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Partial Replacement of Dietary Methionine with Betaine and Choline in Heat-Stressed Broiler Chickens

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We conducted two trials to evaluate the methionine-sparing effects of choline (Chol) and betaine (Bet), and their effects on growth performance and blood antioxidative potential in heat-stressed broiler chickens fed methionine (Met)-deficient diets. We used 360 1-day-old broiler chicks (Ross 308) in a completely randomized study with 5 replicate pens of 12 birds each. After Day 21, we raised the temperature to 35±3℃ using an automated air-forced heater for 12 hours/day from 8 am to 8 pm to expose the birds to heat stress. In Trial 1, the treatments comprised a negative control (control-; 1200 mg/kg Met-deficient), a positive control (control+; recommended level of Met), 280Chol (control- plus 280 mg/kg Chol), 560Chol (control- plus 560 mg/kg Chol), 320Bet (control- plus 320 mg/kg Bet), and 640Bet (control- plus 640 mg/kg Bet); and in Trial 2, the treatments comprised a negative control (control-), a positive control (control+), 140Chol+160Bet (control- plus 140 mg/kg Chol and 160 mg/kg Bet), 280Chol+160Bet (control- plus 280 mg/kg Chol and 160 mg/kg Bet), 140Chol+320Bet (control- plus 140 mg/kg Chol and 320 mg/kg Bet), and 280Chol+320Bet (control- plus 280 mg/kg Chol and 320 mg/kg Bet). Compared with the other treatments, the feed conversion ratio (FCR) was improved in the 280Chol and control+ groups in Trials 1 and 2 ($P<0.05$). In Trial 2, the cost of meat production for the entire experimental period (1−42 days) was higher in the 140Cho+320Bet-fed birds than in the other birds ($P<0.05$), except the control- birds. Supplementing diets with 280 mg/kg of Chol significantly reduced the serum concentration of uric acid compared with the control+ group ($P<0.05$). Our results indicate that the Met requirements of heat-stressed broiler chickens can be reduced by 20% (1200 mg/kg) if the diet is supplemented with 280 mg/kg of Chol.

Key words: acute phase protein, anti-oxidant status, broiler, organs, performance

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Introduction

Heat stress (HS) is defined as a negative balance between the net amount of energy flowing from an animal's body to its surrounding environment and the amount of heat energy produced by the animal (Lara and Rostagno, 2013). The effects of HS on performance in domestic fowl are frequently investigated, and the results have been carefully reviewed by many researchers (Charles, 1986; Mitchell and Carlisle, 1992; Mahmoud and Yaseen, 2005). Broiler chickens are very susceptible to high environmental temperatures because they lack sweat glands and have a high metabolic rate (Geraert *et al*., 1993). The problems associated with HS in broilers include respiratory alkalosis (Teeter *et al*., 1985), decreased performance (Howlider and Rose, 1989; Cooper and Washburn, 1998; Akşit *et al*., 2006), decreased breast yield (Akşit *et al*., 2006), and oxidative stress (Mujahid *et al*., 2005; Lin *et al*., 2006a). Under HS conditions, chickens eliminate excess heat by increasing their respiratory rate, which may result in an acid/base imbalance known as respiratory alkalosis (Borges *et al*., 2004; Toyomizu *et al*., 2005). Moreover, HS leads to increased levels of reactive oxygen species (ROS), possibly due to disruption of the electron transport assemblies of the cell membrane (Ando *et al*., 1997). ROS play an important role in many biological systems, including the body's response to infection, heavy metals, and ethanol toxicity, as well as other conditions (Donati *et al*., 1990).

For many decades, researchers have sought nutritional ways to moderate the negative impact of HS on broilers. Choline (Chol) is a water-soluble, essential quaternary amine that plays an important role as a methyl donor in the conversion of homocysteine to methionine (Met) (Zhang *et al*., 2013). Studies on Chol have mainly focused on its methionine-sparing role (Keshavarz and Austic, 1985; Waldroup *et al*., 2006). However, although Chol is essential, it may also be beneficial for improving the efficiency of

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energy and protein utilization (Rao *et al*., 2001). Chol is readily oxidized to betaine (Bet), which also functions as a methyl donor and an osmolyte (Criag, 2004). Chol also plays an important role in lipid metabolism in the liver, thereby preventing the abnormal accumulation of fat in that organ (Rao *et al*., 2001). Furthermore, Chol acts as an osmolyte; it plays an important role in maintaining normal cell volume and can produce Bet and glycerol-phosphocholine. The latter is a dominant osmolyte in kidney cells, and protects renal medullary cells from hypertonicity of the interstitial fluid (Nakanishi and Burg, 1989).

