, St. Louis, MO, USA) was added as an indirect marker for digestibility analysis. Diet samples for chemical analysis were obtained after the mixing.

Table 1. **Composition (g/kg, as-fed basis) of the experimental diets**

Ingredients	1–3 week	4–5 week
Barley	23.85	—
Corn	35.12	63.72
SBM	35.00	31.5
Vegetable oil	1.38	0.58
Limestone	1.27	0.96
Monocal P	1.85	1.70
Iodized salt	0.26	0.30
Vit-Min premix[1]	0.30	0.40
Lys-HCl	0.34	0.20
L-Arginine	0.13	0.17
D or L-Methionine	0.25	0.18
Threonine	0.13	0.15
Valine	0.12	0.14
Calculated values		
ME, kcal/kg	3050	3150
CP, %	22	20
Ca, %	1.00	0.85
Av P, %	0.50	0.45
Lys, %	1.44	1.21
Met, %	0.56[2]	0.48[3]
Met + Cys, %	0.93	0.82
Thr, %	0.92	0.88
Trp, %	0.27	0.23
Val, %	1.1	1.03
Arg, %	1.5	1.40

[1] Supplied per kilogram of total diets: Fe ($FeSO_4 \cdot H_2O$), 80 mg; Zn ($ZnSO_4 \cdot H_2O$), 80 mg; Mn ($MnSO_4 \cdot H_2O$) 80 mg; Co ($CoSO_4 \cdot H_2O$) 0.5 mg; Cu ($CuSO_4 \cdot H_2O$) 10 mg; Se (Na_2SeO_3) 0.2 mg; I, ($Ca(IO_3) \cdot 2H_2O$) 0.9 mg; Vitamin A, 24,000 IU; Vitamin D_3, 6,000 IU; Vitamin E, 30 IU; Vitamin K, 4 mg; Thiamin, 4 mg; Riboflavin, 12 mg; Pyridoxine, 4 mg; Folacine, 2 mg; Biotin, 0.03 mg; Vitamin B_8, 0.06 mg; Niacin, 90 mg; Pantothenic acid, 30 mg.
[2] Met from ingredient: Synthetic Met=55.5: 44.5
[3] Met from ingredient: Synthetic Met=62.5: 37.5

Heat Stress Protocol

In the thermo-neutral control group, ambient temperature was maintained at 30±1℃ from days 1 to 3 and then gradually decreased to 25±1℃ until the birds were 14 days old. Thereafter, temperature was maintained at 25±1℃ throughout the experiment. Similarly, in the acute heat stress treatment group, ambient temperature was maintained at 30±1℃ from days 1 to 3 and then gradually decreased to 25±1℃ until the birds were 14 days old. On day 14, the broilers were subjected to acute heat stress as follows. The ambient temperature (25℃) was increased over the course of 2 h (in 4℃ increments per hour) until 33℃ (70% humidity). Subsequently, the temperature was held at 33±0.5℃ for 3 h and gradually returned to ambient temperature (25℃). Thereafter, the ambient temperature (25±1℃) was maintained till the end of the experiment. Deep-body temperatures of the broilers during the acute heat stress period (start, 2 h, and 5 h)

were measured (FlashCheck® tip probe thermometer, Weber Scientific, Hamilton, NJ, USA) using a thermistor probe inserted into the bird rectum.

Measurements

Body weights of the broilers were recorded (pen basis) on day 1 of the experiment. Subsequently, weekly body weights were measured until the end of the experiment. Daily weight gain was calculated from the body weights. Pen-wise feed disappearance was measured weekly. Mortality-corrected average daily feed intake and feed conversion ratio were calculated.

At the end of the experiment, randomly selected 30 broilers per treatment (five birds per pen) were examined for the incidence of foot pad dermatitis in both feet. Lesions were scored on a 5-point scale from 0 (no lesion) to 4 (very severe lesions) as described by Butterworth (2013).

Sample Collection

Samples were collected on days 14, 21, and 35 of the experiment. One bird was selected randomly from each cage at a time (12 broilers per treatment) for sample collection. Prior to euthanizing the selected broilers, blood samples were collected into spray-coated K2 EDTA vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) from the brachial vein. The collected blood samples were immediately transported to the laboratory for plasma separation.

