# Physiological and Biochemical Aspects of Methionine Isomers and Precursors in Broilers

by

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## ABSTRACT

Methionine (Met) is an essential amino acid for animals and also the first limiting amino acid in a broiler diet. The dietary supplemental Met sources include the natural isoform L-methionine (L-Met), the synthetic form DL-methionine (DL-Met) and the synthetic Met precursor DL-2-hydroxy-4 (methylthio)-butanoic acid (DL-HMTBA). The objective of this dissertation was to determine the effect of different dietary Met source supplementation and Met deficiency on a series of physiological and biochemical aspects, including growth performance, global DNA methylation and methyltransferase activity, blood antioxidant profile (e.g., acute phase protein, leukocyte count), intestinal nutrient transporter gene expression, Met converting enzyme gene expression and activity, oxidative stress markers, and a potential pathway related to amino acid signaling. To achieve this goal, male Cobb-500 broilers were raised from day of hatch (d0) to d35 post-hatch and fed a diet deficient in methionine and cysteine (Met + Cys) (control) or the same diet supplemented with 0.22% DL-Met, 0.22% L-Met or 0.31% DL-HMTBA (to provide 0.22% DL-Met equivalent) to meet Met + Cys requirements. Tissues (liver, breast muscle, duodenum, jejunum and ileum) and blood samples were collected at various ages from d0 to d35 for analysis. Met supplementation significantly improved body weight gain and feed efficiency compared to the Met deficient group, but no differences were observed among DL-Met, L-Met and DL-HMTBA for growth performance parameters ($P > 0.05$). Met supplementation had no effect on red blood cell packed cell volume, white blood cell differential count, hepatic total

DNA methylation, DNA methyltransferase and Met oxidase activity, and had limited effects on activation of p70S6K, a key amino acid signaling protein ($P > 0.05$). Although dietary Met sources did not change oxidative status of the treated chickens, both L-Met and DL-Met but not DL-HMTBA supplementation decreased the level of acute phase protein serum amyloid A compared to the control group ($P \leq 0.05$). The effect of Met supplementation on gene expression of nutrient transporters and Met converting enzymes were complex and dynamic. Most of the target genes demonstrated tissue- and development-dependent expression patterns, with few significant treatment effects observed. L-Met and DL-Met but not DL-HMTBA supplementation enhanced the neutral amino acid transporters $ATB^{0,+}$ and $B^0AT$ gene expression in various small intestinal segments. All three Met sources increased monocarboxylic acid transporter (MCT1) gene expression in the jejunum. DL-HMTBA and L-Met fed chickens showed greater hepatic L-HMTBA oxidase (HAO1) gene expression. DL-Met increased glutamic-oxaloacetic transaminase 2 (GOT2) gene expression in the duodenum. An in vitro study with tissue explants, however, did not demonstrate a similar gene expression pattern as that in the in vivo study. Lastly, RNA sequencing results illustrated that Met deficiency could lead to many differentially expressed genes but different supplemental Met sources had no influence on hepatic gene expression profiles. In conclusion, as common dietary supplemental Met sources, L-Met, DL-Met and DL-HMTBA exhibited similarity in impacting intestinal amino acid/peptide/monocarboxylic acid transporter gene expression and Met converting enzyme activity. The regulatory roles of Met as an antioxidant and nutrient signaling in cell metabolism were not affected by different dietary supplemental Met sources.

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## ABBREVIATIONS

| | |
|---|---|
| **α-1-AGP** | **alpha-1-acid glycoprotein** |
| **BBMV** | **brush border membrane vesicle** |
| **Cys** | **cysteine** |
| **D-AAOX** | **D-amino acid oxidase** |
| **DEG** | **differentially expressed gene** |
| **D-HADH** | **D-2-hydroxy acid dehydrogenase** |
| **FRAP** | **ferric reducing/antioxidant power** |
| **GIT** | **gastrointestinal tract** |
| **GSH** | **glutathione** |
| **GSSG** | **glutathione disulfide, oxidized form of glutathione** |
| **HMTBA** | **2-hydroxy-4-(methylthio) butanoic acid** |
| **HRP** | **horseradish peroxidase** |
| **KMB** | **2-keto-4 (methylthio) butanoic acid** |
| **LDH** | **lactate dehydrogenase** |
| **L-HAOX** | **L-2-hydroxy acid oxidase** |
| **Met** | **methionine** |
| **MTA** | **5'-deoxy-5'-methylthioadenosine** |
| **mTOR** | **mammalian target of rapamycin** |
| **NLCPAR** | **nonlinear common plateau asymptotic regression model** |
| **RBC-PCV** | **red blood cell packed cell volume** |
| **RBE** | **relative biological efficacy** |

| | |
|---|---|
| **rGSH** | **reduced glutathione** |
| **ROS** | **reactive oxygen species** |
| **SAA** | **serum amyloid A** |
| **SAM** | **S-adenosylmethionine** |
| **TBARS** | **thiobarbituric acid reactive substances** |
| **TGSH** | **total glutathione** |
| **TSAA** | **total dietary sulfur-containing amino acids** |
| **WBC** | **white blood cell** |

# CHAPTER 1 Introduction

Methionine (Met) is a dietary indispensable amino acid because it cannot be synthesized in sufficient amounts to sustain normal growth in mammals and avians. As a result, supplemental Met sources are produced for cost efficiency in the animal nutrition industry, especially for a poultry diet, in which Met is usually the first limiting amino acid. L-methionine (L-Met) is the natural form of Met that can be directly used by animals, while the isoform mixture DL-methionine (DL-Met) and the methionine precursor DL-2-hydroxy-4 (methylthio)-butanoic acid (DL-HMTBA) are two commonly used supplemental Met sources in the poultry industry. DL-Met and DL-HMTBA are mainly absorbed in the small intestine through different mechanisms, and conversion to L-Met is the first step before both of them can be utilized by animals (Figure 1.1).

The literature review in this dissertation will focus on the comparison between these three dietary supplemental Met sources (L-Met, DL-Met and DL-HMTBA) in different species (poultry, swine and ruminants). The different uptake mechanisms of these Met sources, the conversion of D-Met and DL-HMTBA to L-Met, and the relative efficacy especially for DL-Met and DL-HMTBA are introduced in detail, particularly emphasizing on chickens. The knowledge of intestinal amino acid transporters and antioxidant potential of HMTBA will also be covered in this chapter. All of these will provide background support for the remainder of the dissertation.

The major roles that amino acids including Met play in metabolism and cell function regulation are: 1) precursors for protein synthesis, 2) antioxidant function, and 3) nutrient signaling to affect intracellular kinases (Figure 1.1). All of these functions can be influenced by nutritional factors such as dietary supplemental Met sources.

Transmethylation and transsulfuration are two major pathways for Met metabolism (Figure 1.2). The major methyl donor S-adenosylmethionine (SAM) is an intermediate in the transmethylation cycle. Thus Met is associated with DNA methylation and epigenetic regulation of gene expression (Figure 1.2). In addition, the reactive oxygen species (ROS) scavengers cysteine and glutathione are products in the transsulfuration pathway, so Met is also related to oxidative status in animals (Figure 1.2). Therefore, this dissertation will assess the effect of different dietary supplemental Met sources on a series of physiological and biochemical aspects, which include basic growth performance parameters, hepatic DNA methylation and methyltransferase activity, intestinal nutrient transporter gene expression, Met converting enzyme activities, oxidative stress markers and blood antioxidant profiles (e.g., acute phase protein, white blood cell distribution pattern), and potential pathways related to amino acid signaling. In addition to the in vivo work, an in vitro study on nutrient transporters and Met converting enzymes as well as an RNA sequencing assay will be covered in this dissertation.

In recent years, a plethora of research of studies has focused on metabolic mechanisms of sulfur amino acids using mammals as a model, with the aim to improve our understanding of human nutrition and health. In poultry, the relative efficacy between different supplemental Met sources has been a major topic of Met research for the past 50 years. Now more research has focused on the molecular aspects of sulfur amino acid research in livestock species and poultry. Our lab has much experience working on molecular nutrition especially nutrient transporters in poultry. Understanding the effects and corresponding mechanisms of different dietary supplemental Met sources in poultry

will greatly benefit the animal nutrition industry, and also may promote the development of biomedical research on humans.

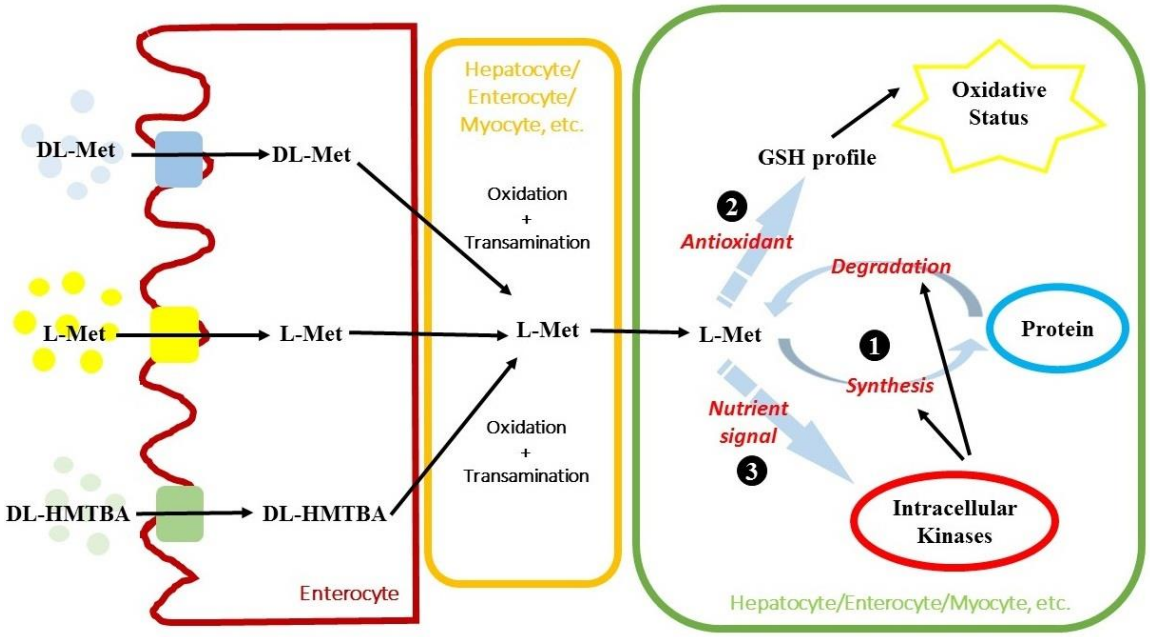


**Figure 1.1** Uptake, metabolism and regulation function in cells of different dietary supplemental methionine sources. DL-Met: DL-methionine; L-Met: L-methionine; DL-HMTBA: DL-2-hydroxy-4 (methylthio) butanoic acid; GSH: glutathione.

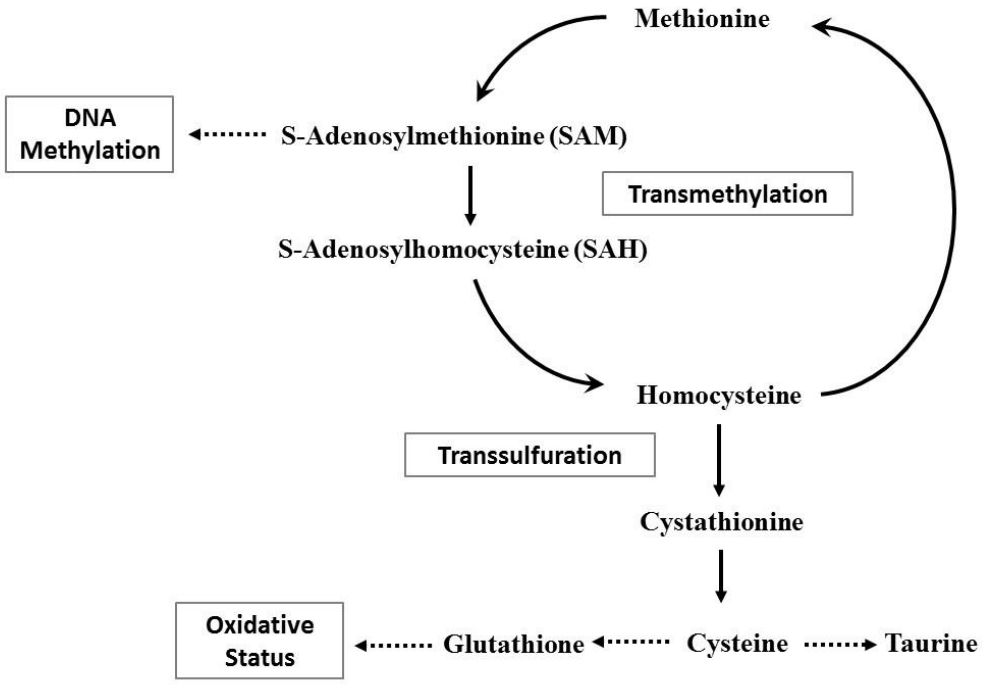


**Figure 1.2** Methionine metabolism through transmethylation cycle and transsulfuration pathway.

# CHAPTER 2 Literature Review

## 2.1 Introduction

In animal diets especially poultry diets, methionine (Met) is considered to be the first limiting amino acid. The dietary supplemental Met sources include L-methionine (L-Met), and its synthetic forms DL-methionine (DL-Met) and DL-2-hydroxy-4 (methylthio) butanoic acid (DL-HMTBA) (Figure 2.1).

As the metabolically active isomer of Met, L-Met can be used as a precursor for protein synthesis, incorporated into the transmethylation pathway of the Met cycle to produce homocysteine, or metabolized through the transsulfuration pathway to produce cysteine and glutathione (Figure 1.2) (Fang et al., 2010b). The gastrointestinal tract (GIT) is the major site for the transmethylation and transsulfuration of dietary Met. The net homocysteine released can induce the formation of reactive oxygen species (ROS) in the mitochondria, contribute to the oxidative stress of the intestinal mucosa, cause an inflammatory response and disrupt the epithelial barrier function in the GIT (Burrin and Stoll, 2007). Thus dietary Met is highly associated with the function and health of the GIT for livestock and humans. Studies also showed that L-Met is an intermediate in the biosynthesis of other important molecules such as carnitine and taurine, and can be converted to S-adenosylmethionine (SAM), which is the major methyl donor in cells, and thus indirectly participates in the epigenetic regulation of gene expression (Obeid, 2013). After absorption in the GIT, biological utilization of both D-Met and DL-HMTBA relies on their conversion to L-Met.

Today L-Met is produced on an industrial-scale by enzymatic reactions using commercial DL-Met as substrates, since a fermentation method is not yet available. DL-Met is exclusively manufactured by chemical synthesis with alkaline hydrolysis as the key step, and sold as powder. In the manufacturing process, a racemic mixture of D- and L-Met is synthesized (Karau and Grayson, 2014). The Met analogue, DL-HMTBA, is available as an 88% liquid concentrate in water as a free acid or as an 84% preparation of the calcium salt (Saunderson, 1985). DL-HMTBA lacks an amino group and thus is not a true amino acid but an amino acid precursor.

There are different absorption, transport and metabolism mechanisms for utilization of different Met sources. There has been a continuing controversy centered on the absorption and bio-efficacy of DL-Met and DL-HMTBA in the past few decades. This review summarizes studies about different absorption, transport and metabolism characteristics of L-Met, DL-Met and DL-HMTBA in several animal models (poultry, swine, and ruminants), and their bio-efficacy in growth and development with a focus on poultry and pigs. The physiology of amino acid and peptide transport and the antioxidant potential of DL-HMTBA will also be reviewed. Understanding the characteristics of different Met sources will greatly benefit the animal nutrition industry as well as bioscience research.

**2.2 Amino acid and peptide absorption and transport in the small intestine**

Small intestine (duodenum, jejunum and ileum) is the major site for nutrient absorption. Dietary protein is digested in the GIT, and the resulting small peptides and amino acids are effectively absorbed into enterocytes via different brushborder membrane transporters, depending on the size and the electrical property of the amino acids (Smith

and Morton, 2010). Most amino acids that enter the enterocyte are then transported out of the cell into the blood stream via various basolateral membrane transporters, except those retained for utilization by the enterocyte (Smith and Morton, 2010). Met is a neutral amino acid, thus the neutral amino acid transporters that have been reported to be associated with Met transport in mammals and avians are summarized in this part (Table 2.1).

Solute carriers (SLCs) comprise the largest family of membrane transport proteins in mammals and avians. Phylogenetic studies have identified at least 384 unique protein sequences forming 52 distinct SLC families (Rask-Andersen et al., 2013). SLCs regulate the transport of several substrates such as inorganic ions, nucleotides, amino acids, neurotransmitters, sugars and fatty acids across biological membranes (Rask-Andersen et al., 2013). SLCs are encoded by a large number of genes.

**The neutral amino acid transporters SAT1, SAT2 and SAT3.** SAT1 (SLC38A1), SAT2 (SLC38A2) and SAT3 (SLC38A3) are co-transporters for $Na^+$ with neutral amino acid uptake driven by the inward directed $Na^+$ gradient across the basolateral membrane of the enterocytes. They have a broad substrate specificity in general, with preference for L-methionine, L-proline, L-serine, but also high affinity for L-asparagine, L-glycine, L-histidine and L-glutamine (Schioth et al., 2013). SAT1 and SAT3 are expressed greater in the nervous system, while SAT2 is ubiquitously expressed in most cell types. Pinilla et al. (2011) suggested that SAT2 plays a role in cell growth and differentiation by signaling through the mTOR (mammalian target of rapamycin) pathway, with a potential function as a transceptor. SAT1 and SAT2 were observed to be highly up-regulated when cells were grown in the absence of essential amino acids,

which is a possible regulatory mechanism utilized by tumor cells under nutritional stress (Jones et al., 2006).

**The neutral amino acid transporter $B^0AT$.** $B^0AT$ (SLC6A19) is the major apical neutral amino acid transporter in kidney and intestine in mouse (Broer, 2008). It is a $Na^+$-dependent transporter, with mRNA localizing to enterocytes with a clear gradient along the villi, being low in the crypts and stronger toward the tips in the small intestine of the mouse (Romeo et al., 2006). $B^0AT$ transports all neutral amino acids, but has more affinity for methionine, leucine, isoleucine and valine (Broer, 2008).

**The neutral and cationic amino acid transporter $ATB^{0,+}$.** $ATB^{0,+}$ (SLC6A14) is a $Na^+$ and $Cl^-$-dependent neutral and cationic amino acid transporter with greatest affinity for large hydrophobic amino acids. It can transport all essential amino acids but not proline, glutamate and aspartate, and is not expressed in kidney (Broer, 2008). A defective $ATB^{0,+}$ transporter could result in embryo undernutrition causing obesity later in human life (Van Winkle et al., 2006). The $ATB^{0,+}$ protein is also a novel and effective drug target for the treatment of estrogen receptor-positive breast cancer (Karunakaran et al., 2011).

**The $b^{0,+}AT$ and rBAT transporter complex.** The heteromeric transporters $b^{0,+}AT$ (SLC7A9) and rBAT (SLC3A1) are the major transporters for cationic amino acids and cysteine in the apical membrane of kidney and intestine in mouse (Broer, 2008). Because of their molecular mass, rBAT is called the heavy chain and $b^{0,+}AT$ the light chain, linked by a disulfide bridge. The rBAT protein alone appears to be relatively unstable and is rapidly degraded; while co-expression of $b^{0,+}AT$ stabilizes rBAT. Physiologically, rBAT/$b^{0,+}AT$ transport neutral amino acids out of the cell into the lumen

in exchange for cationic amino acids and cysteine uptake (Broer, 2008). According to Dave et al. (2004), this complex localized the strongest in ileum, was very weak in duodenum and showed a similar gradient of expression along the villi in the mouse.

**The 4F2hc and system L and $y^+$L transporters.** 4F2hc (SLC3A2) is the heavy chain for system L transporters LAT1 (SLC7A5), LAT2 (SLC7A8) and system $y^+$L transporters $y^+$LAT1 (SLC7A7), $y^+$LAT2 (SLC7A6). The heteromeric transporters 4F2hc/LAT1 and 4F2hc/LAT2 mediate the $Na^+$-independent obligatory exchange of neutral amino acids. 4F2hc/LAT1 has high affinity for large branched chain amino acids and aromatic amino acids, with the affinity being up to 100-fold higher for extracellular amino acids than for intracellular amino acids, especially for L-methionine, L-leucine and L-isoleucine. In contrast, 4F2hc/LAT2 has broad substrate selectivity for all neutral amino acids, including the small ones (Fotiadis et al., 2013). LAT2 also displays lower intracellular affinities. LAT1 and LAT2 can provide neutral branched chain amino acids to activate the mTOR pathway (Fotiadis et al., 2013). Both 4F2hc/$y^+$LAT1 and 4F2hc/$y^+$LAT2 are obligatory exchangers of cationic amino acids ($Na^+$-independent) and neutral amino acids ($Na^+$-dependent), rendering the transporter electroneutral, but 4F2hc/$y^+$LAT2 preferentially mediates the efflux of L-arginine in exchange for L-glutamine (Fotiadis et al., 2013). Under physiological conditions, system $y^+$L transporters would maintain a lower cationic amino acid gradient and a higher neutral amino acid gradient in the cytosol (Broer, 2008). LAT1 is expressed essentially in brain, with very little expression in the intestine. Dave et al. (2004) reported that 4F2hc and LAT2 colocalized completely in all segments of the small intestine and along the length of the

villi, with stronger signals towards the tip of the villi, and $y^+$LAT1 followed a similar distribution pattern as LAT2.

**The di- and tripeptide transporter PepT1.** The peptide transporter PepT1 (SLC15A1) is a proton-dependent, low-affinity, high-capacity transporter. It is primarily expressed in the apical membrane of intestinal and renal epithelial cells. In enterocytes, it is restricted between the crypt-villus junction and villus tip, increasing towards the tip (Gilbert et al., 2008).

## 2.3 Utilization of different dietary supplemental methionine sources in poultry

### 2.3.1 Absorption and transport of dietary L-Met, DL-Met and DL-HMTBA

Since HMTBA does not enter the lumen of the GIT in the form of an amino acid, it is likely absorbed differently from D-Met and L-Met. Knight and Dibner (1984) used both the in vitro intestinal slices model and the in vivo ligated intestinal segments plus crop intubation model to explore the absorption of radioactive HMTBA and L-Met in 8 to 16 day-old chickens. Intestinal uptake of L-Met was inhibited by the electron transport uncoupler 2, 4-dinitrophenol and its uptake conformed to Michaelis-Menten kinetics; whereas, the in vitro uptake of HMTBA was linear relative to concentration. These results led to the conclusion that L-Met absorption was a concentration and energy dependent process while DL-HMTBA absorption was only concentration dependent. The in vivo experiment also illustrated that the absorption rate of the two Met sources varied in different small intestinal locations. L-Met was absorbed more quickly in the ileum, while DL-HMTBA was absorbed more rapidly in the proximal loop of the duodenum and mid-jejunum. Although there is different absorption mechanisms, the two compounds

were absorbed at similar rates, especially at low supplemental concentrations (Dibner and Knight, 1984).

Using the in vitro intestinal slices model, Brachet and Puigserver (1989) reported that HMTBA is absorbed by a combination of diffusion and carrier-mediated uptake. The diffusion plays a major role in HMTBA uptake, particularly at high substrate concentration, which is consistent with the chemical structure of HMTBA as an organic acid (Brachet and Puigserver, 1987; Dibner and Buttin, 2002). At a lower concentration, carrier-mediated transport is more important (Brachet and Puigserver, 1987).

Richards et al. (2005) demonstrated that HMTBA was absorbed along the entire GIT (from crop to cloaca) in chickens, primarily in the proximal GIT prior to the small intestine. They also showed that the uptake rate of DL-HMTBA was equal to or greater than DL-Met using the in vitro intestinal slices model (Richards et al., 2005). However, compared to L-Met, more DL-HMTBA was broken down to non-methionine products and remained unabsorbed during passage down the small intestine in chickens (Maenz and Engele-Schaan, 1996a).

Another popular in vitro model to study nutrient transport is the brush border membrane vesicle (BBMV) model. This model is inappropriate to estimate the HMTBA transport rate since it minimizes uptake by diffusion, which accounts for a large proportion of HMTBA uptake in vivo (Richards et al., 2005). In contrast, the intestinal slices model is more efficient at measuring the uptake rate of HMTBA. However, the BBMV model has proven to be useful to study the carrier-mediated uptake by specific transporters on the intestinal brush border membrane.

Using the BBMV model, Brachet and Puigserver (1989) reported that the transport of both L-HMTBA and D-HMTBA were $Na^+$ independent and electroneutral. L-lactate, a structural analogue, could act as a competitor to L-HMTBA transport, indicating that DL-HMTBA can be taken up by the lactate carrier, which belongs to the proton-linked monocarboxylate transporter family (Brachet and Puigserver, 1989). Maenz and Engele-Schaan (1996b) confirmed this conclusion by showing that DL-HMTBA was absorbed by a $H^+$-dependent, non-stereospecific, monocarboxylic acid transport system.

Similar to the other amino acids, DL-Met is transported primarily by active transport and also by carrier-mediated transport (Brandsch and Brandsch, 2003). Both mechanisms require transporters; while active transport needs ATP hydrolysis to provide energy. Since both D-Met and L-Met can compete for many of the same transporters, and these transporters have a much higher affinity for L-Met than D-Met, the uptake rate of DL-Met is usually slower than L-Met (Richards et al., 2005). Several transport systems were reported to transport DL-Met in chicken jejunum: the $Na^+$-independent $b^{o,+}$ and system L, and the $Na^+$-dependent system $y^+$, system B and system A (Soriano-Garcia et al., 1998). Some of the specific transporters reported to transport Met include $b^{0,+}$AT, LAT1/LAT2, $y^+$LAT1/$y^+$LAT2, $ATB^{0,+}$ and SAT1/SAT2 (Hyde et al., 2003; Broer, 2008) (Table 2.1). The transport system for DL-HMTBA is the monocarboxylate transporter 1 (MCT1) (Martin-Venegas et al., 2007; 2008). MCT1 (SLC16A1) belongs to the SLC16 family and takes lactate, pyruvate and ketone bodies as predominant substrates, with ubiquitous distribution (Halestrap, 2013). Because the transporter MCT1 is $H^+$-dependent, it is coupled with the $Na^+/H^+$ exchanger (NHE3) to maintain the proton

gradient at the brush border membrane of the enterocyte in mammals, according to the study using human Caco-2 cells (Martin-Venegas et al., 2007). No related results have been reported for an in vitro chicken model.

### 2.3.2 Conversion and metabolism of dietary L-Met, DL-Met and DL-HMTBA

For DL-Met, D-Met must be converted to L-Met following transport into the enterocyte. Firstly, D-Met is oxidatively deaminated to the α-keto analogue of L-Met, 2-keto-4 (methylthio) butanoic acid (KMB), by the enzyme D-amino acid oxidase (D-AAOX; EC 1.4.3.3), a peroxisomal oxidase containing flavin adenine dinucleotide (FAD) as a cofactor (Brachet and Puigserver, 1992). Then KMB is converted to L-Met by the transfer of nitrogen from donor amino acids catalyzed by the ubiquitous transaminases. Even though not yet definitively demonstrated, transaminase is not thought to be a limiting step in the conversion process.

L-Met can be directly used to synthesize SAM or can be degraded through pathways such as transamination, or utilized by the bacteria in the lumen of the small intestine (Muramatsu et al., 1987). SAM can undergo the transmethylation pathway to synthesize homocysteine or the transsulfuration pathway, with the products cysteine, glutathione, taurine, etc (Figure 2.2). In skeletal muscle, Met can regulate the ribosomal protein S6 kinase (S6K1) pathway and its translational targets, i.e., ribosomal protein S6 (S6) and eukaryotic elongation factor 2 (eEF2), and regulate protein accretion and synthesis (Barnes et al., 1995). In quail muscle QM7 myoblasts, S6K1 signaling was shut down when incubated in Met-free medium, which was due to uncharged tRNA as well as Met itself. Re-activation of S6K1 can be induced by both L-Met and KMB but not DL-HMTBA or D-Met (Metayer-Coustard et al., 2010).

DL-HMTBA also needs to be transformed to L-Met for utilization. The stereospecific pathway of DL-HMTBA conversion to L-Met was demonstrated by Dibner and Knight using chicken liver homogenates (Dibner and Knight, 1984). The first step is oxidation of the α-hydroxyl group yielding the keto intermediate KMB, catalyzed by two enzyme systems according to the substrate isomers (Figure 2.1). L-HMTBA is the substrate of L-2-hydroxy acid oxidase (L-HAOX; EC 1.1.3.15), a hydrogen peroxide ($H_2O_2$)-producing flavoenzyme found in peroxisomes of liver and kidney in chickens. There are two isoforms of L-HAOX, HAO1 and HAO2. HAO1 is rich in liver and preferentially oxidizes short-chain aliphatic 2-hydroxyacids; while HAO2 is abundant in kidney and catalyzes the oxidation of long-chain aliphatic or aromatic 2-hydroxyacids (Ferjancic-Biagini et al., 1998). Thus HAO1 is assumed to be involved mainly in L-HMTBA oxidation. D-HMTBA is oxidized through an entirely different mechanism, catalyzed by the mitochondrial D-2-hydroxy acid dehydrogenase (D-HADH; EC 1.1.99.6), producing $H_2O_2$ as a byproduct. Because D-HADH is located in mitochondria, D-HMTBA could be used by any organ for protein synthesis, including small intestine and skeletal muscle (Dupuis et al., 1990). FAD and flavin mononucleotide (FMN) are common cofactors for both L-HAOX and D-HADH. L-HAOX also has oxidative decarboxylation activity in vitro, using nicotinamide adenine dinucleotide (NADH) as a cofactor (Ferjancic-Biagini et al., 1995).

In both cases the resulting keto intermediate KMB is transaminated to L-Met by transaminase, the second step in DL-HMTBA metabolism (Figure 2.1). In rat liver, KMB is transaminated by the transaminase using glutamine or asparagine as substrates to produce L-Met (Backlund et al., 1982). In rat skeletal muscle, the branched-chain amino

acids play an important role in transamination (Wu and Thompson, 1989). However, in chicken the physiology is more complicated. Kidney is the most active tissue for conversion of KMB to L-Met, the liver and small intestinal mucosa are intermediate, and skeletal muscle has the lowest activity. All amino acids can serve as substrates for transamination of KMB in chicken. Branched-chain amino acids, glutamic acid and asparagine are more effective substrates in tissues other than the small intestinal mucosa. In mitochondria, the preferred substrates are glutamate in liver mitochondria, isoleucine and alanine in kidney mitochondria and branched-chain amino acids and glutamic acid in skeletal muscle mitochondria (Rangel-Lugo and Austic, 1998). In human Caco-2 cells, the branched-chain amino acid leucine is the preferred amino acid group donor (Martin-Venegas et al., 2011).

Conversion of DL-HMTBA to L-Met occurs principally in liver, because both L-HAOX and D-HADH are highly expressed in liver. Both isomers of DL-HMTBA can be used as Met sources at similar rates for hepatocyte protein synthesis and are biochemically equivalent to DL-Met in chickens (Dibner, 1983). Wang et al. (2001) did not observe any sign of toxicity from infusion of pharmacological levels of DL-HMTBA directly into the broiler hepatic portal vein while feeding a diet containing normal supplemental levels of DL-HMTBA, indicating that liver is a major site for DL-HMTBA removal from circulation and metabolism in chickens. At super physiological concentrations, DL-HMTBA is oxidized principally in skeletal muscle (Dupuis et al., 1989). However, because growing broilers have more than enough biochemical capacity for DL-Met and DL-HMTBA conversion, no accumulation of dietary Met sources might occur (Dibner and Ivey, 1992). DL-HMTBA transformation also takes place in the small

intestine, following the steps of oxidation and transamination (Martin-Venegas et al., 2006; 2011) . The dietary DL-HMTBA supplementation can up-regulate the intestinal oxidation, but the transamination step is not affected by DL-HMTBA availability (Martin-Venegas et al., 2011). Under fasting or Met deficient conditions, brain and liver showed enhanced rates of DL-HMTBA and DL-Met conversion (Saunderson, 1987). In non-ruminants, oxidation is the major pathway for DL-HMTBA catabolism. In ruminants, DL-HMTBA can also be degraded directly by microorganisms.

In addition to access from diet, HMTBA is also a naturally occurring compound in chickens through a salvage pathway to regenerate L-Met from 5'-deoxy-5'-methylthioadenosine (MTA), without KMB production (Backlund et al., 1982; Dibner et al., 1990; Dibner and Ivey, 1990). Even though the naturally occurring HMTBA may not be an obligatory intermediate in the cytosolic MTA salvage pathway, it can still be used as an L-Met precursor in chicken liver (Dibner et al., 1990).