Betaine (Bet), also known as trimethylglycine, is a zwitterionic quaternary ammonium compound and a neutrally charged osmolyte (Yancey *et al*., 1982) that may be beneficial to the performance of broilers under extreme ambient temperatures. Bet has been widely evaluated for its methionine-sparing, choline-sparing (Patil *et al*., 2007), performance-enhancing, acid/base-balancing, and osmolytic (Criag, 2004) effects in broilers (Waldroup and Fritts, 2005). Methionine re-methylation catalyzed by betaine-homocysteine methyl transferase (BHMT) is involved in the regulation of osmosis (Schafer *et al*., 2007). Bet can act as an antioxidant agent in low-methionine diet-induced oxidative stress in broilers (Alirezaei *et al*., 2012). However, methionine has a potentially protective role in the scavenging of free radicals generated indirectly by lead, including those formed during xenobiotic-induced lipid peroxidation and those caused by reduced antioxidant enzyme activities and vitamin levels (Caylak *et al*., 2008). Moreover, Bet can replace up to 25% of Met without adversely affecting the production performance of the broilers, indicating that up to 25% of dietary Met is involved in methylation reactions in broilers (Sun *et al*., 2008). Therefore, the objectives of the present study were to evaluate the effects on production performance, anti-oxidative indices, and certain blood metabolites of supplementing a Met-deficient diet with Chol and Bet in broiler chickens reared in HS conditions.

Materials and Methods

Animals and Diets

This experiment consisted of two trials. In Trial 1, 360 1-day-old male and female broiler chicks (Ross 308) were randomly assigned to 1 of 6 experimental treatment groups: a negative control group (control-; 1200 mg/kg Met-deficient), a positive control group (control+; recommended level of Met, without Met deficiency), a 280Chol group (negative control diet plus 280 mg/kg Chol), a 560Chol group (negative control diet plus 560 mg/kg Chol), a 320Bet group (negative control diet plus 320 mg/kg Bet), and a 640Bet group (negative control diet plus 640 mg/kg Bet) in 5 replicate pens of 12 birds each. In Trial 1, the replacement of Met with Bet or Chol provided a similar number of methyl groups (an equivalence) to that provided by 1200 mg (280Chol and 320Bet) and 2400 mg (560Chol and 640Bet) of methionine as a methyl donor.

In the second trial, we assessed the effects of supplementing a Met-deficient diet (similar to Trial 1) with a combination of Chol and Bet on production performance, certain blood metabolites, liver enzymes, and the blood anti-oxidative status of heat-stressed broiler chickens. We assigned 360 (Ross 308) 1-day-old chicks to each of the 6 experimental treatments in 5 replicate pens of 12 birds each. The treatments comprised control-, control+, 140Chol+160Bet (negative control diet plus 140 mg/kg Chol and 160 mg/kg Bet), 280Chol+160Bet (negative control diet plus 280 mg/kg Chol and 160 mg/kg Bet), 140Chol+320Bet (negative control diet plus 140 mg/kg Chol and 320 mg/kg Bet), and 280Chol+320Bet (control diet plus 280 mg/kg Chol and 320 mg/kg Bet). In Trial 2, the replacement of Met with Bet or Chol provided a similar number of methyl groups (an equivalence) to that provided by 1200 mg (140Chol+160Bet), 1800 mg (280Chol+160Bet and 140Chol+320Bet), and 2400 mg (280Chol+320Bet) of methionine as a methyl donor.

Each pen (1×1.2 m) was provided with a feeder (1 m long) and a bell-type drinker. In order to expose birds to HS in both trials, after Day 21 the house temperature was raised to 35±3℃ by an automated air-forced heater 12 hours/day from 8 am to 8 pm. All birds were maintained under the same temperature condition (under 60% relative humidity) in tunnel ventilated house and lighting control system (23 h light/d- 30 lux) during the entire period of study. The basal diet (control diet) was formulated based on a corn-soybean meal for the starter (0−21 days) and growing (22−42 days) periods. Diets were formulated to meet or slightly exceed all nutrient recommendations (except for Met) for broilers. (Table 1; Aviagen, 2014). Supplemental Bet (Betafin, Biochem, Lohne, Germany, 96% betaine) and choline (Choline chloride, Hylen, Qingdao, China; 60% choline chloride) was substituted for equivalent amounts of corn in the basal diet. Birds had free access to feed and water throughout the experimentation period. Light was continuous during the first day and a 23 h: 1 h lightening: darkness regimen was employed afterwards.

Sample Collection and Analytical Determination

Live body weight (BW) and feed intake (FI) were recorded on a weekly basis, and the data were used to calculate the average daily weight gain (ADWG), the average daily feed intake (ADFI), and the feed conversion ratio (FCR) for each phase. Viability, economic factors including the European efficiency factor (EEF), and the cost of producing 1 kg of live weight (Cm) were calculated using the following formulae (Bera *et al*., 2010; Lup *et al*., 2012):

$$\text{Viability} = \{1 - (\text{n of dead birds/total birds})\} \times 100$$

$$\text{EEF} = \{\text{BW} \times \text{viability} \times 100/(\text{FCR} \times \text{age})\}$$

where EEF is the European efficiency factor and BW is the average body weight (kg). Age was measured in days. The cost of producing 1 kg of live weight was calculated as:

$$\text{Cm} = \text{FCR} \times \text{Cf}$$

where Cm is the cost of producing 1 kg of live weight, FCR is the feed conversion ratio, and Cf is the cost of feed per kg.