The broilers were euthanized via cervical dislocation and sacrificed by cutting the carotid artery and jugular vein, followed by bleeding. Abdominal incisions were made after the sacrifice and the ileum was separated from the gastrointestinal tract. The ileum was defined as the segment of small intestine that extended from Meckel's diverticulum to the ileocecal junction (Incharoen *et al*., 2010). The removed ileal samples (3 cm piece) were flushed with ice-cold phosphate-buffered saline (PBS, pH 7.4) and fixed in 10% formaldehyde until microscopic slide preparation.

The remaining digesta of the ileal segment was gently stripped into labeled plastic containers and quickly stored at −20℃ until further analysis.

Sample Preparation and Laboratory Analyses

Blood samples were centrifuged (Micro 12, Hanil Science Co. Ltd., Korea) at 3,000×*g* for 10 min at 4℃ and the plasma was separated. Blood urea nitrogen (BUN) levels and creatinine concentration were analyzed using an automatic biochemistry analyzer 7020 (Hitachi, Tokyo, Japan). Briefly, BUN level was quantified using the urease enzymatic kinetic method (Morishita *et al*., 1997) and plasma creatinine concentration using the Jaffe assay (Peake and Whiting, 2006).

Formaldehyde-fixed ileal tissue samples were used for sample preparation. Ring-shaped ileal tissue samples were excised and dehydrated, followed by impregnation in paraffin wax. Six transverse sections (4−6 μm) were cut from each of these using a microtome, stained with hematoxylin and eosin, and mounted on glass slides. Histological indices were measured using NIS-Elements Viewer software (Version: 4.20; NIS Elements, Nikon, Tokyo, Japan) and an inverted microscope (Nikon) with a calibrated eyepiece graticule. The heights of 10 well-oriented villi and their associated crypts were measured and used for analysis.

The collected digesta samples were pre-dried for 24 h at 55℃ and the samples were ground through a 0.75-mm sieve (ZM 200 Ultra-Centrifugal Mill, Retsch GmbH & Co. KG, Haan, Germany). Using the fine ground samples, levels of dry matter, crude protein (N×6.25, macro-Kjeldahl), and gross energy were analyzed according to the methodologies of AOAC (1995). The chromium oxide concentration in the samples was also analyzed (Fenton and Fenton, 1979). Nutrient digestibility was calculated as described by Huang *et al*. (2005) using the following equation.

$$\text{Apparent digestibility coefficient} = 1-\left[\frac{(Indicator_{Diet}\times Nutriet_{Digesta})}{(Indicator_{Digesta}\times Nutrient_{Diet})}\right]$$

Calculations and Statistical Analysis

The results were analyzed as completely randomized design, using general linear model (GLM) procedure of two-way analysis of variance (ANOVA) of the SPSS software (Version 21; IBM SPSS 2012). The pen was used as the experimental unit for growth performance. Selected individual broilers were considered experimental units for the incidence of foot pad dermatitis, blood parameters, gut morphology, and nutrient digestibility. Dietary treatments (methionine isomers) and heat stress condition were considered as the two main effects.

The incidence of foot pad dermatitis was recorded as multinomial scales and then transformed to a binomial scale as many zero values were present in the data set. On this binomial scale, food pad scores were classified as 1 (no lesion: score 1), 2 (initial lesion: scores 1 and 2), and 3 (severe lesions: scores 3 and 4). The diet-directed response data were analyzed using the $\chi 2$ test (Martins *et al*., 2016). The means were separated using Tukey's multiple range test procedures of the SPSS software (Version 21; IBM SPSS 2012) when treatment effect was significant ($P<0.05$).

Results

We observed heavy panting, dispersed distribution within the cage, and higher rectal temperature ($P<0.05$; Table 2), which showed that the broilers were sufficiently heat-stressed.

Growth Performance

The effect of dietary methionine isomers (i.e., D- and L-isomers) and acute heat stress on the growth performance of broilers from the time of hatching to 35 days of age is shown in Table 3. No interaction was observed between diet and heat stress with respect to growth performance. Broilers fed L-methionine-supplemented diet showed better ($P<0.05$) average daily gain and feed conversion ratio than broilers fed D-methionine-supplemented diet during the starter period (1−21 days), irrespective of exposure to acute heat stress. However, diet did not affect ($P>0.05$) feed intake during the experimental period. Broilers subjected to acute heat stress had lower ($P<0.05$) daily gain on days 21 and 28, during the starter period (1−21 days) and during the entire duration of

Table 2. **Effect of dietary feed-grade methionine isomers and the duration of heat stress on rectal temperature of broilers[1]**