### 2.3.3 The bio-efficacy of L-Met, DL-Met and DL-HMTBA in poultry

The DL-HMTBA and DL-Met have been commercially available and used in animal production systems for over 50 years. Both liquid form of DL-HMTBA (Alimet) and dry/calcium salt form of DL-HMTBA (MHA) were proved nutritionally effective in broiler chickens (Bishop and Halloran, 1977; Waldroup et al., 1981). Many studies have already shown that there are no significant differences between those two commercial Met sources for body weight gain and feed conversion ratio in broiler chickens fed traditional corn-soybean meal (Elkin and Hester, 1983; Garlich, 1985; Liu et al., 2007; Zou et al., 2015). However, the relative biological efficacy (RBE) of DL-HMTBA and DL-Met remains a controversy. Although initial studies showed that these two

commercial Met sources provided similar quantities of Met activity (Bird, 1952), it was shown later that DL-HMTBA is a little less effective than L-Met or DL-Met in purified diets at total dietary sulfur-containing amino acid (TSAA) deficient levels (Smith, 1966; Katz and Baker, 1975), due to rate limiting DL-HMTBA metabolism (Saunderson, 1985).

To estimate the RBE, a dose-response trial is commonly used and DL-HMTBA must function at a dilution of DL-Met (e.g., 65% DL-Met) with the same form of dose-response and same plateau. The conflicting conclusions drawn from different studies could be related to the statistical models used (Agostini et al., 2016). The slope-ratio and parallel line assays (linear response) were used in earlier studies. Littell et al. (1997) proposed the nonlinear common plateau asymptotic regression model (NLCPAR) and fitted to the treatment means of body weight gain and feed conversion ratio. Rate parameter estimates were obtained from the NLCPAR models for each product and the ratio of these parameter estimates was then interpreted as the RBE of the test product relative to the standard for the entire dose range. Following this model, Lemme et al. (2002) reported 72% weight gain, 51% feed conversion, 48% carcass yield and 60% breast muscle yield (an average of 62%) of liquid DL-HMTBA compared to DL-Met using an exponential model.

However, Kratzer and Littell (2006) questioned this approach with a common plateau since the two products do not fit the same dose response profile due to their different absorption and metabolic pathways. Vazquez-Anon et al. (2006) imposed linear, quadratic and exponential equations to body weight gain and selected the best model (with best goodness of fit) to estimate the gain responses after feeding different doses of DL-HMTBA vs. DL-Met to male chickens for 6-7 weeks. They concluded that DL-

HMTBA outperformed those for DL-Met at TSAA levels near the maximum gain response (commercial levels), while DL-Met outperformed DL-HMTBA at TSAA deficient levels (Vazquez-Anon et al., 2006). Agostini et al. (2016) further showed the above conclusions over a practical range of doses using 0 to 28 day-old broiler chickens, taking TSAA into account. They showed that DL-HMTBA outperformed DL-Met above requirement levels, whereas the opposite was observed below the TSAA requirement, but the latter was only manifest in females. Similar results were observed in turkeys, with feeding supplemental DL-HMTBA to young male turkeys for 21 days leading to lower growth compared with DL-Met at TSAA deficient levels (Gonzales-Esquerra et al., 2007). This understanding leads to a statistical approach in which predictions are developed for each Met source independently, and predicted differences are determined along the dose response. Roughly speaking, if a sufficiently wide range of concentrations are fed, a quadratic equation can fit the data. If supplemental concentrations are low relative to peak response, a linear equation may fit the data better. Alternatively, if supplemental concentrations are approaching peak response but are insufficient to create a decline in performance, exponential equations may provide a better goodness of fit (Kratzer and Littell, 2006; Gonzales-Esquerra et al., 2007).

Even though, Hoehler (2006) argued that Kratzer and Littell (2006) who defined RBE as the ratio of induced responses at a given supplementation level misunderstood the original concepts of RBE. Littell et al. (1997) proposed RBE based on the ratio of supplementation levels at a given response level. Hoehler (2006) insisted that there was no problem to use the NLCPAR model. Guided by this thought, Elwert et al. (2008) assessed the RBE of calcium salt of DL-HMTBA in comparison to DL-Met using the

NLCPAR model in both male Ross 308 and Cobb 500 chickens fed a TSAA deficient diet for 38 days. Their results suggested an average of 63% RBE (66% body weight gain, 64% feed efficiency and 54% breast muscle meat yield) of calcium salt of DL-HMTBA in relation to DL-Met (Elwert et al., 2008). Sauer et al. (2008) used meta-analysis including data out of 40 experiments from peer reviewed publications with the NLCPAR model to illustrate 79% and 87% RBE of DL-HMTBA over DL-Met for the average daily gain and gain to feed ratio, respectively. Vedenov and Pesti (2010) applied an economic analysis to these meta-analyses and reported relative economic values (cost ratio, DL-HMTBA: DL-Met) between 81%-86%, depending on the value of a broiler and the cost of feed and DL-Met. However, the authors concluded that these results were questionable because the profit-maximizing levels of DL-Met and DL-HMTBA in this trial were so far above the levels studied in most of the trials (Vedenov and Pesti, 2010).

In addition to the effects on regular growth performance parameters, e.g., body weight gain, feed conversion ratio and breast muscle yield, DL-HMTBA and DL-Met may also differ in effects on fat deposition. Esteve-Garcia and Llaurado (1997) showed that DL-HMTBA supplementation resulted in greater abdominal fat deposition at 41 days for male broilers deficient in TSAA, even if live body weight was the same.

The RBE of DL-Met and L-Met was not different in both purified and practical-type low-protein diets of varying TSAA contents fed to chickens from 8 to 20 days (Dilger and Baker, 2007). Furthermore, the addition of 0.2% L-cysteine in a TSAA deficient diet improved body weight gain efficiency but was associated with anorexic behavior in the chickens, which was thought to be due to a unique nutritional imbalance. The improvement in gain to feed ratio mediated through reduced feed intake is a rare

event in nutritional studies (Dilger and Baker, 2007). More research is needed to provide a physiological basis for this phenomenon.

## 2.4 Utilization of different dietary supplemental methionine sources in other livestock species

### 2.4.1. Utilization of dietary L-Met, DL-Met and DL-HMTBA in swine

Methionine is considered to be one of the most limiting amino acids for swine especially for piglets. Studies on dietary Met metabolism and systemic homocysteine regulation using swine as models may benefit the clinical treatment of cardiovascular disease and stroke (Bauchart-Thevret et al., 2009a).

Some early studies showed that DL-HMTBA and DL-Met provided equimolar levels of Met activity in early-weaned pigs (Knight et al., 1998). Recently, Jendza et al. (2011) indicated that when cost per mole of Met activity is not different, DL-HMTBA is a better choice in low-fiber pig diets due to increased apparent ileal digestibility of acid and neutral detergent fiber and several other amino acids. DL-Met, however, is preferred in high-fiber pig diets considering the negative interaction between DL-HMTBA and wheat middlings on digestibility of other amino acids.

The conversion of DL-HMTBA and D-Met to L-Met in swine follows the same pathways as those in chickens, and is also tissue-specific. Liver and kidney are the major sites for DL-HMTBA conversion, with the highest L-HAOX and D-HADH activity and mRNA abundance. The stomach can also convert DL-HMTBA to L-Met, while the small intestine, with higher D-AAOX expression, contains a relatively higher capacity to convert D-Met than to convert DL-HMTBA (Fang et al., 2010a). However, liver and GIT are still the major organs involved in dietary Met utilization in piglets. Both dietary Met

status and sources can affect the utilization process. With sufficient Met supply, the GIT metabolizes 20% of dietary Met intake by transmethylating it to homocysteine and then transsulfurating to cysteine in neonatal pigs. In contrast, TSAA deficiency coordinates Met metabolism, such that protein synthesis is preserved over Met transmethylation and the Met pool is preserved by up-regulation of homocysteine re-methylation and suppression of transsulfuration (Bauchart-Thevret et al., 2009b). Supplementation of dietary DL-HMTBA, which is all absorbed by the end of the duodenum in pigs (Jendza et al., 2011), increased circulating plasma taurine concentrations compared to DL-Met supplementation, indicating the greater potential of DL-HMTBA over DL-Met to promote the transsulfuration of dietary Met (Fang et al., 2010a). Besides, dietary DL-HMTBA supplementation may also up-regulate portal blood flow and net portal absorption of amino acids in piglets (Fang et al., 2009). All of these may imply a beneficial nutritional effect of DL-HMTBA on swine growth.

### 2.4.2 Utilization of dietary DL-HMTBA in ruminants

The absorption and metabolism of DL-HMTBA in ruminants are different from that in non-ruminants. There are several destinations in the GIT for the dietary DL-HMTBA after oral administration. First, DL-HMTBA can be directly absorbed, mainly through the ruminal, omasal or abomasal epithelia. McCollum et al. (2000) clearly indicated that the former two sites account for at least a portion of the absorption of DL-HMTBA in sheep. The absorption amount is determined by several factors such as the retention time (the inverse of liquid outflow rate) within the rumen (Lobley et al., 2006b). Second, the forestomach tissues may convert DL-HMTBA to L-Met during the absorption process (Lobley et al., 2006a). This would increase the net availability of Met

for absorption or support tissue protein synthesis (Lobley et al., 2006b). But it is still unknown whether this conversion within the digestive tract is dose dependent or represents a fixed value linked to the enzyme capacity within the tissues. Third, microbes in the rumen may degrade DL-HMTBA and the products would continue to form during passage of fluid between the rumen and abomasum.

Lobley et al. (2006b) pointed out that the majority of dietary DL-HMTBA has been either oxidized or converted to other products, only less than 30% of the oral dose being absorbed as DL-HMTBA. The DL-HMTBA converting enzymes were found in ruminal and omasal epithelia, liver and kidney in sheep, demonstrating the possible metabolic sites of DL-HMTBA in ruminants (McCollum et al., 2000). Actually, the non-hepatic tissues act as major sites for L-Met synthesis from DL-HMTBA (Lobley et al., 2006b; Lapierre et al., 2011). Approximately 65%-75% of absorbed DL-HMTBA passed beyond the liver for subsequent metabolism by peripheral tissues in lambs (Wester et al., 2006). Most of the synthesized L-Met is preferably retained to support tissue protein synthesis with little returned to the plasma. As a consequence, only a small increase in plasma Met was observed even when enough dietary DL-HMTBA was supplemented. This would yield an energy savings through using passive diffusion of DL-HMTBA rather than active transport of Met (McCollum et al., 2000). This also indicates that the plasma Met concentrations cannot accurately predict Met availability in ruminants (Lobley et al., 2006a).

In ruminants, the hepatic DL-HMTBA can undergo similar fates as in non-ruminants, such as catabolism (oxidation), conversion to L-Met and export or use by cells, or conversion to other metabolic products such as cysteine and glutathione through

the transsulfuration pathway (Wester et al., 2006). To ensure maintenance of aminoacidemia and prevent Met toxicity, the liver plays a key role in removal of extra Met (Lapierre et al., 2011). There also appears to be preferential use of D-HMTBA by ruminant tissue, with D-HADH more active (by 45% to 75%) than L-HAOX in ovine omasum, rumen and kidney but not in liver (Lobley et al., 2006a).

In lactating dairy cows, dietary DL-HMTBA supplementation has been proposed as a means to increase milk protein yield, considering that it is more resistant to rumen microbial degradation than DL-Met (Noftsger et al., 2005). Approximately 15% of Met incorporated into milk protein originated from direct conversion of DL-HMTBA to L-Met, and the remaining 85% was provided indirectly, where L-Met synthesized from DL-HMTBA within peripheral tissues was used to support intracellular protein synthesis, allowing L-Met released from protein breakdown to be exported for use by the mammary gland (Lapierre et al., 2011).

**2.5 Antioxidant potential of DL-HMTBA**

It is now well established that DL-HMTBA is a safe and efficacious precursor of Met widely used in animal diets (Dilger et al., 2007). Furthermore, DL-HMTBA has potential antioxidant effects. Martin-Venegas et al. (2013) observed higher taurine and cysteine levels in chicken enterocytes using DL-HMTBA as a dietary supplemental Met source compared to DL-Met, indicating that DL-HMTBA is preferentially metabolized through the transsulfuration pathway. Thus DL-HMTBA can theoretically reduce the net homocysteine level produced by Met metabolism via the transmethylation pathway and protect the intestinal epithelial barrier function.

To illustrate the potential antioxidant effect of DL-HMTBA, Willemsen et al. (2011) compared the effects of dietary DL-HMTBA or DL-Met supplementation on broiler chickens exposed to heat stress (32°C-33°C). High temperature exposure can impair growth performance and induce oxidative stress in poultry. However, the negative effects on reduced body weight gain and feed intake were less pronounced with DL-HMTBA supplementation. Also, at 6 week of age, both the plasma thiobarbituric acid reactive substances (TBARS) level resulting from lipid peroxidation and the hepatic ratio of oxidized glutathione (GSSG) to reduced GSH (rGSH) were significantly lower in DL-HMTBA supplemented chickens compared to DL-Met group. Thus, DL-HMTBA fed chickens may have developed a better nonenzymatic antioxidant defense mechanism against heat stress.

DL-HMTBA supplementation mitigated the decreased feed utilization and decreased humoral and nonspecific immunocompetence of broiler chickens due to Met deficiency (Zhang and Guo, 2008), and also mitigated the impaired body weight gain due to low protein diet (Swennen et al., 2011). Furthermore, Xie et al. (2007) showed that excess Met sources led to toxicity in ducks. But the plasma homocysteine level of ducks fed DL-HMTBA supplemented diets was significantly lower than ducks fed equimolar DL-Met supplemented diets, suggesting reduced toxicity of DL-HMTBA relative to DL-Met. A more recent study on turbot fish also demonstrated a higher serum ascorbic acid concentration associated with dietary DL-HMTBA supplementation, suggesting an antioxidant activity and immune function of DL-HMTBA in fish (Ma et al., 2013).

In mammals, Tang et al. (2011) showed that supplementation of 0.2% DL-HMTBA brought about a significant improvement in antioxidant defenses in high fat

diet-fed male C57BL/6 mice. Excessive fat intake can induce the hyper-secretion of insulin, which then increases feed intake and subsequently increases electron flow along the mitochondrial respiratory chain, resulting in oxidative stress. The supplementation of 0.2% DL-HMTBA can restore these changes, indicating the potential of DL-HMTBA as a dietary supplement to considerably improve certain metabolic disorders and address the redox imbalance.

Some more recent studies showed that dietary DL-HMTBA supplementation had a positive effect on microbial protein synthesis in the rumen of dairy cows (Lee et al., 2015), and can improve the digestibility of phosphorus in a corn-soybean diet if supplied together with micro-minerals such as zinc, copper and manganese in piglets (Liu et al., 2014).

In human Caco-2 cells, pre-incubation with DL-HMTBA partially prevented the inflammation induced by $H_2O_2$ or inflammatory cytokine tumor necrosis factor α, while pre-incubation with DL-Met did not significantly improve the antioxidant capacity of the Caco-2 cells (Martin-Venegas et al., 2013). The protective role of DL-HMTBA on intestinal epithelial barrier function was reflected by higher level of taurine and reduced GSH in this experiment. Also taurine was reported to have the capacity to protect epithelial barrier function from disruption by oxidative stress generated by docosahexaenoic acid enrichment (Martin-Venegas et al., 2013). This may partially explain the antioxidant effect of DL-HMTBA.

Although it still remains unknown whether the human GIT is able to take advantage of DL-HMTBA, DL-HMTBA is currently being marketed as an enteral product, in conjunction with a low-protein diet, for patients suffering with chronic renal

insufficiency (Feiten et al., 2005). Moreover, the protection of barrier function by dietary DL-HMTBA reduced the passage of macromolecules such as antigens and pathogens through the para-cellular pathway, thus contributing to the quality of animal products for human consumption (Martin-Venegas et al., 2013).

**2.6 Summary and perspective**

In this review, I summarized the absorption, transport, metabolism and bio-efficiency of three dietary Met sources, L-Met, DL-Met and DL-HMTBA in different species including poultry, swine and ruminants. DL-HMTBA is an efficacious Met precursor in the promotion of growth in chickens and pigs. It is absorbed mainly by diffusion, while uptake of D-Met and L-Met takes place via multiple carrier-mediated systems. DL-HMTBA is absorbed along the entire GIT, especially the upper GIT. Intestine, liver and kidney all can remove D-Met and DL-HMTBA from circulation and metabolize them to L-Met through oxidation and transamination.

Dietary Met is indispensable in the animal nutrition industry, and can be utilized to promote animal growth and development and improve the quality of animal products for human consumption. Compared to DL-Met, dietary DL-HMTBA supplementation also has potential benefits in anti-oxidation. DL-HMTBA is promising as a feed additive to alleviate some metabolic diseases to improve both animal performance and human health.

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**Table 2.1** Small intestinal amino acid transporters related to methionine transport in mammals and avians.

| System | Transporter | SLC name[1] | Location[2] | Description/gene function |
|---|---|---|---|---|
| A | SAT1 | SLC38A1 | BL | $Na^+$-dependent short-chained neutral amino acid transporter. |
| | SAT2 | SLC38A2 | BL | $Na^+$-dependent neutral amino acid transporter. Sensitive to low pH. Ubiquitous expression. |
| | SAT3 | SLC38A3 | BL | $Na^+$-dependent neutral amino acid transporter. |
| B | $B^0AT$ | SLC6A19 | BBM | $Na^+$-dependent neutral amino acid transporter. |
| $B^{0,+}$ | $ATB^{0,+}$ | SLC6A14 | BBM | $Na^+$-dependent neutral and cationic amino acid transporter. |
| $b^{0,+}$ | $b^{0,+}AT$ | SLC7A9 | BBM | $Na^+$-independent cationic and neutral amino acid exchanger. |
| | rBAT | SLC3A1 | / | Heavy chain corresponding to the $b^{0,+}$ transport system, dimerizes with $b^{0,+}AT$ |
| L | LAT1 | SLC7A5 | BL | $Na^+$-independent neutral amino acid transporter, exchanger for large hydrophobic amino acids. Ubiquitous expression. |
| | LAT2 | SLC7A8 | BL | Same as LAT1. Not present in the chicken genome. |
| $y^+L$ | $y^+LAT1$ | SLC7A7 | BL | $Na^+$-independent cationic/ $Na^+$-dependent neutral amino acid exchanger. |
| | $y^+LAT2$ | SLC7A6 | BL | $Na^+$-independent cationic/ $Na^+$-dependent neutral amino acid exchanger. |
| / | 4F2hc | SLC3A2 | / | 4F2 cell-surface antigen heavy chain, dimerizes with LAT1, $y^+LAT1$ and $y^+LAT2$. |

1 SLC: solute carrier family.

2 BBM: brushborder membrane; BL: basolateral membrane.

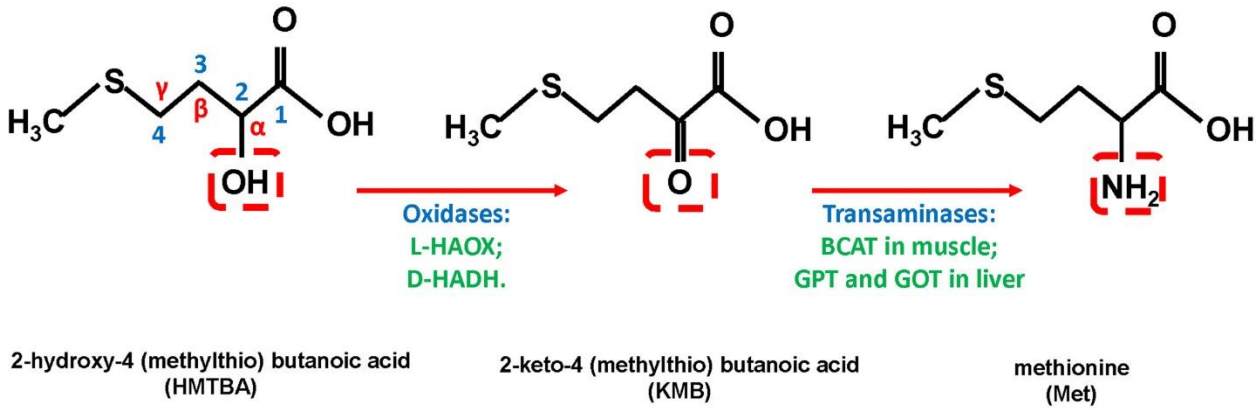


**Figure 2.1** Chemical structures of 2-hydroxy-4 (methylthio) butanoic acid, 2-keto-4 (methylthio) butanoic acid and methionine. The α-hydroxyl group in DL-HMTBA can be oxidized to the α-keto group in KMB by L-HAOX or D-HADH, and can then be converted to α-amino group by transaminases. The major transaminases reported in animals include BCAT in skeletal muscle, with amino group donors of branched-chain amino acids, and GPT and GOT in liver, with amino group donors of glutamate. BCAT: branched-chain amino acid transaminase; D-HADH: D-2-hydroxy acid dehydrogenase; GPT: glutamate pyruvate transaminase; GOT: glutamic oxaloacetic acid transaminase; L-HAOX: L-2-hydroxy acid oxidase.

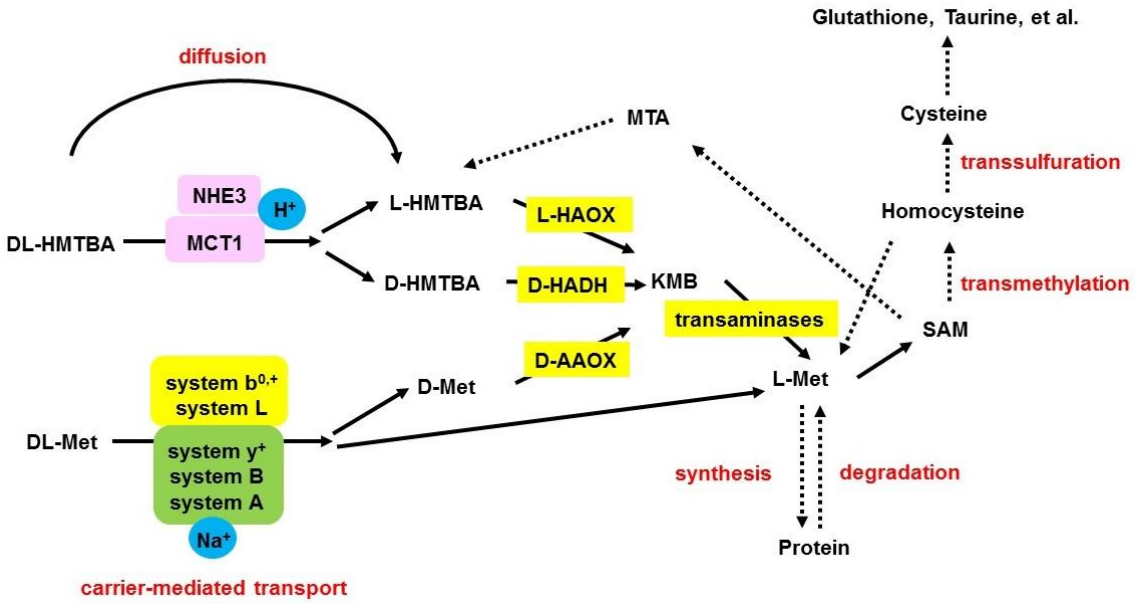


**Figure 2.2** Absorption, transport and metabolism of L-Met, D-Met and DL-HMTBA in non-ruminants. L-Met, D-Met and DL-HMTBA can be absorbed through diffusion or multiple carrier-mediated transport systems in the small intestine. D-Met and DL-HMTBA must be metabolized to L-Met for utilization. The product L-Met can be used for protein synthesis, or undergo transmethylation or transsulfuration. D-AAOX: D-amino acid oxidase; D-HADH: D-2-hydroxy acid dehydrogenase; D-Met: D-methionine; HMTBA: 2-hydroxy-4 (methylthio) butanoic acid; KMB: 2-keto-4 (methylthio) butanoic acid; L-HAOX: L-2-hydroxy acid oxidase; L-Met: L-methionine; MCT1: monocarboxylate transporter 1; MTA: 5'-deoxy-5'-methylthioadenosine; NHE3: $Na^+/H^+$ exchanger; SAM: S-adenosylmethionine.

# CHAPTER 3 DNA Methylation, Blood Profile and Intestinal Nutrient Transporter Gene Expression in Broiler Chickens Fed Methionine Isomers or Precursors

## 3.1 Abstract

Methionine (Met) is generally the first limiting amino acid in corn-soybean based diets for poultry. The objective of this study was to determine the effect of supplementation of L-methionine (L-Met), DL-methionine (DL-Met) and the methionine analogue, DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA), and Met deficiency on DNA methylation, blood profile and intestinal nutrient transporter gene expression of broiler chickens. Male Cobb-500 broilers were fed from day of hatch (d0) to d35 post-hatch using a diet deficient in methionine plus cysteine (Met + Cys) (control), or the same diet supplemented with 0.22% DL-Met, 0.22% L-Met or 0.31% DL-HMTBA to meet the Met + Cys requirements. Tissue (liver, duodenum, jejunum and ileum) and blood samples were collected at various ages, from d0 to d35. Performance of the birds, blood parameters (e.g., acute phase proteins, white blood cell counts), mRNA expression of intestinal nutrient transporters and DNA methylation properties of liver tissues were examined. Both body weight and feed efficiency were improved in Met supplemented groups compared to the control group. No significant differences were observed among DL-Met, L-Met and DL-HMTBA for growth performance parameters. L-Met and DL-Met supplementation decreased the acute phase protein, serum amyloid A, while DL-HMTBA had no effect. Met supplementation had no effect on red blood cell packed cell volume, white blood cell differentiation count, hepatic total DNA methylation or DNA methyltransferase (DNMT) activity. L-Met and DL-Met, but not DL-HMTBA,

supplementation resulted in enhanced expression of the $ATB^{0,+}$ and $B^0AT$ transporters in various small intestinal segments. All Met sources increased expression of MCT1 in the jejunum. In conclusion, Met supplementation improved growth performance of male broilers. Met supplementation was also associated with changes in intestinal nutrient transporter gene expression in certain segments and ages, suggesting that intestinal amino acid absorptive function can be regulated by the source of amino acid and effects are complex and dynamic.

## 3.2 Introduction

Methionine (Met) is an essential amino acid in mammals and is the first limiting amino acid in a broiler diet and thus its uptake from the diet is required for growth and development. Met is supplemented in feed as L-methionine (L-Met), DL-methionine (DL-Met, a mixture of the L- and D- enantiomers) and the methionine analogue, DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). DL-HMTBA lacks an amino group and thus is not an amino acid but an amino acid precursor that needs to be converted to L-Met (Dibner and Knight, 1984). Many studies have addressed the relative efficiency of these Met sources (Lemme et al., 2002; Hoehler et al., 2005; Elwert et al., 2008; Sauer et al., 2008). Met is a precursor for the synthesis of proteins and serves as the predominant amino acid for translation initiation. In addition, Met is an intermediate in the biosynthesis of other important molecules such as cysteine, carnitine and taurine and can be converted to S-adenosylmethionine (SAM), which serves as a methyl donor.

The assimilation of dietary nutrients occurs mainly in the small intestine. Dietary proteins are first digested through enzymatic hydrolysis to generate absorbable end products, including free amino acids and peptides. These nutrients are taken up by epithelial cells that line the small intestine by a variety of transporters. Once inside the epithelial cells, these nutrients are either used for cell metabolism or transported out of the cell and into the blood for delivery to other cells and tissues (Broer, 2008; Gilbert et al., 2008).

The amino acid and peptide transporters are members of the Solute Carrier (SLC) gene series, which includes 52 families and 395 transporter genes in the human genome (Hediger et al., 2013). These transporters are located in the brush border membrane for

transport of amino acids from the intestinal lumen to the inside of the intestinal epithelial cells and on the basolateral membrane for transport of amino acids from the inside of the epithelial cell to the blood or vice versa. In mammals, the amino acid transporters act in a $Na^+$-dependent or $Na^+$-independent manner. At the brush border membrane, free Met is transported by $Na^+$-dependent neutral amino acid transporters $B^0AT$, $Na^+$-dependent neutral and cationic amino acid transporter $ATB^{0,+}$, and $Na^+$-independent cationic and neutral amino acid transporter $b^{0,+}AT$ (Hyde et al., 2003; Pramod et al., 2013). Furthermore, at the brush border membrane, the $H^+$-dependent peptide transporter PepT1 transports a wide variety of di- and tripeptides into the intestinal epithelial cells (Gilbert et al., 2008; Smith et al., 2013). At the basolateral membrane, Met is transported by $Na^+$-dependent neutral amino acid transporters SAT1, SAT2, and SAT3, $Na^+$-independent neutral amino acid transporters LAT1 and LAT2, and $Na^+$-dependent neutral amino acid transporters $y^+$LAT1 and $y^+$LAT2. The amino acid transporters $b^{0,+}AT$, LAT1, LAT2, $y^+$LAT1 and $y^+$LAT2 are heterodimeric transporters: $b^{0,+}AT$ complexes with rBAT and LAT1, LAT2, $y^+$LAT1 and $y^+$LAT2 complex with 4F2hc (Broer, 2008; Fotiadis et al., 2013; Broer, 2014). A search of the chicken genome (Ensembl, release 83 – December 2015) did not reveal a chicken ortholog to LAT2. In chickens, Met is transported by the $Na^+$-independent systems $b^{0,+}$ and L, and the $Na^+$-dependent systems $y^+$, B and A (Soriano-Garcia et al., 1998). In contrast, DL-HMTBA transport was reported to be associated with the proton dependent monocarboxylate transporter 1 (MCT1) and the $Na^+$-$H^+$ exchanger (NHE3) (Martin-Venegas et al., 2007; 2008) .

Methylation is a covalent modification that is critical for regulating gene transcriptional activity. DNA methylation involves the addition of a methyl group by

DNA methyltransferases (DNMTs) to the pyrimidine ring of cytosine at the 5-carbon, which results in 5-methylcytosine at CpG dinucleotides (reviewed by Breiling and Lyko, 2015) . Hypermethylation of DNA, particularly at dense regions of CpGs called CpG islands, serves as a major transcriptional silencing mechanism (Gilbert and Liu, 2012). From a nutritional perspective, one-carbon metabolism is controlled by availability of essential nutrients. Dietary deficiencies or excesses in methyl donors and enzymatic cofactors may lead to global changes in DNA methylation patterns. Met is a precursor for the biosynthesis of SAM, which is a vital methyl donor in more than 80 different biological reactions including methylation of DNA, RNA and proteins.

Acute phase proteins are blood proteins, which serve as biomarkers of infection, inflammation and stress (Murata, 2007; Eckersall and Bell, 2010). The acute phase protein levels after a single stimulus remain unchanged for 48 h or longer, and thus can be used for assessment of general health in animals (Gruys et al., 2005). Serum amyloid A (SAA) and alpha -1-acid glycoprotein (α-1-AGP) are two acute phase proteins commonly studied in chickens. Furthermore, the number of lymphocytes would decrease and the number of heterophils would increase in response to stress in chicken blood samples (Gross and Siegel, 1983). Thus, evaluation of the heterophil/lymphocyte ratio also acts as a measure of the stress response in chickens. Red blood cell packed cell volume (RBC-PCV) is the volume percentage of red blood cells in whole blood. A low RBC-PCV value is an indicator of anemia.

The objective of this study was to determine the effects of different dietary Met source supplementation and Met deficiency on intestinal amino acid/peptide/monocarboxylic acid transporter gene expression, global DNA methylation,

white blood cell differentiation count and expression of acute phase proteins in male broiler chickens. We hypothesized that dietary Met supplementation would increase hepatic DNA methylation, decrease expression of acute phase proteins and alter the expression profiles of intestinal nutrient transporter genes.

## 3.3 Materials and Methods

### 3.3.1 Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee at Virginia Tech. Mixed sex, day of hatch Cobb 500 broiler chicks were sourced locally (Tyson Food Inc., Crewe, VA). Eight day-old chicks were identified as males by visual sexing of gonadal tissue and used for collection of liver and intestinal tissue. A blood sample was drawn from approximately 700 chicks, which were then randomly distributed into heated floor pens with new wood shavings based on body weight and given one of the four experimental diets: Control (C): deficient in dietary Met + Cys, without any supplementation of Met sources; Treatment 1 (DL-Met): control diet + 0.22% DL-Met; Treatment 2 (L-Met): control diet + 0.22% L-Met and Treatment 3 (DL-HMTBA): control diet + 0.31% DL-HMTBA (to provide 0.22% DL-Met equivalent). The supplementation calculated for L-Met was recalculated for DL-Met and DL-HMTBA on an equimolar basis, which resulted in levels of 0.22% for DL-Met or L-Met and 0.31% for DL-HMTBA.