At 28 (7 days after heat treatment) and 42 days of age, two birds from each pen were randomly selected for blood sampling via a wing vein. The serum was harvested by centrifuging the blood samples at 3000×*g* for 20 min at 4℃

Table 1. **Feed ingredients and nutrient composition of basal diet (control) during different growth periods**

Feed ingredients (g/kg)	Starter (0−21 days)	Grower (22−42 days)
Corn grain[1]	499.7	585.8
Soybean meal	436.4	341.2
Soybean oil	23.1	40.5
Ca-carbonate	13.1	11.0
Di-calcium phosphate	12.8	7.7
DL-methionine	1.8	0.9
L-lysine HCl	2.0	1.2
L-threonine	1.1	0.5
Na-bicarbonate	1.7	1.8
Salt	2.7	2.7
Vitamins and minerals Premix[2]	5.0	5.0
Multi-enzyme[3]	0.5	0.5
Phytase[4]	0.1	0.1
Analyses (calculated g/kg)		
ME (MJ/kg)	12.55	13.39
Crude protein	232.3	200.0
Methionine	4.88	3.67
Methionine + cysteine	8.30	7.10
Lysine	12.80	10.30
Threonine	8.60	6.90
Calcium	10	7.9
Available phosphorus	5.0	3.95
Sodium	1.6	1.6

[1] Dietary treatments were added as part of corn grain.
[2] Vitamin and mineral premix provided per kg of diet: Mn (from $MnSO_4 \cdot H_2O$), 90 mg; Zn (from ZnO), 85 mg; Fe (from $FeSO_4 \cdot 7H_2O$), 54 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 9 mg; I (from $Ca(IO_3)_2 \cdot H_2O$), 1.8 mg; Se, 0.35 mg; Mo, 0.16 mg; vitamin A (from retinyl acetate), 12,500 IU; cholecalciferol, 3500 IU; vitamin E (from DL-α-tocopheryl acetate), 40 IU; vitamin B12, 0.03 mg; riboflavin, 6.4 mg; nicotinamide, 50 mg; calcium pantothenate, 28 mg; menadione (from menadione dimethyl-pyrimidinol), 3.5 mg; folic acid, 1.2 mg; thiamine, 3 mg; pyridoxine, 7.8 mg; biotin, 0.25 mg; ethoxyquin, 85 mg.
[3] Kemzyme Dry (Kemin Industries, Inc.).
[4] Phyzyme XP 5000 (Biochem, Germany).

using a benchtop centrifuge (Pars Azma, Tehran, Iran), and stored at −20℃ until required. We determined serum levels of total protein (TP), albumin (ALB), glucose (GLU), total cholesterol (CHL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), triglycerides (TG), calcium (Ca), phosphorus (P), uric acid, alkaline phosphatase (ALP), aspartate amino transferase (AST), and alanine amino transferase (ALT) using commercial kits (Pars Azmoon, Tehran, Iran). Non-esterified fatty acids (NEFAs; FA115, Randox, Crumlin, UK), total antioxidant status (TAS; NX2332, Randox), glutathione peroxidase (GPx; RS 506-supplementary pack MS 181, Randox), and superoxide dismutase (SOD; SD 125, Randox) were assayed colorimetrically using the reagent kits indicated and an Alcyon 300 automated biochemical analyzer (Abbott Laboratories, Illinois, US) following the manufacturers' instructions. The serum amyloid A (SAA) concentration was measured with a CSB- EQ027630CH kit (Cusabio, China) equipped with an enzyme-linked immunosorbent assay (ELISA) reader machine (Stat Fax 4200, Awareness Technology Inc. Palm City, FL, US). The serum concentration of ceruloplasmin (CEP) was measured using the method described by Sunderman and Nomoto (1970). All analyses were performed twice.

When they were 42 days old, two birds from each pen were randomly selected to provide blood samples. They were slaughtered, processed, and the proportional weight of the internal organs (as a percentage of the carcass weight) including the proventriculus, gizzard, liver, spleen, heart, and bursa of Fabricius were recorded. The total lipid content of the liver was assayed according to the method described by Folch *et al*. (1957).

Statistical Analysis

The data were analyzed using the GLM procedure of SAS 9.1 (SAS, 2003) according to the following model:

$$Yij = \mu + Ti + eij$$

where μ is the overall mean, T_i is the fixed effect of treatments, and e_{ij} is the random residual error. Means were partitioned using Duncan's Multiple Range Test (Duncan, 1955). For all statistical analyses, significance was declared

at $P \leq 0.05$, unless otherwise stated.

Results and Discussion

Production Performance

In Trials 1 and 2, the overall ADFI and ADWG were not affected by dietary treatments ($P > 0.05$; Tables 2 and 3).