Item	Start (25℃)		2 h (33℃)		5 h (33℃)		SEM[2]	P-value		
	L-Methionine	D-Methionine	L-Methionine	D-Methionine	L-Methionine	D-Methionine		Time[3]	Diet[4]	Time×Diet
Temperature, ℃	41.22	41.34	41.61	41.55	41.48	41.41	0.027	0.001	0.984	0.305

[1] Values are the mean of six replicates per treatment.
[2] Pooled standard error of mean
[3] Time of the heat stress (0 h, 2 h, and 5 h)
[4] Dietary methionine isomers

Table 3. **Effect of dietary feed-grade methionine isomers and heat stress on growth performance of broilers[1]**

Item	Acute heat stress		Thermo-neutral control		SEM[2]	P-value		
	L-Methionine	D-Methionine	L-Methionine	D-Methionine		HS[3]	Diet[4]	HS×Diet
Avg. daily gain, g								
Day 7	22.75	21.80	23.09	22.46	0.24	0.320	0.121	0.736
Day 14	50.85	48.32	49.88	49.68	0.66	0.883	0.313	0.389
Day 21	71.42	67.69	74.53	74.37	1.02	0.026	0.351	0.391
Day 28	76.48	74.82	85.47	84.09	1.20	0.001	0.533	0.954
Day 35	74.35	69.65	71.70	71.54	2.25	0.934	0.595	0.620
Day 1−21	48.34	45.93	49.16	48.84	0.29	0.004	0.029	0.088
Day 22−35	75.41	72.23	78.59	77.82	1.19	0.080	0.414	0.617
Day 1−35	59.17	56.46	60.93	60.43	0.51	0.011	0.134	0.296
Avg. daily feed intake, g								
Day 7	25.8	24.8	25.2	24.7	0.24	0.491	0.156	0.598
Day 14	67.6	67.2	67.9	67.6	0.78	0.828	0.794	0.965
Day 21	103.7	102.1	115.4	115.1	1.55	0.001	0.758	0.822
Day 28	131.6	129.1	132.3	132.2	1.81	0.602	0.716	0.743
Day 35	127.0	123.0	126.3	125.6	2.14	0.824	0.595	0.703
Day 1−21	65.7	64.7	69.5	69.1	0.45	0.001	0.445	0.703
Day 22−35	129.3	126.1	129.3	128.9	1.33	0.594	0.501	0.598
Day 1−35	91.2	89.2	93.4	93.1	0.65	0.029	0.385	0.554
Feed conversion ratio, g/g								
Day 7	1.13	1.14	1.09	1.10	0.01	0.077	0.233	0.621
Day 14	1.33	1.39	1.36	1.37	0.01	0.864	0.214	0.266
Day 21	1.45	1.51	1.55	1.55	0.02	0.026	0.282	0.390
Day 28	1.73	1.73	1.55	1.58	0.03	0.006	0.770	0.841
Day 35	1.72	1.78	1.79	1.80	0.04	0.617	0.686	0.773
Day 1−21	1.30	1.35	1.33	1.34	0.01	0.289	0.048	0.143
Day 22−35	1.72	1.76	1.67	1.69	0.03	0.235	0.625	0.845
Day 1−35	1.47	1.51	1.47	1.48	0.01	0.465	0.303	0.513

[1] Values are the mean of six replicates per treatment.
[2] Pooled standard error of mean
[3] Heat stress condition (acute heat stress vs thermo-neutral control)
[4] Dietary methionine isomers

the experiment (1−35 days) than broilers subjected to thermo-neutral treatment, irrespective of the presence of methionine isomers in the diet. Furthermore, compared to the thermo-neutral treatment, acute heat stress reduced ($P<0.05$) feed intake on day 21 during the starter period (1−21 days) and during the entire duration of the experiment (1−35 days). In addition, acute heat stress decreased feed efficiency independent of the presence of methionine isomers in the diet on days 21 and 28.

