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Original Research Article

Antioxidant response and bioavailability of methionine hydroxy analog relative to DL-methionine in broiler chickens

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ABSTRACT

This study was designed to compare the effect of methionine (Met) sources (DL-methionine [DLM] and DL-2-hydroxy-4-methylthio-butanoic acid [HMTBa]) and their supplementation levels on broiler growth performance and redox state. A 2 × 2 factorial arrangement was used with 2 sources (DLM and HMTBa) and 2 supplementation levels (0.05% and 0.25%) of Met. A total of 480 one-day-old broiler chicks were randomly divided into 4 treatments with 8 replicates per treatment (15 birds per replicate). The experiment lasted for 21 d. Broiler growth performance, redox capacity, redox-related genes expression, and Met transporters in different tissues were tested. Broilers fed high Met supplementation levels had improved ($P < 0.05$) body weight (BW), average daily gain (ADG) and feed conversion ratio (FCR). Similarly, broilers fed high Met levels had better ($P < 0.05$) antioxidant abilities in the serum, small intestine, and liver. Whereas, interactive effects ($P < 0.05$) were also observed between Met sources and levels. Compared with DLM, birds fed HMTBa diets had decreased ($P < 0.05$) total glutathione (T-GSH) and oxidized glutathione (GSSG) contents in duodenum, ileum, and liver. Similarly, broilers fed HMTBa supplemented diets had increased ($P < 0.05$) thioredoxin (*Trx*) gene expression in the duodenum and ileum, but decreased ($P < 0.05$) glutaredoxin (*Grx*), glutathione reductase (*GSR*), and glutathione synthetase (*GSS*) genes expression. Furthermore, lower gene expression of Na^+ and Cl^- dependent neutral and cationic amino acid transporter ($ATB^{0,+}$), and Na^+ dependent neutral amino acid transporter (B^0AT) in the duodenum brush border, but higher gene expression of diamine acetyltransferase 1 (*SAT1*) and Na^+-independent branched-chain and aromatic amino acid transporter (*LAT1*) in the jejunum and ileum basement membrane along with higher expression of the proton dependent monocarboxylate transporter 1 (*MCT1*) gene in the ileum were detected in birds fed HMTBa diets. In conclusion, DLM can be effectively used in glutathione synthesis to exert antioxidant functions, whereas HMTBa favors S-adenosylmethionine (SAM) synthesis and thus stimulates antioxidant-related genes expression.

1. Introduction

Methionine (Met) is involved in several crucial functions in poultry, which include protein synthesis, lipid metabolism, innate immune system regulation, digestive functions, and altering the level of S-adenosylmethionine (SAM), thereby changing gene expression by DNA methylation reactions (Martínez et al., 2017; Pan et al., 2017; Obata et al., 2018; Song et al., 2018; Zhang, 2018).

DL-methionine (DLM) and DL-2-hydroxy-4-methylthio-butanoic acid (HMTBa) are 2 commonly used Met additives (Agostini et al., 2015). DL-methionine and HMTBa are structurally different molecules with different physiological characteristics (Tang et al., 2011; Zhang et al., 2016), and have different optimum dietary levels in poultry. Better growth performance, improved feed conversion ratio (FCR), and lower mortality have been reported in broilers under heat stress or when birds were fed low protein diets supplemented with HMTBa compared with DLM (Swennen et al., 2011; Willemsen et al., 2011). DL-2-hydroxy-4-methylthio-

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butanoic acid and DLM can produce cysteine (Cys), a precursor of glutathione (GSH), which can then be converted into taurine and GSH (Willemsen et al., 2011; Wen et al., 2017). However, HMTBa exhibits greater conversion efficiency (Martin-Venegas et al., 2006). DL-2-hydroxy-4-methylthio-butanoic acid supplementation produces less homocysteine in the plasma than DLM (Xie et al., 2007) and improves L-methionine conversion for effective utilization in chicken livers (Dibner and Knight, 1984). Furthermore, Met serves as an antioxidant, thereby eliminating reactive oxygen species (ROS) by Met residues or through GSH synthesis (Wen et al., 2017; Zeitz et al., 2018).

The significance of Met sources in animal production necessitates an in-depth investigation about their role in antioxidant response in broilers. Therefore, the present research evaluated the effect of different Met sources and levels in broiler diets on growth performance, redox status in different tissues, metabolism pathways and absorption processes.

2. Materials and methods

All the procedures adopted in carrying out this experiment were approved by the China Agricultural University and conducted in accordance with the Guidelines for Experimental Animals.

2.1. Birds and experimental diets

DL-methionine (99%, Sumitomo Chemical, Japan) and HMTBa (effective content 88%; Adisseo, France) were obtained from the market.

A 2 × 2 factorial experiment with 2 sources (DLM and HMTBa) and 2 supplementation levels (0.05% and 0.25%) of Met was conducted. A total of 480 one-day-old male AA + broiler chicks with similar body weight (BW) were randomly divided into 4 treatments. Each treatment consisted of 8 replicates (15 chickens per replicate). A corn-soybean meal-based diet without supplementation of any synthetic Met served as a basal diet. Synthetic Met (DLM and HMTBa) were added to the basal diet on an equimolar basis at 0.05% and 0.25% Met equivalents. The experimental diets were formulated in accordance with the Feeding Standards of Broilers (NY/T 33-2004). All the nutrients (except Met) met the broiler starter phase requirement (Table 1).