In Trial 1, supplementing Met-deficient (control-) diets with 280 mg/kg Chol, 560 mg/kg Chol, and 320 mg/kg Bet resulted in similar overall FCRs (Days 1−42) compared with the control+ group (Table 2), which agreed with the findings reported by Sun *et al*. (2008). However, the control- diet with 640 mg/kg of Bet significantly increased the overall FCR of the birds compared with the control+ group (Table 2; $P < 0.05$). Furthermore, only the FCR 640 mg/kg Bet-fed and control+-fed birds exhibited significant differences ($P < 0.05$). In Trial 2, significant differences were observed in the FCR for the starting period as well as for the overall FCR (1−42 days) among the dietary groups (Table 3, $P < 0.05$). Feeding birds with 140Chol+160Bet, 280Chol+160Bet, and 280Cho+320Bet diets resulted in similar overall FCRs compared with the control+ birds (Table 3. $P > 0.05$). The results showed that 20% of dietary Met, which is probably needed for methylation processes in broiler chickens, can be replaced by 280 mg/kg Chol or 320 mg/kg Bet (as a methyl group donor; Trial 1), or by a combination of 140Chol+160Bet (Trial 2), with no adverse effects on ADFI, ADWG, or FCR in growing birds. In agreement with the findings reported by Sun *et al*. (2008) and Esteve-Garcia and Mack (2000), the methyl donor compounds used in the present study successfully replaced Met, and increased the weight of the broiler chicks. Azadmanesh and Jahanian (2014) found that dietary supplementation with Chol reduced FCR and ADFI in broiler chicks reared under normal conditions. Moreover, Rao *et al*. (2011) reported that supplementing Met-deficient diets with Bet increased feed efficiency and weight gain.

In Trial 1, EEF, Cm in the starter and grower periods, and viability were not affected by the treatments (Table 2). However, overall Cm (1−42 days) was lower in the 280Chol group compared with the other treatments, with the single exception of 320Bet-fed birds ($P < 0.05$). In Trial 2, viability and Cm in the grower period were not affected by dietary treatments (Table 3), but control+ and 280Chol+320Bet-fed chicks had a higher EEF and a lower Cm (1−21 days) than the control- chicks ($P < 0.05$). In Trial 2, the overall EEFs (1−42 days) were similar in the control+ birds and those fed Met-deficient diets supplemented with combinations of Chol and Bet (Table 3). The cost of producing 1 kg of live weight was similar in chicks fed control+ diets and those fed Met-deficient diets supplemented with combinations of Chol and Bet (Table 3). According to the results given in Tables 2 and 3, 20% of the dietary Met requirements could be replaced with each level of 280 mg/kg of Chol and/or Bet (with the exception of 640Bet and 140Chol+320Bet) tested in this experiment, with no negative effects on production performance. Interestingly, the 280Chol-fed birds had a significantly lower Cm, even compared with the control+ birds. This reduction in Cm (0.26$) was owing to lower feed costs and improved FCRs, which are economically beneficial for the producer.

Organ Weights

In Trial 1, the relative weight of a thigh plus drumstick was lower in the 280Chol-fed birds than in the control chicks ($P < 0.05$), but was similar among the birds receiving other dietary treatments (Table 4). The relative weight of the proventriculus increased in birds fed with the diet containing 640Bet compared with the control-, control+, and 560Chol-fed birds ($P < 0.05$), but was similar to the other birds (Table 4). In Trial 2, the relative weight of the gizzard was greater in birds fed diets fortified with 280Chol+320Bet compared with the other birds with the exception of birds fed on a 280Cho+160Bet diet (Table 5). The relative weight of the gastrointestinal tract is a well-known indicator of its development (Nir *et al*., 1994; Hetland *et al*., 2003). An increase in the relative weight of the proventriculus in chickens fed a 640Bet (Trial 1) should be considered an indicator of greater anatomical development and increased acid and enzyme secretion in the same segment. An increase in the relative weight of the gizzard in the chicks fed 280Chol+320Bet (Trial 2) should have resulted in increased gizzard movement, and consequently increased feed digestibility and improved production performance, but such an effect was not observed in the present study. In Trial 2, the relative weight of the bursa of Fabricius increased in the chicks fed with 140Chol+320Bet (equivalent to 1800 mg of Met) and 280 Chol+320Bet (equivalent to 2400 mg of Met) compared with those fed Met-deficient diets supplemented with either 140 mg/kg Bet plus 160 mg/kg Bet or with 280 mg/kg Chol plus 160 mg/kg Bet ($P < 0.05$). Moreover, the relative weight of the bursa of Fabricius was numerically higher in chicks fed 140Chol+320Bet and 280Chol+320Bet than in the control+ birds (22.2% higher; Table 5). This indicates that in heat-stressed chicks, supplementing a Met-deficient diet with at least 320 mg/kg Bet in combination with Chol may improve the immune response. Swain and Johri (2000) reported that Bet and Chol improve the immune response by acting as coccidiostat enhancers in broilers. Interestingly, the relative liver weight was greater in the 280Chol-fed chicks than in the Bet-fed chicks ($P < 0.05$). This was unexpected because we anticipated a lower total lipid content of the liver in the Chol-fed chicks than in the other birds. Rao *et al*. (2001) observed that liver fat content decreased in broiler breeders fed a Met-deficient diet supplemented with Chol. Neto *et al*. (2000) reported that the level of liver lipids was not affected by dietary Bet supplementation. However, we observed a reduction in liver lipid content in birds fed with 280Chol+160Bet (Trial 2).