Table 4. **Effect of dietary feed-grade methionine isomers and heat stress on blood metabolite levels of broilers[1]**

Item	Acute heat stress		Thermo-neutral control		SEM[2]	P-value		
	L-Methionine	D-Methionine	L-Methionine	D-Methionine		HS[3]	Diet[4]	HS×Diet
Blood urea nitrogen, mg/dL								
Day 14	0.98	1.71	0.47	0.87	0.068	0.006	0.019	0.472
Day 21	1.44[b]	0.82[ab]	0.48[a]	0.79[ab]	0.107	0.032	0.490	0.042
Day 35	0.65	0.53	0.53	0.60	0.032	0.836	0.825	0.412
Creatinine, mg/dL								
Day 14	0.28	0.28	0.26	0.29	0.009	0.852	0.405	0.405
Day 21	0.29[b]	0.30[b]	0.28[b]	0.22[a]	0.006	0.001	0.118	0.018
Day 35	0.22	0.24	0.23	0.23	0.008	0.837	0.413	0.413

[1] Values are the mean of six replicates per treatment.
[2] Pooled standard error of mean
[3] Heat stress condition (acute heat stress vs. thermo-neutral control)
[4] Dietary methionine isomers
[a, b] Means in the same row with different superscripts differ significantly ($P<0.05$).

Table 5. **Effect of feed-grade methionine isomers in diet and heat stress on gut morphology of broilers[1]**

Item	Acute heat stress		Thermo-neutral control		SEM[2]	P value		
	L-Methionine	D-Methionine	L-Methionine	D-Methionine		HS[3]	Diet[4]	HS×Diet
Day 14								
Villous height	499.60	327.58	505.28	465.16	19.42	0.102	0.026	0.128
Crypt depth	62.47	69.27	66.02	70.49	4.42	0.794	0.542	0.899
V:C[5]	8.38	4.74	7.79	7.03	0.52	0.431	0.065	0.199
Day 21								
Villous height	502.937	377.58	571.95	565.16	12.25	0.003	0.062	0.088
Crypt depth	71.46	77.37	63.69	66.35	1.72	0.026	0.247	0.649
V:C	7.037	4.97	8.983	8.55	0.29	0.001	0.062	0.194
Day 35								
Villous height	902.51	847.64	977.53	945.86	64.24	0.519	0.745	0.930
Crypt depth	111.53	113.03	116.20	119.82	2.117	0.213	0.562	0.808
V:C	8.03	7.50	8.41	7.74	0.447	0.736	0.520	0.941

[1] Values are the mean of six (10 measurements each) replicates per treatment.
[2] Pooled standard error of mean
[3] Heat stress condition (acute heat stress vs. thermo-neutral control)
[4] Dietary methionine isomers
[5] Villous height: Crypt depth ratio

Blood Parameters

Broilers subjected to acute heat stress showed higher ($P<0.05$) BUN levels on days 14 and 21 than those under thermo-neutral conditions, and an interaction was observed between diet and heat stress ($P<0.05$) on day 21 (Table 4). The BUN level of broilers fed L-methionine-supplemented diet was lower ($P<0.05$) that that of broilers fed D-methionine diet, irrespective of exposure to acute heat stress.

On day 21, broilers reared under thermo-neutral conditions and fed D-methionine-supplemented diet showed lower ($P<0.05$) creatinine kinase level than broilers subjected to other treatments, which was indicative of an interaction between heat stress and diet ($P<0.05$).

Gut Morphology

On day 14, broilers fed L-methionine-supplemented diet showed longer ($P<0.05$) villi than broilers fed D-methionine-supplemented diet, irrespective of acute heat stress (Table 5). On day 21, broilers subjected to acute heat stress showed shorter ($P<0.05$) villi and higher ($P<0.05$) crypt depth, and therefore, lower ($P<0.05$) villus height to crypt depth ratio than broilers reared under thermo-neutral conditions, irrespective of the diet. No differences in ileal morphology ($P>0.05$) were observed between broilers on dietary methionine isomers or under heat stress on day 35.

Table 6. **Effect of dietary feed-grade methionine isomers and heat stress on nutrient (ileal) digestibility of broilers[1]**

Item	Acute heat stress		Thermo-neutral control		SEM[2]	P value		
	L-Methionine	D-Methionine	L-Methionine	D-Methionine		HS[3]	Diet[4]	HS×Diet
Dry Matter								
Day 14	0.74	0.73	0.76	0.75	0.003	0.008	0.095	0.773
Day 21	0.76	0.76	0.76	0.75	0.003	0.773	0.565	0.391
Day 35	0.77	0.78	0.78	0.77	0.002	0.503	0.503	0.054
Crude protein								
Day 14	0.68	0.66	0.70	0.68	0.005	0.043	0.087	0.999
Day 21	0.69	0.67	0.71	0.69	0.005	0.043	0.087	0.999
Day 35	0.72	0.71	0.74	0.72	0.005	0.130	0.261	0.605
Energy								
Day 14	0.74	0.73	0.74	0.76	0.003	0.027	0.383	0.089
Day 21	0.78	0.77	0.80	0.79	0.004	0.028	0.195	0.935
Day 35	0.84	0.82	0.82	0.83	0.004	0.589	0.658	0.193