The sex of the chicks was determined using a PCR-based method. Briefly, 2-5 μL of blood was processed using the Phusion Blood Direct PCR Kit (Thermo Scientific, Waltham, MA) and PCR was performed using primers for the W chromosome and the tyrosinase gene (Gilbert et al., 2007). On d3, female chicks and chicks with an ambiguous

PCR test were removed from the pens. The remaining 242 male chicks were redistributed within their treatment group into 6 replicate pens per treatment based on body weight (~10 chickens/pen, see Appendix B, Table B for details) and fed the experimental diets until d26. Due to limitations in availability of the research facility, chickens were moved from floor pens to cages (2 chickens/cage, 15 cages/treatment) and fed the 4 experimental diets. Because there were only 2 chickens per cage, this was considered insufficient for calculating feed conversion efficiency and thus feed intake and feed conversion efficiency are only reported to d26, while the chickens were being raised in floor pens.

All pens had 24h lighting and the temperature was set at 80°F. Chickens were given ad libitum access to feed and water. A starter diet was fed from 0 to 10 d, a grower diet from 11 to 21 d, and a finisher diet from 22 to 35 d. Feed was produced by Research Diet Services BV (Utrecht, Netherlands). Basal diets were formulated to meet the broilers' requirements (Aminochick® 2.0, Evonik Nutrition & Care GmbH, Germany) except for Met + Cys. Three Met sources (DL-Met (Evonik), L-Met (Evonik-Rexim), HMTBA (MHA-FA, Novus)) were supplemented to the basal diets in order to meet or slightly exceed the broilers Met + Cys requirements (see Appendix A, Table A).

Individual body weights of all chicks in a pen were recorded on d10, d15, d21, d26 and d35. Feed intake and feed conversion efficiency (feed intake/body weight gain) of each pen were calculated at the end of the different diet phases while in floor pens (d10, 21, 26), but were only reported from d10 to d26 because both males and females were present in the pens from d0 to d3 and thus feed intake for just males could not be determined for d0 to d10. Abdominal fat weight, breast muscle weight, organ (kidney,

liver, heart and small intestine) weight and carcass weight (body weight minus the head and internal organs) were collected at d10, 21, 26 and 35.

At d0, 3, 5, 10, 21, 26, 35, 6 birds per treatment were euthanized by cervical dislocation and intestine and liver were collected. The intestine was separated into duodenum, jejunum, and ileum. The proximal enlarged loop of the small intestine was taken as the duodenum. The rest of the small intestine was divided by Meckel's diverticulum into proximal and distal portions, corresponding to the jejunum and ileum, respectively. Digesta were gently squeezed out of the intestine and segments were rinsed three times in PBS ($NaH_2PO_4$ 1.47 mmol/L, $Na_2HPO_4$ 8.09 mmol/L, NaCl 145 mmol/L). Whole segments were stored in RNAlater (Life Technologies, Carlsbad, CA) at -80°C. Liver samples were snap-frozen in liquid nitrogen and then stored at -80°C. At d21 and 26, whole blood was collected from the jugular vein using syringes and then kept on ice in blood vials with EDTA as anticoagulant. Plasma was obtained using 2 mL whole blood by centrifugation and then stored at -80°C.

### 3.3.2 Dry matter digestibility

Titanium dioxide ($TiO_2$) was added to feed as an indigestible marker. A total of 16 digesta samples (n=4) from 4 different finisher feed samples (C, DL-Met, L-Met and DL-HMTBA) were collected from ileum at d26 and then freeze-dried. The $TiO_2$ concentration were determined following the protocol of Boguhn et al. (2009). Briefly, dry samples were digested with sulfuric acid and nitric acid, and then Ti was measured via inductively coupled plasma atomic emission spectroscopy (ICP-AES) by VT Soils Lab. Dry matter digestibility was calculated as the ratio of $TiO_2$ in diet to $TiO_2$ in dry digesta (Boguhn et al., 2009).

### 3.3.3 Global DNA methylation and DNA methyltransferase activity

Genomic DNA was isolated from six liver samples per treatment on d0, 3, 5, 10, 21, 26 and 35 (n=6) using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Global DNA methylation was quantified using the MethylFlash$^{TM}$ Methylated DNA Colorimetric Quantification kit (Epigentek, Farmingdale, NY), following the manufacturer's instructions. A total of 100 ng DNA was added to strip wells and the methylated fraction of DNA was quantified colorimetrically with a multi-mode plate reader (Infinite M200 Pro, Tecan, Morrisville, NC). The amount of methylated DNA is proportional to the optical density measured at 450 nm.

Nuclear extracts were prepared from 6 liver samples per treatment on d0, 3, 5, 10, 21, 26 and 35 (n=6) using the EpiQuik™ Nuclear Extraction Kit I (Epigentek) according to the manufacturer's protocol and stored at -80°C. Total DNA methyltransferase (DNMT) activity was measured using the EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric, Epigentek), according to the manufacturer's protocol. A total of 4-20 µg nuclear extracts were added to microplate wells and the amount of methylated DNA, which is proportional to enzyme activity, was measured through an ELISA-like reaction by reading the absorbance at 450 nm using an Infinite M200 Pro multiplate reader.

### 3.3.4 Blood profiling

Red blood cell packed cell volume (RBC-PCV) was measured in fresh blood samples from d21, 26 and 35 (n=6). StatSpin™ Glass Microhematocrit Tubes (40 mm, Fisher Scientific, USA) were used to collect fresh blood and then centrifuged in a

microhematocrit (StatSpin, USA). RBC-PCV was calculated using a micro-capillary reader (DAMON/IEC Division, USA).

White blood cell differentiation count was also assayed in fresh blood samples from d21, 26 and 35 (n=6). Individual blood smears were prepared on glass slides and Wright-Giemsa differential was used to stain the slides. Three images were captured per slide with a Nikon Eclipse 80i microscope and DS-Ri1 color camera (Nikon, Japan). The numbers of eosinophil and heterophil, lymphocyte, basophil and monocyte were counted per 100 white blood cells per image and the average percentages were calculated.

Analysis of acute phase proteins alpha-1-acid glycoprotein (α-1-AGP) and serum amyloid A (SAA) in plasma on d21 and 26 (n=6) were assayed using the Chicken α-1-AGP ELISA Kit (Life Diagnostics, West Chester, PA) and Chicken SAA ELISA Kit (Life Diagnostics), respectively, following the manufacturer's protocol. Briefly, the diluted samples were incubated in the corresponding antibody-coated microtiter wells together with horseradish peroxidase (HRP) conjugate and protein was measured by reading the absorbance at 450 nm using an Infinite M200 Pro multiplate reader.

### 3.3.5 Nutrient transporter gene expression

The small intestinal segments (duodenum, jejunum, and ileum) from five birds per treatment on d0, 3, 5, 10, 21, 26 and 35 were minced using razor blades and homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH) using 5 mm stainless steel beads (Qiagen) and a Tissue Lyser II (Qiagen). Total RNA was isolated following the manufacturer's instructions (Molecular Research Center). The Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA) was used for total RNA purification. The total RNA samples were evaluated for quality by agarose-formaldehyde gel

electrophoresis and concentration and purity assessed by spectrophotometry at 260/280/230 nm with a NanoDrop$^{TM}$ 1000 (Thermo Scientific, Waltham, MA).

First-strand cDNA was synthesized from 500 ng total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). Reactions were performed under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Expression of 14 transporter genes $ATB^{0,+}$, $b^{0,+}AT$, $B^{0}AT$, 4F2hc, LAT1, MCT1, NHE3, PepT1, rBAT, SAT1, SAT2, SAT3, $y^{+}LAT1$ and $y^{+}LAT2$, and 6 reference genes β-actin, hexose-6-phosphate dehydrogenase (H6PD), lactate dehydrogenase A (LDHA), ribosomal protein L4 (PRL4), ribosomal protein (large, P0) (PRLP0) and ribosomal protein (large, P1) (PRLP1) were assayed using relative quantification real time PCR. Primers designed for real time PCR are listed in Table 3.1 and were validated for amplification efficiency before use (90-115% efficiency). Real-time PCR was performed in duplicate in 10 µL volume reactions that contained 5 µL Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µL each of 5 µM forward and reverse primers and 3 µL of 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR was performed under the following conditions: 95°C for 20 s and 40 cycles of 90°C for 3 s plus 60°C for 30 s. A dissociation step consisting of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s was performed at the end of each PCR reaction to verify that a single amplicon was generated.

Stability of reference genes was tested using geNorm. Three reference genes (PRL4, PRLP0 and PRLP1) that showed the highest stability were selected from a panel of 6 reference genes (β-actin, PRL4, PRLP0, PRLP1, H6PD and LDHA) (Vandesompele et al., 2002). The Cq values of each target gene were analyzed using algorithms provided

by Hellemans et al. (2007). Briefly, the average Cq value was calculated for PCR replicates, then transformed into relative quantities (RQs) using $(1+e)^{-\Delta Cq}$, where $\Delta Cq$= Cq (target sample) – Cq (average). The normalization factor (NF) for each sample was calculated based on the geometric mean of RQs of the three reference genes for that sample. The normalized relative quantities (NRQs) equals RQ/NF (sample specific), which was used for statistical analysis.

### 3.3.6 Data analysis

There were three statistical models for mRNA expression data, which considered: 1) the main effects of treatment, age, small intestinal segment and their interactions; 2) the main effects of treatment and age and the treatment × age interaction within each small intestinal segment (duodenum, jejunum, and ileum) and 3) the main effects of treatment and intestinal segment and the treatment × segment interaction, within each age (d3, d5, d10, d21, d26, and d35). Data were checked for normality and homogeneity of variances. Split-plot design was considered when analyzing data from model 1 and 3, with chicks as the whole plot expressing the error term for testing the effects of treatment, the segment as the split plot and chicks as the random effect. Data from model 2 were analyzed by two-way ANOVA. Contrasts were used to separate treatment means.

For the other parameters, the statistical models included the main effects of treatment, age and their interactions, or the effect of treatment within each age. Data were checked for normality and homogeneity of variances and analyzed by ANOVA. Means were separated using Tukey's Test.

JMP Pro version 11.0 (SAS Institute, Cary, NC) was used for statistical analysis. Data are presented as least square means ± SEM and statistical significance assigned at $P < 0.05$.

## 3.4 Results

### 3.4.1 Growth performance

Average body weight of the chickens fed the control (C) and treatment diets (DL-Met, L-Met, and DL-HMTBA) from d10 to d35 are shown in Table 3.2. Analysis of body weight within each time point (d10, 15, 21, 26, 35) showed an increase for DL-Met, L-Met, and DL-HMTBA compared to C ($P < 0.001$). However, there was no difference among DL-Met, L-Met, and DL-HMTBA-fed chickens.

There was no difference between the four treatment groups for average feed intake from d10 to d26 (Table 3.2). Feed conversion ratios were measured on a pen basis and are shown in Table 3.2. The feed conversion ratios of chickens fed DL-Met, L-Met and DL-HMTBA were less than C for d10 to d26 ($P < 0.001$), but there was no difference between DL-Met, L-Met and DL-HMTBA.

The average dry matter digestibility for chickens fed C, DL-Met, L-Met and DL-HMTBA at d26 are also shown in Table 3.2. There was no significant difference between these four treatment groups.

Because there were no differences between three Met supplementation groups for body weight or feed conversion ratio, we examined potential treatment effects on relative organ weights (organ weight/live weight) and carcass weights (Table 3.3). The relative small intestinal weights were measured only at d21, d26 and d35. Relative breast muscle weight (breast muscle weight/live weight) was improved for dietary Met supplemented

groups (DL-Met, L-Met and DL-HMTBA) compared to the control group at d10 ($P < 0.001$), d21 ($P < 0.001$), d26 ($P < 0.001$) and d35 ($P < 0.001$). There was no difference between DL-Met, L-Met, and DL-HMTBA at any time point. Relative kidney weight (kidney weight/live weight) was decreased for DL-HMTBA compared to C at d21 ($P = 0.027$) and decreased for DL-Met compared to C at d35 ($P = 0.017$). Relative liver weight (liver weight/live weight) was decreased for L-Met and DL-HMTBA compared to C at d21 ($P = 0.014$). Relative carcass weight (carcass weight/live weight) was lower for L-Met than DL-Met and C at d26 ($P = 0.008$); but by d35 relative carcass weight for DL-Met, L-Met, and DL-HMTBA were all lower than C ($P = 0.009$). At d35, relative jejunum weight was lower in L-Met than C ($P = 0.025$) and relative ileum weight was lower in DL-Met than C ($P = 0.016$). There was no difference due to treatment effect at all the time points for relative duodenum weight, relative abdominal fat weight and relative heart weight.

### 3.4.2 Blood profile

There was no significant treatment effect for RBC-PCV, eosinophil + heterophil, lymphocyte, basophil and monocyte percentages and the (eosinophil + heterophil)/lymphocyte ratio (Table 3.4). There was, however, an effect of age with the percentage of eosinophil + heterophil and (eosinophil + heterophil)/lymphocyte ratio greater at d26 compared to d21 and d35 ($P = 0.008$ and 0.014, respectively).

As another indicator of stress, plasma levels of acute phase proteins were measured (Table 3.5). There was a significant treatment × age interaction for serum amyloid A (SAA) level ($P = 0.007$). To further clarify, analysis was performed within each time period (d21 and d26). SAA was lower in DL-Met and L-Met than in C at d21

($P$ = 0.014). There was no treatment main effect at d26. The level of alpha-1-acid glycoprotein was significantly greater at d26 compared to d21 ($P$ = 0.005), but there was no effect due to treatment.

### 3.4.3 Global DNA methylation and DNA methyltransferase (DNMT) activity

No significant differences were seen among C, DL-Met, L-Met and DL-HMTBA groups for both hepatic total DNA methylation and DNMT activity. However, hepatic DNA methylation and DNMT activity changed with time, where DNMT activity increased from d5 to d10 (Figure 3.1A, $P < 0.001$); whereas DNA methylation decreased from d10 to d26 and d35 (Figure 3.1B, $P$ = 0.001).

### 3.4.4 Nutrient transporter gene expression

Gene expression was analyzed using three statistical models as described in Material and Methods. For Model 1, there was significant age × segment interaction effects for all the genes (Table 3.6). There was a treatment main effect for 4F2hc expression ($P$ = 0.030). Although the control group had greater 4F2hc expression tendency compared to DL-Met and L-Met supplemented groups, no statistically significant differences were detected among the four treatment groups using Tukey's HSD test. There was a treatment × segment interaction for SAT2 expression ($P$ = 0.040). No statistically significant differences were seen among the treatments within each segment. However, when the data were analyzed within duodenum, SAT2 expression in DL-Met chickens was greater than DL-HMTBA using student t-test ($P$ = 0.034). There was a three-way treatment × age × segment interaction for $ATB^{0,+}$ expression ($P$ = 0.002).

For model 2, both DL-Met and L-Met groups had greater $ATB^{0,+}$ mRNA abundance compared to C in duodenum (Figure 3.2A, $P = 0.039$). All three Met supplemented groups (DL-Met, L-Met and DL-HMTBA) had greater MCT1 expression compared to the control group in jejunum (Figure 3.2B, $P = 0.039$). There were significant treatment × age interactions for LAT1, $ATB^{0,+}$, and $B^0AT$ expression (Table 3.7). In duodenum, L-Met chickens had greater LAT1 expression than C, DL-Met and DL-HMTBA chickens at d3 and C chickens had greater LAT1 expression than L-Met and DL-HMTBA chickens at d10. In jejunum, DL-Met, L-Met and DL-HMTBA chickens had greater $ATB^{0,+}$ expression than C chickens at d5, and DL-Met chickens had greater $B^0AT$ expression than C, L-Met and DL-HMTBA chickens at d26. In ileum at d5, DL-Met chickens had greater $ATB^{0,+}$ expression compared to C and L-Met chickens and DL-HMTBA chickens had greater $ATB^{0,+}$ expression compared to C chickens. At d21, DL-Met chickens had greater expression of $ATB^{0,+}$ than C, L-Met and DL-HMTBA chickens.

For Model 3, DL-Met chickens had greater $ATB^{0,+}$ abundance than C at d5 (Figure 3.3A, $P = 0.048$). At d21, both DL-Met and L-Met chickens had greater NHE3 mRNA abundance than DL-HMTBA (Figure 3.3B, $P = 0.021$), while at d35, DL-HMTBA chickens had greater SAT1 abundance than DL-Met and L-Met groups (Figure 3.3C, $P = 0.039$). There were significant treatment × segment interactions for 4F2hc, $b^{0,+}AT$, $ATB^{0,+}$, $B^0AT$, LAT1 and SAT2 expression (Table 3.8). At d3, DL-HMTBA chickens had greater 4F2hc mRNA abundance than C, DL-Met and L-Met chickens and greater $b^{0,+}AT$ mRNA abundance than DL-Met and L-Met chickens in ileum at d3. In addition, C chickens had greater $b^{0,+}AT$ expression than L-Met chickens in ileum. At d21,

DL-Met chickens had greater $ATB^{0,+}$ mRNA abundance than C, L-Met and DL-HMTBA chickens in ileum. At d26, DL-Met chickens had greater $B^0AT$ mRNA abundance than C, L-Met and DL-HMTBA chickens in jejunum and C chickens had greater LAT1 mRNA abundance than DL-Met, L-Met and DL-HMTBA chickens in ileum. At d35, DL-Met chickens had greater $ATB^{0,+}$ mRNA abundance than C chickens in duodenum while L-Met chickens had greater $ATB^{0,+}$ mRNA abundance than DL-Met and DL-HMTBA chickens in ileum. DL-Met chickens had greater SAT2 expression than C and DL-HMTBA chickens in duodenum at d35.

**3.5 Discussion**

Methionine is the first limiting amino acid in a broiler corn-soybean meal based diet. These diets are commonly supplemented with Met sources such as DL-Met or DL-HMTBA to meet the recommended amounts for the particular growth phase. This study examined the differential effects of Met supplements DL-Met, L-Met and DL-HMTBA on global DNA methylation, levels of stress indicators (acute phase proteins, white blood cell differential) and expression of selective intestinal amino acid transporters.

The growth performance data showed that Met supplementation improved body weight gain, feed efficiency and relative breast muscle weight. The three diets with Met source supplementation were designed to meet the requirements of broiler chickens, so these groups should not be limiting in methionine according to the analytic values in diets. In our trial, the diets were formulated based on a relative bio-efficacy of 65% for DL-HMTBA compared to DL-Met, which was reported by Lemme et al. (2002). Our results showed no difference between the three supplementary Met sources, which are consistent with those bio-efficacy values.

Little is known about DNA methylation in birds. L-Met can be directly converted into S-adenosylmethionine (SAM), the major methyl donor in cells. Methyl group donor deficiency has been shown to up-regulate DNA methyltransferase (DNMT) but does not increase genomic DNA hypomethylation in rat liver (Kim, 2004). Thus, we hypothesized that Met supplementation would increase total DNA methylation in the liver. Our results showed that Met supplementation had no significant effects on hepatic DNA methyltransferase (DNMT) activity or global DNA methylation. DNA methylation regulation is a complex network, which may not be affected by either dietary Met+Cys level or Met source. However, we observed that hepatic global DNA methylation decreased from d10 to d26 and d35, while DNMT activity increased from d5 to d10 and then remained constant. This was partially in accordance with the observation of Gryzinska et al. (2013) who reported a decrease in global DNA methylation with age in the blood of 32-week-old compared to 1-day-old hens.

A Met-deficient diet could cause physiological stress, which could be manifested as a change in white blood cell numbers or increase in acute phase proteins. For white blood cell differentiation count, the heterophil/lymphocyte ratio is often used as an indicator of disease or stress (Gross and Siegel, 1983). In chickens eosinophils represent only 1% of the white blood cells (Scanes and Sturkie, 2015) and are difficult to discern from heterophils, therefore only the eosinophil + heterophil percentage and the (eosinophil + heterophil)/lymphocyte ratio have been reported. However, no significant differences among the treatment groups were observed, indicating that there were no difference in stress, as measured by white blood cells, between control and Met supplemented groups.

Determination of acute phase protein levels can help in monitoring the health of animals (Murata, 2007). Serum amyloid A (SAA) is a positive acute phase reactant expressed primarily in the liver that circulates in blood. In chickens, SAA gene expression may increase more than 100-fold following infection (Matulova et al., 2013). Alpha -1-acid glycoprotein (α-1-AGP) is also an acute phase protein that is elevated in chicken serum due to injury, infection or disease. The amount of α-1-AGP may increase 5-fold or more in chickens during an acute phase response (Holt and Gast, 2002). In our trial, SAA was decreased in DL-Met and L-Met fed birds compared to the control at d21. These results indicate that our control diet, which was Met-deficient, may have induced stress in the chickens as revealed by high SAA concentrations, which can be alleviated by L-Met and DL-Met supplementation. SAA concentration in DL-HMTBA fed birds was intermediate between control and L-Met and DL-Met. Shakeri et al. (2014) reported that stress induced by high stocking density of broilers led to an increase in the acute phase protein α-1-AGP, however, we did not see a difference in α-1-AGP concentrations.

Supplementation of the diets with L-Met, DL-Met or DL-HMTBA altered the expression of some intestinal nutrient transporter genes. Many of these changes were revealed as treatment × age and treatment × intestinal segment interactions. The two Met transporters that were most responsive to Met supplementation were $ATB^{0,+}$ (SLC6A14) and $B^0AT$ (SLC6A19). For example, DL-Met supplementation resulted in increased expression of $B^0AT$ in the jejunum and duodenum and increased $ATB^{0,+}$ at d5; whereas L-Met supplementation increased $ATB^{0,+}$ in the duodenum. Both $ATB^{0,+}$ and $B^0AT$ are brush border membrane transporters of neutral/cationic and neutral amino acids, respectively. Upregulation of $ATB^{0,+}$ and $B^0AT$ by DL-Met would lead to enhanced

uptake of not only Met but also other neutral and cationic amino acids. The transport kinetics of D-Met and L-Met are comparable using human Caco-2 cells and a perfused rat intestinal model (Zheng et al., 1994). Maenz and Engele-Schaan (1996) reported that L- and D-Met are transported by the broad specificity system B type transporter in chicken brush border membrane vesicles. Martin-Venegas et al. (2009) further showed that systems L and $B^{0,+}$ are responsible for L-Met transport while only system $B^{0,+}$ is responsible for D-Met transport in human Caco-2 cells. In addition, $ATB^{0,+}$ can transport L-enantiomers of neutral and cationic amino acids as well as D-enantiomers such as D-Ser, D-Ala, D-Met, D-Leu and D-Trp (Hatanaka et al., 2002). These results are in accordance with our findings that DL-Met and L-Met supplementation altered expression of $ATB^{0,+}$ and $B^0AT$. Ferraris and Diamond (1989) have proposed a biphasic model for the uptake of essential amino acids such as Met. Below maintenance levels of dietary nitrogen (0-18%), the uptake of Met decreased or remained constant with increasing dietary nitrogen; whereas above maintenance levels (>18%), uptake of Met increased with increasing dietary nitrogen. Although the dietary protein levels decrease in the starter, grower and finisher diets, the protein requirement levels also decrease. However, this may partially explain the temporal changes in transporter expression. Thus the regulation of expression of transporters for a substrate such as Met are expected to be complex and may depend on the balance and availability of other amino acids in the diet.

In contrast to L-Met and DL-Met, DL-HMTBA as a Met precursor had minor effects on the regulation on $ATB^{0,+}$ and $B^0AT$. Only the brush border membrane transporter $b^{0,+}AT$ (SLC7A9) was expressed greater in the ileum at d3 by HMTBA supplementation but not by DL-Met and L-Met supplementation. In fact, $b^{0,+}AT$ was

downregulated in the ileum of L-Met chickens compared to control chickens. In contrast, in growing pigs, the addition of Lys, Thr and Met to a wheat diet deficient in these three amino acids caused an upregulation of $b^{0,+}$AT in the jejunum (Garcia-Villalobos et al., 2012).

We hypothesized that changes in the expression of Met transporters may have a pleiotropic effect on the expression of the peptide transporter PepT1. Shiraga et al. (1999) showed that various amino acids, such as Arg, Phe, and Lys, increased promoter activity of rat PepT1, although Met was not one of the amino acids tested in this study. Our results showed that PepT1 was not changed by the different Met supplementation, even though expression of Met transporters was changed.

The transport system for DL-HMTBA is the monocarboxylate transporter MCT1 (SLC16A1), which is coupled to the $Na^+/H^+$ exchanger NHE3 (SLC9A3) to maintain the proton gradient at the brush border membrane of the enterocyte (Martin-Venegas et al., 2007). Martin-Venegas et al. (2014) further showed that MCT1 is upregulated in Caco-2 cells by DL-HMTBA. In our study, MCT1 expression was upregulated in the jejunum of DL-Met, L-Met and DL-HMTBA chickens compared to control chickens. This was a surprising result because we expected an increase in MCT1 in the presence of DL-HMTBA and not DL-Met and L-Met. The mechanism by which L-Met and DL-Met are regulating MCT1 remains to be elucidated. NHE3 expression did not show a corresponding main effect of DL-Met, L-Met, and DL-HMTBA compared to the control like MCT1 gene expression. This is not surprising because the activity of many transporters are coupled to the proton gradient (Thwaites and Anderson, 2007).

The amino acid transporters SAT1, SAT2, and SAT3 are members of the SLC38A family and are responsible for System A and System B transport of small neutral amino acids such as Gln, Met and Ala (Schioth et al., 2013). There was increased expression of SAT1 in DL-HMTBA chickens compared to DL-Met and L-Met chickens, but not compared to control chickens. SAT2 expression was greater in the duodenum of DL-Met chickens compared to control and DL-HMTBA chickens at d35. SAT2 is a basolateral membrane transporter that also plays a role as an amino acid sensor, mediating its transceptor signaling through the mTOR pathway (Pinilla et al., 2011). An increase in SAT2 would lead to downstream signaling through mTORC1 resulting in increased protein synthesis (Taylor, 2014).

In summary, supplementation of feed with L-Met, DL-Met or DL-HMTBA improved growth rate, feed efficiency, and breast muscle yield equally compared to a control diet that was deficient in Met. There were no differences in white blood cell differentiation count, DNA methylation or DNA methyltransferase activity between control and Met supplemented diets. One acute phase protein (serum amyloid A) was increased in control compared to DL-Met and L-Met but not by DL-HMTBA at d26. Met supplementation did not affect expression of basolateral transporters but did result in enhanced expression of brush border membrane transporters, for example, the $ATB^{0,+}$ transporter in the duodenum and MCT1 in the jejunum. Moreover, DL-HMTBA does not affect the expression of transporters in a similar manner as DL-Met and L-Met. These results suggest that DL-Met, L-Met and DL-HMTBA improve growth performance but do not have the same influence on biochemical pathways. This study can help to further

understand different dietary Met sources in the animal nutrition industry, which then can be better utilized to promote animal growth and development.

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**Table 3.1** Primers used for quantitative real-time PCR.

| SLC[1] name | Gene | GenBank ID | Description/gene function | Primer Sequence (5' → 3'), sense/antisense[4] | bp [2] | Mean Cq value[3] | Efficiency |
|---|---|---|---|---|---|---|---|
| SLC3A1 | rBAT | XM_426125.4 | Heavy chain corresponding to the $b^{o,+}$ transport system, dimerizes with $b^{o,+}$AT | CCCGCCGTTCAACAAGAG/ AATTAAATCCATCGACTCCTTTGC | 70 | 22.5 | 100.6 |
| SLC3A2 | 4F2hc | XM_426125.4 | 4F2 cell-surface antigen heavy chain, dimerizes with LAT1, $y^+$LAT1 and $y^+$LAT2 | GGTTTCAGCTCAGTCGCAATC/ CGCAGTCCTGCCAGATGTAGT | 63 | 23.0 | 103.6 |
| SLC6A14 | $ATB^{0,+}$ | XM_004940661.1 | $Na^+$- and $Cl^-$-dependent neutral and cationic amino acid transporter | TGGCAACATCGTGTGGTACCT/ AGGCAGCTCCAACGATCATC | 65 | 27.0 | 108.7 |
| SLC6A19 | $B^0$AT | XM_419056.4 | $Na^+$-dependent neutral amino acid transporter | GGGTTTTGTGTTGGCTTAGGAA/ TCCATGGCTCTGGCAGAGAT | 60 | 21.1 | 93.0 |
| SLC7A5 | LAT1 | NM_001030579.1 | $Na^+$-independent large neutral amino acid transporter, dimerizes with 4F2hc | GATTGCAACGGGTGATGTGA/ CCCCACACCCACTTTTGTTT | 70 | 26.4 | 96.8 |
| SLC7A6 | $y^+$LAT2 | XM_001231336.3 | $Na^+$-independent cationic & $Na^+$-dependent neutral amino acid transporter, dimerizes with 4F2hc | GCCCTGTCAGTAAATCAGACAAGA / TTCAGTTGCATTGTGTTTTGGTT | 82 | 23.2 | 98.6 |
| SLC7A7 | $y^+$LAT1 | XM_418326.4 | $Na^+$-independent cationic & $Na^+$-dependent neutral amino acid transporter, dimerizes with 4F2hc | CAGAAAACCTCAGAGCTCCCTTT/ TGAGTACAGAGCCAGCGCAAT | 71 | 28.3 | 111.3 |
| SLC7A9 | $b^{0,+}$AT | NM_001199133.1 | $Na^+$-independent cationic and neutral amino acid transporter, dimerizes with rBAT | CAGTAGTGAATTCTCTGAGTGTGA AGCT/ GCAATGATTGCCACAACTACCA | 88 | 22.3 | 103.6 |
| SLC9A3 | NHE3 | XM_004935075.1 | $Na^+$-$H^+$ exchanger | AGGCTGGACCGGTTTGC/ TCCCGAATACTTTTCCTCCTTTG | 61 | 22.6 | 94.5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SLC15A1 | PepT1 | NM_204365.1 | $H^+$-dependent transporter of di- and tripeptides | CCCCTGAGGAGGATCACTGTT/ CAAAAGAGCAGCAGCAACGA | 66 | 20.8 | 96.0 |
| SLC16A1 | MCT1 | NM_001006323.1 | Monocarboxylic acid transporter | AGCAGCATCCTGGTGAACAAG/ AGGCACCCACCCACGAT | 59 | 25.7 | 95.8 |
| SLC38A1 | SAT1 | NM_001199603.1 | $Na^+$-dependent neutral amino acid transporter | CACAGTGCCAGTGCTGTTTTTC/ TGTTTTTCTTGCCAGCTCGAA | 61 | 29.3 | 96.5 |
| SLC38A2 | SAT2 | NM_001030741.1 | $Na^+$-dependent neutral amino acid transporter | TGCAACGCTGGCACCTT/ GGTTTGCATGCATCACTGATTG | 57 | 23.5 | 97.6 |
| SLC38A3 | SAT3 | NM_001199549.1 | $Na^+$-dependent neutral amino acid transporter | TCTTCATCCTTCCCTCTGCATT/ GAGACCTCATGGGCTCCTTCT | 60 | 27.0 | 93.3 |
| | β-actin | NM_205518.1 | Beta-actin | GTCCACCGCAAATGCTTCTAA/ TGCGCATTTATGGGTTTTGTT | 78 | 17.7 | 113.8 |
| | H6PD | XM_425746.4 | Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) | GACAGAGCCCCTTGGTCTCA/ TCAGAAGCCAGCTGGGAAAT | 58 | 24.3 | 105.9 |
| | LDHA | NM_205284.1 | Lactate dehydrogenase A | CACAAAGAGGAGCACGCTCAT/ CTGCACCCACACCAACCA | 58 | 20.6 | 110.9 |
| | PRL4 | NM_001007479.1 | Ribosomal protein L4 | TCAAGGCGCCCATTCG/ TGCGCAGGTTGGTGTGAA | 54 | 23.1 | 112.0 |
| | PRLP0 | NM_204987.1 | Ribosomal protein, large, P0 | GCGATTGCTCCCTGTGATG/ TCTCAGGTCCGAGACCAGTGT | 58 | 20.5 | 111.9 |
| | PRLP1 | NM_205322.1 | Ribosomal protein, large, P1 | TCTCCACGACGACGAAGTCA/ CCGCCGCCTTGATGAG | 62 | 20.9 | 97.3 |

[1] SLC: solute carrier family.
[2] Amplicon size.
[3] Cq = quantification cycle.
[4] Primers for rBAT, $B^0AT$, LAT1, $y^+LAT2$, $y^+LAT1$, $b^{0,+}AT$, PepT1 and β-actin were those used in Gilbert et al. (2007). The other primers were designed by Primer Express 3.0 software (Applied Biosystems, Grand Island, NY).