Table 1
Ingredients composition (calculated and analyzed nutrients) of the experimental diets for broilers at 1 to 21 d of age (%, as-fed basis).

Ingredients	Content	Calculated nutrients	Content
Maize	54.50	ME, Mcal/kg	2.97
Soybean meal	37.00	Crude protein	21.53
Soybean oil	2.50	Calcium	1.00
Wheat middling	2.00	Available phosphorus	0.45
Dicalcium phosphate	1.85	Lysine	1.29
Limestone	1.24	Methionine	0.32
Salt	0.35	Cysteine	0.34
Choline chloride 50%	0.20	Threonine	0.82
Trace mineral premix[1]	0.20	Tryptophan	0.26
L-lysine HCl 78%	0.13		
Vitamin premix[2]	0.03		
Total	100		

ME = metabolizable energy.
[1] Trace mineral supplied per kilogram of diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.
[2] Vitamin premix supplied per kilogram of diet: vitamin A, 12,500 IU; vitamin D_3, 2,500 IU; vitamin E, 18.75 mg; vitamin K_3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; nicotinic acid, 50 mg.

2.2. Animal management

All the birds were raised on wire net floors in an environmentally controlled house under 23 h continuous light and 1 h darkness. The room temperature was initially set at 33 °C and then gradually reduced according to the age of the birds until reaching 23 °C on d 21. Feed and water were provided *ad libitum*. The birds were weighed on d 1 and then on d 21 after 6 h fasting to calculate body weight gain. Feed intake from d 1 to 21 was also recorded to calculate average daily gain (ADG), average daily feed intake (ADFI), FCR, and European composite index (ECI).

2.3. Sample collection

On d 21, one bird per replicate from each treatment was anesthetized with 50 mg/kg pentobarbital sodium (Sigma, St. Louis, MO, USA). Blood was collected from the jugular vein, and stored at −20 °C after centrifugation (3,000 × *g* for 10 min at 4 °C). Liver samples (2 g) were collected, 8 to 10 mm segments of the small intestine were opened and flushed with cold phosphate buffered saline. Mucosa from the middle duodenum, jejunum, and ileum was collected by gentle scraping with a microscope slide. Liver samples were snap-frozen in liquid N_2, and stored at −80 °C for further analysis.

2.4. Redox balance

An enzyme recycling method was used to test the GSH content in serum, mucosa and liver samples according to the manufacturer's recommendations (T-GSH assay kit; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total glutathione (T-GSH) was tested using a bicinchoninic acid (BCA) Protein Assay Kit (CWBIO, Beijing, China), and expressed relative to the protein concentration in the samples. Glutathione peroxidase (GPx) activity in the plasma, mucosa, and liver was measured by kits (Nanjing Institute of Bioengineering, Nanjing, China). Glutathione and oxidized glutathione (GSSG) contents in the serum, mucosa, and liver were measured using a Pik Wan-day Biotechnology Company kit.

2.5. Small intestinal met transporter and antioxidant-related gene expression

The total RNA of the duodenum, jejunum, ileum, and liver was extracted using Trizol reagent (TAKARA, Japan). The specific procedure has been described in the supplementary material of the kit. The RNA purity and concentration was determined by a nucleic acid analyzer (Nano-drop 2000). cDNA was synthesized using a reverse transcription kit (TAKARA, Japan) and the reversed product was stored in a refrigerator at −30 °C. Fluorescence quantitative PCR was performed according to the SYBP Premix ExTaq (TAKARA, Japan) manual. The reaction instrument was a 7500-fluorescence detection system (Applied Biosystems). The PCR conditions were as follows: 95 °C pre-denaturation for 30 s, denatured at 95 °C for 5 s, 40 cycles of reaction. After PCR amplification, the dissolution curve was observed and agarose gel electrophoresis was used to identify whether the amplified gene fragment was as designed to verify the specificity of the amplified product. The amino acid and antioxidant-related genes of Na^+ dependent neutral amino acid transporter (*B^0AT*), Na^+ and Cl^- dependent neutral and cationic amino acid transporter (*$ATB^{0,+}$*), Na^+-independent cationic and zwitterionic amino acid transporter (*$b^{0,+}AT$*), diamine acetyltransferase 1 (*SAT1*), Na^+-independent branched-chain and aromatic amino acid transporter (*LAT1*), Na^+-independent cationic and Na^+-dependent neutral

amino acid (y^+ *LAT2*), proton dependent monocarboxylate transporter 1 (*MCT1*), Na^+-H^+ exchanger (*NHE3*), methionine adenosyltransferase 1 (*MAT1*), methionine sulfoxide reductase *A* (*MrsA*), thioredoxin (*Trx*), glutaredoxin (*Grx*), glutathione reductase (*GSR*), glutathione synthetase (*GSS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers are shown in Table 2. The results of the genes expression were analyzed by $2^{-\triangle\triangle CT}$.