Blood Parameters

The blood biochemical factors assessed in Trials 1 and 2 were similar among the dietary treatments, with the single exception of LDL (Tables 6 and 7, respectively). Feeding chickens a Met-deficient diet supplemented with 320 mg/kg of Bet elevated the serum LDL concentration by approximately 66 and 55% compared with those grown on a control+ diet or a diet supplemented with 560 mg/kg of Chol, respec-

Table 2. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on feed intake, weight gain, feed conversion ratio, European efficiency factor, the cost of producing 1 kg of live weight, and viability in broiler chicks (Trial 1)**

Parameter	Treatments						SEM	P-value
	Control-	Control+	280Chol	560Chol	320Bet	640Bet		
ADFI, g								
1–21 days	33.2	32.9	30.8	35.1	34.5	34.8	0.65	0.4490
21–42 days	145.7	141.7	143.6	150.9	145.6	139.6	1.77	0.5759
1–42 days	87.7	87.3	84.4	91.4	89.5	86.7	0.84	0.2549
ADWG, g								
1–21 days	27.4	29.3	26.0	29.1	28.7	28.0	0.43	0.2201
21–42 days	71.1	71.0	75.0	74.7	71.0	68.4	1.27	0.6983
1–42 days	48.5	50.2	49.3	51.2	49.6	48.0	0.61	0.7493
FCR, g/g								
1–21 days	1.21	1.13	1.18	1.21	1.20	1.24	0.016	0.4154
21–42 days	2.06	2.00	1.92	2.03	2.06	2.05	0.020	0.3079
1–42 days	1.81^{a}	1.72^{bc}	1.71^{c}	1.76^{abc}	1.79^{ab}	1.80^{a}	0.010	0.0314
EEF								
1–42 days	281.0	300.4	291.5	285.7	283.1	280.5	4.56	0.8334
C_m ($/kg)								
1–21 days	19702	18362	18854	18740	19177	19923	251.1	0.4428
22–42 days	30661	30281	29033	31097	31200	31008	288.9	0.3264
1–42 days	27371^{a}	27147^{a}	26225^{b}	27455^{a}	26951^{ab}	27336^{a}	131.5	0.0415
Viability (%)								
1–42 days	100	100	97.9	97.9	97.9	97.9	0.65	0.8424

ADFI, average daily feed intake; ADWG, average daily weight gain; FCR, feed conversion ratio; Chol, choline; Bet, betaine; EEF, European efficiency factor; Cm, the cost of producing 1 kg of meat.
[a–c] Mean values within a row with no common superscript differ significantly ($P<0.05$).

Table 3. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on feed intake, weight gain, feed conversion ratio, European efficiency factor, the cost of producing 1 kg of live weight, and viability in broiler chicks (Trial 2)**

Parameter	Treatments						SEM	P-value
	Control-	Control+	140 Chol+ 160 Bet	280 Chol+ 160 Bet	140 Chol+ 320 Bet	280 Chol+ 320 Bet		
ADFI, g								
1–21 days	32.0	33.1	30.4	32.1	35.5	32.6	0.71	0.5141
21–42 days	139.4	145.5	144.8	139.9	147.3	148.1	1.71	0.6094
1–42 days	84.5	88.8	86.4	85.0	90.3	88.5	0.90	0.3859
ADWG, g								
1–21 days	25.3	29.5	26.3	25.7	29.0	27.8	0.57	0.1344
21–42 days	69.1	75.0	74.5	71.1	68.8	74.2	1.22	0.5319
1–42 days	46.7	52.0	49.9	47.9	48.6	50.2	0.69	0.3148
FCR, g/g								
1–21 days	1.28^{a}	1.14^{c}	1.15^{c}	1.25^{ab}	1.22^{abc}	1.17^{bc}	0.016	0.0292
21–42 days	2.02	1.95	1.95	1.97	2.16	2.00	0.025	0.1071
1–42 days	1.83^{ab}	1.71^{c}	1.73^{bc}	1.77^{abc}	1.87^{a}	1.76^{bc}	0.016	0.0319
EEF								
1–42 days	261.6^{b}	313.6^{a}	294.9^{ab}	286.5^{ab}	289.8^{ab}	297.6^{a}	5.13	0.0391
C_m ($/kg)								
1–21 days	20341^{a}	18435^{c}	18395^{c}	20039^{ab}	19543^{abc}	18736^{bc}	234.7	0.026
22–42 days	30943	29952	30093	30485	33479	31039	376.1	0.074
1–42 days	27915^{ab}	26643^{b}	26961^{b}	27632^{b}	29153^{a}	27518^{b}	237.1	0.026
Viability (%)								
1–42 days	100	100	97.9	100	100	100	0.32	0.37

ADFI, average daily feed intake; ADWG, average daily weight gain; FCR, feed conversion ratio; Chol, choline; Bet, betaine; 1$=35700 Rail; EEF, European efficiency factor; Cm, the cost of producing 1 kg of meat.
[a–c] Mean values within a row with different superscripts differ significantly ($P<0.05$).