**Table 3.2** Mean body weight, feed conversion ratio, feed intake, average dry matter digestibility and mortality of male broiler chickens fed a diet with or without methionine supplementation until post hatch day 35.

| Treatment[1] | Mean body weight (g) | | | | | Feed conversion ratio[2] (d10 to d26) | Feed intake[2] (kg/bird, d10 to d26) | Average dry matter digestibility[3] (d26) | Mortality (d0 to d26) |
|---|---|---|---|---|---|---|---|---|---|
| | d10 (n=50~55) | d15 (n=43~50) | d21 (n=42~50) | d26 (n=36~44) | d35 (n=29~30) | | | | |
| C | 266.0±5.3$^{b}$ | 514.4±9.9$^{b}$ | 928.0±16.6$^{b}$ | 1235.0±27.2$^{b}$ | 1771.2±53.0$^{b}$ | 1.90±0.06$^{a}$ | 1.64±0.04 | 0.27 | 1.75% |
| DL-Met | 311.2±4.3$^{a}$ | 618.4±8.3$^{a}$ | 1131.0±16.5$^{a}$ | 1544.9±26.1$^{a}$ | 2303.8±62.3$^{a}$ | 1.44±0.01$^{b}$ | 1.70±0.02 | 0.26 | 3.08% |
| L-Met | 308.2±3.6$^{a}$ | 612.2±10.6$^{a}$ | 1112.6±25.3$^{a}$ | 1608.3±22.8$^{a}$ | 2390.2±54.5$^{a}$ | 1.43±0.02$^{b}$ | 1.70±0.04 | 0.27 | 1.69% |
| DL-HMTBA | 304.6±4.2$^{a}$ | 605.1±8.4$^{a}$ | 1113.0±14.4$^{a}$ | 1547.8±22.8$^{a}$ | 2300.1±66.3$^{a}$ | 1.43±0.01$^{b}$ | 1.66±0.04 | 0.38 | 4.92% |

[1] C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: diet supplemented with 0.22% DL-methionine; L-Met: diet supplemented with 0.22% L-methionine; DL-HMTBA: diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

[2] Feed conversion ratio = feed intake (g) / body weight gain (g). Feed intake was only reported from d10 to d26 because both males and females were present in the pens from d0 to d3 and thus feed intake for just males could not be determined for d0 to d10.

[3] Dry matter digestibility = $TiO_2$ in diet / $TiO_2$ in dry digesta.

Data are shown as least squares means ± standard errors of the means. Means in a column without a common letter differ, $P < 0.05$.

**Table 3.3** Relative breast muscle, kidney, liver, carcass, empty small intestine, abdominal fat and heart weight of male broiler chickens fed a diet with or without methionine supplementation at post-hatch days 10, 21, 26 and 35.

| Treatment | Relative breast muscle weight[1] (%) | | | | Relative kidney weight[2] (%) | | | | Relative liver weight[3] (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | d10 | d21 | d26 | d35 | d10 | d21 | d26 | d35 | d10 | d21 | d26 | d35 |
| C | 11.1±0.36$^{b}$ | 15.4±0.41$^{b}$ | 18.2±0.32$^{b}$ | 18.1±0.28$^{b}$ | 0.87±0.07 | 0.51±0.05$^{a}$ | 0.66±0.04 | 0.56±0.03$^{a}$ | 4.11±0.35 | 2.77±0.17$^{a}$ | 2.39±0.11 | 2.31±0.14 |
| DL-Met | 14.6±0.30$^{a}$ | 20.0±0.43$^{a}$ | 22.7±0.46$^{a}$ | 25.4±1.07$^{a}$ | 0.75±0.07 | 0.43±0.05$^{ab}$ | 0.58±0.06 | 0.45±0.03$^{b}$ | 3.76±0.04 | 2.23±0.11$^{ab}$ | 2.25±0.07 | 2.04±0.12 |
| L-Met | 13.1±0.66$^{a}$ | 20.3±0.35$^{a}$ | 22.5±0.81$^{a}$ | 24.3±1.31$^{a}$ | 0.71±0.06 | 0.38±0.04$^{ab}$ | 0.55±0.08 | 0.47±0.02$^{ab}$ | 3.65±0.20 | 2.15±0.16$^{b}$ | 2.42±0.14 | 1.94±0.09 |
| DL-HMTBA | 13.5±0.37$^{a}$ | 19.6±0.49$^{a}$ | 22.8±0.57$^{a}$ | 22.8±1.18$^{a}$ | 0.69±0.08 | 0.31±0.03$^{b}$ | 0.53±0.07 | 0.52±0.02$^{ab}$ | 3.63±0.16 | 2.16±0.10$^{b}$ | 2.22±0.12 | 2.26±0.16 |

| Treatment | Relative carcass weight[4] (%) | | | | Relative jejunum weight[5] (%) | | | Relative ileum weight[6] (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | d10 | d21 | d26 | d35 | d21[7] | d26 | d35 | d21 | d26 | d35 |
| C | 56.8 ± 2.91 | 60.1 ± 0.38 | 60.8 ± 0.98$^{a}$ | 61.3 ± 0.34$^{a}$ | 1.60 ± 0.16 | 1.31 ± 0.10 | 1.38 ± 0.06$^{a}$ | 1.25 ± 0.02 | 1.16 ± 0.05 | 1.09 ± 0.05$^{a}$ |
| DL-Met | 55.0 ± 0.54 | 60.0 ± 0.65 | 59.8 ± 0.88$^{a}$ | 56.0 ± 1.51$^{b}$ | 1.29 ± 0.06 | 1.18 ± 0.02 | 1.16 ± 0.06$^{ab}$ | 1.12 ± 0.06 | 1.02 ± 0.05 | 0.88 ± 0.05$^{b}$ |
| L-Met | 54.1 ± 0.95 | 59.6 ± 0.33 | 56.6 ± 0.72$^{b}$ | 57.2 ± 1.16$^{b}$ | 1.27 ± 0.06 | 1.18 ± 0.08 | 1.13 ± 0.05$^{b}$ | 1.08 ± 0.04 | 1.03 ± 0.04 | 0.92 ± 0.03$^{ab}$ |
| DL-HMTBA | 54.6 ± 0.40 | 58.4 ± 2.01 | 58.6 ± 0.51$^{ab}$ | 56.9 ± 0.83$^{b}$ | 1.30 ± 0.06 | 1.17 ± 0.06 | 1.18 ± 0.05$^{ab}$ | 1.12 ± 0.06 | 1.05 ± 0.04 | 0.95 ± 0.04$^{ab}$ |

| Treatment | Relative abdominal fat weight[8] (%) | | | | Relative heart weight[9] (%) | | | | Relative duodenum weight[10] (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | d10 | d21 | d26 | d35 | d10 | d21 | d26 | d35 | d21 | d26 | d35 |
| C | 0.65 ± 0.13 | 1.43 ± 0.10 | 1.45 ± 0.12 | 1.60 ± 0.18 | 0.82 ± 0.03 | 0.77 ± 0.04 | 0.85 ± 0.09 | 0.66 ± 0.03 | 0.72 ± 0.06 | 0.78 ± 0.07 | 1.09 ± 0.04 |
| DL-Met | 0.91 ± 0.09 | 1.32 ± 0.12 | 1.51 ± 0.09 | 1.29 ± 0.10 | 0.86 ± 0.04 | 0.65 ± 0.02 | 0.66 ± 0.03 | 0.60 ± 0.05 | 0.62 ± 0.04 | 0.68 ± 0.03 | 0.64 ± 0.05 |
| L-Met | 0.97 ± 0.16 | 1.23 ± 0.08 | 1.41 ± 0.13 | 1.29 ± 0.17 | 0.76 ± 0.04 | 0.74 ± 0.07 | 0.85 ± 0.11 | 0.57 ± 0.03 | 0.64 ± 0.04 | 0.72 ± 0.04 | 0.61 ± 0.02 |
| DL-HMTBA | 0.79 ± 0.03 | 1.19 ± 0.10 | 1.48 ± 0.18 | 1.44 ± 0.11 | 0.89 ± 0.03 | 0.74 ± 0.05 | 0.71 ± 0.07 | 0.56 ± 0.06 | 0.74 ± 0.02 | 0.68 ± 0.04 | 0.62 ± 0.02 |

[1] C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

[2] Relative breast muscle weight: breast muscle weight/live weight; [3] Relative kidney weight: kidney weight/live weight; [4] Relative liver weight: liver weight/live weight; [5] Relative carcass weight: carcass weight/live weight, carcass weight equals to body weight minus the head and internal organs. [5] Relative jejunum weight: empty jejunum weight/live weight; [6] Relative ileum weight: empty ileum weight/live weight. [7] The relative jejunum/ileum/duodenum weight data are reported only for d21, d26 and d35; [8] Relative abdominal fat weight: abdominal fat weight/live weight; [9] Relative heart weight: heart weight/live weight; [10] Relative duodenum weight: empty duodenum weight/live weight.

Data are shown as least squares means ± standard errors of the means. Means in a column without a common letter differ, $P < 0.05$. n=6.

**Table 3.4** Red blood cell packed cell volume (RBC-PCV) and white blood cell differential count of male broiler chickens fed a diet with or without methionine supplementation at post-hatch days 21, 26 and 35.

| Day | Treatment[1] | RBC-PCV (%) | Eosinophil + Heterophil (%) | Lymphocyte (%) | Basophil (%) | Monocyte (%) | (Eosinophil + Heterophil) / Lymphocyte |
|---|---|---|---|---|---|---|---|
| 21 | C | 29.7 | 40.7 | 49.8 | 4.3 | 5.2 | 0.83 |
| | DL-Met | 27.1 | 42.2 | 41.8 | 6.8 | 9.2 | 0.91 |
| | L-Met | 31.0 | 37.8 | 48.3 | 7.3 | 6.5 | 0.82 |
| | DL-HMTBA | 31.1 | 41.6 | 49.6 | 4.8 | 4.2 | 0.87 |
| | SEM | 1.62 | 2.7 | 3.0 | 1.2 | 1.6 | 0.08 |
| | *P*-value | 0.31 | 0.67 | 0.22 | 0.25 | 0.19 | 0.89 |
| 26 | C | 30.6 | 46.1 | 44.8 | 7.3 | 1.8 | 1.07 |
| | DL-Met | 30.4 | 44.7 | 44.5 | 6.7 | 4.2 | 1.12 |
| | L-Met | 28.7 | 41.7 | 48.2 | 5.5 | 4.7 | 0.90 |
| | DL-HMTBA | 29.6 | 54.7 | 36.7 | 5.0 | 3.7 | 1.37 |
| | SEM | 1.20 | 4.2 | 3.7 | 1.1 | 1.1 | 0.16 |
| | *P*-value | 0.67 | 0.18 | 0.20 | 0.45 | 0.29 | 0.28 |
| 35 | C | 31.4 | 37.6 | 53.4 | 3.6 | 6.0 | 0.62 |
| | DL-Met | 29.0 | 41.4 | 51.4 | 2.8 | 5.5 | 0.84 |
| | L-Met | 31.2 | 41.8 | 49.7 | 4.2 | 4.3 | 0.86 |
| | DL-HMTBA | 32.6 | 39.0 | 50.7 | 4.5 | 5.8 | 0.80 |
| | SEM | 1.19 | 2.8 | 3.1 | 0.7 | 1.4 | 0.11 |
| | *P*-value | 0.21 | 0.68 | 0.86 | 0.41 | 0.82 | 0.44 |

[1] C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

Data are shown as least squares means ± standard errors of the means. Means in a column without a common letter differ, $P < 0.05$. n=6.

**Table 3.5** Acute phase protein level of male broiler chickens fed a diet with or without methionine supplementation at post-hatch days 21 and 26.

| Day | Treatment[1] | Serum amyloid A (SAA) (ng/ml) | α-1-acid glycoprotein (α-1-AGP) (mg/ml) |
|---|---|---|---|
| 21 | C | 88.4[a] | 0.23 |
| | DL-Met | 37.4[b] | 0.14 |
| | L-Met | 25.8[b] | 0.20 |
| | DL-HMTBA | 43.7[ab] | 0.22 |
| | SEM | 12.5 | 0.03 |
| | *P*-value | 0.01 | 0.27 |
| 26 | C | 44.1 | 0.31 |
| | DL-Met | 32.9 | 0.24 |
| | L-Met | 71.3 | 0.29 |
| | DL-HMTBA | 47.2 | 0.26 |
| | SEM | 11.2 | 0.04 |
| | *P*-value | 0.14 | 0.61 |

[1] C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

Data are shown as least squares means ± standard errors of the means. Means in a column without a common letter differ, $P < 0.05$. n=6.

**Table 3.6** Relative small intestinal nutrient transporter gene expression using the whole statistical model[1]. Male broiler chickens were fed a diet with or without methionine supplementation until post-hatch day 35.

| Item | | Relative Gene Expression[3] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SAT1 | SAT2 | SAT3 | $ATB^{0,+}$ | $B^{0}AT$ | $b^{0,+}AT$ | rBAT | PepT1 | LAT1 | $y^{+}LAT1$ | $y^{+}LAT2$ | 4F2hc | MCT1 | NHE3 |
| Treatment (n=5) | C | 1.49 | 1.17 | 1.28 | 1.21 | 1.36 | 1.40 | 1.30 | 1.43 | 1.22 | 1.21 | 1.12 | 1.35 | 1.10 | 1.16 |
| | DL-Met | 1.25 | 1.17 | 1.24 | 2.07 | 1.44 | 1.26 | 1.15 | 1.38 | 1.14 | 1.14 | 1.18 | 1.11 | 1.17 | 1.34 |
| | L-Met | 1.46 | 1.23 | 1.33 | 1.73 | 1.25 | 1.28 | 1.15 | 1.23 | 1.26 | 1.27 | 1.11 | 1.11 | 1.22 | 1.21 |
| | DL-HMTBA | 1.35 | 1.17 | 1.27 | 1.59 | 1.29 | 1.37 | 1.19 | 1.29 | 1.19 | 1.12 | 1.13 | 1.27 | 1.23 | 1.33 |
| | SEM | 0.08 | 0.07 | 0.06 | 0.17 | 0.10 | 0.11 | 0.07 | 0.11 | 0.06 | 0.09 | 0.07 | 0.07 | 0.06 | 0.09 |
| | *P*-value | 0.16 | 0.93 | 0.78 | 0.01 | 0.58 | 0.75 | 0.47 | 0.54 | 0.64 | 0.65 | 0.86 | 0.03 | 0.32 | 0.40 |
| Segment (n=5) | Duodenum | 0.83 | 1.29 | 0.71 | 1.22 | 0.94 | 1.13 | 0.98 | 1.80 | 0.93 | 0.99 | 0.94 | 1.03 | 1.44 | 1.40 |
| | Jejunum | 1.56 | 1.11 | 1.28 | 1.21 | 0.99 | 0.98 | 1.16 | 1.32 | 1.33 | 1.30 | 1.24 | 1.05 | 1.12 | 1.51 |
| | Ileum | 1.78 | 1.15 | 1.84 | 2.52 | 2.08 | 1.87 | 1.47 | 0.87 | 1.35 | 1.26 | 1.22 | 1.54 | 0.99 | 0.88 |
| | SEM | 0.07 | 0.05 | 0.06 | 0.10 | 0.08 | 0.07 | 0.06 | 0.07 | 0.05 | 0.05 | 0.04 | 0.06 | 0.04 | 0.07 |
| | *P*-value | <0.01 | 0.03 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Age (n=5) | Day 3 | 1.49 | 1.39 | 1.23 | 1.18 | 1.46 | 1.58 | 1.41 | 1.24 | 1.34 | 1.40 | 1.00 | 0.87 | 0.94 | 0.98 |
| | Day 5 | 2.32 | 1.39 | 1.61 | 2.36 | 1.25 | 1.26 | 1.08 | 1.36 | 1.21 | 1.36 | 1.48 | 1.88 | 1.62 | 1.52 |
| | Day 10 | 1.89 | 1.61 | 1.54 | 1.78 | 1.80 | 1.63 | 1.48 | 1.46 | 1.66 | 1.82 | 1.42 | 1.01 | 1.00 | 1.38 |
| | Day 21 | 0.69 | 1.07 | 1.34 | 2.65 | 1.36 | 1.19 | 1.20 | 1.49 | 1.15 | 0.90 | 1.09 | 1.12 | 1.14 | 1.38 |
| | Day 26 | 0.86 | 0.88 | 1.14 | 1.22 | 1.35 | 1.18 | 1.04 | 1.46 | 0.91 | 0.83 | 0.99 | 1.24 | 1.15 | 1.32 |
| | Day 35 | 1.08 | 0.76 | 0.80 | 0.72 | 0.82 | 1.12 | 0.99 | 0.97 | 0.93 | 0.80 | 0.82 | 1.12 | 1.24 | 1.01 |
| | SEM | 0.10 | 0.08 | 0.08 | 0.21 | 0.13 | 0.13 | 0.09 | 0.13 | 0.08 | 0.11 | 0.08 | 0.09 | 0.07 | 0.11 |
| | *P*-value | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.02 | <0.01 | 0.05 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Interaction[2] | T×S | 0.60 | 0.04 | 0.79 | 0.02 | 0.96 | 0.77 | 0.81 | 0.79 | 0.82 | 0.68 | 0.83 | 0.80 | 0.23 | 0.61 |
| | S×A | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.1 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| | T×A | 0.14 | 0.30 | 0.12 | <0.01 | 0.96 | 0.96 | 0.96 | 0.20 | 0.07 | 0.83 | 0.54 | 0.31 | 0.83 | 0.30 |
| | T×S×A | 0.99 | 0.58 | 0.78 | <0.01 | 0.24 | 0.46 | 0.71 | 0.40 | 0.63 | 0.48 | 0.73 | 0.97 | 0.95 | 0.98 |

[1] The whole statistical model included main effects of treatment (C, DL-Met, L-Met, DL-HMTBA), age (d3, 5, 10, 21, 26, 35), and small intestine segment (duodenum, jejunum, ileum) and their interactions.

[2] For the interaction, T, S and A represent the effects of treatment, small intestine segment and age, respectively.

[3] Data are shown as least squares means ± standard errors of the mean. Means in a column without a common letter differ, $P < 0.05$. n=5.

**Table 3.7** Contrasts for significant treatment × age interaction effects for LAT1, $ATB^{0,+}$ and $B^0AT$ expression analyzed within each small intestinal segment. Male broiler chickens were fed a diet with or without methionine supplementation until post-hatch day 35.

| | Treatment[1] | | | | | | Significant *P*-values for Contrasts[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | DL-Met | L-Met | DL-HMTBA | SEM[2] | *P*-value[3] | DL-Met vs. L-Met | DL-Met vs. DL-HMTBA | L-Met vs. DL-HMTBA | C vs. DL-Met | C vs. L-Met | C vs. DL-HMTBA |
| **Duodenum**, *LAT1*[5] | | | | | 0.10 | 0.033 | | | | | | |
| Day 3 | 0.91 | 0.82 | 1.74 | 0.83 | | | 0.002 | | 0.002 | | 0.004 | |
| Day 5 | 0.66 | 0.82 | 0.70 | 0.91 | | | | | | | | |
| Day 10 | 1.68 | 1.15 | 1.03 | 0.82 | | | | | | | 0.023 | 0.003 |
| Day 21 | 1.07 | 1.06 | 1.29 | 0.99 | | | | | | | | |
| Day 26 | 0.42 | 0.51 | 0.61 | 0.75 | | | | | | | | |
| Day 35 | 0.79 | 1.02 | 0.75 | 1.00 | | | | | | | | |
| **Jejunum**, $ATB^{0,+}$ | | | | | 0.20 | 0.033 | | | | | | |
| Day 3 | 0.68 | 1.14 | 1.68 | 0.66 | | | | | | | | |
| Day 5 | 0.60 | 3.44 | 2.83 | 3.00 | | | | | | <0.001 | <0.001 | <0.001 |
| Day 10 | 1.37 | 1.26 | 0.85 | 1.74 | | | | | | | | |
| Day 21 | 0.87 | 1.75 | 1.36 | 1.14 | | | | | | | | |
| Day 26 | 0.51 | 0.49 | 0.58 | 1.05 | | | | | | | | |
| Day 35 | 0.48 | 0.68 | 0.54 | 0.52 | | | | | | | | |
| **Jejunum**, $B^0AT$ | | | | | 0.16 | 0.038 | | | | | | |
| Day 3 | 0.71 | 0.51 | 0.83 | 0.55 | | | | | | | | |
| Day 5 | 0.85 | 0.84 | 0.50 | 0.83 | | | | | | | | |
| Day 10 | 0.69 | 0.39 | 0.52 | 0.95 | | | | | | | | |
| Day 21 | 1.26 | 1.40 | 1.53 | 1.00 | | | | | | | | |
| Day 26 | 1.21 | 2.92 | 1.20 | 1.24 | | | <0.001 | <0.001 | | <0.001 | | |
| Day 35 | 1.46 | 0.81 | 0.82 | 0.75 | | | | | | | | |

| | Treatment[1] | | | | | | Significant *P*-values for Contrasts[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | DL-Met | L-Met | DL-HMTBA | SEM[2] | *P*-value[3] | DL-Met vs. L-Met | DL-Met vs. DL-HMTBA | L-Met vs. DL-HMTBA | C vs. DL-Met | C vs. L-Met | C vs. DL-HMTBA |
| **Ileum**, *ATB$^{0,+}$* | | | | | 0.36 | <0.001 | | | | | | |
| Day 3 | 0.54 | 0.83 | 1.43 | 0.90 | | | | | | | | |
| Day 5 | 0.93 | 4.49 | 1.99 | 3.06 | | | 0.015 | | | <0.001 | | 0.037 |
| Day 10 | 2.47 | 0.90 | 2.32 | 3.08 | | | | | | | | |
| Day 21 | 5.45 | 9.56 | 4.17 | 3.65 | | | <0.001 | <0.001 | | <0.001 | | |
| Day 26 | 3.09 | 1.99 | 2.26 | 1.73 | | | | | | | | |
| Day 35 | 0.94 | 0.51 | 1.59 | 0.72 | | | | | | | | |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d3, 5, 10, 21, 26, 35) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

[2,3] These values are pooled SEM of the mean and the *P*-value for the interaction, respectively.

[4] All contrasts were tested but for brevity only significant differences are displayed.

[5] LAT1: $Na^+$-independent large neutral amino acid transporter; $ATB^{0,+}$: $Na^+$- and $Cl^-$-dependent neutral and cationic amino acid transporter; $B^0AT$: $Na^+$-dependent neutral amino acid transporter.

**Table 3.8** Contrasts for significant treatment × segment interaction effects for 4F2hc, $b^{0,+}AT$, $ATB^{0,+}$, $B^0AT$, LAT1 and SAT2 expression analyzed within each growth period. Male broiler chickens were fed a diet with or without methionine supplementation until post-hatch day 35.

| | Treatment[1] | | | | | | Significant *P*-values for Contrasts[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | DL-Met | L-Met | DL-HMTBA | SEM[2] | *P*-value[3] | DL-Met vs. L-Met | DL-Met vs. DL-HMTBA | L-Met vs. DL-HMTBA | C vs. DL-Met | C vs. L-Met | C vs. DL-HMTBA |
| **Day 3**, *4F2hc*[5] | | | | | 0.05 | 0.009 | | | | | | |
| Duodenum | 0.89 | 0.84 | 0.77 | 0.83 | | | | | | | | |
| Jejunum | 0.50 | 0.51 | 0.72 | 0.54 | | | | | | | | |
| Ileum | 1.15 | 1.07 | 0.97 | 1.61 | | | | <0.001 | <0.001 | | | 0.003 |
| **Day 3**, *$b^{0,+}AT$* | | | | | 0.16 | 0.037 | | | | | | |
| Duodenum | 1.00 | 1.18 | 1.29 | 1.27 | | | | | | | | |
| Jejunum | 0.70 | 0.65 | 0.86 | 0.83 | | | | | | | | |
| Ileum | 3.07 | 2.59 | 1.72 | 3.76 | | | | 0.014 | <0.001 | | 0.005 | |
| **Day 21**, *$ATB^{0,+}$* | | | | | 0.45 | 0.013 | | | | | | |
| Duodenum | 0.45 | 0.70 | 1.68 | 1.18 | | | | | | | | |
| Jejunum | 0.87 | 1.75 | 1.36 | 1.14 | | | | | | | | |
| Ileum | 5.45 | 9.56 | 4.17 | 3.65 | | | <0.001 | <0.001 | | 0.005 | | |
| **Day 26**, *$B^0AT$* | | | | | 0.19 | 0.044 | | | | | | |
| Duodenum | 0.99 | 0.96 | 0.65 | 0.76 | | | | | | | | |
| Jejunum | 1.21 | 2.92 | 1.20 | 1.24 | | | 0.004 | 0.005 | | 0.004 | | |
| Ileum | 2.20 | 1.12 | 1.58 | 1.30 | | | | | | | | |

| | Treatment[1] | | | | | | Significant *P*-values for Contrasts[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | DL-Met | L-Met | DL-HMTBA | SEM[2] | *P*-value[3] | DL-Met vs. L-Met | DL-Met vs. DL-HMTBA | L-Met vs. DL-HMTBA | C vs. DL-Met | C vs. L-Met | C vs. DL-HMTBA |
| **Day 26**, *LAT1* | | | | | 0.07 | 0.002 | | | | | | |
| Duodenum | 0.42 | 0.51 | 0.61 | 0.75 | | | | | | | | |
| Jejunum | 1.95 | 1.00 | 1.14 | 1.04 | | | | | | | | |
| Ileum | 1.08 | 0.80 | 0.81 | 0.77 | | | | | | <0.001 | <0.001 | <0.001 |
| **Day 35**, *ATB$^{0,+}$* | | | | | 0.11 | 0.033 | | | | | | |
| Duodenum | 0.28 | 1.02 | 0.57 | 0.83 | | | | | | 0.003 | | |
| Jejunum | 0.48 | 0.68 | 0.54 | 0.52 | | | | | | | | |
| Ileum | 0.94 | 0.51 | 1.59 | 0.72 | | | 0.002 | | 0.010 | | | |
| **Day 35**, *SAT2* | | | | | 0.06 | 0.039 | | | | | | |
| Duodenum | 0.72 | 1.39 | 1.07 | 0.80 | | | | 0.003 | | <0.001 | | |
| Jejunum | 0.73 | 0.63 | 0.80 | 0.80 | | | | | | | | |
| Ileum | 0.57 | 0.52 | 0.54 | 0.58 | | | | | | | | |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of small intestine segment (duodenum, jejunum, ileum) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

[2,3] These values are pooled SEM of the mean and the *P*-value for the interaction, respectively.

[4] All contrasts were tested but for brevity only significant differences are displayed.

[5] 4F2hc: 4F2 cell-surface antigen heavy chain; $b^{0,+}AT$: $Na^+$-independent cationic and neutral amino acid transporter; $ATB^{0,+}$: $Na^+$- and $Cl^-$-dependent neutral and cationic amino acid transporter; $B^0AT$: $Na^+$-dependent neutral amino acid transporter; LAT1: $Na^+$-independent large neutral amino acid transporter; SAT2: $Na^+$-dependent neutral amino acid transporter.

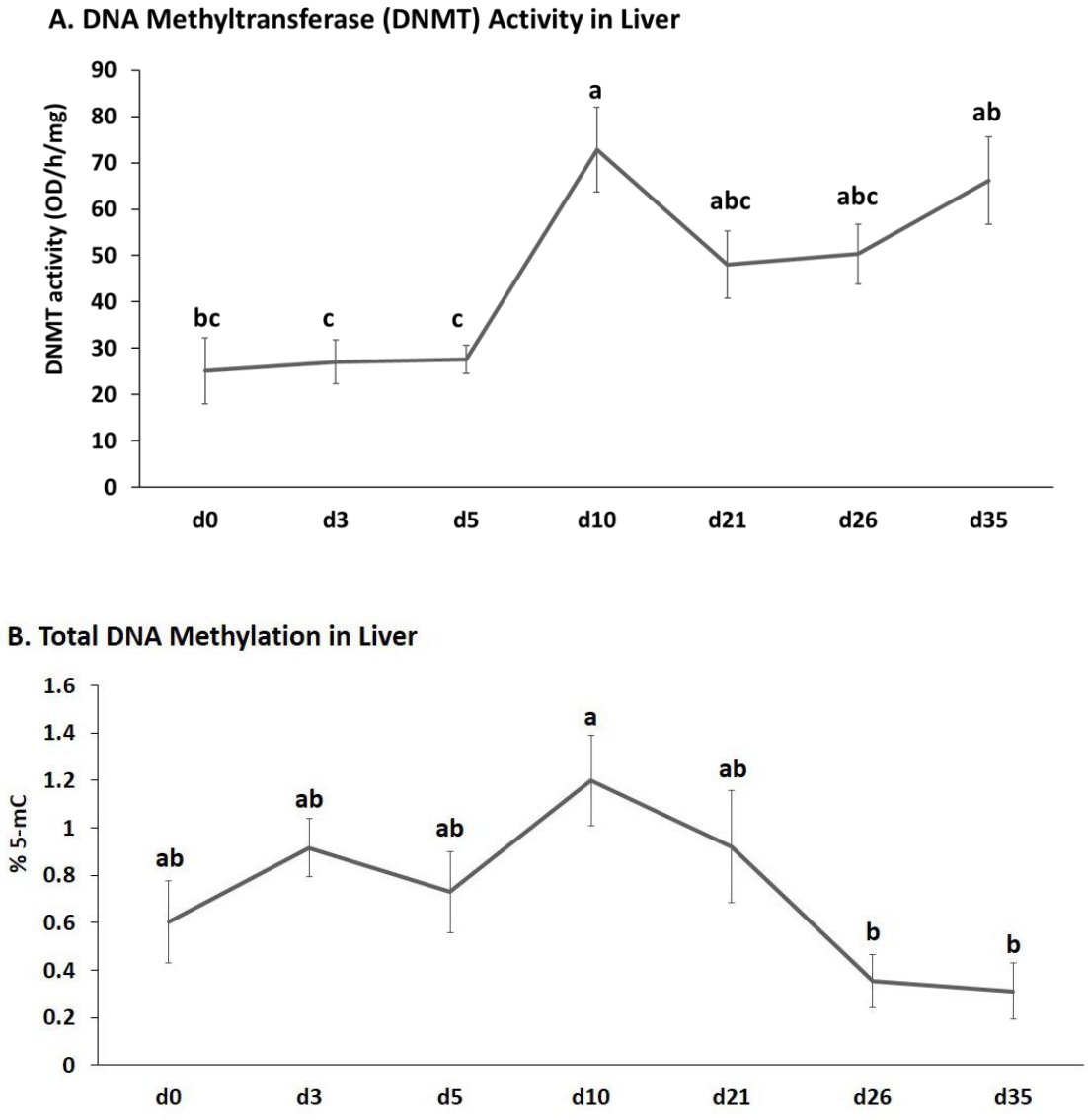


**Figure 3.1** Temporal changes in DNA methyltransferase activity and total DNA methylation in liver of chickens. **(A)** Hepatic DNA methyltransferase (DNMT) activity and **(B)** total DNA methylation in liver in male broiler chickens at post-hatch days 0, 3, 5, 10, 21, 26 and 35. Because there was no difference between four treatment groups, we pooled them (n=24; 4 treatments each of 6 replicates). Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant difference, $P < 0.05$.