2.6. Statistical analysis

Two-factor analysis was performed using the general linear model (GLM) procedure of SPSS 23.0 (IBM, Armonk, New York, US). The results were expressed as means. Grubbs method was used to eliminate the abnormal values of data. Significant means were separated by Duncan's Multiple Range test method. The significance level was $P \leq 0.05$, whereas $P < 0.1$ was considered as a tendency of significant difference.

3. Results

3.1. Growth performance

Growth performance results are presented in Table 3. A source × level interaction was observed on ADFI, BW, and ADG, and these indexes improved ($P < 0.05$) when broilers were fed diets with higher HMTBa supplementation levels. Similarly, high Met supplementation levels improved ($P < 0.05$) BW, ADG, FCR and ECI of broilers.

3.2. Antioxidant properties in the serum, mucosa and liver

An antioxidant property in broiler serum and livers is presented in Table 4. In the serum, no source × level interaction on the GPx activity was observed. The Met source did not affect the antioxidant property. However, GPx activity in the serum was increased ($P < 0.05$) with high Met levels.

In the liver, the antioxidant status was affected by the dietary Met supplementation level and source. There was an interaction ($P < 0.05$) on T-GSH, and GSSG. Total glutathione and GSSG were higher ($P < 0.05$) in the 0.25% DLM group than that of the 0.05% DLM group and both HMTBa groups. Methionine sources affected ($P < 0.05$) the hepatic GSH content. DL-methionine supplementation increased ($P < 0.05$) GSH, GSSG, and T-GSH contents. High dietary Met supplementation levels improved ($P < 0.05$) GSH:GSSG. Glutathione peroxidase activity in the liver remained unaffected by the Met source, although a slight improvement ($P = 0.054$) was observed in the liver with higher Met levels.

There was no interaction between the Met source and level on the T-GSH, GSH, and GSSG contents (Table 5). DL-methionine increased ($P < 0.05$) the T-GSH and GSSG contents in the duodenal mucosa, and GSSG in the jejunal mucosa, and T-GSH, GSSG, GSH in the ileal mucosa compared with HMTBa. However, DLM decreased ($P < 0.05$) the GSH:GSSG in the jejunal mucosa.

The GSH content and GSH:GSSG in the duodenal mucosa, and T-GSH and GSSG contents in the jejunal mucosa decreased ($P < 0.05$) with increasing Met levels. However, GSH:GSSG in the jejunal mucosa increased ($P < 0.05$) with high Met supplementation levels.

3.3. Antioxidant-related gene expression in the liver and intestine

There was an interaction between the Met source and level on *Grx* gene expression in the ileum and *Trx* gene expression in the liver. DL-2-hydroxy-4-methylthio-butanoic acid supplementation at 0.25% increased ($P < 0.05$) *Grx* gene expression compared with 0.05% or 0.25% DLM supplementation. The addition of 0.25% DLM decreased ($P < 0.05$) *Trx* gene expression in the liver compared with 0.05% DLM, or 0.25% HTMBA supplementation (Table 6).

Methionine sources affected antioxidant gene expression in broiler intestinal mucosa and liver. Dietary supplementation with HMTBa increased ($P < 0.05$) *Trx* gene expression in the duodenum, *Trx*, *Grx*, *GSR*, and *GSS* gene expression in the ileum, and *MAT1* gene expression in the liver than that of DLM.

Methionine levels had no effect on antioxidant gene expression in broiler intestinal mucosa and liver, except *MAT1* gene expression, which decreased ($P < 0.05$) with increasing Met levels in the liver.

3.4. Amino acid transporter-related gene expression in the intestine

There was an interaction ($P < 0.05$) between the Met sources and levels on $b^{0,+}AT$ and *MCT1* genes expression in the duodenum, and *SAT1* and *LAT1* in the ileum. Proton dependent monocarboxylate transporter 1 and $b^{0,+}AT$ gene expression in the duodenum decreased ($P < 0.05$) with increasing DLM levels, however,

Table 2
Primer sequences of fluorescence quantitative PCR.