Table 4. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on organ weight and total lipid content of the liver in broiler chicks (Trial 1)**

Parameter	Treatments						SEM	P-value
	Control	Control+	280Chol	560Chol	320Bet	640Bet		
Feathers removed*	92.8	93.9	93.7	93.2	93.4	93.4	0.19	0.6742
Carcass	69.1	71.1	70.8	69.7	69.8	70.4	0.30	0.3687
Breast	23.7	25.0	24.3	24.9	23.8	23.9	0.26	0.5569
Thigh + drumstick	20.5^{a}	19.8^{ab}	19.2^{b}	19.7^{ab}	19.7^{ab}	20.6^{a}	0.14	0.0283
Back	24.6	23.4	24.2	23.8	23.7	24.9	0.19	0.1182
Digestive system	13.2	12.6	13.6	13.4	13.6	12.7	0.15	0.2172
Gizzard	3.09	3.05	3.13	3.02	3.08	2.79	0.057	0.6241
Proventriculus	0.46^{b}	0.45^{b}	0.52^{ab}	0.47^{b}	0.51^{ab}	0.55^{a}	0.010	0.0171
Liver	2.57^{b}	2.68^{ab}	2.94^{a}	2.96^{a}	2.52^{b}	2.40^{b}	0.050	0.0023
Spleen	0.14	0.12	0.11	0.14	0.11	0.13	0.005	0.3844
Heart	0.53	0.48	0.53	0.50	0.51	0.55	0.009	0.2736
Bursa Fabricius	0.11	0.09	0.11	0.11	0.09	0.11	0.006	0.5468
Total lipid content of liver (%)	26.5^{ab}	24.5^{ab}	33.1^{a}	25.0^{ab}	22.7^{b}	18.7^{b}	1.13	0.0176

* Relative weight to live body weight for feathers removed, and carcass weight and relative weight to carcass weight for others; Chol, choline; Bet, betaine.

$^{a-c}$ Mean values within a row with different superscripts differ significantly ($P<0.05$). Values represent 4 replicates of 2 birds per replicate for each treatment.

Table 5. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on organ weight and total liver lipid concentration in broiler (Trial 2)**

Parameter	Treatments						SEM	P-value
	Control-	Control+	140Chol+ 160Bet	280Chol+ 160Bet	140Chol+ 320Bet	280Chol+ 320Bet		
Feathers removed*	92.7	93.7	93.4	94.5	93.0	93.3	0.48	0.9397
Carcass	68.7	67.4	71.6	71.4	70.2	69.8	0.75	0.5828
Breast	23.8	24.6	26.1	26.0	25.2	25.1	0.30	0.2110
Thigh + drumstick	20.1	19.8	19.7	20.0	20.0	19.8	0.10	0.8597
Back	24.4	23.2	24.6	24.3	24.3	24.8	0.24	0.5115
Digestive system	13.4	13.2	12.2	13.2	12.7	13.7	0.18	0.2726
Gizzard	3.00^{bc}	3.03^{bc}	2.78^{c}	3.21^{ab}	3.00^{bc}	3.44^{a}	0.056	0.0208
Proventriculus	0.46	0.5	0.48	0.5	0.48	0.53	0.013	0.6830
Liver	2.63	2.65	2.4	2.38	2.55	2.69	0.037	0.0503
Spleen	0.14	0.12	0.1	0.12	0.14	0.12	0.005	0.1749
Heart	0.51	0.48	0.5	0.52	0.49	0.51	0.008	0.6675
Bursa of Fabricius	0.08^{ab}	0.09^{ab}	0.07^{b}	0.06^{b}	0.11^{a}	0.11^{a}	0.005	0.0068
Total lipid content of liver (%)	24.4^{a}	25.9^{a}	22.6^{a}	14.8^{b}	21.7^{a}	21.0^{a}	0.97	0.0153

* Relative weight to live body weight for feathers removed, and carcass weight and relative weight to carcass weight for others; Chol, choline; Bet, betaine.

$^{a-c}$ Mean values within a row with different superscripts differ significantly ($P<0.05$). Values represent 4 replicates of 2 birds per replicate for each treatment.

tively ($P<0.05$; Table 6). Habibian *et al*. (2014) showed that serum concentrations of total cholesterol and LDL increased in heat-stressed broilers, owing to the lipotropic effect of betaine (Cholewa *et al*., 2014). Betaine can stimulate lipolysis and inhibit lipogenesis via gene expression and the subsequent activity of lipolytic-/lipogenic-related proteins (Cholewa *et al*., 2014).

We did not observe any significant changes in the activities of liver enzymes (ALT, AST, and ALP), or in hemoglobin and malondialdehyde (MDA) concentrations in the present study ($P>0.05$; Table 8 and Table 9). Owing to a higher blood uric acid concentration, the serum total antioxidant status (Trial 1) was significantly greater in the control+ chicks than in the other chicks, with the exception of

Table 6. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on blood parameters in broilers (Trial 1)**

Parameter	Treatments						SEM	P-value
	Control-	Control+	280 Chol	560 Chol	320 Bet	640 Bet		
ALB (g/dL)	1.48	1.43	1.44	1.46	1.58	1.44	0.019	0.1860
TP (g/dL)	3.46	3.43	3.65	3.54	3.58	3.20	0.045	0.0860
GLU (mg/dL)	191.2	186.9	198.5	201.7	198.4	189.0	2.79	0.5834
Ca (mg/dL)	8.90	8.95	9.11	9.58	8.49	9.28	0.261	0.8940
P (mg/dL)	4.77	4.53	4.90	4.46	4.09	4.96	0.108	0.1915
TG (mg/dL)	36.7	46.9	36.9	46.2	48.5	44.6	1.94	0.2965
CHL (mg/dL)	130.1	127.3	128.4	127.3	133.6	116.3	2.43	0.2655
HDL (mg/dL)	81.2	81.2	80.8	77.0	78.0	67.5	2.01	0.1670
LDL (mg/dL)	41.5^{ab}	30.9^{b}	40.1^{ab}	33.1^{b}	51.3^{a}	40.5^{ab}	1.79	0.0091
VLDL (mg/dL)	7.69	9.38	7.37	9.25	9.71	8.92	0.386	0.4109
NEFA (mmol/L)	0.38	0.37	0.35	0.37	0.43	0.42	0.015	0.6490