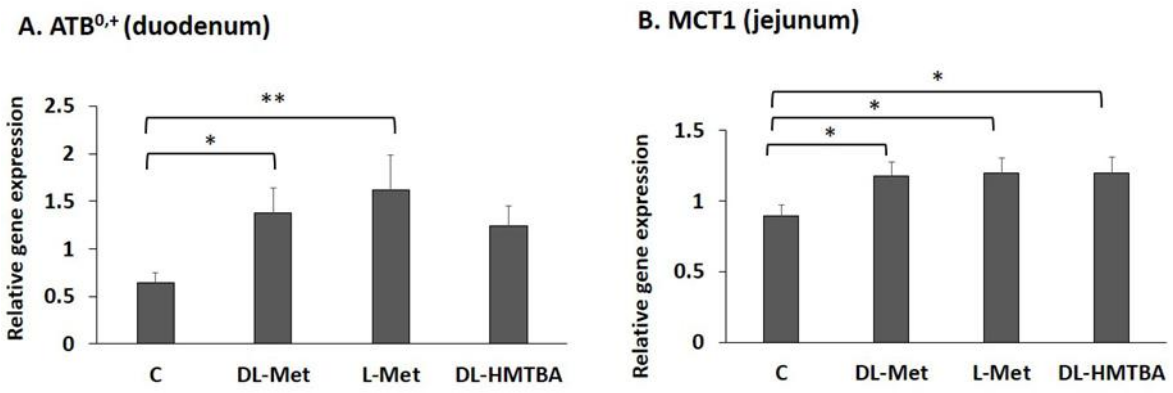


**Figure 3.2** Relative gene expression of intestinal nutrient transporters from chickens fed different methionine sources analyzed by segment. **(A)** Relative $ATB^{0,+}$ mRNA abundance in duodenum and **(B)** MCT1 mRNA abundance in jejunum in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=5). Figure shows main effect of treatment within each small intestine segment across all the ages. Values represent least squares means ± SEM. Means were compared using contrasts. * $P < 0.05$, ** $P < 0.01$.

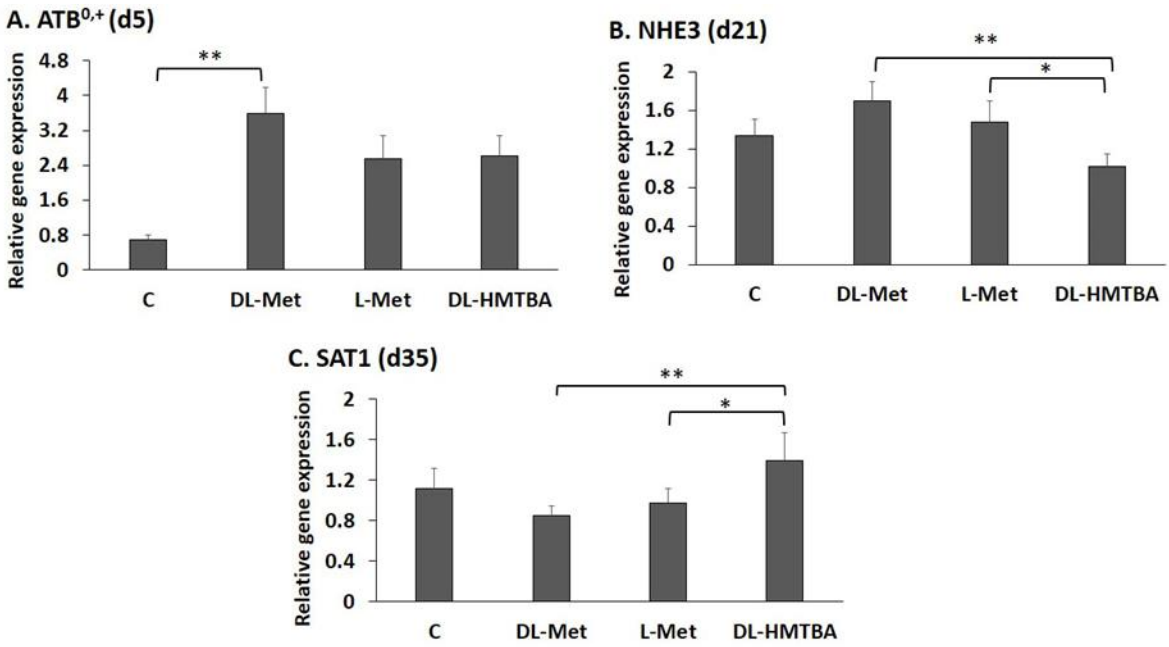


**Figure 3.3** Relative gene expression of intestinal nutrient transporters from chickens fed different methionine sources analyzed by age. **(A)** Relative $ATB^{0,+}$ mRNA abundance at post-hatch day 5 (d5), **(B)** NHE3 mRNA abundance at d21 and **(C)** SAT1 mRNA abundance at d35 in small intestine in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=5). Figure shows main effect of treatment within each age across all the small intestine segments. Values represent least squares means ± SEM. Means were compared using contrasts. * $P < 0.05$, ** $P < 0.01$.

# CHAPTER 4 Methionine Converting Enzymes, Oxidative Stress Markers and Amino Acid Signaling in Broiler Chickens Fed Methionine Isomers or Precursors

## 4.1 Abstract

The common dietary supplemental methionine (Met) sources include DL-Met and the analog DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). D-Met and DL-HMTBA must be converted to L-Met through oxidation and transamination for utilization. Met also plays an important role in the defense against oxidative stress. The objective of this study was to determine the effect of different dietary Met source supplementation and Met deficiency on mRNA expression and localization and enzyme activity of Met converting enzymes, as well as oxidative status and amino acid signaling pathway of broiler chickens. Liver, breast muscle, duodenum, jejunum and ileum were collected at days 10, 21 and 26 from male Cobb 500 chickens fed a diet deficient in sulfur amino acids (control), or the control diet supplemented with DL-Met, L-Met or DL-HMTBA to meet requirements (n=5). The mRNA abundance of three oxidases and four transaminases was measured by real-time PCR, and the oxidase activity was measured using colorimetric assays. Markers of oxidative stress including glutathione (GSH) profile, protein carbonyl, thiobarbituric acid reactive substances (TBARS) and ferric reducing/antioxidant power (FRAP) were assayed using commercial kits. The expression of the effector gene p70S6K in the SAT2/mTOR pathway was assayed using western blot. Both mRNA and protein of the Met converting enzymes showed tissue- and development-specific expression. DL-HMTBA and L-Met enhanced hepatic L-HMTBA oxidase (L-2-hydroxy acid oxidase, HAO1) gene expression, all three Met sources

decreased D-Met oxidase (D-aspartate oxidase, DAO) gene expression, while DL-Met increased glutamic-oxaloacetic transaminase 2 gene expression in duodenum. However, there was no significant differences for the three oxidase activities among treatment groups. There were significant tissue effects for all the oxidative stress markers independent of treatment. Total GSH (TGSH) and reduced GSH (rGSH) levels varied among groups in breast muscle and ileum. Nevertheless, the ratio of rGSH/TGSH, which actually defines the oxidative status, was not affected by Met sources. Protein carbonyl levels also varied among groups in jejunum and muscle tissue, but these values are in the normal physiological range. TBARS and FRAP levels were not different between groups in different tissues. In addition, DL-HMTBA decreased phospho-p70S6K protein expression in jejunum, which was inconsistent with the increase in SAT2 gene expression. In conclusion, there was complex regulation of Met converting enzyme and the SAT2/mTOR/p70S6K pathway by dietary Met sources. Different Met sources and Met deficiency did not alter oxidative status of the treated chickens, but L-Met and DL-HMTBA supplementation to a Met-deficient diet can increase the intracellular antioxidant capacity produced by GSH in breast muscle.

## 4.2 Introduction

Methionine (Met) is the first limiting amino acid in broiler chicken diets. It is also a precursor for the synthesis of proteins and serves as the predominant amino acid for translation initiation. In addition, Met participates in methyl group metabolism and thus is involved in synthesis of the other sulfur amino acids, especially cysteine. Cysteine is an essential component for glutathione (GSH) and taurine synthesis, which play a vital role in host defense against oxidative stress (Metayer et al., 2008). Thus, Met deficiency results in reduced protein accretion and growth rate as well as impaired immune competence (Zhang and Guo, 2008).

Dietary supplemental Met sources are provided in the feed for cost efficiency. Two common Met sources are DL-Met and DL-Met hydroxy analog, which is also known as DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). Dietary DL-HMTBA is added either as a free acid or as a calcium salt. DL-HMTBA lacks an amino group and thus is not a true amino acid but an amino acid precursor.

DL-Met and DL- HMTBA are transported by different transporter systems in the small intestine. DL-Met is transported by the $Na^+$-independent systems $b^{0,+}$ and L, and the $Na^+$-dependent systems $y^+$, B and A (Soriano-Garcia et al., 1998). In contrast, DL-HMTBA transport was mediated by the monocarboxylate transporter 1 (MCT1) and coupled to the $Na^+$-$H^+$ exchanger (NHE3) (Martin-Venegas et al., 2007; 2008) .

Both D-Met and DL-HMTBA must be converted to L-Met for utilization at the cellular level. D-Met is first oxidized to 2-keto-4 (methylthio) butanoic acid (KMB) by the enzyme D-amino acid oxidase (D-AAOX; EC 1.4.3.3), which is a peroxisomal enzyme containing flavin adenine dinucleotide (FAD) as a cofactor. In rat liver, KMB is

transaminated by the enzyme transaminase using glutamine or asparagine as amino donors to produce L-Met (Backlund et al., 1982). In contrast, in chicken liver, leucine, isoleucine and valine act as amino donors for Met synthesis while glutamine is much less effective (Gordon and Sizer, 1965). Transaminase is ubiquitous and is not thought to be a limiting step in the transformation process, although this has not been definitively demonstrated.

D-HMTBA and L-HMTBA are transformed to KMB using two different enzymatic systems, which are located in two different cell organelles (Dibner and Knight, 1984). L-2-hydroxy acid oxidase (L-HAOX; EC 1.1.3.15) is mainly located in the hepatic and renal peroxisomes of chickens and rats, and catalyzes the conversion of L-HMTBA to KMB. There are two isoforms of L-HAOX, HAO1 and HAO2. HAO1 is believed to be involved mainly in L-HMTBA oxidation because it preferentially oxidizes short-chain aliphatic 2-hydroxyacids (Ferjancic-Biagini et al., 1998). D-2-hydroxy acid dehydrogenase (D-HADH; EC 1.1.99.6) is located in the mitochondria in various tissues, and catalyzes the conversion of D-HMTBA to KMB. FAD and flavin mononucleotide (FMN) are common cofactors for both L-HAOX and D-HADH, and hydrogen peroxide ($H_2O_2$) is the common byproduct. In both cases the resulting KMB is transaminated to L-Met by transaminase. Conversion of DL-HMTBA to Met occurs principally in the liver. However, this conversion also occurs in the small intestine (Martin-Venegas et al., 2006; 2011) .

Met is directly related to synthesis of GSH and cysteine, which are direct scavengers of reactive oxygen species (ROS) and thus can alleviate the deleterious effect of lipid peroxidation and protein oxidation (Swennen et al., 2011). Therefore, it is

reasonable to hypothesize that dietary Met supplementation is beneficial in alleviating oxidative stress. The commonly used oxidative stress markers include the glutathione (GSH) profile, protein carbonyl, thiobarbituric acid reactive substances (TBARS) and ferric reducing /antioxidant power (FRAP). GSH is the key antioxidant in animal tissues, which can easily reduce the disulfide bonds in proteins to cysteine and in the process be converted to the oxidized form GSSG. In normal tissues, most of the glutathione is present as the reduced form (rGSH) compared to the oxidized form (GSSG). Thus a decrease in the rGSH/total GSH (TGSH) ratio is an indicator of oxidative stress. Protein carbonyl content is the most commonly used marker of protein oxidation, whereas TBARS is the most widely employed assay used to determine lipid peroxidation. FRAP is usually used to represent antioxidant capacity.

The availability of amino acids is monitored by cellular amino acid sensors. For example, the sodium-dependent neutral amino acid transporter-2 (SAT2, SLC38A2), serves as an amino acid transporter as well as an amino acid sensor and has been termed a transceptor. SAT2 is a member of the System A amino acid transporters, which mediates its transceptor signaling through the mTOR pathway (Pinilla et al., 2011). Amino acids can also act as a nutrient signal to regulate intracellular kinases. A key signaling kinase downstream of mTOR is p70S6K, which is a serine/threonine kinase that phosphorylates the 70-kDa ribosomal protein S6. Elevated levels of mTOR activity are associated with increased phosphorylation of p70S6K, which in turn phosphorylates S6, resulting in increased protein synthesis and cell proliferation.

The objective of this study was to determine the effect of different dietary Met source supplementation and Met deficiency on gene expression of Met converting

enzymes (oxidases and transaminases), enzymatic activity and mRNA localization of Met oxidases, markers of oxidative stress including GSH profile, protein carbonyl, TBARS and FRAP levels, and expression of the effector genes (p70S6K) in the amino acid signaling (SAT2/mTOR) pathway in male broiler chickens. We hypothesized that dietary Met supplementation could alter the expression profiles of Met converting enzymes, oxidative stress markers and the p70S6K protein expression.

## 4.3 Materials and Methods

### 4.3.1 Animals

Details about the animals used in this study are the same as described in 3.3.1.

### 4.3.2 Met converting enzyme gene expression

Liver, breast muscle and three small intestinal segments (duodenum, jejunum, and ileum) from five birds per treatment on d10, 21 and 26 were used in this assay. Total RNA was isolated and purified and first-strand cDNA was synthesized, following the same protocol as described in section 3.3.5. Expression of three oxidase genes: D-amino acid oxidase (DAO), L-2-hydroxy acid oxidase (HAO1) and D-2-hydroxy acid dehydrogenase (LDHD) and four transaminase genes: alanine-glyoxylate aminotransferase (AGXT), branched chain amino acid transaminase (BCAT1), glutamic-oxaloacetic transaminase (GOT2) and ornithine aminotransferase (OAT), and 3 reference genes: ribosomal protein L4 (PRL4), ribosomal protein large, P0 (PRLP0) and ribosomal protein large, P1 (PRLP1) were assayed using relative quantification real time PCR. Primers designed for real time PCR are listed in Table 4.1 and were validated for amplification efficiency before use (90-115% efficiency). Real-time PCR was performed and relative gene expression was calculated as described in section 3.3.5.

### 4.3.3 Enzymatic activity of Met oxidases

Liver, breast muscle and three small intestinal segments (duodenum, jejunum, and ileum) from five birds per treatment on d10, 21 and 26 were prepared as described by Dibner and Knight (1984). Approximately 0.1g of tissue sample was homogenized in 1 ml of homogenizing buffer using 5 mm stainless steel beads and Tissue Lyser II. The homogenizing buffer consisted of 0.25 M sucrose, 1 mM phenyl methylsulfonyl fluoride and 0.02 M potassium phosphate, pH 7.5. Mitochondrial and peroxisomal fractions were prepared by differential centrifugation. Tissue homogenates were centrifuged at 350 × g for 10 min to remove cells and cell debris. The supernatant was centrifuged at 3,000 × g for 15 min to enrich for mitochondria in the pellet, which was subsequently resuspended and washed twice in 0.01 M Tris, pH 6.8. The supernatant from the 3,000 × g step was centrifuged at 22,000 × g for 15 min to yield a peroxisome enriched pellet. The peroxisome pellet was resuspended and washed twice in 0.01 M Tris, pH 7.5. Protein concentration in both fractions was measured using the Bradford assay (Sigma-Aldrich, St. Louis, MO).

L-HAOX activity was measured in the peroxisome fraction. 0.1 ml (approximately 0.2 mg protein) of peroxisome fraction was added to a reaction buffer containing 0.75 ml 0.01 M Tris, pH 7.5, 2.5 μmol L-HMTBA, 0.25 μmol flavin adenine dinucleotide (FAD) and 0.15 μmol cycloserine. D-AAOX activity was measured in the mitochondrial fraction. 0.1 ml (approximately 0.2 mg protein) of mitochondrial fraction was added to a reaction buffer containing 0.75 ml 0.01 M Tris, pH 7.5, 2.5 μmol D-Met, 0.25 μmol FAD and 0.15 μmol cycloserine. D-HADH activity was measured in the mitochondrial fraction. 0.1 ml (approximately 0.2 mg protein) of mitochondrial fraction

was added in a reaction buffer containing 0.75 ml 0.01 M Tris, pH 8.24, 2.5 μmol D-HMTBA and 0.5 μmol phenazine methosulfate as an electron acceptor. All the enzyme reactions were incubated at 37ºC for 90 min on a rotary shaker. Purified L-HMTBA and D-HMTBA were chemically synthesized and provided by Dr. Kevin J. T. Noonan (Carnegie Mellon University, Pittsburgh, PA).

Enzyme reactions were stopped by adding 0.75 ml of cold 10% trichloroacetic acid and incubated on ice for 10 minutes. The produced 2-keto-4 (methylthio) butanoic acid (KMB) was detected using a spectrophotometric assay. Briefly, the enzyme reactions were centrifuged at 12,000 × g for 3 min. A 200μl aliquot of supernatant was transferred to a 96-well plate, and 60 μl of 0.25% dinitrophenylhydrazine in 2 N HCl was added. After a 10 min incubation at room temperature, 140 μl of 4 N NaOH was added and the keto acid was quantified colorimetrically with a multi-mode plate reader (Infinite M200 Pro, Tecan, Morrisville, NC). The amount of KMB is proportional to the optical density measured at 435 nm. One unit of enzyme activity was defined as the amount yielding 1 μg of KMB per milligram of protein under standard reaction conditions.

### 4.3.4 In situ hybridization of Met oxidases

Liver, breast muscle and three small intestinal segments (duodenum, jejunum, and ileum) from four treatment groups were collected at d10 (n=1). Samples were rinsed in PBS and submerged and fixed in 10% neutral-buffered formalin at 4ºC for overnight incubation. Samples were then dehydrated in 70% ethanol, paraffin embedded by Histo-Scientific Research Lab (Mount Jackson, VA) and sectioned at 6 μm using a HM355 S rotary microtome (Microm International GmbH, Germany). The RNAscope® assay (Wang et al., 2012) was used for in situ RNA analysis of L-HMTBA oxidase L-2-

hydroxy acid oxidase (HAO1) and D-HMTBA oxidase D-2-hydroxy acid dehydrogenase (LDHD) in the formalin-fixed paraffin-embedded (FFPE) samples. Probes for HAO1 and LDHD were synthesized by Advanced Cell Diagnostics (Hayward, CA). The RNAscope® assay was conducted using the corresponding commercial kits provided by Advanced Cell Diagnostics, following the manufacturer's protocol. Briefly, the prepared sections were deparaffinized using xylene, and pretreated with hydrogen peroxide, retrieval reagent and protease to expose the target RNA. Gene-specific "Z"-shape probe pairs were used to hybridize to the target mRNA. Probes were then hybridized to a cascade of signal amplification molecules to culminate in binding of HRP-labeled probes, and 3,3'-diaminobenzidine (DAB) was added to detect the target. Slides were counterstained with 50% hematoxylin and 0.02% ammonia water, and mounted for visualization. Images were captured at 400 × magnification with a Nikon Eclipse 80i microscope and DS-Ri1 super high-definition cooled color camera head, and analyzed by NIS-Elements Advanced Research Software (Nikon). The target mRNA was revealed as brown dots. PPIB (peptidylprolyl isomerase B) was used as a positive control. Bacterial gene dapB (dihydrodipicolinate reductase) was used as a negative control.

### 4.3.5 Assays for oxidative stress markers

Markers of oxidative stress including total glutathione (TGSH), oxidized glutathione (GSSG), reduced glutathione (rGSH), protein carbonyl, thiobarbituric acid reactive substances (TBARS) and ferric reducing/antioxidant power (FRAP) were assayed in liver, breast muscle and three small intestinal segments (duodenum, jejunum, and ileum) from five birds per treatment on d10 and d26 using commercially available ELISA kits.

TGSH, GSSG and rGSH were measured using an enzymatic recycling method provided by the Glutathione Assay Kit (Cayman Chemical Company, Ann Arbor, MI), following the manufacturer's protocol. Briefly, 0.2 g samples were first deproteinized, and then the GSH in the sample was allowed to react with DTNB (5,5'-dithio-bis-2-(nitrobenzoic acid), Ellman's reagent) to produce the yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) was reduced by glutathione reductase to recycle the GSH and produce more TNB. The amount of TNB production, quantified colorimetrically with the Infinite M200 Pro at 410 nm, was directly proportional to the concentration of TGSH in the sample. GSH was easily oxidized to the disulfide dimer GSSG by glutathione peroxidase. Quantification of GSSG, exclusive of GSH, was accomplished by first derivatizing GSH with 2-vinylpyridine, then following the above enzymatic method. rGSH levels were calculated by substracting twice the GSSG concentration from the TGSH concentration. To better represent the redox status, the rGSH to TGSH ratio and rGSH to GSSG ratio were also calculated.

Protein carbonyl was measured using the Protein Carbonyl Colorimetric Assay Kit (Cayman Chemical Company), following the manufacturer's protocol. Briefly, 0.2 g samples were used and nucleic acids were first removed using streptomycin sulfate. Then 2,4-dinitrophenylhydrazine (DNPH) was added to react with protein carbonyls, forming a Schiff base to produce hydrazine, which was quantified colorimetrically with the Infinite M200 Pro at 367 nm.

TBARS was measured using the TBARS Assay Kit (Cayman Chemical Company), following the manufacturer's protocol. Briefly, the MDA-TBA adduct formed

by the reaction of malondialdehyde (MDA) and TBA at 100°C and acidic conditions was quantified colorimetrically with the Infinite M200 Pro at 535 nm.

FRAP was measured using the DetectX® FRAP™ Colorimetric Detection Kit (Arbor Assays, Ann Arbor, MI), following the manufacturer's protocol. Briefly, ferric iron ($Fe^{3+}$) can be reduced by electron-donating antioxidants present within the sample to its ferrous form ($Fe^{2+}$). The iron colorimetric probe complex then can develop a dark blue color product upon reduction, which was measured with the Infinite M200 Pro at 560 nm.

### 4.3.6 Western blot assay for p70S6K expression

Liver, breast muscle and three small intestinal segments (duodenum, jejunum, and ileum) from 4 birds per treatment on d10, 21 and 26 were used for western blot assay. Approximately 0.1 g tissue was minced using razor blades in liquid nitrogen and homogenized in RIPA buffer (Sigma-Aldrich) with Halt Protease Inhibitor (Thermo Scientific) using 5 mm stainless steel beads and a Tissue Lyser II. Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Nineteen micrograms of protein (9.5 µL at 2 µg/µL) were added to an equal volume of TruSep SDS Sample Buffer (NuSep, Bogart, GA) with 1 µL 2-mercaptoethanol, vortexed, and boiled for 5 min at 95ºC. Samples were loaded onto a 10% Criterion™ TGX™ Precast Midi Protein Gel (Bio-Rad, Hercules, CA). The HyperPAGE II Prestained Protein Marker (Bioline USA, Taunton, MA) was loaded in the first well of each gel. Samples from the same replicate and the same tissue were run as duplicate gels. One replicate was used for p70S6K protein detection and the other for phospho-p70S6K protein detection. Gels were run at 200 V for 40 min and then equilibrated for 10 min in cold CAPS transfer buffer (10×CAPS stock buffer, 10%

methanol). Immun-Blot™ PVDF membranes (Bio-Rad) were pre-wetted in methanol and placed together with 3 MM cellulose chromatography papers (GE Healthcare Life Sciences, Pittsburgh, PA) in transfer buffer for 10 min prior to transfer. Transfer sandwiches were set up for a semi-dry transfer using the Trans-Blot® Turbo™ Blotting System (Bio-Rad). Transfers were performed at 25V, 2.5A for 6 min at constant current. Following transfer, membranes were cut at 55kD based on the molecular weight marker to separate target protein (p70S6K or phospho-p70S6K, larger than 55kD) and internal control protein (smaller than 55kD). Cut membranes were blocked simultaneously in 5% Bovine Serum Albumin (BSA; Sigma-Aldrich) in TBST (30 mM Tris, 200 mM NaCl, 0.1 % Tween-20) for 1 h at room temperature on a rocker. After blocking solution was poured off, membranes were rinsed 2×5 min in TBST at room temperature on a rocker and primary antibodies diluted in 5% BSA in TBST were added. The rabbit anti human p70S6K antibody (C-Terminus, cross-reacts with chicken; LifeSpan BioSciences, Seattle, WA, catalog # LS-C287402) and the rabbit anti human phospho-p70S6K antibody (phospho T389, cross-reacts with chicken; Abcam, Cambridge, MA, catalog # ab126818) were used to detect the target proteins at 1:1000 dilution. The rabbit anti human GAPDH antibody (cross-reacts with chicken; Abcam, catalog # ab9485) diluted 1:2500 was used as an internal standard in breast muscle and the rabbit anti human beta-actin antibody (cross-reacts with chicken; Cell Signaling Technology, Danvers, MA, catalog # 4970) diluted 1:1000 was used as internal standards in liver and small intestinal tissues. Membranes were incubated in the primary antibody solutions overnight at 4°C on a rocker. Antibody solutions were poured off and membranes were washed 2×5 min, 1×15 min and 2×5 min in TBST at room temperature on a rocker. The secondary antibody

solution consisted of HRP-linked goat anti-rabbit IgG (Cell Signaling Technology, catalog # 7074), which was diluted 1:2000 in 3% BSA in TBST. Membranes were incubated in the secondary antibody solution for 1 h at room temperature on a rocker. After secondary antibody was poured off, membranes were washed 2×5 min, 1×15 min and 2×5 min in TBST at room temperature on a rocker.

Following the last wash, membranes were incubated in Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) for 5 min (0.1mL/$cm^2$ membrane). Chemiluminescence was imaged on membranes using a Chemi-Doc™ XPS+ imaging system (Bio-Rad) with an exposure time of 60 sec. Bands were quantified by volume tool using Image Lab™ software (Bio-Rad). The expression of p70S6K and phospho-p70S6K were normalized to the internal control from the same gel. The ratio of phospho-p70S6K expression to p70S6K expression was calculated.

**4.3.7 Data analysis**

For the Met converting enzyme gene expression assay, there were three statistical models for the mRNA expression data, which considered: 1) the main effects of treatment, age and tissue and their two-way and three-way interactions; 2) the main effects of treatment and age and the treatment × age interaction within each tissue (liver, breast muscle, duodenum, jejunum, and ileum) and 3) the main effects of treatment and tissue and the treatment × tissue interaction, within each age (d10, d21 and d26). For the Met oxidase activity assay, statistical models 2 and 3 were used. Data were checked for normality and homogeneity of variances. Split-plot design was considered when analyzing data from model 1 and 3, with chicks as the whole plot, the tissue as the split

plot and chicks as the random effect. Data from model 2 were analyzed by two-way ANOVA. Contrasts were used to separate treatment means.

For parameters in the other assays, data were analyzed within each tissue (liver, breast muscle, duodenum, jejunum and ileum). The statistical models included the main effects of treatment, age and their interactions. All the data were checked for normality and homogeneity of variances and analyzed by ANOVA. Means were separated using Tukey's Test.

JMP Pro version 11.0 (SAS Institute, Cary, NC) was used for statistical analysis. Data are presented as least square means ± SEM and statistical significance assigned at $P < 0.05$.

## 4.4 Results

### 4.4.1 Met converting enzyme gene expression

Data were analyzed using three statistical models, as described in Materials and Methods. For model 1, there were significant tissue × age interactions for all the genes. In addition, there was a significant treatment × tissue interaction for mRNA expression of HAO1, a significant treatment × age interaction for mRNA expression of DAO, and a significant treatment × tissue × age three-way interacton for mRNA expression of BCAT1. Data are shown in Table 4.2.

For the age effects, all of the measured mRNAs except BCAT1 had greater expression at day 10 than day 26 (Table 4.2). BCAT1 had greater mRNA expression at d21 than d26. For the tissue effects, both L-HMTBA oxidase (HAO1) mRNA and D-HMTBA oxidase (LDHD) mRNA were more abundant in liver than other tissues, while D-Met oxidase (DAO) mRNA was more abundant in muscle, followed by liver, and three

segments of small intestine (Figure 4.1). Among the 4 transaminase mRNAs, BCAT1 was expressed greatest in muscle and AGXT was expressed greatest in liver compared to other tissues. GOT2 and OAT were expressed greater in liver and muscle compared to the intestinal tissues.

For model 2, chickens fed DL-Met had lower hepatic HAO1 mRNA expression compared to L-Met and DL-HMTBA fed chickens ($P = 0.033$, Figure 4.2A), all three groups with Met supplementation had lower DAO mRNA expression compared to control group ($P = 0.003$, Figure 4.2B), while chickens fed DL-Met had greater GOT2 mRNA expression relative to the other treatment groups and control in duodenum ($P = 0.043$, Figure 4.2C). There were significant treatment × age interaction effects for AGXT, BCAT1 and GOT2 expression (Table 4.3). At d10, in breast muscle, DL-HMTBA fed chickens had lower AGXT mRNA expression compared to the other three groups, and both control and DL-Met groups showed greater AGXT mRNA expression compared to L-Met group. However, DL-HMTBA group had greater BCAT1 mRNA expression compared to control and L-Met groups at d10; while L-Met group had greater BCAT1 mRNA expression compared to control and DL-Met groups at d21. In jejunum, DL-Met fed chickens had greater GOT2 mRNA expression compared to the other three groups at d10.

For model 3, chickens fed DL-Met had greater GOT2 mRNA expression compared to the other three treatment groups ($P = 0.044$, Figure 4.3). There were significant treatment × tissue interaction effects for HAO1, BCAT1 and DAO mRNA expression (Table 4.4). At d10, both L-Met and DL-HMTBA groups showed greater HAO1 mRNA expression compared to control and DL-Met groups in liver. DL-HMTBA

fed chickens had greater BCAT1 mRNA expression compared to the other three groups and DL-Met fed chickens had greater BCAT1 mRNA expression compared to control and L-Met groups in breast muscle. At d21, L-Met group had greater BCAT1 mRNA expression compared to the other three groups and DL-HMTBA group had greater BCAT1 mRNA expression compared to DL-Met group in breast muscle. At d26, control group had greater DAO mRNA abundance compared to the other three groups in both liver and breast muscle, and L-Met fed chickens had greater DAO mRNA expression compared to DL-HMTBA group in breast muscle.

### 4.4.2 Enzymatic activity of Met oxidases

To measure oxidase activity, the optimal incubation time was determined first by incubating the hepatic enzymatic reaction buffer at 37$^{o}$C and detecting the produced KMB at 0, 30, 60, 90 and 120 min. After 90 min incubation, KMB production reached a peak for all three oxidases (Figure 4.4) and thus an incubation time of 90 min was chosen.

When normalized to per milligram of protein, only D-HADH activity decreased from d10 to d26 (from 14.8 to 10.1 μg KMB/mg protein, $P = 0.038$, other data not shown). No age effect was observed for L-HAOX and D-AAOX activity. Ileum had the lowest L-HAOX activity compared to the other tissues (Figure 4.5). Jejunum had greater D-HADH activity but lower D-AAOX activity compared to duodenum, liver and breast muscle (Figure 4.5). Ileum also had greater D-HADH activity and lower D-AAOX activity compared to liver (Figure 4.5).

Analyzing within each tissue, no significant differences were seen among C, DL-Met, L-Met and DL-HMTBA groups for all three oxidase activities (Table 4.5).

However, there were some age effects, which are shown in Table 4.5. D-AAOX increased from d10 to d21 in duodenum, decreased from d10 to d26 in jejunum and decreased from d10 and d21 to d26 in ileum. L-HAOX increased from d10 to d21 and d26 in duodenum, and decreased from d10 to d21 and d26 in breast muscle. D-HADH decreased from d10 to d21 and d26 in jejunum.

Analyzing within each age, DL-HMTBA fed chickens had decreased L-HAOX activity compared to control at d26 (Table 4.6). There were also tissue main effects, which are shown in Table 4.6. At d10, jejunum had greater D-HADH activity compared to duodenum, liver and breast muscle, and ileum had greater D-HADH activity compared to duodenum. At d21, the D-AAOX activity was lower in jejunum than duodenum and breast muscle. The D-HADH activity was lower in liver than duodenum, jejunum and ileum. At d26, the D-AAOX activity was greater in liver compared to jejunum and ileum. The L-HAOX activity was lower in breast muscle compared to duodenum, jejunum and liver.

### 4.4.3 In situ hybridization of Met oxidases

HAO1 and LDHD mRNA were localized in liver, breast muscle, duodenum, jejunum and ileum from 10-day-old chickens fed four treatments (C, DL-Met, L-Met and DL-HMTBA) (Figure 4.6). Liver showed much more abundant HAO1 mRNA expression compared to the other tissues (Figure 4.6A). No obvious differences were observed for HAO1 mRNA abundance between breast muscle and small intestinal segments. LDHD mRNA expression showed similar distribution pattern as HAO1 (Figure 4.6B). Neither gene showed obvious differences among the four treatment groups (Figure 4.6A-B). In small intestine, both HAO1 and LDHD mRNA were primarily expressed in epithelia and

in crypts, with decreasing signals towards the tip along the length of the villus (Figure 4.6C). Since it is difficult to quantify the results, only qualitative assessment was made here.