Target	Accession No.	Forward (5′ to 3′)	Reverse (5′ to 3′)
B^0AT	XM_419056.4	GGGTTTTGTGTTGGCTTAGGAA	TCCATGGCTCTGGCAGAGAT
$ATB^{0,+}$	XM_004940661.1	TGGCAACATCGTGTGGTACCT	AGGCAGCTCCAACGATCATC
$b^{0,+}AT$	NM_004935075.1	CAGTAGTGAATTCTCTGAGT GTGAAGCT	GCAATGATTGCCAC AACTACCA
SAT1	NM_001199603.1	CACAGTGCCAGTGCTGTTTTT C	TGTTTTTCTTGCCAGCTCGAA
LAT1	NM_001030579.1	GATTGCAACGGGTGATGTGA	CCCCACACCCACTTTTGTTT
y^+LAT2	XM_001231336.3	GCCCTGTCAGTAAATCAGACA AGA	TTCAGTTGCATTGTGTTT TGGTT
MCT1	NM_001006323.1	AGCAGCATCCTGGTGAACAAG	AGGCACCCACCCACGAT
NHE3	XM_001199133.1	AGGCTGGACCGGTTTGC	TCCCGAATACTTTTCCTCCTTTG
MAT1	NM_001199519.1	CCAGGTTAGCAGAGCTGAGG	ATGGACACGCACTGGTATGA
MrsA	XM_004935891.2	CTGGCCACACAGAGGTTGTA	AGTCATTACCTTGCCGCATT
Trx	NM_205453.1	GATTTCTCTGCCACATGGTGT	ATCTTGGGCATCATCCACAT
Grx	NM_205160.1	CCGTCCCTCGTGTGTTTATT	CACCAGAGCACCAATTTGAC
GSR	XM_015276627.1	GTGGTTACGGCAAGTTCACC	CACAGTCAGGAGGGACCACT
GSS	XM_425692.5	TTGCTGGGCTGTACTCACTG	ACAGGTTGTTCCCTCCTCCT
GAPDH	K01458	TGCTGCCCAGAACATCATCC	ACGGCAGGTCAGGTCAACAA

B^0AT = Na^+ dependent neutral amino acid transporter; $ATB^{0,+}$ = Na^+ and Cl^- dependent neutral and cationic amino acid transporter; $b^{0,+}AT$ = Na^+-independent cationic and zwitterionic amino acid transporter; *SAT1* = diamine acetyltransferase 1; LAT1 = Na^+-independent branched-chain and aromatic amino acid transporter; y^+LAT2 = Na^+-independent cationic & Na^+-dependent neutral amino acid; *MCT1* = proton dependent monocarboxylate transporter 1; *NHE3* = Na^+-H^+ exchanger; *MAT1* = methionine adenosyltransferase *1*; *MrsA* = methionine sulfoxide reductase *A*; *Trx* = thioredoxin; *Grx* = glutaredoxin; *GSR* = glutathione reductase; *GSS* = glutathione synthetase; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

Table 3
Effects of 2 sources and levels of methionine on the growth performance in broilers on d 21.

Source	Level, %	BW, g	ADG, g	ADFI, g	FCR	European composite index
DLM	0.05	795.09^{ab}	35.76^{ab}	54.15^{ab}	1.55	219.80
	0.25	798.38^{ab}	35.93^{ab}	53.32^{ab}	1.49	230.55
HMTBa	0.05	779.29^{a}	35.02^{a}	53.03^{a}	1.52	217.99
	0.25	815.78^{b}	37.10^{b}	54.47^{b}	1.48	240.04
SEM		5.23	0.25	0.33	0.01	3.86
Main effect						
Source	DLM	796.73	35.84	54.00	1.51	227.77
	HMTBa	801.09	36.06	53.85	1.50	230.30
Level, %	0.05	787.19^{a}	35.39^{a}	53.85	1.52^{b}	221.49^{a}
	0.25	810.64^{b}	36.52^{b}	53.99	1.48^{a}	236.58^{b}
P-value						
Source		0.648	0.628	0.821	0.477	0.733
Level		0.020	0.019	0.823	0.016	0.049
Source × Level		0.042	0.042	0.029	0.929	0.204

ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio; DLM = DL-methionine; HMTBa = DL-2-hydroxy-4-methylthio-butanoic acid; BW = body weight.
[a, b] Within a column, means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Table 4
Effects of 2 sources and levels of methionine on the antioxidant property in serum and liver of broilers on d 21.

Source	Level, %	Serum	Liver				
		GPx, U/mL	GPx, U/mg protein	T-GSH, nmol/g	GSSG, nmol/g	GSH, nmol/g	GSH:GSSG
DLM	0.05	1,665.89	30.74	1,733.23^{a}	428.81^{a}	875.61	4.10
	0.25	2,639.79	34.96	2,132.34^{b}	635.82^{b}	860.70	3.35
HMTBa	0.05	1,699.77	32.47	1,512.09^{a}	423.15^{a}	665.79	3.63
	0.25	2,457.07	35.63	1,720.89^{a}	422.82^{a}	715.79	3.32
SEM		59.22	0.80	63.37	23.94	32.80	0.06
Main effect							
Source	DLM	2,152.84	33.35	1,932.79^{b}	532.32^{b}	868.15^{b}	3.73
	HMTBa	2,078.42	33.93	1,475.47^{a}	427.66^{a}	620.14^{a}	3.48
Level, %	0.05	1,682.83^{a}	31.99	1,622.66	425.98^{a}	770.70	3.86^{b}
	0.25	2,548.43^{b}	35.29	1,785.60	534.00^{b}	717.60	3.35^{a}
P-value							
Source		0.540	0.724	<0.001	0.002	<0.001	0.079
Level		<0.001	0.054	0.095	0.002	0.332	0.001
Source × Level		0.374	0.955	0.019	0.004	0.484	0.100

GPx = glutathione peroxidase; T-GSH = total glutathione; GSSG = glutathione disulfide; GSH = glutathione; DLM = DL-methionine; HMTBa = DL-2-hydroxy-4-methylthio-butanoic acid.
[a, b] Within a column, means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

no differences were found between different HMTBa supplementation levels. Diamine acetyltransferase 1 gene expression increased ($P < 0.05$) with increasing DLM supplementation levels, whereas, it decreased ($P < 0.05$) with increasing HMTBa supplementation levels. No differences between 0.25% DLM and HMTBa were noted. Na^+-independent branched-chain and aromatic amino acid transporter gene expression decreased ($P < 0.05$) with increasing HMTBa levels, however, there were no differences between 2 levels of DLM (Table 7).