[1] Values are the mean of six replicates per treatment.
[2] Pooled standard error of mean
[3] Heat stress condition (acute heat stress vs. thermo-neutral control)
[4] Dietary methionine isomers

Table 7. **Effect of dietary feed-grade methionine isomers on incidence of foot pad demerits in 35-day-old broilers**

Diet	Thermo-neutral control		Acute heat stress	
	D-Methionine	L-Methionine	D-Methionine	L-Methionine
Scores[1]				
1	15	23	15	24
2	7	6	8	2
3	8	1	7	4
P value[2]	0.027		0.039	

[1] Scores: 1=No lesions, 2=initial lesion, 3=severe lesion
[2] Chi-square tests - $\chi 2$ for the effect of dietary methionine isomers for each heat stress condition.

Nutrient Digestibility

Broilers subjected to acute heat stress had consistently reduced ($P<0.05$) dry matter, crude protein content, and energy digestibility on day 14, and lower ($P<0.05$) crude protein and energy digestibility on day 21 than broilers under thermo-neutral treatment, irrespective of the methionine isomer present in the diet (Table 6).

Incidence of Foot Pad Dermatitis

Higher occurrence ($P<0.05$) of foot pad dermatitis was detected in broilers fed D-methionine-supplemented diet than in broilers fed L-methionine-supplemented diet, irrespective of the thermal conditions. Furthermore, broilers fed D-methionine-supplemented diet had more severe lesions (i.e., score ≥ 3) than broilers fed L-methionine-supplemented diet.

Discussion

This study was designed for investigating the differential effects of D- and L- methionine on growth performance, along with intestinal responses and nutrient digestibility of broilers under acute heat stress. The acute heat stress conditions were designed to mimic the sudden temperature variations caused by heat waves that directly affect poultry production in the field.

Our results showed improved growth performance (i.e., average daily gain and feed conversion ratio) in broilers fed the L-methionine-supplemented diet during the starter period (from day 1 to 21); however, there was no significant effect during the grower period (from day 21 to 35). The diet-related growth performance response supports the hypothesis that L-methionine is better utilized by young broilers than D-methionine. Furthermore, our results are consistent with those of previous studies (Marrett and Sunde, 1965, Shen *et al*., 2015), which indicated that young broilers fed L-methionine-supplemented diet showed enhanced growth performance compared to broilers fed D-methionine diet. These observations can be ascribed to a lower D-amino acid

oxidase level in young broilers, which is responsible for converting D-methionine to L-methionine (D'Aniello *et al*., 1993; Shen *et al*., 2015). D-amino acid oxidase converts D-methionine to α-keto-γ-methiolbutyrate, followed by transamination to L-methionine, which is biologically available to birds (Lewis, 2003; Fang *et al*., 2010, Kong *et al*., 2016). One possible mechanism underlying the observed effect of diet on growth is that improved utilization of L-methionine may enhance intestinal development and thereby improve growth performance (Shen *et al*., 2015). The appearance of longer villi and the tendency of lower villus height to crypt depth ratio in the starter period, as observed in this study, supports this mechanism. Although a reduction in the growth performance (i.e., average daily gain, average daily feed intake, and feed conversion ratio) of broilers was observed under acute heat stress on days 21 and 28, no effect was detected on day 35. Our results collectively suggest that the effect of acute heat stress on growth performance may be alleviated within a few weeks after the event. Consistent with this interpretation, Gonzalez-Esquerra and Leeson (2005) demonstrated that the reduction in growth performance was severe at the time of heat exposure; however, the impairment of growth performance reduced with time. The poor growth performance observed under acute heat stress in broilers may be ascribed to higher energy loss via increased respiration, reduced feed intake, and retardation of nutrient digestibility (Mello *et al*., 2015). Consistent with this, the combination of shorter villi and higher crypt depth, leading to a higher villus height to crypt depth ratio and lower nutrient digestibility, were associated with reduced growth performance of broiler chickens under acute heat stress in this study.