### 4.4.4 Assays for oxidative stress markers

The oxidative stress markers were measured in different tissues and there was a significant tissue effect independent of treatment and age. For the reduced glutathione (rGSH) to total glutathione (TGSH) ratio, which directly defines oxidative stress, duodenum showed the lowest rGSH/TGSH ratio, and breast muscle showed the highest rGSH/TGSH ratio compared to the other tissues (Figure 4.7A, $P < 0.001$). In addition, duodenum also had lower TBARS level which defines lipid peroxidation compared to jejunum, ileum and liver (Figure 4.7C, $P < 0.001$); and liver had the highest protein carbonyl level which defines protein oxidation compared to the other tissues (Figure 4.7B, $P < 0.001$). For the antioxidant capacity marker FRAP, the tissue distribution pattern is: liver > breast muscle > duodenum > jejunum = ileum (Figure 4.7D, $P < 0.001$).

The oxidative stress marker levels of the glutathione profile, protein carbonyl, TBARS and FRAP were analyzed within each tissue. The glutathione profile includes TGSH level, the oxidized glutathione (GSSG) level, rGSH level, ratio of rGSH to GSSG and ratio of rGSH to TGSH (Table 4.7). When analyzed within each tissue, there were significant treatment main effects for TGSH (Table 4.7A) and rGSH (Table 4.7C) levels in breast muscle. L-Met and DL-HMTBA fed chickens showed greater TGSH and rGSH levels compared to the control group, and L-Met fed chickens also showed greater TGSH and rGSH levels than the DL-Met group in breast muscle (Figure 4.8A and 4.8B, $P = <0.001$). There was significant treatment × age interaction effects for TGSH in jejunum

and ileum (Table 4.7A), GSSG in jejunum (Table 4.7B), and rGSH in ileum (Table 4.7C). At d26, L-Met fed chickens showed greater GSSG level compared to the control and DL-Met groups in jejunum (Figure 4.8E, $P = 0.001$). At d26, L-Met group also had greater TGSH and rGSH levels compared to the DL-Met group in ileum (Figure 4.8C and 4.6D, $P = 0.013$ and $P = 0.024$, respectively). However, there was no significant effect related to treatment for the ratio of rGSH to GSSG (Table 4.7D), and the ratio of rGSH to TGSH (Table 4.7E).

Protein carbonyl levels varied only in jejunum and breast muscle (Table 4.8). In jejunum, DL-Met group also had greater protein carbonyl level compared to DL-HMTBA group (Figure 4.9A, $P = 0.050$). There was significant treatment × age interaction effect for protein carbonyl level in breast muscle. At d26, L-Met group showed greater protein carbonyl level compared to control and DL-HMTBA group, and DL-Met group showed greater protein carbonyl level compared to DL-HMTBA group in breast muscle (Figure 4.9B, $P = 0.012$).

However, there was no significant difference for TBARS and FRAP levels between the four treatment groups in different tissues (Tables 4.9 and 4.10).

### 4.4.5 Western blot assay for p70S6K expression

SAT2 mRNA expression in different tissues at d10, 21 and 26 was assayed using the protocol listed in section 3.3.5. Differences in the expression of SAT2 mRNA in liver, breast muscle and intestine (duodenum, jejunum and ileum) at each age are shown in Figure 4.10. At d10, liver, duodenum and jejunum had greater SAT2 mRNA abundance than ileum and breast muscle. At d21, ileum had greater SAT2 mRNA expression than

duodenum, jejunum and breast muscle. At d26, breast muscle had the lowest SAT2 mRNA abundance than other tissues.

Representative western blot results for proteins p70S6K and phospho-p70S6K (T389) expression in liver, breast muscle, duodenum, jejunum and ileum are shown in Figure 4.11A-E. When analyzed within each tissue, protein expression of p70S6K and phospho-p70S6K are summarized in Table 4.11 and Table 4.12, respectively. The only significant treatment effect was that DL-HMTBA fed chickens showed lower phospho-p70S6K (T389) expression compared to the control group in jejunum ($P = 0.020$).

**4.5 Discussion**

DL-Met and DL-HMTBA are two most commonly used dietary supplemental Met sources. At the cellular level, both D-Met and DL-HMTBA are converted to L-Met for utilization through oxidation and transamination. However, the biochemical conversion takes place through different pathways. To determine the effect of different dietary Met source supplementation and Met deficiency on these pathways, the mRNA expression and enzymatic activities of key oxidases and transaminases were measured.

For mRNA expression, DL-HMTBA fed chickens showed greater HAO1 mRNA expression in liver compared to DL-Met and control groups. This was expected since HAO1 encodes the L-HMTBA oxidase, L-2-hydroxy acid oxidase (L-HAOX). Interestingly, L-Met fed chickens also had greater HAO1 mRNA expression compared to DL-Met and control groups. Ferjancic-Biagini et al. (1998) reported that L-HAOX also has an oxidative decarboxylation activity in vitro, with NADH as a cofactor. Thus L-HAOX may play a role in catalyzing more substrates than expected. In addition, chickens deficient in Met showed the greatest DAO mRNA expression compared to the other three

groups in liver and breast muscle at d26, indicating the downregulation of the D-Met oxidase gene when Met requirement is satisfied. No significant differences were seen among treatments for mRNA abundance of LDHD, the gene encoding D-HMTBA oxidase D-2-hydroxy acid dehydrogenase (D-HADH).

For the enzymatic activity, no significant effects appeared associated with the treatment effects, except for the decreased L-HAOX activity in DL-HMTBA group compared to control at d26. It's difficult to provide an explanation for the only treatment effect, since we did not expect decreased L-HMTBA oxidase activity with DL-HMTBA supplementation. However, the overall trend of non-significant treatment effect may be due to the excess of the enzyme concentration: the concentration of substrates is the rate limiting factor in these oxidative reactions. The post-transcriptional regulation by molecules such as tRNA and post-translational modification such as phosphorylation and glycosylation to make a mature enzyme are all possible factors that can explain the difference between mRNA and enzymatic activity. Fang et al. (2010) examined the three oxidase activities in piglets supplemented with DL-Met and DL-HMTBA. They observed increased L-HAOX and D-HADH activity in stomach and increased D-AAOX activity in kidney and duodenum with DL-Met supplementation compared to DL-HMTBA supplementation. No dietary treatment effects were shown in liver, muscle and other segments of intestine, which is similar to our results.

In addition to the treatment effects, the oxidase expression also showed significant tissue and age effects. Liver is the tissue with the greatest HAO1 and LDHD mRNA expression, while breast muscle had the greatest DAO mRNA expression. Liver also showed more abundant DAO mRNA expression compared to small intestine. Based on

the enzymatic activity results, liver is one of the tissues with the greatest L-HAOX and D-AAOX activity, and high D-AAOX activity was also observed in breast muscle. These results suggested a positive correlation between mRNA abundance and enzyme activity. Duodenum also had high D-AAOX activity. All these tissue distribution patterns were in accordance with the reports that both D-Met and DL-HMTBA were converted to L-Met mainly in liver and kidney, whereas the D-amino acid oxidases also catalyzed oxidation of D-Met in chicken small intestine (Brachet and Puigserver, 1992). However, the mRNA expression and enzyme activity of D-HMTBA oxidase exhibited a negative correlation: liver had the greatest LDHD mRNA expression, but the lowest D-HADH activity. Jejunum and ileum were the tissues with the highest D-HADH activity. More work is needed to explore the underlying regulation mechanisms. D-AAOX and L-HAOX are mainly produced in peroxisome and D-HADH is mainly produced in mitochondria. Liver is the tissue with abundant peroxisome and mitochondria (De Duve and Baudhuin, 1966), which provides the biological basis for our tissue distribution results. Similar to the other amino acid, D-Met is mainly absorbed in the small intestine through carrier-mediated transport. As an organic acid, DL-HMTBA can be taken up along the entire gastrointestinal tract, primarily in the proximal part in chickens, relying on a combination of diffusion and monocarboxylic acid transporter-mediated uptake. Thus digestive physiology can explain the higher levels of Met oxidase activity in duodenum and jejunum compared to ileum, especially for L-HAOX. Although breast muscle requires a large amount of L-Met for protein synthesis, the activities for all three oxidases were not the greatest in breast muscle. It can be assumed that not much DL-HMTBA and D-Met would reach the breast muscle in broilers fed a standard diet. Breast muscle can directly

utilize the L-Met that was produced in small intestine and liver. However, Dupuis et al. (1989) suggested that at high concentrations, DL-HMTBA would be oxidized principally in skeletal muscle. Thus skeletal muscle may have great potential for Met precursor conversion. All the oxidases and the transaminases except for branched chain amino acid transaminase (BCAT1) showed greater mRNA expression at d10 relative to d26, indicating that younger chickens had more Met converting capacity, which is reasonable since more L-Met is needed for protein synthesis and other metabolic activities during the grower phase.

The in situ hybridization analysis of the HAO1 and LDHD mRNA further confirmed the gene expression results from the real-time PCR assay. Both HAO1 and LDHD had greatest expression in liver compared to muscle and small intestine. No obvious treatment effects were observed for mRNA expression of both oxidases. Furthermore, there were some new findings related to the localization of these two genes. In all three segments (duodenum, jejunum and ileum) of the small intestine, the mRNA of these two oxidases are primarily expressed in epithelia and in crypts, with decreasing expression towards the villus tip. No one has reported the localization of DL-HMTBA oxidases. To my knowledge, it appears those two oxidases are mainly produced in enterocytes aggregating in crypts. Martin-Venegas, et al. (2011) suggested the complete conversion of DL-HMTBA to L-Met takes place in the intestinal epithelium in human Caco2 cells, while our in situ results indicated that liver has more capacity to oxidize DL-HMTBA than small intestine.

Transamination is not assumed to be the limiting step in the Met converting process. A wide variety of amino acids can serve as substrates for transamination of

KMB to form L-Met in chicken, such as branched chain amino acids and glutamic acid in skeletal muscle, glutamate in liver, isolecucine and alanine in kidney, and all amino acids expect alanine in intestinal mucosa (Rangel-Lugo and Austic, 1998). Considering the diversity of transaminases, only four most commonly used transaminases were chosen for the gene expression assay. The mRNA expression of glutamic-oxaloacetic transaminase 2 (GOT2), alanine-glyoxylate aminotransferase (AGXT) and BCAT1 showed significant treatment effects. For example, DL-Met fed chickens had greater GOT2 mRNA expression compared to the other three groups in duodenum and in jejunum at d10. In breast muscle, L-Met fed chickens had the greatest BCAT1 mRNA expression at d21, while DL-HMTBA fed chickens had the greatest BCAT1 mRNA expression at d10, and DL-HMTBA group also had the lowest AGXT mRNA expression at d10. Aspartate and glutamate are major substrates for GOT2 and branched chain amino acids (leucine, isoleucine and valine) are major substrates for BCAT1. Thus it is possible that aspartate and glutamate are better substrates for KMB transamination in DL-Met fed broilers in duodenum and in jejunum at d10, while in breast muscle, branched chain amino acids are better substrates in L-Met fed chickens at d21 and in DL-HMTBA fed chickens at d10. To test these hypotheses, the transaminases activity can be measured through chromatography in a subsequent study. There were also significant tissue and age effects for transaminase gene expression. All of these results illustrate the complexity and dynamics of Met converting enzyme regulation by the dietary source of Met.

After L-Met is formed from DL-HMTBA or D-Met, this nutritionally indispensable amino acid can be incorporated into several metabolic pathways, e.g., protein synthesis, transmethylation to form S-adenosylmethionine (SAM) or

transsulfuration to form L-cysteine (L-Cys). L-Cys can be directly incorporated into glutathione (GSH) or catabolized to taurine, and all of which have a crucial role in oxidative stress reactions since they all have the capacity to affect the cellular redox status (Shoveller et al., 2005). Cys and GSH can also function as direct scavengers of ROS, which is formed at the mitochondrial level to produce deleterious effects such as lipid and protein oxidation and DNA strand breaks, which affect metabolic processes (Metayer et al., 2008). Thus dietary Met supplementation is related to regulation of oxidative stress. In this study, we measured the GSH profile, protein oxidation, lipid peroxidation and ferric reducing /antioxidant power (FRAP) in liver, breast muscle and small intestine of chickens fed different dietary supplemental Met sources or a diet deficient in Met. Overall, duodenum showed the most unbalanced thiol redox status reflected by the lowest rGSH/TGSH ratio compared to the other tissues. However, jejunum and ileum demonstrated higher lipid peroxidation and lower FRAP than duodenum. Liver showed greater protein oxidation and lipid peroxidation, but also had higher FRAP. Breast muscle was the tissue with the most balanced thiol redox status. Considering the treatment effects within each tissue, the L-Met and DL-HMTBA supplemented chickens showed greater TGSH and rGSH levels compared to the control group in breast muscle. Also at d26, L-Met group had greater GSSG than control and DL-Met groups in the jejunum, and greater TGSH and rGSH than DL-Met in the ileum. Nevertheless, the ratios of rGSH/GSSG and rGSH/TGSH, which actually define the oxidative status, were not affected by Met sources. Although L-Met and DL-HMTBA supplementation to Met deficient diets did improve the intracellular antioxidant capacity in breast muscle induced by TGSH. As a protein oxidation marker, the protein carbonyl

levels varied among groups only in jejunum and muscle tissue, but these values were still in normal physiological ranges (refer to Xie et al. (2015) for the normal values of protein carbonyl). Furthermore, values of TBARS, an indicator of lipid oxidants, and FRAP, reflecting antioxidant capacity, were not different between groups in different tissues, indicating that different Met sources did not alter oxidative status of the treated chickens. However, with application of some more environmental stress factors, DL-HMTBA supplementation was reported to be beneficial in reducing oxidative stress. For example, DL-HMTBA is more effective in alleviating oxidative stress in broiler chickens induced by heat stress (Willemsen et al., 2011) or low protein diet (Swennen et al., 2011), which is revealed by reduced lipid peroxidation and more favorable rGSH/TGSH ratios compared to the DL-Met group. Also DL-HMTBA supplementation markedly decreased the serum protein carbonyl level and increased serum TGSH level at a low ambient temperature in broilers (Yang et al., 2016).

Amino acids including Met are a potential nutrient signal and regulator for mRNA translation and proteolysis (Yoshizawa, 2004; Nakashima et al., 2005; Kimball and Jefferson, 2006). The initiation step of mRNA translation needs the initiator methionyl-tRNA (Met-tRNAi), and may be inhibited by Met deficiency. With the potential effect on intracellular kinases, Met was reported to regulate p70S6K phosphorylation and protein synthesis in an avian myoblast cell line (QM7) of quail origin: only KMB but not D-Met or DL-HMTBA can activate the p70S6K pathway (Metayer-Coustard et al., 2010). Thus the effects of Met deficiency and different supplemental Met sources on p70S6K expression in broilers was of interest. In our study described in chapter 3, we observed greater SAT2 mRNA expression in the duodenum of DL-Met fed chickens compared to

control and DL-HMTBA groups at d35. SAT2 is a basolateral membrane transporter that also plays a role as an amino acid sensor, mediating its transceptor signaling through the mTOR pathway. An increase in SAT2 would lead to downstream signaling through mTORC1 resulting in increased protein synthesis (Taylor, 2014) and p70S6K is the key downstream protein in the SAT2/mTOR pathway. Based on the SAT2 mRNA expression results, p70S6K and phospho-p70S6K protein expression was measured in chickens fed different Met sources and hypothesized that DL-Met fed chickens would show increased phospho-p70S6K expression compared to control and DL-HMTBA groups in duodenum. However, increased phospho-p70S6K expression in control compared to DL-HMTBA fed chickens in jejunum was observed. The results in jejunum were unexpected, which may be due to the involvement of other transcriptional regulators in the SAT2-mTOR-p70S6K pathway, or because the SAT2 effect is age-dependent. The more activated p70S6K state, which means more protein synthesis in Met deficient group compared to DL-HMTBA group in jejunum, was also a new finding. We have no further explanation for this result.

In summary, there was regulation of Met converting enzymes by the dietary supplemental Met sources. Both mRNA expression and enzymatic activity of the three Met oxidases were tissue- and development-specific. Different Met sources and Met deficiency did not alter oxidative status of the treated chickens, but an increase in thiol redox status such as TGSH or rGSH can reveal the effects of sulfur amino acid diets. The amino acid sensing and protein synthesis pathway (SAT2/mTOR/p70S6K) in jejunum was also affected by dietary Met sources. Future studies are needed to further understand

how different dietary supplemental Met sources work at the molecular level to benefit animals.

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**Table 4.1** Primers used for quantitative real time PCR for Met converting enzyme genes and reference genes.

| Gene | GenBank ID | Description/gene function | Primer Sequence (5' → 3'), sense/antisense | bp [1] | Mean Cq value[2] | Efficiency |
|---|---|---|---|---|---|---|
| DAO[3] | XM_015284572.1 | D-aspartate oxidase (D-AAOX) | CCAACACGACAAGCGATGTG/ GGTGTGCCTGGGTAGGTATG | 61 | 29.7 | 93.5 |
| HAO1 | NM_001199442.2 | L-2-hydroxy acid oxidase (L-HAOX) (hydroxyacid oxidase 1 or glycolate oxidase 1) | GTGACTGTGGACACGCCATT/ GGAACTTGTTGCGCACATCA | 58 | 28.9 | 112.7 |
| LDHD | XM_015279229.1 | D-2-hydroxy acid dehydrogenase (D-HADH) (lactate dehydrogenase D) | CACGTGGGCGATGGTAACT/ GTCGTCAGCGCGGAAGA | 51 | 28.1 | 108.3 |
| AGXT | XM_003641735.2 | Alanine-glyoxylate aminotransferase | GATGCTGAGGAGGAAGACGAA/ TTGCCAGACAGCCCATGTC | 59 | 30.3 | 112.4 |
| BCAT1 | XM_004937974.1 | Branched chain amino acid transaminase | GGGCAAGCATTTGCACATT/ TCAGGATTTGGGTGGTTAACTGA | 62 | 26.4 | 114.5 |
| GOT2 | NM_205523.1 | Glutamic-oxaloacetic transaminase 2 | GGGAGCATACCGGGATGAC/ GCCTTGCGAACGCAGTTC | 57 | 28.5 | 91.0 |
| OAT | NM_001006567.1 | Ornithine aminotransferase | TGCGTGATAATGGGCTCCTT/ GGGCCAGCCGGATGA | 57 | 24.4 | 115.0 |
| PRL4 | NM_001007479.1 | Ribosomal protein L4 | TCAAGGCGCCCATTCG/ TGCGCAGGTTGGTGTGAA | 54 | 23.0 | 112.0 |
| PRLP0 | NM_204987.1 | Ribosomal protein, large, P0 | GCGATTGCTCCCTGTGATG/ TCTCAGGTCCGAGACCAGTGT | 58 | 20.6 | 111.9 |
| PRLP1 | NM_205322.1 | Ribosomal protein, large, P1 | TCTCCACGACGACGAAGTCA/ CCGCCGCCTTGATGAG | 62 | 20.6 | 97.3 |

[1] Amplicon size. [2] Cq = quantification cycle. [3] The D-aspartate oxidase has a symbol DDO in GenBank. For the D-amino acid oxidase (DAO) in chicken (Gallus gallus) (GenBank ID: NC_006102.2), the record was removed from NCBI for unknown reasons, and D-aspartate oxidase is recorded to encode D-Met oxidase in chicken instead. Thus we kept the symbol DAO here to represent D-aspartate oxidase gene. See Appendix C for the relative gene expression of the original DAO gene.

**Table 4.2** Relative Met converting enzyme gene expression using a statistical model that included main effects of treatment (C, DL-Met, L-Met, DL-HMTBA), age (d10, 21, 26), and tissue (liver, muscle, duodenum, jejunum, ileum) and their interactions.

| | | Relative Gene Expression[2] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *HAO1* | *LDHD* | *DAO* | *GOT2* | *AGXT* | *OAT* | *BCAT1* |
| Treatment (n=5) | C | 9.61 | 1.14 | 3.03 | 1.19 | 13.41 | 1.37 | 2.43 |
| | DL-Met | 8.65 | 1.15 | 2.25 | 1.47 | 14.04 | 1.36 | 2.63 |
| | L-Met | 11.46 | 1.19 | 3.01 | 1.30 | 13.02 | 1.21 | 2.88 |
| | DL-HMTBA | 11.77 | 1.14 | 2.42 | 1.33 | 12.95 | 1.27 | 2.94 |
| | SEM | 0.85 | 0.07 | 0.24 | 0.09 | 1.41 | 0.08 | 0.17 |
| | *P*-value | 0.03 | 0.93 | 0.04 | 0.24 | 0.95 | 0.43 | 0.14 |
| Tissue (n=5) | Duodenum | 0.90 | 0.82 | 0.96 | 0.74 | 0.35 | 0.67 | 0.37 |
| | Jejunum | 0.21 | 1.10 | 0.93 | 0.77 | 0.42 | 0.68 | 0.53 |
| | Ileum | 0.43 | 0.93 | 0.28 | 0.66 | 1.02 | 1.04 | 0.93 |
| | Muscle | 2.47 | 0.85 | 8.92 | 1.83 | 0.77 | 1.92 | 10.75 |
| | Liver | 47.86 | 2.06 | 2.28 | 2.61 | 64.21 | 2.20 | 1.03 |
| | SEM | 0.95 | 0.07 | 0.26 | 0.09 | 1.59 | 0.09 | 0.20 |
| | *P*-value | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Age (n=5) | Day 10 | 19.6 | 1.42 | 3.27 | 1.55 | 20.13 | 1.92 | 3.13 |
| | Day 21 | 6.49 | 1.27 | 3.20 | 1.16 | 10.75 | 1.21 | 3.87 |
| | Day 26 | 5.02 | 0.77 | 1.56 | 1.27 | 9.17 | 0.77 | 1.17 |
| | SEM | 0.74 | 0.06 | 0.20 | 0.08 | 1.22 | 0.07 | 0.15 |
| | *P*-value | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Interaction[1] | Trt×T | <0.01 | 0.99 | 0.38 | 0.81 | 1.00 | 0.93 | 0.09 |
| | T×A | <0.01 | 0.02 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| | Trt×A | 0.19 | 0.95 | 0.02 | 0.36 | 0.95 | 1.00 | 0.02 |
| | Trt×T×A | 0.06 | 0.30 | 0.37 | 0.37 | 1.00 | 1.00 | <0.01 |

[1] For the interaction, Trt, T and A represent the effects of treatment, tissue and age, respectively.

[2] Data are shown as least squares means ± standard errors of the means.

**Table 4.3** Contrasts for significant treatment × age interaction effects for AGXT, BCAT1 and GOT2 expression analyzed within different tissues. Male broiler chickens were fed different methionine sources until post-hatch day 35.

| | Treatment[1] | | | | | | Significant *P*-values for Contrasts[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | DL-Met | L-Met | DL-HMTBA | SEM[2] | *P*-value[3] | DL-Met vs. L-Met | DL-Met vs. DL-HMTBA | L-Met vs. DL-HMTBA | C vs. DL-Met | C vs. L-Met | C vs. DL-HMTBA |
| Muscle, AGXT[5] | | | | | 0.15 | 0.001 | | | | | | |
| Day 10 | 2.15 | 2.45 | 1.41 | 0.36 | | | 0.006 | <0.001 | 0.006 | | 0.047 | <0.001 |
| Day 21 | 0.74 | 0.42 | 0.16 | 0.41 | | | | | | | | |
| Day 26 | 0.38 | 0.14 | 0.29 | 0.32 | | | | | | | | |
| Muscle, BCAT1 | | | | | 0.87 | 0.044 | | | | | | |
| Day 10 | 9.15 | 12.67 | 10.08 | 15.32 | | | | | 0.017 | | | 0.006 |
| Day 21 | 15.08 | 14.36 | 20.37 | 17.01 | | | 0.010 | | | | 0.023 | |
| Day 26 | 3.74 | 4.08 | 4.07 | 3.10 | | | | | | | | |
| Jejunum, GOT2 | | | | | 0.08 | 0.031 | | | | | | |
| Day 10 | 0.89 | 1.37 | 0.81 | 0.67 | | | 0.011 | 0.002 | | 0.029 | | |
| Day 21 | 0.72 | 0.63 | 0.87 | 0.84 | | | | | | | | |
| Day 26 | 0.55 | 0.46 | 0.67 | 0.73 | | | | | | | | |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 21, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. [2,3] These values are pooled SEM of the mean and the *P*-value for the interaction, respectively. [4] All contrasts were tested but for brevity only significant differences are displayed. [5] AGXT: alanine-glyoxylate aminotransferase; BCAT1: branched chain amino acid transaminase; GOT2: glutamic-oxaloacetic transaminase 2.

**Table 4.4** Contrasts for significant treatment × tissue interaction effects for HAO1, BCAT1 and DAO expression analyzed within each age (d10, 21 and 26). Male broiler chickens were fed a diet with or without methionine supplementation until post-hatch day 35.

| | Treatment[1] | | | | | | Significant *P*-values for Contrasts[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | DL-Met | L-Met | DL-HMTBA | SEM[2] | *P*-value[3] | DL-Met vs. L-Met | DL-Met vs. DL-HMTBA | L-Met vs. DL-HMTBA | C vs. DL-Met | C vs. L-Met | C vs. DL-HMTBA |
| **Day 10**, HAO1[5] | | | | | 1.85 | 0.001 | | | | | | |
| Liver | 80.43 | 75.15 | 106.13 | 109.12 | | | <0.001 | <0.001 | | | <0.001 | <0.001 |
| Breast muscle | 3.11 | 2.97 | 2.30 | 1.28 | | | | | | | | |
| Duodenum | 2.02 | 2.27 | 2.31 | 2.77 | | | | | | | | |
| Jejunum | 0.18 | 0.29 | 0.15 | 0.25 | | | | | | | | |
| Ileum | 0.42 | 0.37 | 0.25 | 0.51 | | | | | | | | |
| **Day 10**, BCAT1 | | | | | 1.36 | 0.045 | | | | | | |
| Liver | 2.01 | 1.76 | 1.80 | 2.16 | | | | | | | | |
| Breast muscle | 9.15 | 12.67 | 10.08 | 15.32 | | | 0.037 | 0.033 | <0.001 | 0.005 | | <0.001 |
| Duodenum | 0.42 | 0.38 | 0.45 | 0.33 | | | | | | | | |
| Jejunum | 0.63 | 0.71 | 0.63 | 0.81 | | | | | | | | |
| Ileum | 0.72 | 0.83 | 0.86 | 0.95 | | | | | | | | |
| **Day 21**, BCAT1 | | | | | 0.33 | 0.020 | | | | | | |
| Liver | 0.50 | 0.63 | 0.61 | 0.62 | | | | | | | | |
| Breast muscle | 15.08 | 14.36 | 20.37 | 17.00 | | | <0.001 | 0.016 | 0.004 | | <0.001 | |
| Duodenum | 0.57 | 0.37 | 0.38 | 0.42 | | | | | | | | |
| Jejunum | 0.49 | 0.50 | 0.62 | 0.44 | | | | | | | | |
| Ileum | 1.06 | 1.29 | 1.01 | 1.03 | | | | | | | | |
| **Day 26**, DAO | | | | | 0.51 | 0.002 | | | | | | |
| Liver | 3.82 | 1.01 | 2.33 | 1.14 | | | | | | <0.001 | 0.043 | <0.001 |
| Breast muscle | 7.10 | 3.39 | 3.90 | 2.24 | | | | | 0.024 | <0.001 | <0.001 | <0.001 |
| Duodenum | 0.86 | 0.61 | 0.70 | 0.50 | | | | | | | | |
| Jejunum | 1.37 | 0.62 | 0.74 | 0.47 | | | | | | | | |
| Ileum | 0.13 | 0.11 | 0.08 | 0.07 | | | | | | | | |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 21, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. [2,3] These values are pooled SEM of the mean and the *P*-value for the interaction, respectively. [4] All contrasts were tested but for brevity only significant differences are displayed. [5] HAO1: L-2-hydroxy acid oxidase; BCAT1: branched chain amino acid transaminase. DAO: D-aspartate oxidase.

**Table 4.5** Methionine oxidase activity analyzed within each tissue (duodenum, jejunum, ileum, liver and breast muscle). Male broiler chickens were fed a diet with or without methionine supplementation until post-hatch day 26.

| Items | | Enzyme activity [1, 2, 3] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | D-amino acid oxidase (D-AAOX) | | | | | L-2-hydroxy acid oxidase (L-HAOX) | | | | | D-2-hydroxy acid dehydrogenase (D-HADH) | | | | |
| | | Duodenum | Jejunum | Ileum | Liver | Muscle | Duodenum | Jejunum | Ileum | Liver | Muscle | Duodenum | Jejunum | Ileum | Liver | Muscle |
| Treatment (n=5) | C | 33.8 | 13.5 | 16.0 | 49.5 | 30.1 | 45.3 | ND | 28.8 | 49.1 | 43.7 | 6.5 | 19.1 | 18.1 | 10.6 | 14.7 |
| | DL-Met | 34.0 | 14.5 | 20.2 | 22.1 | 33.6 | 45.3 | 43.8 | 20.6 | 35.1 | 42.9 | 14.0 | 19.5 | 16.4 | 6.6 | 6.7 |
| | L-Met | 29.2 | 8.7 | 20.7 | 42.5 | 32.8 | 29.6 | 33.4 | 28.4 | 56.2 | 29.9 | 7.4 | 18.2 | 13.3 | 6.8 | 11.4 |
| | DL-HMTBA | 27.3 | 12.7 | 20.8 | 22.8 | 32.4 | 37.2 | 31.2 | 12.6 | 52.6 | 33.8 | 12.6 | 16.6 | 16.3 | 4.8 | 8.6 |
| | SEM | 5.1 | 2.9 | 5.1 | 9.2 | 5.5 | 4.9 | - | 5.5 | 12.9 | 5.2 | 3.5 | 2.9 | 3.2 | 2.8 | 2.8 |
| | *P*-value | 0.74 | 0.55 | 0.88 | 0.09 | 0.97 | 0.09 | - | 0.12 | 0.68 | 0.20 | 0.32 | 0.89 | 0.77 | 0.47 | 0.22 |
| Age (n=5) | day 10 | 21.3$^{b}$ | 14.6$^{a}$ | 23.3$^{a}$ | 25.4 | 30.5 | 21.0$^{b}$ | ND | 18.2 | 49.5 | 64.7$^{a}$ | 5.5 | 29.1$^{a}$ | 18.8 | 11.5 | 11.1 |
| | day 21 | 39.1$^{a}$ | 14.5$^{ab}$ | 26.4$^{a}$ | 31.2 | 35.1 | 47.6$^{a}$ | 34.7 | 19.6 | 43.0 | 24.6$^{b}$ | 14.1 | 16.8$^{b}$ | 14.9 | 3.6 | 11.4 |
| | day 26 | 32.9$^{ab}$ | 7.9$^{b}$ | 8.6$^{b}$ | 46.1 | 31.0 | 49.4$^{a}$ | 45.6 | 30.0 | 52.3 | 23.4$^{b}$ | 10.8 | 9.2$^{b}$ | 14.3 | 7.1 | 8.5 |
| | SEM | 4.4 | 2.4 | 4.3 | 8.0 | 4.8 | 4.2 | - | 4.7 | 11.2 | 4.5 | 3.0 | 2.5 | 2.8 | 2.4 | 2.3 |
| | *P*-value | 0.03 | 0.04 | 0.02 | 0.18 | 0.76 | <0.001 | - | 0.23 | 0.84 | <0.001 | 0.13 | <0.001 | 0.50 | 0.09 | 0.66 |
| Interaction (n=5) | Treatment ×Age | 0.85 | 0.44 | 0.94 | 0.33 | 0.35 | 0.89 | - | 0.38 | 0.42 | 0.96 | 0.16 | 0.86 | 0.30 | 0.59 | 0.92 |

[1] Values represent enzyme activity normalized to per milligram of protein (μg/mg protein). [2] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 21, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$. [3] ND: not detectable.