Methionine sources affected amino acid transporter-related gene expression in the intestine. DL-2-hydroxy-4-methylthio-butanoic acid supplementation increased ($P < 0.05$) *SAT1* and *LAT1* gene expression in the jejunum, and *MCT1* gene expression in the ileum ($P < 0.05$). Amino acid transporter-related gene expression in the intestine was also affected by Met levels. $b^{0,+}$ *AT* gene expression in the jejunum, and *MCT1* gene expression in the ileum increased ($P < 0.05$) with increasing Met levels.

4. Discussion

Methionine plays an important role in numerous biological processes (Métayer et al., 2008). Considerable research has demonstrated that optimum Met levels in poultry diets can improve growth performance, but there is continued controversy about the efficacy of synthetic Met sources i.e., HMTBa and DLM continues to persist (Richards et al., 2005; Sauer et al., 2008). It has been reported that both a deficiency and excess of dietary Met leads to poor broiler performance (Song et al., 2018; Zeitz et al., 2018).

Regarding Met, when dietary Met is supplemented at low levels, HMTBa is channelized to generate more Cys and taurine (Martin-Venegas et al., 2006). More than 50% Cys is used by the gastrointestinal tract (Bauchart Thevret et al., 2011), leaving less Met for other organs, which subsequently affects growth performance (Conde-Aguilera et al., 2013). Regarding sulfur amino acids (TSAA), HMTBa outperforms DLM when TSAA in the diet meets or exceeds the requirement, whereas DLM yields better results when the diet is low in TSAA (Vazquez-Anon et al., 2006). Kim et al. (1997) reported that the biological requirement for total Met of broilers is 138 mg/d. Ekmay et al. (2016) recommended a Cys requirement is of 30.5 mg/d and TSAA requirement is of 132.25 mg/d for broilers. Notably, the Met intake (199 to 297 mg/d) in this study was higher than the recommendation. The biological efficiency of HMTBa has been reported to be 81% of the DLM value for ADG (Sauer et al., 2008). Vazquez-Anon et al. (2006) also reported that HMTBa may exert a similar effect on performance compared with DLM at high Met levels. Therefore, it was not unexpected that there was no difference in performance between DLM and HMTBa supplemented diets.

Apart from its role as an amino acid, Met is an important intermediate in methylation reactions and can be converted to Cys,

Table 5
Effects of 2 sources and levels of methionine on the glutathione levels in intestinal mucosa of broilers on d 21.

Source	Level, %	Duodenal mucosa				Jejunal mucosa				Ileal mucosa			
		T-GSH, nmol/g	GSSG, nmol/g	GSH, nmol/g	GSH:GSSG	T-GSH, nmol/g	GSSG, nmol/g	GSH, nmol/g	GSH:GSSG	T-GSH, nmol/g	GSSG, nmol/g	GSH, nmol/g	GSH:GSSG
DLM	0.05	1,266.04	541.78	182.48	2.47	970.55	290.42	389.71	3.38	966.08	239.04	488.00	4.12
	0.25	1,260.19	569.24	68.68	2.13	734.70	203.29	328.12	3.73	889.10	216.68	455.74	4.15
HMTBa	0.05	981.87	424.49	142.89	2.36	833.33	210.02	413.29	3.98	669.07	161.04	346.98	4.15
	0.25	947.22	466.19	14.84	2.04	666.78	152.95	360.88	4.56	551.39	152.94	245.52	3.61
SEM		41.81	23.52	25.11	0.06	37.64	13.67	17.26	0.12	41.81	10.21	25.16	0.10
Main effect													
Source	DLM	1,252.39^{b}	563.41^{b}	125.58	2.30	852.62	246.85^{b}	358.91	3.56^{a}	927.59^{b}	227.86^{b}	471.87^{b}	4.14
	HMTBa	969.54^{a}	445.34^{a}	78.87	2.20	750.05	181.48^{a}	387.09	4.27^{b}	610.23^{a}	156.99^{a}	296.25^{a}	3.88
Level, %	0.05	1,128.95	483.14	162.68^{b}	2.42^{b}	901.94^{b}	250.22^{b}	401.50	3.68^{a}	817.57	200.04	417.49	4.14
	0.25	1,092.98	525.61	41.76^{a}	2.09^{a}	700.74^{a}	178.12^{a}	344.50	4.15^{b}	720.25	184.81	350.63	3.88
P-value													
Source		<0.001	0.015	0.325	0.366	0.134	0.006	0.418	0.001	<0.001	<0.001	<0.001	0.187
Level		0.609	0.357	0.015	0.004	0.005	0.003	0.107	0.028	0.125	0.364	0.095	0.189
Source × Level		0.902	0.986	0.880	0.946	0.606	0.499	0.894	0.592	0.744	0.669	0.379	0.151

T-GSH = total glutathione; GSSG = glutathione; GSH = glutathione; DLM = DL-methionine; HMTBa = DL-2-hydroxy-4-methylthio-butanoic acid.
[a, b] Within a column, means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Table 6
Effects of 2 sources and levels of methionine on the antioxidant-related gene expression in intestine and liver of broilers on d 21.