Changes in the levels of dietary amino acids may rapidly alter nitrogen metabolism of animals (Tian *et al*., 2016). Efficient nitrogen utilization and low urea synthesis contribute to reduction of plasma urea nitrogen levels (Brown and Cline, 1974; Tian *et al*., 2016). Therefore, plasma urea nitrogen levels can be used as an indirect indicator of the metabolic efficiency of dietary amino acids (Wickramasuriya *et al*., 2016). The conversion efficiency of D- to L-methionine may be correlated with BUN level (Tian *et al*., 2016). In our study, higher BUN level was observed in broilers fed D-methionine-supplemented diet than those fed L-methionine diet on day 14. This indicates that D-amino acid oxidase is less abundant in young broilers and that they are therefore unable to efficiently convert D-methionine to L-methionine. An interaction between methionine isomers and thermal conditions was observed on day 21, although the mechanism underlying this observation is unclear. Plasma creatinine kinase activity increases under acute heat stress, which is indicative of heat stress-induced myopathy (Sandercock *et al*., 2001; Willemsen *et al*., 2011). Del Vesco *et al*. (2015) observed higher plasma creatinine concentrations when broilers were subjected to acute heat stress. Consistent with this, our results demonstrated that broilers subjected to acute heat stress had significantly higher plasma creatinine levels on day 21, although the effect of acute heat stress was not observed on day 35. Higher protein turnover or breakdown rates, in association with renal deficiency, contribute to higher blood levels of creatinine (Biolo *et al*., 1998; Del Vesco *et al*., 2015). We observed an interaction between methionine isomers and thermal conditions on plasma creatinine levels on day 21, although the mechanism underlying this interaction is unknown.

Studies show that methionine has beneficial effects on growth and development of gut structure, which can be the attributed to its antioxidative properties (Shen *et al*., 2014; Shen *et al*., 2015). As a direct source of methionine, L-methionine has an advantageous effect on gastrointestinal tract development (Shen *et al*., 2015). Consistent with this, our results demonstrated that on day 14, broilers fed L-methionine-supplemented diet had longer villi than broilers fed D-methionine-supplemented diet, and L-methionine-fed broilers also showed a tendency to develop higher villus height to crypt depth ratio on days 14 and 21. Heat stress alters gut morphology and reduces the functional barrier by altering intestinal metabolism (Pearce *et al*., 2012; Song *et al*., 2014). A previous study demonstrated that heat stress reduces villus height and increases crypt depth in the small intestine of broilers (Song *et al*., 2014). In the present study, broilers subjected to acute heat stress showed shorter villi and higher crypt depth, leading to lower villus height to crypt depth ratio, than broilers in the thermo-neutral control group on day 21. Changes in the gut morphology of broilers subjected to heat stress could be due to intestinal ischemia, lesions induced by toxin accumulation, and epithelial shedding (Song *et al*., 2014).

We observed that the diet supplemented with L-methionine did not alter the digestibility of nutrients compared to the diet containing D-methionine, irrespective of thermal conditions. However, our results indicated that acute heat stress consistently reduced the dry matter and crude protein content, and energy digestibility. As shown previously (Faria Filho *et al*., 2007), changes in nutrient digestibility may occur in broilers in response to heat exposure. Our observations could be the result of reduced intestinal integrity in response to excessive heat exposure and the resulting changes in post-absorptive energetics. Surprisingly, similar nutrient digestibility was observed among broilers subjected to acute heat stress and in the thermo-neutral control group on day 35. These observations suggested that the reduction in nutrient digestibility was only present for a limited period of time after heat exposure.

Foot pad dermatitis is a significant problem in poultry production, which is caused by microbial action and broiler litter conditions, and affects both animal welfare and the economics of production (Shepherd and Fairchild, 2010; El-Wahab, 2015). Dietary methionine exhibits a significant relationship with foot pad dermatitis in poultry (Murillo and Jensen, 1976; Shepherd and Fairchild, 2010). In the present study, broilers fed L-methionine-supplemented diet showed lower incidence of foot pad dermatitis than broilers fed D-methionine-supplemented diet. This can be due to the wound healing properties of methionine (Perez-Tamayo and Ihnen, 1953). In addition to the level of occurrence, the severity

scores of foot pad dermatitis were higher in broilers fed D-methionine diet than in those fed L-methionine diet, suggesting that L-methionine reduced both the incidence and severity of foot pad dermatitis in broilers.

In conclusion, broilers fed L-methionine-supplemented diet showed improved growth performance and feed efficiency during the starter period and had a significantly lower incidence of foot pad dermatitis on day 35, irrespective of heat stress conditions. Furthermore, acute heat stress impeded growth performance, nutrient digestibility, and gut development of broilers, irrespective of the methionine isomer present in the diet.

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