**Table 4.6** Methionine oxidase activity analyzed within each age (day 10, 21 and 26). Male broiler chickens were fed a diet with or without methionine supplementation until post-hatch day 26.

| | | Enzyme activity [1, 2, 3] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | D-amino acid oxidase (D-AAOX) | | | L-2-hydroxy acid oxidase (L-HAOX) | | | D-2-hydroxy acid dehydrogenase (D-HADH) | | |
| | | day 10 | day 21 | day 26 | day 10 | day 21 | day 26 | day 10 | day 21 | day 26 |
| Treatment (n=5) | C | 23.2 | 30.8 | 31.8 | ND | 37.1 | 47.6$^{a}$ | 17.5 | 15.7 | 8.4 |
| | DL-Met | 29.4 | 25.1 | 20.2 | 39.8 | 31.5 | 41.3$^{ab}$ | 15.0 | 11.3 | 11.7 |
| | L-Met | 19.1 | 30.8 | 30.4 | 34.5 | 29.4 | 42.6$^{ab}$ | 13.0 | 10.2 | 11.0 |
| | DL-HMTBA | 20.4 | 30.3 | 18.9 | 33.7 | 37.6 | 29.1$^{b}$ | 15.0 | 11.4 | 8.9 |
| | SEM | 4.7 | 4.5 | 5.9 | - | 7.0 | 4.7 | 2.6 | 2.2 | 2.3 |
| | *P*-value | 0.39 | 0.77 | 0.29 | - | 0.80 | 0.04 | 0.69 | 0.31 | 0.67 |
| Tissue (n=5) | Duodenum | 21.3 | 39.1$^{a}$ | 32.9$^{ab}$ | 21.0 | 47.6 | 49.4$^{a}$ | 5.51$^{c}$ | 14.1$^{a}$ | 10.8 |
| | Jejunum | 14.6 | 14.5$^{b}$ | 7.9$^{b}$ | ND | 34.7 | 45.6$^{a}$ | 29.1$^{a}$ | 16.8$^{a}$ | 9.2 |
| | Ileum | 23.3 | 26.4$^{ab}$ | 8.6$^{bc}$ | 18.2 | 19.5 | 30.0$^{ab}$ | 18.8$^{ab}$ | 14.9$^{a}$ | 14.3 |
| | Liver | 25.4 | 31.2$^{ab}$ | 46.1$^{a}$ | 49.5 | 43.0 | 52.3$^{a}$ | 11.0$^{bc}$ | 3.6$^{b}$ | 7.1 |
| | Muscle | 30.5 | 35.1$^{a}$ | 31.0$^{abc}$ | 64.7 | 24.6 | 23.4$^{b}$ | 11.1$^{bc}$ | 11.4$^{ab}$ | 8.5 |
| | SEM | 5.1 | 5.0 | 6.6 | - | 7.7 | 5.1 | 2.9 | 2.4 | 2.5 |
| | *P*-value | 0.37 | <0.01 | <0.01 | - | 0.07 | <0.01 | <0.01 | <0.01 | 0.31 |
| Interaction (n=5) | Treatment ×Tissue | 0.88 | 0.97 | 0.14 | - | 0.30 | 0.49 | 0.87 | 0.45 | 0.67 |

[1] Values represent enzyme activity normalized to per milligram of protein (μg/mg protein). [2] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of tissue (duodenum, jejunum, ileum, liver, breast muscle) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$. [3] ND: not detectable.

**Table 4.7** Glutathione profile in liver, breast muscle and three small intestinal segments from chickens fed a diet with or without methionine supplementation at post-hatch days 10 and 26. **(A)** The glutathione profile includes total glutathione (TGSH), **(B)** oxidized glutathione (GSSG), **(C)** reduced glutathione (rGSH), **(D)** ratio of rGSH to GSSG and **(E)** ratio of rGSH to TGSH.

**(A)**

| Items | | Total glutathione (TGSH) (nmol/g tissue)[1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 30.23 | 77.49 | 128.47 | 413.83 | 155.62$^{c}$ |
| | DL-Met | 29.14 | 91.23 | 132.83 | 530.29 | 186.43$^{bc}$ |
| | L-Met | 30.63 | 89.42 | 153.79 | 503.02 | 276.79$^{a}$ |
| | DL-HMTBA | 28.95 | 82.74 | 138.58 | 445.54 | 235.36$^{ab}$ |
| | SEM | 2.98 | 8.75 | 11.16 | 45.04 | 18.98 |
| | *P*-value | 0.97 | 0.67 | 0.40 | 0.26 | <0.01 |
| Age (n=5) | Day 10 | 39.59$^{a}$ | 92.59 | 141.54 | 501.98 | 203.14 |
| | Day 26 | 19.89$^{b}$ | 77.85 | 135.29 | 444.36 | 223.95 |
| | SEM | 2.10 | 6.19 | 7.89 | 31.86 | 13.42 |
| | *P*-value | <0.01 | 0.10 | 0.58 | 0.21 | 0.28 |
| Interaction (n=5) | Treatment ×Age | 0.20 | 0.01 | 0.01 | 0.07 | 0.38 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**(B)**

| Items | | GSSG (nmol/g tissue) [1,2] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 10.69 | 7.32 | 6.65 | 31.68 | ND |
| | DL-Met | 10.51 | 8.90 | 7.31 | 37.89 | 1.43 |
| | L-Met | 10.11 | 11.34 | 9.63 | 34.97 | 1.28 |
| | DL-HMTBA | 10.01 | 8.11 | 8.79 | 39.76 | 1.09 |
| | SEM | 1.27 | 1.25 | 1.37 | 3.37 | - |
| | *P*-value | 0.98 | 0.14 | 0.41 | 0.36 | - |
| Age (n=5) | Day 10 | 14.35[a] | 9.06 | 6.72 | 34.77 | 0.57 |
| | Day 26 | 6.30[b] | 8.77 | 9.47 | 37.37 | 1.96 |
| | SEM | 0.90 | 0.88 | 0.97 | 2.38 | 0.67 |
| | *P*-value | <0.01 | 0.82 | 0.05 | 0.45 | 0.22 |
| Interaction (n=5) | Treatment ×Age | 0.18 | <0.01 | 0.10 | 0.16 | - |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.
[2] ND: not detectable.

**(C)**

| Items | | Reduced GSH (rGSH) (nmol/g tissue) [1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 8.86 | 62.85 | 115.16 | 350.47 | 155.62$^{c}$ |
| | DL-Met | 7.59 | 73.44 | 118.21 | 454.51 | 185.86$^{bc}$ |
| | L-Met | 9.01 | 66.73 | 134.54 | 433.09 | 275.99$^{a}$ |
| | DL-HMTBA | 8.93 | 66.52 | 121.67 | 367.02 | 234.92$^{ab}$ |
| | SEM | 2.01 | 7.62 | 10.14 | 44.34 | 18.96 |
| | *P*-value | 0.95 | 0.80 | 0.54 | 0.29 | <0.01 |
| Age (n=5) | Day 10 | 10.17 | 74.47 | 128.10 | 432.92 | 202.90 |
| | Day 26 | 7.02 | 60.30 | 116.69 | 269.62 | 231.87 |
| | SEM | 1.42 | 5.39 | 7.17 | 31.36 | 14.34 |
| | *P*-value | 0.13 | 0.072 | 0.27 | 0.16 | 0.16 |
| Interaction (n=5) | Treatment ×Age | 0.60 | 0.07 | 0.02 | 0.12 | 0.31 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**(D)**

| Items | | Ratio of rGSH to GSSG[1,2] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 0.88 | 9.99 | 18.49 | 11.39 | ND |
| | DL-Met | 1.06 | 10.50 | 17.00 | 12.47 | 264.55 |
| | L-Met | 0.98 | 6.82 | 17.49 | 13.48 | 585.15 |
| | DL-HMTBA | 1.19 | 9.79 | 16.51 | 11.63 | 216.09 |
| | SEM | 0.28 | 1.77 | 2.11 | 1.71 | - |
| | *P*-value | 0.87 | 0.46 | 0.91 | 0.82 | - |
| Age (n=5) | Day 10 | 0.78 | 10.10 | 19.87[a] | 14.17[a] | 554.13 |
| | Day 26 | 1.28 | 8.45 | 14.87[b] | 10.32[b] | 156.39 |
| | SEM | 0.20 | 1.25 | 1.50 | 1.21 | 136.6 |
| | *P*-value | 0.08 | 0.36 | 0.03 | 0.03 | 0.11 |
| Interaction (n=5) | Treatment ×Age | 0.77 | 0.16 | 0.42 | 0.94 | - |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.
[2] ND: not detectable.

**(E)**

| Items | | Ratio of rGSH to TGSH[1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 0.28 | 0.79 | 0.90 | 0.83 | 1.000 |
| | DL-Met | 0.25 | 0.81 | 0.90 | 0.85 | 0.996 |
| | L-Met | 0.34 | 0.75 | 0.88 | 0.85 | 0.996 |
| | DL-HMTBA | 0.32 | 0.79 | 0.88 | 0.82 | 0.998 |
| | SEM | 0.06 | 0.02 | 0.02 | 0.02 | 0.002 |
| | *P*-value | 0.68 | 0.42 | 0.85 | 0.68 | 0.47 |
| Age (n=5) | Day 10 | 0.27 | 0.80 | 0.91 | 0.86 | 0.999 |
| | Day 26 | 0.32 | 0.77 | 0.87 | 0.82 | 0.996 |
| | SEM | 0.04 | 0.02 | 0.01 | 0.02 | 0.001 |
| | *P*-value | 0.41 | 0.31 | 0.08 | 0.10 | 0.15 |
| Interaction (n=5) | Treatment ×Age | 0.84 | 0.21 | 0.50 | 0.93 | 0.65 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**Table 4.8** Protein carbonyl level in liver, breast muscle and three small intestinal segments from chickens fed a diet with or without methionine supplementation at post-hatch days 10 and 26.

| Items | | Protein carbonyl (nmol/g tissue) [1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 8.42 | 6.45$^{ab}$ | 6.63 | 16.70 | 5.37 |
| | DL-Met | 7.69 | 8.04$^{a}$ | 6.06 | 19.54 | 7.55 |
| | L-Met | 8.38 | 6.95$^{ab}$ | 5.54 | 19.53 | 9.35 |
| | DL-HMTBA | 8.29 | 5.79$^{b}$ | 7.88 | 19.59 | 4.53 |
| | SEM | 1.37 | 0.56 | 0.75 | 1.78 | 0.74 |
| | *P*-value | 0.98 | 0.05 | 0.17 | 0.62 | <0.01 |
| Age (n=5) | Day 10 | 8.41 | 5.40$^{b}$ | 5.73$^{b}$ | 16.21$^{b}$ | 5.14 |
| | Day 26 | 7.98 | 8.61$^{a}$ | 7.32$^{a}$ | 21.47$^{a}$ | 8.25 |
| | SEM | 0.97 | 0.48 | 0.53 | 1.27 | 0.53 |
| | *P*-value | 0.75 | <0.01 | 0.04 | <0.01 | <0.01 |
| Interaction (n=5) | Treatment ×Age | 0.89 | 0.23 | 0.90 | 0.98 | 0.01 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**Table 4.9** Thiobarbituric acid reactive substances (TBARS) level in liver, breast muscle and three small intestinal segments from chickens fed a diet with or without methionine supplementation at post-hatch days 10 and 26.

| Items | | TBARS (MDA, μM) [1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 3.89 | 6.66 | 7.75 | 8.02 | 4.93 |
| | DL-Met | 3.89 | 5.44 | 9.13 | 8.28 | 5.63 |
| | L-Met | 5.09 | 5.85 | 5.13 | 7.91 | 4.88 |
| | DL-HMTBA | 4.21 | 6.67 | 4.87 | 7.65 | 6.94 |
| | SEM | 0.46 | 0.77 | 1.63 | 0.64 | 0.83 |
| | *P*-value | 0.22 | 0.60 | 0.20 | 0.92 | 0.29 |
| Age (n=5) | Day 10 | 4.91[a] | 7.22[a] | 5.06[b] | 7.44 | 4.97 |
| | Day 26 | 3.63[b] | 5.09[b] | 8.39[a] | 8.49 | 6.22 |
| | SEM | 0.33 | 0.55 | 1.15 | 0.45 | 0.59 |
| | *P*-value | 0.01 | 0.01 | 0.05 | 0.11 | 0.14 |
| Interaction (n=5) | Treatment ×Age | 0.51 | 0.50 | 0.44 | 0.47 | 0.82 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**Table 4.10** Ferric reducing/antioxidant power (FRAP) level in liver, breast muscle and three small intestinal segments from chickens fed a diet with or without methionine supplementation at post-hatch days 10 and 26.

| Items | | FRAP ($FeCl_2$, μM) [1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=5) | C | 709.3 | 492.0 | 391.6 | 1707.3 | 1565.6 |
| | DL-Met | 665.5 | 489.4 | 406.4 | 1860.9 | 901.1 |
| | L-Met | 779.1 | 439.6 | 370.9 | 1726.9 | 1127.0 |
| | DL-HMTBA | 650.4 | 432.3 | 412.0 | 1914.5 | 1226.6 |
| | SEM | 65.1 | 42.7 | 25.4 | 128.2 | 174.1 |
| | *P*-value | 0.53 | 0.65 | 0.67 | 0.61 | 0.08 |
| Age (n=5) | Day 10 | 564.8$^{b}$ | 371.2$^{b}$ | 335.0$^{b}$ | 2080.4$^{a}$ | 1238.0 |
| | Day 26 | 837.4$^{a}$ | 555.4$^{a}$ | 455.5$^{a}$ | 1524.4$^{b}$ | 1172.1 |
| | SEM | 46.0 | 30.22 | 18.0 | 90.6 | 123.1 |
| | *P*-value | <0.01 | <0.01 | <0.01 | <0.01 | 0.71 |
| Interaction (n=5) | Treatment ×Age | 0.36 | 0.90 | 0.25 | 0.95 | 0.64 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**Table 4.11** p70S6K protein expression in liver, breast muscle and three segments of small intestine from chickens fed a diet with or without methionine supplementation at post-hatch days 10, 21 and 26.

| P70S6K | | Ratio to loading control[1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=4) | C | 0.52 | 0.23 | 0.43 | 0.95 | 1.02 |
| | DL-Met | 0.41 | 0.34 | 0.37 | 1.10 | 1.00 |
| | L-Met | 0.41 | 0.29 | 0.45 | 0.98 | 0.90 |
| | DL-HMTBA | 0.41 | 0.41 | 0.39 | 0.78 | 1.04 |
| | SEM | 0.09 | 0.07 | 0.12 | 0.26 | 0.15 |
| | *P*-value | 0.84 | 0.32 | 0.95 | 0.86 | 0.92 |
| Age (n=4) | Day 10 | 0.57 | 0.54[a] | 0.43 | 0.77 | 1.00 |
| | Day 21 | 0.45 | 0.29[b] | 0.38 | 1.22 | 1.05 |
| | Day 26 | 0.30 | 0.13[b] | 0.43 | 0.87 | 0.92 |
| | SEM | 0.08 | 0.06 | 0.10 | 0.23 | 0.13 |
| | *P*-value | 0.09 | <0.01 | 0.93 | 0.36 | 0.79 |
| Interaction (n=4) | Treatment ×Age | 0.87 | 0.65 | 0.93 | 0.42 | 0.54 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 21, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

**Table 4.12** Phospho-p70S6K (T389) protein expression in liver, breast muscle and three segments of small intestine from chickens fed a diet with or without methionine supplementation at post-hatch days 10, 21 and 26.

| Phospho-p70S6K (T389) | | Ratio to p70S6K[1] | | | | |
|---|---|---|---|---|---|---|
| | | Duodenum | Jejunum | Ileum | Liver | Breast muscle |
| Treatment (n=4) | C | 0.71 | 1.42[a] | 0.64 | 0.73 | 0.19 |
| | DL-Met | 0.72 | 0.68[ab] | 0.59 | 0.54 | 0.22 |
| | L-Met | 0.78 | 0.58[ab] | 0.31 | 0.80 | 0.28 |
| | DL-HMTBA | 1.05 | 0.37[b] | 0.46 | 0.75 | 0.25 |
| | SEM | 0.21 | 0.23 | 0.21 | 0.18 | 0.05 |
| | *P*-value | 0.62 | 0.02 | 0.69 | 0.77 | 0.55 |
| Age (n=4) | Day 10 | 0.61 | 0.18[d] | 0.42 | 0.73 | 0.21 |
| | Day 21 | 0.68 | 0.49[d] | 0.31 | 0.62 | 0.22 |
| | Day 26 | 1.15 | 1.61[c] | 0.77 | 0.76 | 0.28 |
| | SEM | 0.18 | 0.20 | 0.17 | 0.16 | 0.04 |
| | *P*-value | 0.10 | <0.01 | 0.18 | 0.80 | 0.49 |
| Interaction (n=4) | Treatment ×Age | 0.96 | 0.41 | 0.68 | 0.77 | 0.86 |

[1] Data presented are least squares means and standard errors of the mean. The statistical model included main effect of treatment (C, DL-Met, L-Met, DL-HMTBA), main effect of age (d10, 21, 26) and their interactions. C: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid. Means in a column without a common letter differ, $P < 0.05$.

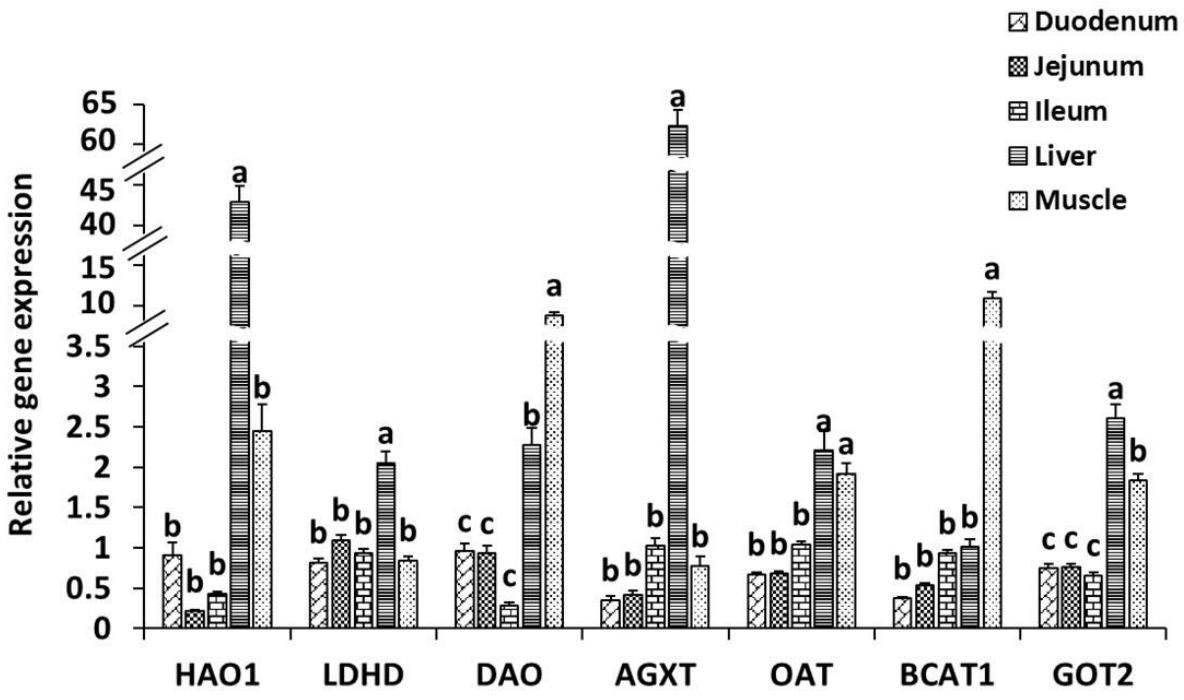



**Figure 4.1** Tissue distribution of mRNA expression for methionine oxidases and transaminases. HAO1: L-2-hydroxy acid oxidase 1; LDHD: D-2-hydroxy acid dehydrogenase; DAO: D-aspartate oxidase; AGXT: alanine-glyoxylate aminotransferase; OAT: ornithine aminotransferase; BCAT1: branched chain amino acid transaminase; GOT2: glutamic-oxaloacetic transaminase 2. Figure shows tissue effect within each gene independent of treatment and age (n=60). Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters within each gene represent significant differences, $P < 0.05$.

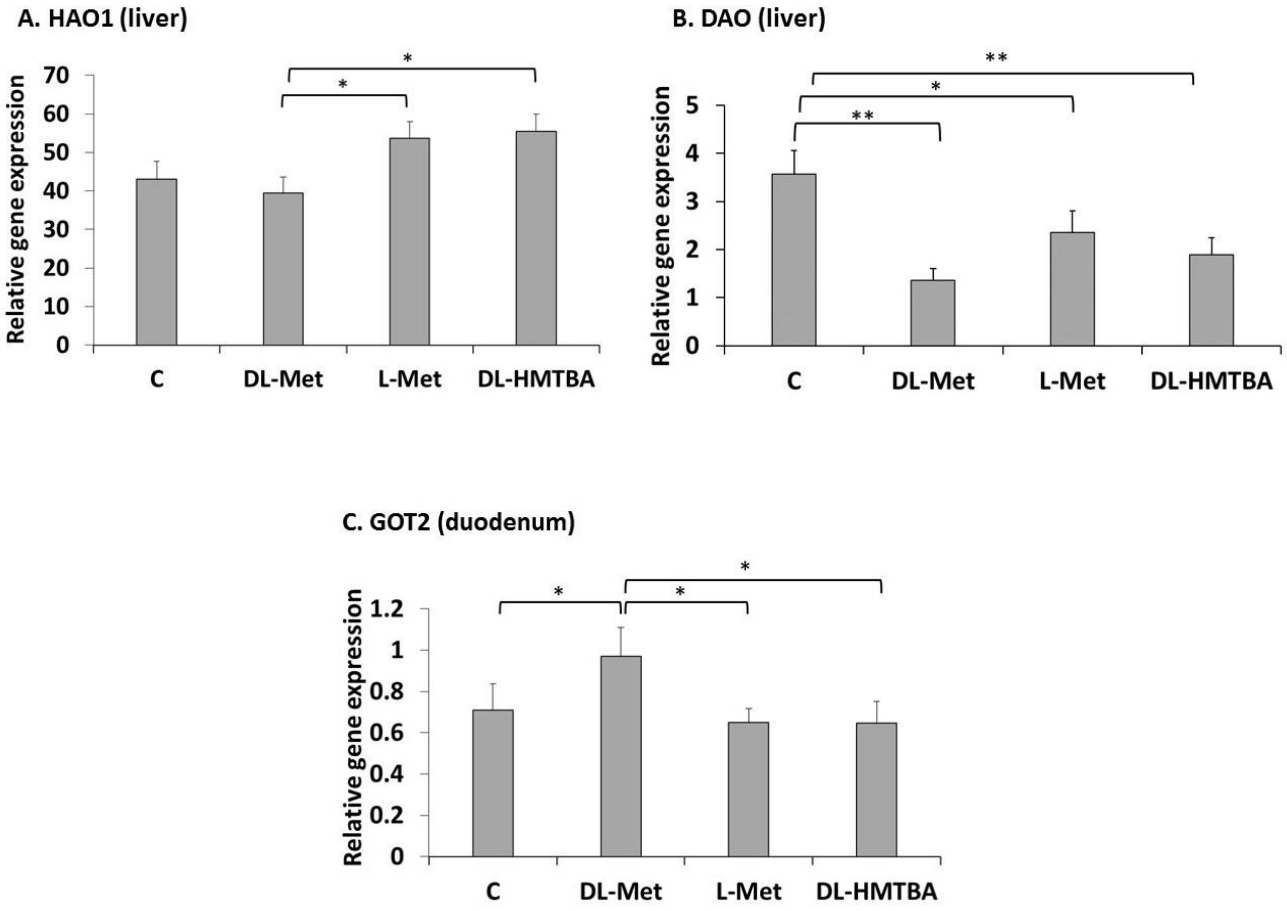



**Figure 4.2** Relative gene expression of methionine converting enzymes from chickens fed different methionine sources analyzed by tissue. Relative HAO1 (L-2-hydroxy acid oxidase 1) (A) and DAO (D-aspartate oxidase) (B) mRNA abundance in liver and GOT2 (glutamic-oxaloacetic transaminase 2) mRNA abundance in duodenum (C) in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=5). Figure shows main effect of treatment within each tissue across all ages. Values represent least squares means ± SEM. Means were compared using contrasts. * $P < 0.05$, ** $P < 0.01$.

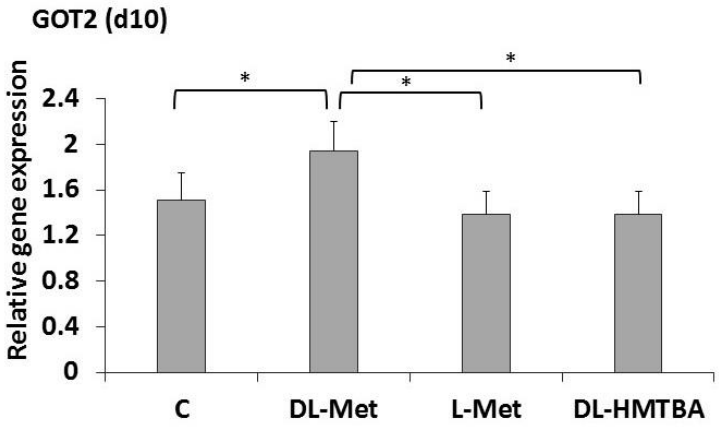



**Figure 4.3** Relative gene expression of methionine converting enzymes from chickens fed different methionine sources analyzed by age. Relative GOT2 (glutamic-oxaloacetic transaminase 2) mRNA abundance at d10 in small intestine in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=5). Figure shows main effect of treatment within each age across all tissues (liver, breast muscle, duodenum, jejunum and ileum). Values represent least squares means ± SEM. Means were compared using contrasts. * $P < 0.05$.

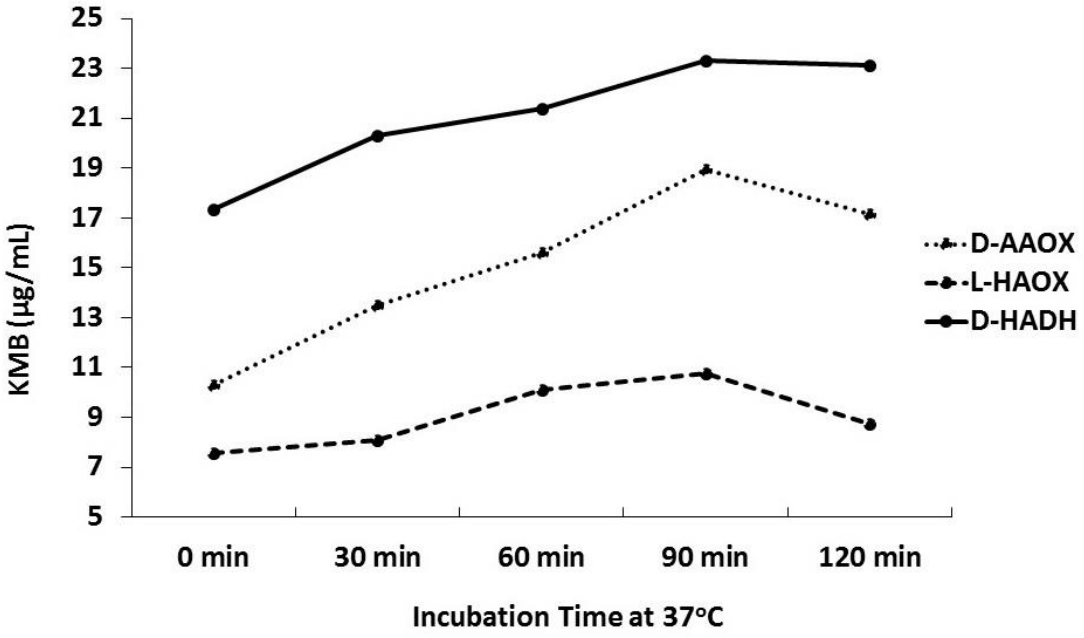



**Figure 4.4** Hepatic methionine oxidase activity under different incubation times. D-AAOX (D-amino acid oxidase), L-HAOX (L-2-hydroxy acid oxidase) and D-HADH (D-2-hydroxy acid dehydrogenase) activity were measured in chicken liver extracts after the reaction was incubated at 37ºC for 0, 30, 60, 90 and 120 min. KMB: 2-keto-4 (methylthio) butanoic acid, which is the product of those oxidation reaction.

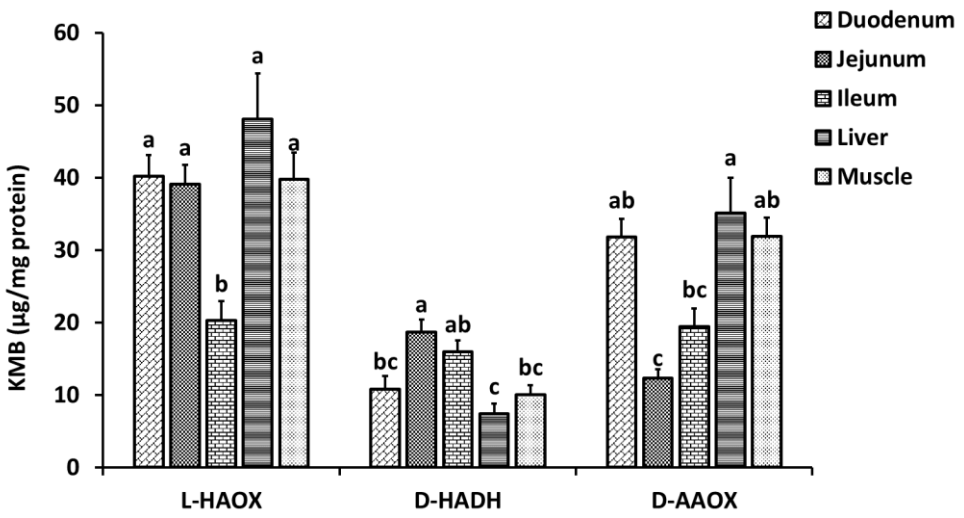
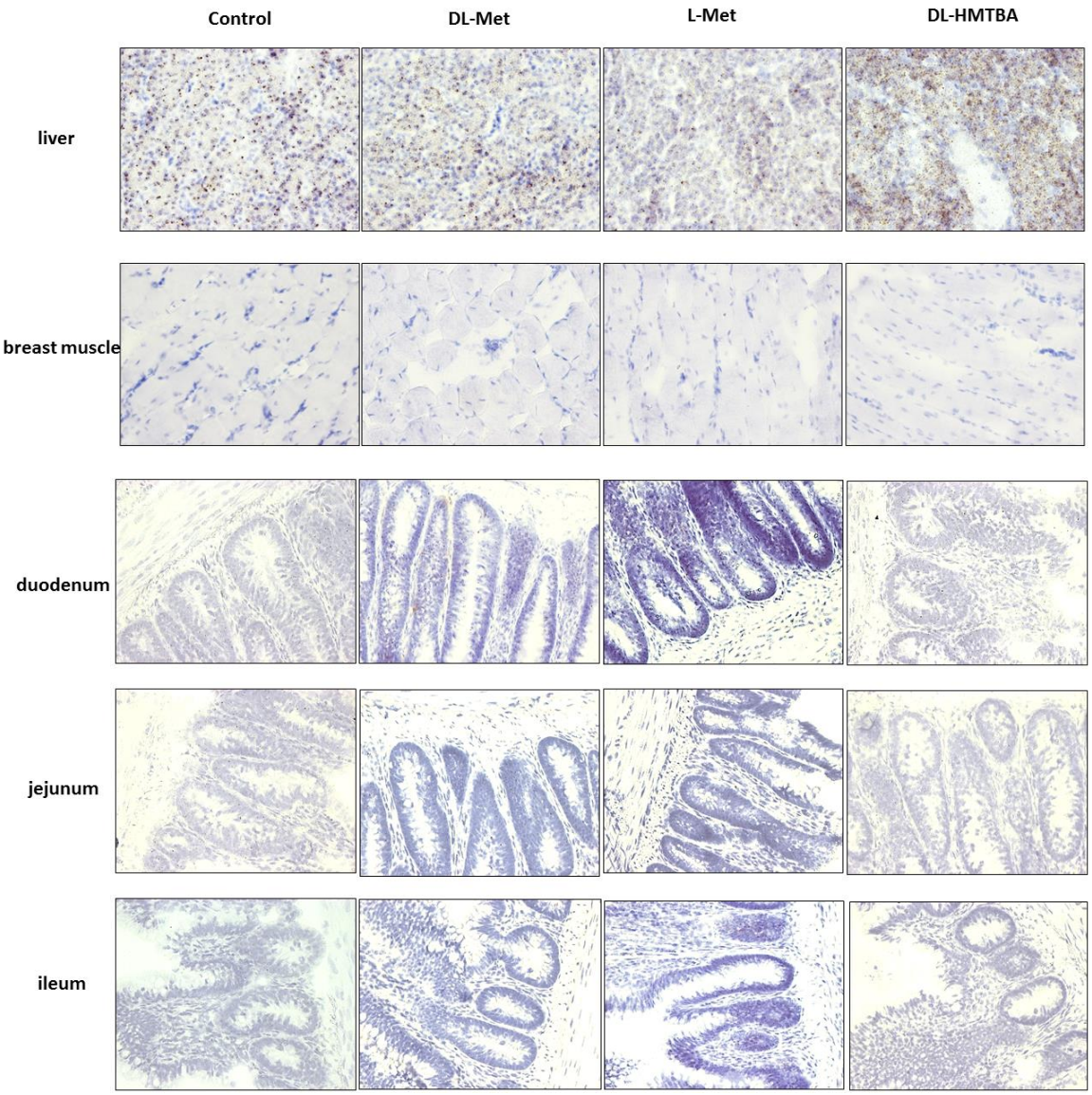
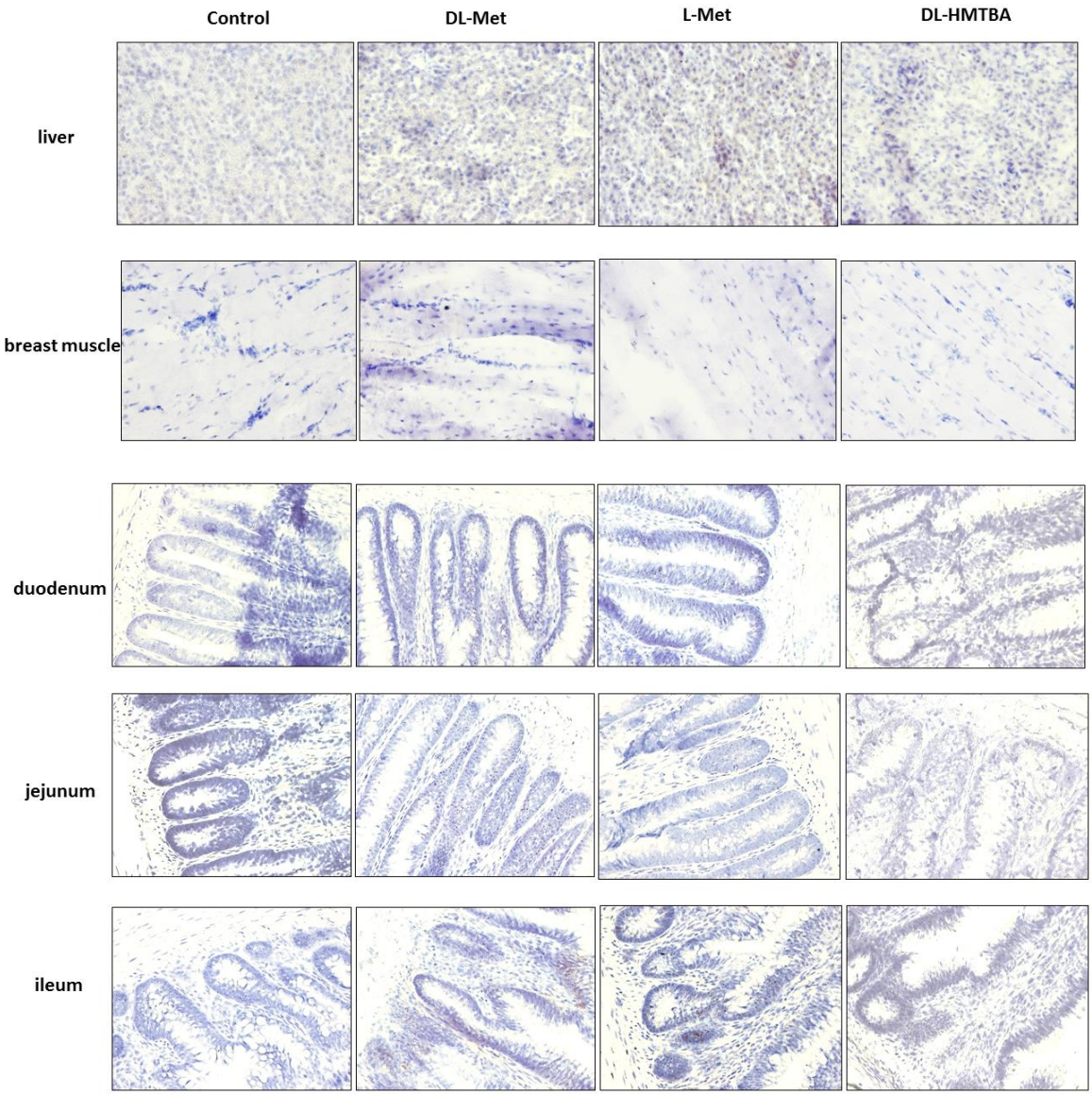



**Figure 4.5** Tissue distribution of methionine oxidase activity normalized to per milligram of protein. D-AAOX: D amino acid oxidase; L-HAOX: L-2-hydroxy acid oxidase; D-HADH: D-2-hydroxy acid dehydrogenase. Figure shows tissue effect within each enzyme independent of treatment and age (n=60). Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters within each enzyme represent significant differences, $P < 0.05$.