Source	Level, %	Duodenum						Jejunum						Ileum						Liver					
		MAT1	*MrsA*	*Trx*	*Grx*	*GSR*	*GSS*	*MAT1*	*MrsA*	*Trx*	*Grx*	*GSR*	*GSS*	*MAT1*	*MrsA*	*Trx*	*Grx*	*GSR*	*GSS*	*MAT1*	*MrsA*	*Trx*	*Grx*	*GSR*	*GSS*
DLM	0.05	1.02	1.03	0.74	0.89	1.21	1.13	1.14	0.96	1.05	1.06	0.94	1.03	1.01	1.02	1.01	1.01^{a}	1.03	1.05	1.03	1.06	1.02^{b}	1.07	1.03	1.06
	0.25	0.98	0.78	0.72	0.74	1.19	0.97	0.96	1.06	0.85	0.96	1.02	1.14	0.91	0.98	0.97	0.87^{a}	1.02	1.14	0.81	1.01	0.73^{a}	0.67	0.90	0.90
HMTBa	0.05	0.71	0.90	0.79	0.81	1.38	1.18	1.24	0.86	0.97	0.78	1.02	1.09	1.05	1.00	1.07	1.06^{ab}	1.18	1.36	1.25	1.31	0.86^{ab}	2.12	0.97	0.84
	0.25	0.95	1.04	0.94	0.86	1.47	1.35	1.16	1.11	1.07	0.99	1.22	1.37	0.74	1.28	1.32	1.37^{b}	1.37	1.56	1.11	1.10	0.95^{b}	0.65	1.23	1.20
SEM								0.09	0.05	0.06	0.07	0.06	0.07	0.05	0.05	0.05	0.06	0.06	0.07	0.06	0.04	0.04	0.17	0.06	0.07
Main effect																									
Source	DLM	1.07	0.84	0.72^{a}	0.81	1.30	1.12	1.20	0.96	0.88	0.86	0.94	0.97	0.94	0.97	0.94^{a}	0.92^{a}	0.97^{a}	1.04^{a}	0.94^{a}	1.04	0.90	0.83	0.99	1.00
	HMTBa	0.81	0.96	0.86^{b}	0.83	1.49	1.32	1.18	0.93	0.98	0.83	1.06	1.12	0.89	1.16	0.89^{b}	1.26^{b}	1.28^{b}	1.48^{b}	1.16^{b}	1.16	0.90	1.23	1.14	0.97
Level, %	0.05	0.91	0.91	0.76	0.80	1.43	1.29	0.92	1.35	0.95	0.82	0.94	1.01	1.02	0.98	1.01	1.02	1.05	1.15	1.17^{b}	1.14	0.97	1.41b	1.06	1.00
	0.25	0.96	0.89	0.82	0.85	1.36	1.16	0.97	1.04	0.90	0.86	1.05	1.08	0.81	1.15	1.14	1.17	1.20	1.37	0.93^{a}	1.06	0.83	0.64a	1.07	0.97
P-value																									
Source		0.135	0.256	0.032	0.833	0.340	0.258	0.904	0.719	0.480	0.828	0.325	0.174	0.583	0.058	0.016	0.008	0.018	0.003	0.047	0.078	0.920	0.151	0.185	0.800
Level		0.754	0.818	0.357	0.617	0.719	0.442	0.125	0.652	0.725	0.738	0.349	0.535	0.051	0.089	0.150	0.211	0.247	0.106	0.044	0.201	0.055	0.009	0.939	0.808
Source × Level		0.552	0.079	0.129	0.927	0.078	0.053	0.341	0.481	0.924	0.939	0.624	0.596	0.198	0.137	0.181	0.042	0.732	0.902	0.820	0.568	0.005	0.102	0.106	0.220

MAT1 = methionine adenosyltransferase 1; *MrsA* = methionine sulfoxide reductase *A*; *Trx* = thioredoxin; *Grx* = glutaredoxin; *GSR* = glutathione reductase; *GSS* = glutathione synthetase; DLM = DL-methionine; HMTBa = DL-2-hydroxy-4-methylthio-butanoic acid.
[a, b] Within a column, means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Table 7
Effects of 2 sources and levels of methionine on the amino acid transports gene expression in the intestine of broilers on d 21.