ALB, albumin; TP, total protein; GLU, glucose; Ca, calcium; P, phosphorus; TG, triglyceride; CHL, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; NEFA, non-esterified fatty acid; Chol, choline; Bet, betaine.
[a–c] Mean values within a row with different superscripts differ significantly ($P<0.05$). Values represent 4 replicates of 2 birds per replicate for each treatment.

Table 7. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on blood parameters in broilers (Trial 2)**

Parameter	Treatments						SEM	P-value
	Control	Control+	140 Chol+ 160 Bet	280 Chol+ 160 Bet	140 Chol+ 320 Bet	280 Chol+ 320 Bet		
ALB (g/dL)	1.48	1.36	1.35	1.47	1.56	1.46	0.023	0.0874
TP (g/dL)	3.55	3.29	3.36	3.50	3.71	3.45	0.050	0.2038
GLU (mg/dL)	196.6	189.5	191.1	200.5	198.6	213.2	3.25	0.2895
Ca (mg/dL)	8.88	8.95	9.33	9.23	9.50	9.49	0.252	0.9693
P (mg/dL)	8.80	4.50	4.65	5.18	4.74	4.69	0.117	0.7148
TG (mg/dL)	40.9	47.1	42.5	43.6	45.9	39.2	1.66	0.7517
CHL (mg/dL)	129.3	126.3	124.0	122.0	131.9	125.2	2.96	0.9595
HDL (mg/dL)	76.3	60.5	69.3	76.1	84.2	73.9	2.29	0.5635
LDL (mg/dL)	44.8	36.4	46.2	37.3	38.5	38.4	2.26	0.7206
VLDL (mg/dL)	8.18	9.42	8.50	8.71	9.17	7.85	0.332	0.7517
NEFA (mmol/L)	0.34	0.39	0.52	0.40	0.53	0.47	0.027	0.2392

ALB, albumin; TP, total protein; GLU, glucose; Ca, calcium; P, phosphorus; TG, triglyceride; CHL, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; NEFA, non-esterified fatty acid; Chol, choline; Bet, betaine.
[a–c] Mean values within a row with different superscripts differ significantly ($P<0.05$). Values represent 4 replicates of 2 birds per replicate for each treatment.

the 320Bet-fed birds ($P<0.05$). Glutathione peroxidase activity increased in the birds fed a Met-deficient diet supplemented with 320 mg/kg Bet ($P<0.05$), but supplementation with 640 mg/kg Bet caused no further elevation in the activity of GPx (Table 8).

In Trial 2, GPx activity increased in the birds fed 140Chol +160Bet and 140Chol+320Bet diets (Table 9). The glutathione antioxidant enzymatic system plays an important role in cellular defense against reactive oxygen species (ROS) (Alirezaei *et al*., 2012). Glutathione peroxidase (GPx) and superoxide dismutase (SOD), which are responsible for the destruction of peroxides, have specific roles in protecting tissues from oxidative damage (Sun *et al*., 2008; Ganesan *et al*., 2011). A reduction in the activity of these enzymes may lead to the formation of O_2- and H_2O_2 when birds experience oxidative stress, which in turn can lead to increased hydroxyl radical formation with harmful consequences (Kalra *et al*., 1988). In the Met-deficient diets, there was increased excretion of nitrogen in the form of uric acid, probably due to an amino acids imbalance. Excess or imbalanced dietary amino acids split into a carbon skeleton and ammonia, which birds convert to uric acid (Namroud *et al*., 2008), a well-known and powerful antioxidant agent (Glantzounis *et al*., 2005; Maiuolo *et al*., 2016). Therefore, the higher total antioxidant status observed in the birds fed a Met-sufficient diet (control+) in Trial 1 can be attributed to the increased

Table 8. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on blood liver enzymes and status of antioxidative factors in broilers (Trial 1)**