Source	Level, %	Duodenum							Jejunum							Ileum						
		B^0AT	$ATB^{0,+}$	$b^{0,+}AT$	*SAT1*	*LAT1*	y^+LAT2	*MCT1*	*NHE3*	B^0AT	$ATB^{0,+}$	$b^{0,+}AT$	*SAT1*	*LAT1*	y^+LAT2	*MCT1*	*NHE3*	B^0AT	$ATB^{0,+}$	$b^{0,+}AT$	*SAT1*	*LAT1*	y^+LAT2	*MCT1*	*NHE3*
DLM	0.05	1.06	1.24	1.03[b]	0.93	1.11	0.94	1.08	1.35	1.09	1.21	0.91	0.95	1.07	1.03	1.08	1.04	0.94	0.93	0.95	1.01[a]	1.04[a]	1.04	1.09	1.07
	0.25	0.88	0.71	0.59[a]	0.80	0.95	0.69	0.76	1.07	0.88	1.41	1.11	0.89	1.07	0.91	1.16	1.08	1.02	2.10	0.79	1.28[bc]	1.41[a]	0.96	1.72	1.15
HMTBa	0.05	1.01	0.37	0.72[a]	1.04	1.21	0.88	1.15	1.47	0.53	0.88	0.59	1.13	1.84	0.75	1.40	1.13	0.90	2.91	0.57	1.47[c]	1.83[b]	1.01	1.98	1.38
	0.25	1.12	0.49	0.60[a]	1.10	1.39	0.82	1.21	1.34	0.88	1.05	0.86	1.19	1.55	1.04	1.60	1.64	1.41	1.94	0.95	1.21[ab]	1.32[a]	1.21	2.30	1.50
SEM		0.07	0.14	0.05	0.06	0.08	0.05	0.09	0.11	0.09	0.13	0.10	0.04	0.10	0.06	0.10	0.11	0.09	0.34	0.08	0.05	0.08	0.08	0.11	0.10
Main effect																									
Source	DLM	0.95	1.13	0.76[b]	0.82	1.01	0.76	0.96	1.16	0.86	1.09	0.93	0.92a	1.02a	0.87	1.05	0.96	0.96	1.54	0.83	1.16[a]	1.21[a]	0.95	1.40[a]	1.05
	HMTBa	1.05	0.44	0.60[a]	1.04	1.30	0.81	1.14	1.33	0.68	0.93	0.69	1.11b	1.58b	0.84	1.38	1.29	1.22	2.37	0.82	1.34[b]	1.56[b]	1.15	2.16[b]	1.44
Level, %	0.05	1.09	0.85	0.75	0.96	1.22	0.86	1.13	1.31	0.70	0.95	0.74	1.02	1.36	0.83	1.20	1.04	0.90[a]	1.95	0.98	1.26	1.41	0.98	1.53[a]	1.16
	0.25	0.91	0.72	0.60	0.89	1.09	0.71	0.97	1.17	0.84	1.07	0.88	1.01	1.24	0.88	1.23	1.22	1.28[b]	1.96	1.13	1.25	1.35	1.13	2.03[b]	1.33
P-value																									
Source		0.613	0.067	0.033	0.130	0.174	0.692	0.340	0.446	0.324	0.542	0.316	0.015	0.020	0.856	0.072	0.160	0.120	0.261	0.912	0.030	0.021	0.231	<0.001	0.062
Level		0.352	0.719	0.051	0.623	0.532	0.170	0.400	0.510	0.444	0.648	0.547	0.967	0.610	0.675	0.864	0.427	0.026	0.990	0.160	0.848	0.663	0.352	0.013	0.421
Source × Level		0.072	0.508	0.035	0.174	0.115	0.094	0.042	0.455	0.622	0.998	0.774	0.460	0.107	0.745	0.269	0.837	0.113	0.136	0.054	0.005	0.003	0.416	0.427	0.865

B^0AT = Na^+ dependent neutral amino acid transporter; $ATB^{0,+}$ = Na^+ and Cl^- dependent neutral and cationic amino acid transporter; $b^{0,+}AT$ = Na^+-independent cationic and zwitterionic amino acid transporter; *SAT1* = diamine acetyltransferase 1; LAT1 = Na^+-independent branched-chain and aromatic amino acid transporter; y^+LAT2 = Na^+-independent cationic & Na^+-dependent neutral amino acid; *MCT1* = proton dependent monocarboxylate transporter 1; *NHE3* = Na^+-H^+ exchanger; DLM = DL-methionine; HMTBa = DL-2-hydroxy-4-methylthio-butanoic acid.
[a, b] Within a column, means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

which is required for GSH and taurine synthesis. Both GSH and taurine are important components in defense against oxidative stress. The antioxidant defense of the body relies both on non-enzymatic as well as enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT) and GPx. Glutathione provides protection against cellular oxidants through non-enzymatic interactions and enzymatic-mediated mechanisms. The present study showed that the liver and intestinal GSH system was influenced by the Met sources and levels in the diet. Xie et al. (2007) reported that the addition of high Met levels resulted in high homocysteine levels, which is toxic and results in neurodegenerative disease by impeding endothelium functions and ROS generation (Tyagi et al., 2009). The Met metabolite, SAM can enter the irreversible transsulfuration pathway to form Cys, a constituent of GSH (Zeitz et al., 2017). The present findings showed that HMTBa has a protective role on the intestinal epithelial barrier function, which is correlated with reduced GSH. Broilers fed HMTBa diets developed a better non-enzymatic antioxidant defense mechanism, which was consistent with the results of Willemsen et al. (2011). Previous research has shown that HMTBa tends to metabolize in the sulfur pathway to improve the antioxidant capacity of body (Martin-Venegas et al., 2006; Jankowski et al., 2017). Hence, it can be inferred that HMTBa might be more beneficial than DLM in protecting cells from ROS. In addition, a reduction of the GSH:GSSG pool is associated with growth stimulation by nutrients. The GSSG content in the liver and intestinal mucosa was higher in DLM at the 0.25% level. Cellular GSSG levels are normally kept low by the action of GSH reductase, as high GSSG levels are toxic to cells. Additionally, HMTBa at different supplementation levels could maintain a stable GSSG level, which indicated that HMTBa yields better results in response to oxidative stress.

Under lower Met conditions, antioxidant defense systems in broilers were activated, which was evident by a lower GPx content in the serum and higher GSH:GSSG in liver. Wu et al. (2012) reported that a Met deficiency reduced the GPx and SOD activities and increased the MDA content in the spleens of broilers, but high dietary Met levels enhanced the serum SOD activity and ferric reducing ability, whereas, the MDA content was reduced (Jankowski et al., 2017; Zduńczyk et al., 2017). Consistent with previous studies, we found that the addition of high Met supplementation levels could strengthen the antioxidant system by enhancing SOD activity in the duodenum, CAT in the ileum, as well as GPx activity and total antioxidant capacity in the serum.

Thioredoxin, as an important enzyme-regulated protein, aids in alleviating intracellular oxidative stress, controls growth and programmed cell death, participates in DNA synthesis, reduces the disulfide bond in the protein thereby, playing an important role in the body's antioxidant system (Saitoh et al., 1998). Gasparino et al. (2018) reported that HMTBa can alleviate heat-induced oxidative damage by improving *Trx* in broilers. Our findings are supported by their work, where HMTBa enhanced *Trx* gene expression in broiler duodenum. However, no effect was observed on GSH in this study, which suggested that HMTBa controls the oxidative balance of cells by stimulating *Trx* gene expression in the duodenum rather than solely being involved GSH production. S-adenosylmethionine, synthesized by Met and ATP through L-methionine S-adenosyltransferase (MAT) catalysis, is an important methyl donor that can improve SOD activity (Zhang et al., 2016). We also found that HMTBa supplementation can improve *MAT1* expression in the liver, but decrease T-GSH, GSSG, and GSH contents. The underlying reason may be the participation of HMTBa in another metabolic pathway to produce SAM, whereas DLM generally generates GSH. We also found that compared with HMTBa, DLM supplementation can enhance GSSG and T-GSH contents in the duodenum and increase GSSG content and decrease GSH:GSSG in the jejunum.

Different mechanisms for cellular absorption, transport, metabolism and bio-efficiency exist for these 2 Met sources. Moreover, there are differences in their utilization among various species such as chickens, pigs and ruminants. As a Met precursor, HMTBa is absorbed mainly by MCT1, coupled with the NHE3 activity, and DLM uptake occurs via multiple carrier-mediated systems. The liver, kidneys and small intestine can metabolize D-Met and HMTBa to L-Met through oxidation and transamination. Unlike DLM, which can only be transported into the body via active transport, HMTBa can either be absorbed into the body by active transport or by passive diffusion through the intestinal epithelial cells into the bloodstream and transported to other tissues (Zhang et al., 2016). The major Met transporter present on the brush border of the intestinal epithelial cells include B^0AT, $ATB^{0,+}$and $b^{0,+}AT$ (Pramod et al., 2013). The transporters present on the basal membrane mainly include SAT1, SAT2 and SAT3, and LAT1 and LAT2, and y^+ LAT1 and y^+ LAT2. It was noted that dietary supplementation with DLM enhanced $ATB^{0,+}$ and B^0AT expression in the duodenum, whereas dietary HMTBa supplementation mainly improved the expression of *SAT1* and *LAT1* in the jejunum and *SAT1*, *LAT1* and *MCT1* in the ileum, which showed that DLM is mainly absorbed in the duodenum whereas the jejunum and ileum are the main sites HMTBa absorption. This concurs with Martin-Venegas et al. (2006), who also reported that the jejunum and ileum had higher HMTBa absorption. In addition, DLM can improve the transporters present on the brush border, whereas, HMTBa tends to enhance the transporters on the basement membrane. Thus, transporters present on the basement membrane transport HMTBa more efficiently to blood to participate in the metabolism. In this way, more HMTBa escapes the first-pass effect in the gastrointestinal tract and is utilized by whole body tissues (Fang et al., 2010).

5. Conclusion

DL-methionine is mainly absorbed in the duodenum and favors GSH synthesis, whereas HMTBa is primarily absorbed in the jejunum and ileum and generates SAM; thus, stimulates *Trx* expression, which in turn participates in oxidation resistance.

Conflicts 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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