Parameter	Treatments						SEM	P-value
	Control-	Control +	280 Chol	560 Chol	320 Bet	640 Bet		
ALP (U/L)	5373	4576	5741	5063	5365	4666	298.8	0.8790
AST (U/L)	222.9	250.7	263.5	258.7	267.9	263.6	8.35	0.5961
ALT (U/L)	10.5	8.9	9.1	11.4	9.6	11.5	0.83	0.9153
TAS (mmol/L)	1.32^{b}	1.70^{a}	1.22^{b}	1.27^{b}	1.48^{ab}	1.30^{b}	0.047	0.0179
SOD (U/g Hb)	100.1	97.7	120.5	102.4	118.2	94.7	4.19	0.3586
GPx (U/g Hb)	42.9^{b}	48.7^{b}	49.0^{b}	49.5^{b}	70.7^{a}	55.2^{ab}	2.56	0.0310
MDA (nmol/mL)	1.86	2.04	2.29	1.89	1.90	1.83	0.072	0.5020
Uric acid (mg/dL)	2.28^{b}	3.71^{a}	1.91^{b}	2.77^{ab}	2.71^{ab}	2.41^{b}	0.151	0.0079
Hb (g/dL)	10.4	11.3	10.6	11.1	11.0	11.2	0.18	0.6186
SAA (μg/mL)	2.63	2.91	3.03	3.03	3.08	2.90	0.083	0.6545
CEP (μg/L)	1486	1461	1470	1510	1389	1384	21.8	0.4953

ALP, alkaline phosphatase; AST, aspartate amino transferase; ALT, alanine amino transferase; TAS, total antioxidant status; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde; Hb, hemoglobin; SAA, serum amyloid A; CEP, ceruloplasmin; Chol, choline; Bet, betaine.

[a–c] Mean values within a row with different superscripts differ significantly ($P<0.05$). Values represent 4 replicates of 2 birds per replicate for each treatment.

Table 9. **Effect of replacing methionine with different levels of betaine and choline (mg/kg) on blood liver enzymes and status of antioxidative factors in broilers (Trial 2)**

Parameter	Treatments						SEM	P-value
	Control-	Control+	140 Chol+ 160 Bet	280 Chol+ 160 Bet	140 Chol+ 320 Bet	280 Chol+ 320 Bet		
ALP (U/L)	5368	4249	4024	5737	5687	5433	306.0	0.4361
AST (U/L)	219.1	242.7	218.4	247.0	234.7	251.1	8.16	0.7898
ALT (U/L)	11.8	8.7	6.0	9.6	10.6	13.1	0.98	0.1970
TAS (mmol/L)	1.48	1.51	1.44	1.71	1.37	1.48	0.053	0.6865
SOD (U/g Hb)	92.2	89.4	118.4	93.3	100.7	79.2	4.58	0.2207
GPx (U/g Hb)	41.9^{b}	43.0^{b}	65.2^{a}	52.5^{ab}	68.3^{a}	47.1^{b}	2.93	0.0119
MDA (nmol/mL)	2.13	2.00	2.26	1.84	1.91	2.01	0.085	0.8069
Uric acid (mg/dL)	2.78	3.21	2.91	3.55	2.75	3.15	0.175	0.8145
Hb (g/dL)	9.2	11.3	13.3	11.0	13.0	11.3	0.57	0.3484
SAA (μg/mL)	2.08	2.33	2.14	2.37	2.25	2.41	0.082	0.8512
CEP (μg/L)	1479	1451	1431	1413	1501	1442	26.6	0.9595

ALP, alkaline phosphatase; AST, aspartate amino transferase; ALT, alanine amino transferase; TAS, total antioxidant status; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde; Hb, hemoglobin; SAA, serum amyloid A; CEP, ceruloplasmin; Chol, choline; Bet, betaine.

[a–c] Mean values within a row with different superscripts differ significantly ($P<0.05$). Values represent 4 replicates of 2 birds per replicate for each treatment.

levels of circulatory uric acid (Table 8). Uric acid, which is the most abundant aqueous antioxidant, accounts for up to 60% of plasma antioxidative capacity in birds. It is a free radical scavenger that stabilizes vitamin C in the serum, mostly owing to its iron-chelating properties, and quenches peroxynitrite, a potentially harmful oxidant, resulting in the formation of a stable nitric oxide (NO) donor *in vitro* (Maiuolo *et al*., 2016).

Our results showed that TAS is not an appropriate indication of antioxidant capacity in birds fed amino acid-deficient or imbalanced diets because of the elevated serum concentration of uric acid, which acts as an antioxidant. Therefore, under such conditions, the activities of antioxidant enzymes such as GPx and SOD should be considered antioxidative indices.

In Trials 1 and 2 (Tables 8 and 9), the serum SAA and CEP concentrations were not affected by the dietary treatments ($P>0.05$), but were slightly higher than the normal ranges reported for chickens (Nazifi *et al*., 2011; O'Reilly and Eckersall, 2014). Heat stress increases the serum concentration of ceruloplasmin in broilers (Lin *et al*., 2006b). Serum amyloid A and CEP are acute-phase proteins, and

their circulatory concentrations are supposed to increase in animals subjected to external or internal challenges such as infection, inflammation, surgical trauma, or stress (Eckersall, 2004).

In conclusion, the present study showed that up to 20% of the dietary Met requirements of broiler chickens exposed to heat stress can be fulfilled by Chol (280Chol and 560Chol) and Bet (320Bet and 140Chol+160Bet), without adversely affecting production performance. The cost of meat production per kg decreased in birds fed the Met-deficient diet supplemented with 280 mg/kg of Chol. Supplementing diets with 280 mg Chol and 640 mg/kg of Bet reduces the serum uric acid concentration, which in turn may reduce its excretion, thereby reducing ammonia emission in poultry houses and nitrogen emission into the environment. However, the latter warrants further investigation.

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