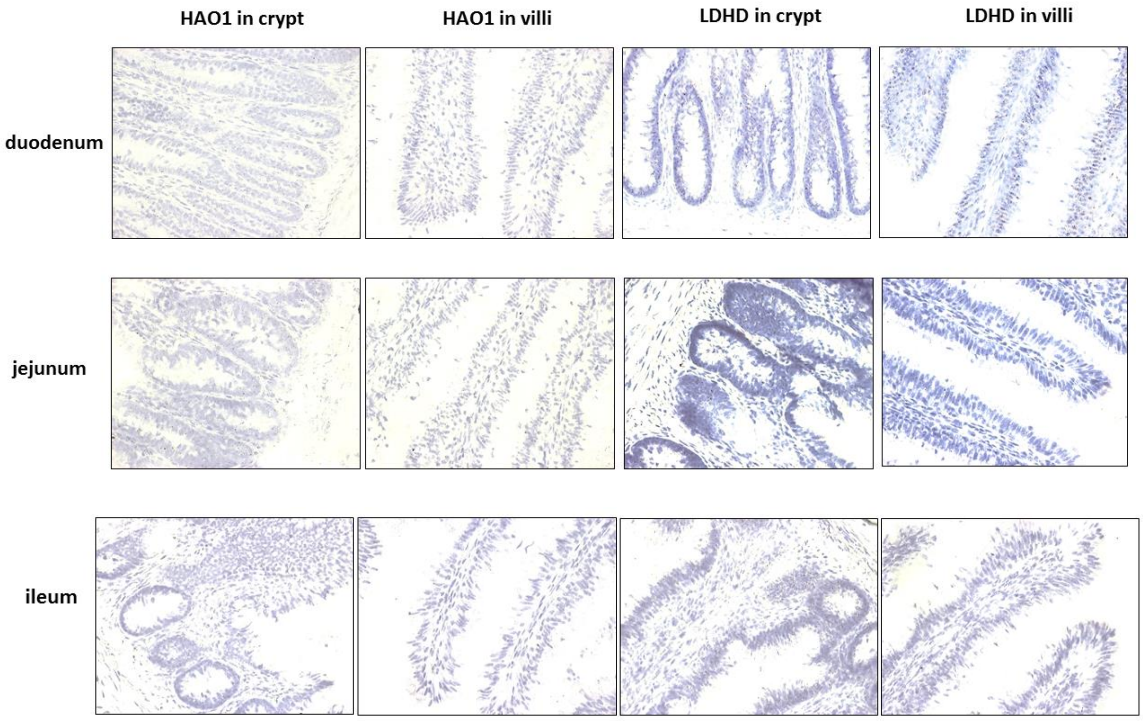
**A. HAO1**


Control
DL-Met
L-Met
DL-HMTBA
liver
breast muscle
duodenum
jejunum
ileum

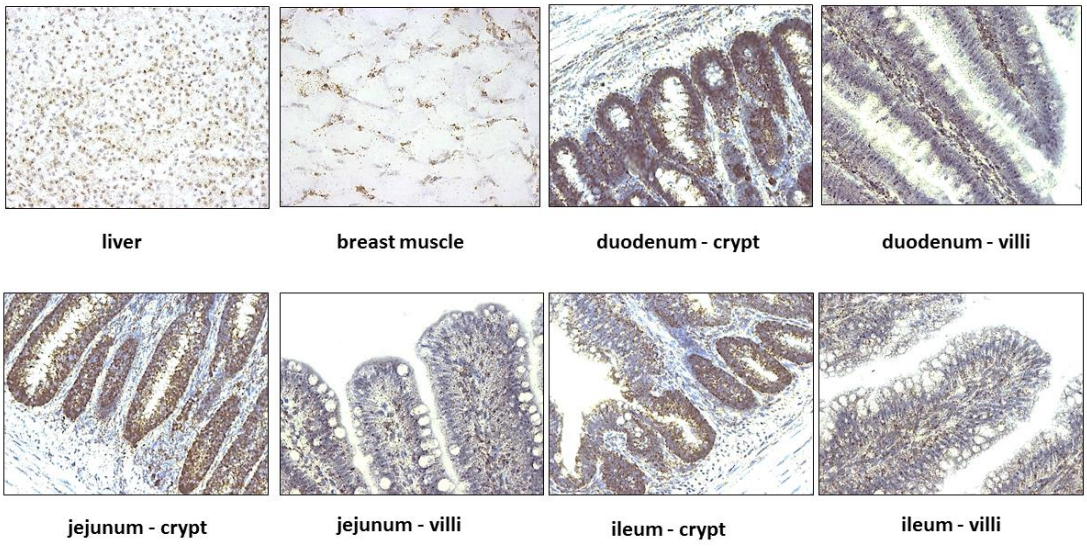
**B. LDHD**


Control
DL-Met
L-Met
DL-HMTBA
liver
breast muscle
duodenum
jejunum
ileum

## C. Crypt and villi in small intestine




## D. Positive control (PPIB)



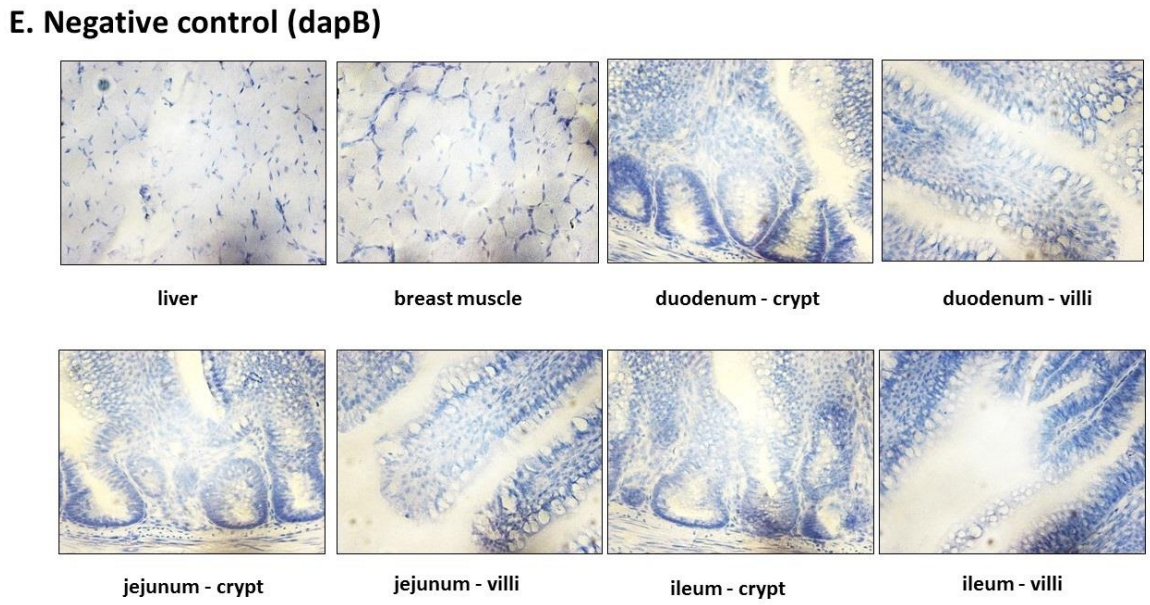
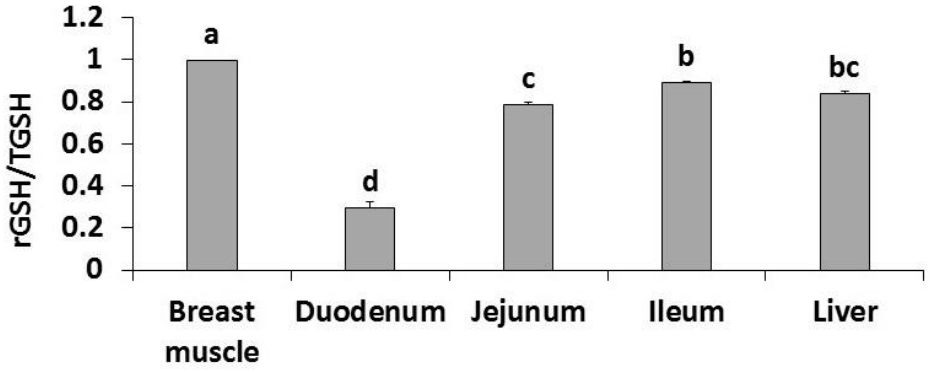
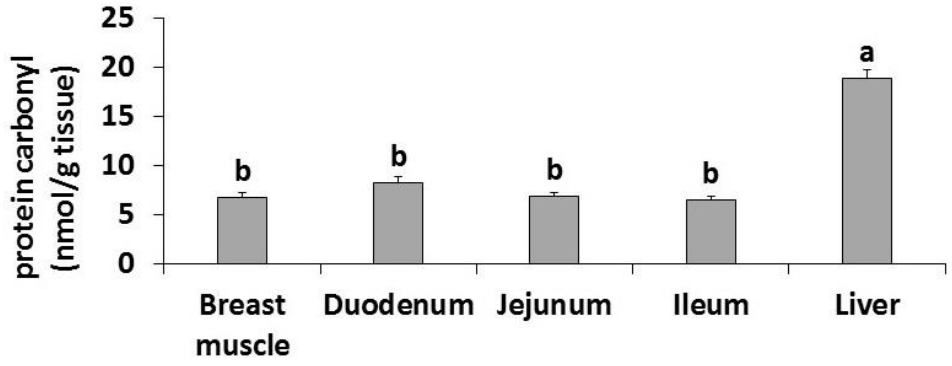



**Figure 4.6** In situ hybridization of HAO1 and LDHD mRNA in different tissues from chickens fed different methionine sources. **(A)** L-HMTBA oxidase (L-2-hydroxy acid oxidase, HAO1) localization and **(B)** D-HMTBA oxidase (D-2-hydroxy acid dehydrogenase, LDHD) localization in liver, breast muscle, duodenum, jejunum and ileum from 10-day-old male broiler chickens fed the diet deficient in Met+Cys (Control), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA). **(C)** The different distribution pattern of HAO1 and LDHD between small intestine crypt and villus was compared. **(D)** PPIB (peptidylprolyl isomerase B) was used as a positive control and **(E)** bacterial dapB (dihydrodipicolinate reductase) was used as a negative control. Brown dots represent the target mRNA. Images were captured at 400 × magnification.

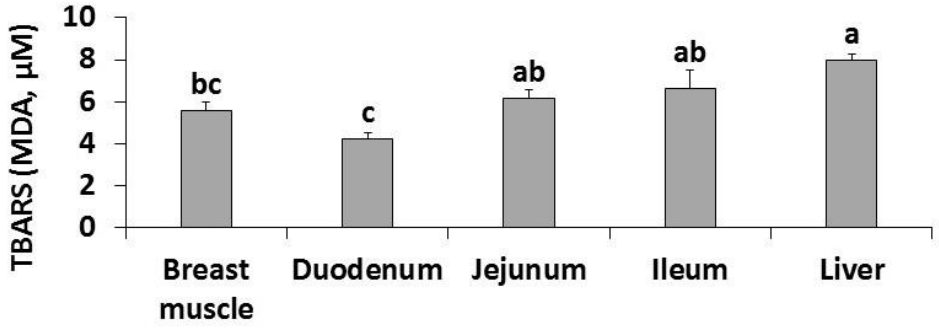
A. rGSH/TGSH




B. protein carbonyl




C. TBARS




D. FRAP

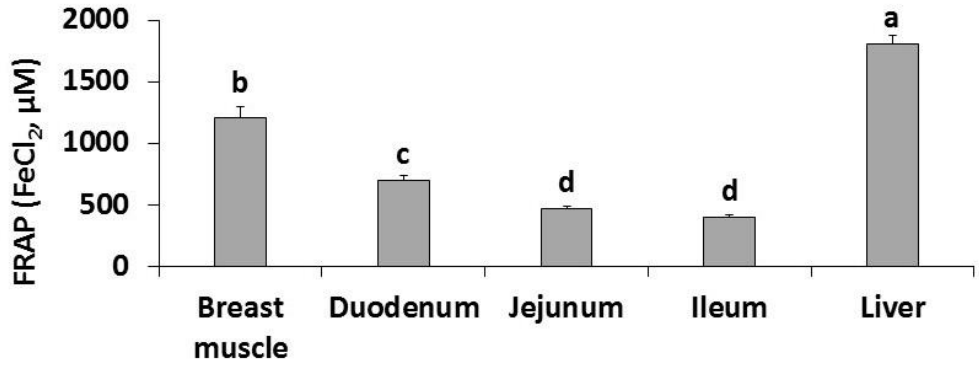


**Figure 4.7** Tissue distribution of oxidative stress markers from chickens fed different methionine sources at post-hatch days 10 and 26. **(A)** Reduced glutathione (rGSH) to total glutathione (TGSH) ratio, **(B)** protein carbonyl level, **(C)** thiobarbituric acid reactive substances (TBARS) level and **(D)** ferric reducing/antioxidant power (FRAP) level in liver, breast muscle, duodenum, jejunum and ileum from male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=40). Figures show main effect of tissue independent of treatment and age. Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant differences, $P < 0.05$.

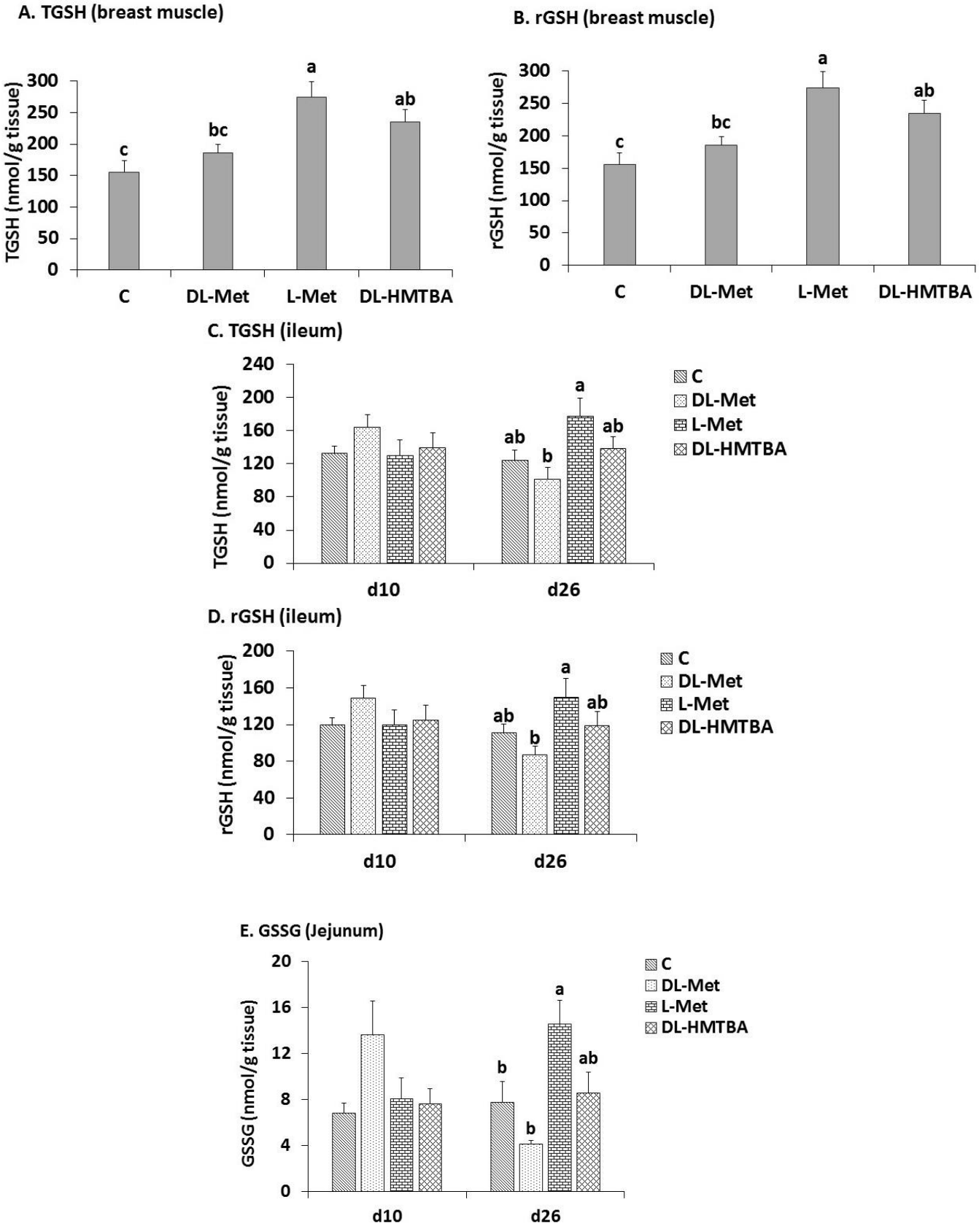

A. TGSH (breast muscle)
TGSH (nmol/g tissue)
300
250
200
150
100
50
0
c
bc
a
ab
C
DL-Met
L-Met
DL-HMTBA
B. rGSH (breast muscle)
rGSH (nmol/g tissue)
300
250
200
150
100
50
0
c
bc
a
ab
C
DL-Met
L-Met
DL-HMTBA
C. TGSH (ileum)
TGSH (nmol/g tissue)
240
200
160
120
80
40
0
ab
b
a
ab
d10
d26
C
DL-Met
L-Met
DL-HMTBA
D. rGSH (ileum)
rGSH (nmol/g tissue)
200
160
120
80
40
0
ab
b
a
ab
d10
d26
C
DL-Met
L-Met
DL-HMTBA
E. GSSG (Jejunum)
GSSG (nmol/g tissue)
20
16
12
8
4
0
b
b
a
ab
d10
d26
C
DL-Met
L-Met
DL-HMTBA

**Figure 4.8** Glutathione profile from chickens fed different methionine sources at post-hatch days 10 and 26 analyzed by tissue. **(A)** Total glutathione (TGSH) level in breast muscle, **(B)** reduced glutathione (rGSH) level in breast muscle, **(C)** TGSH level in ileum, **(D)** rGSH level in ileum and **(E)** oxidized glutathione (GSSG) level in jejunum in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=5). Figure **(A)** and **(B)** show main effect of treatment within each tissue across all the ages. Figure **(C) (D)** and **(E)** show the treatment × age interaction effect within each tissue. Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant differences, $P < 0.05$.

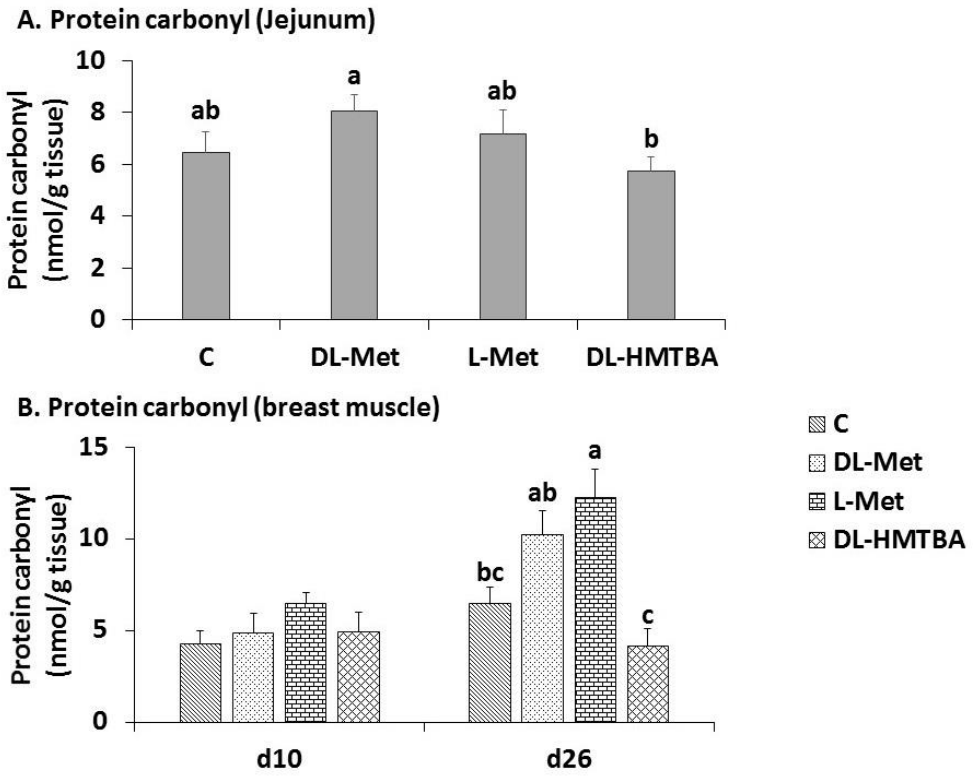



**Figure 4.9** Protein carbonyl levels from chickens fed different methionine sources at post-hatch days 10 and 26 analyzed by tissue. Protein carbonyl level in jejunum **(A)** and in breast muscle **(B)** in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=5). Figure **(A)** shows main effect of treatment within each tissue across all the ages. Figure **(B)** shows the treatment × age interaction effect within each tissue. Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant differences, $P < 0.05$.

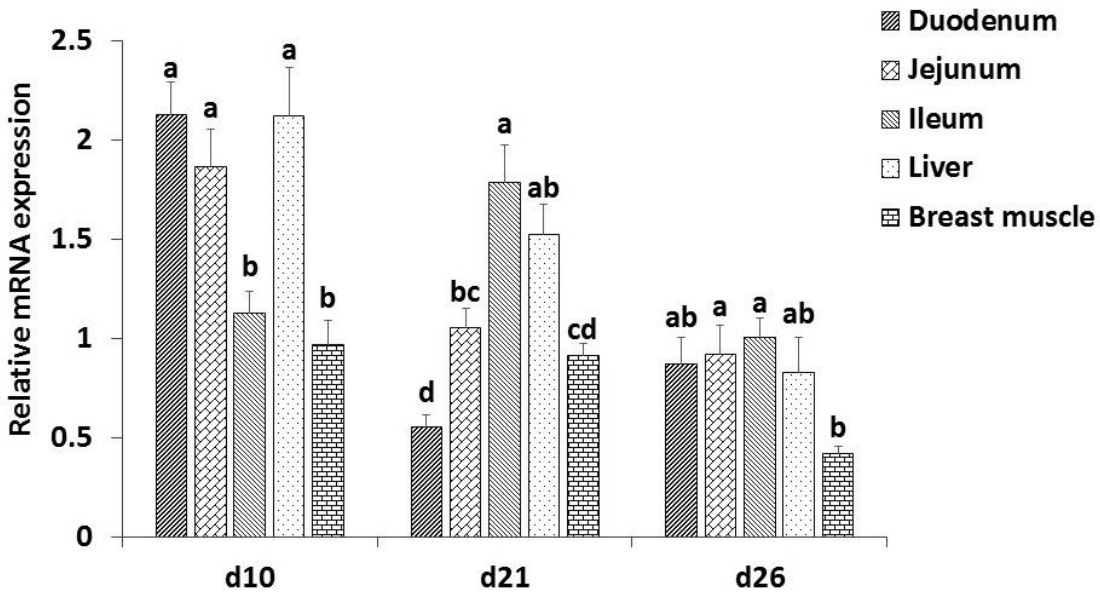
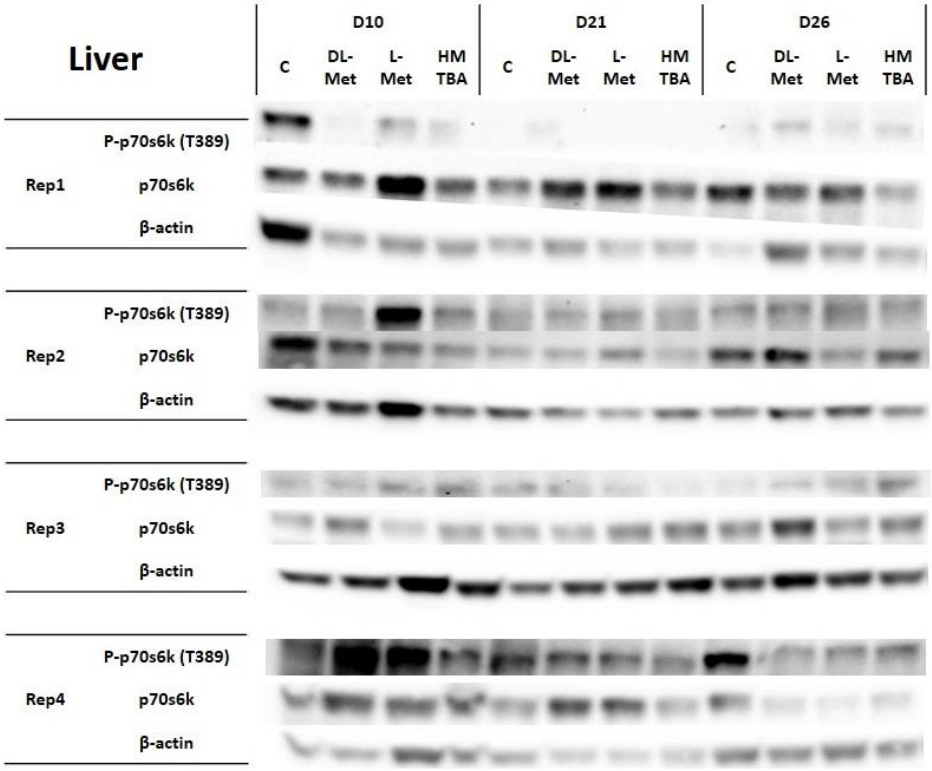
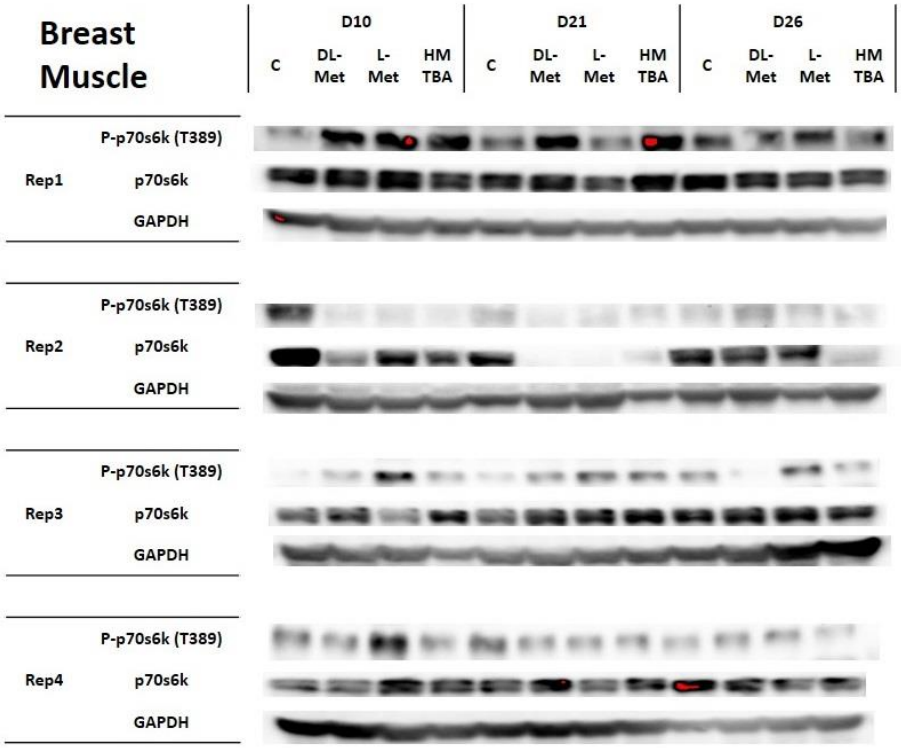



**Figure 4.10** Relative SAT2 mRNA expression in different tissues from chickens fed different methionine sources at post-hatch days 10, 21 and 26. Figure shows tissue effect within each day (n=5). Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters within each age (d10, 21 and 26) represent significant differences, $P < 0.05$.

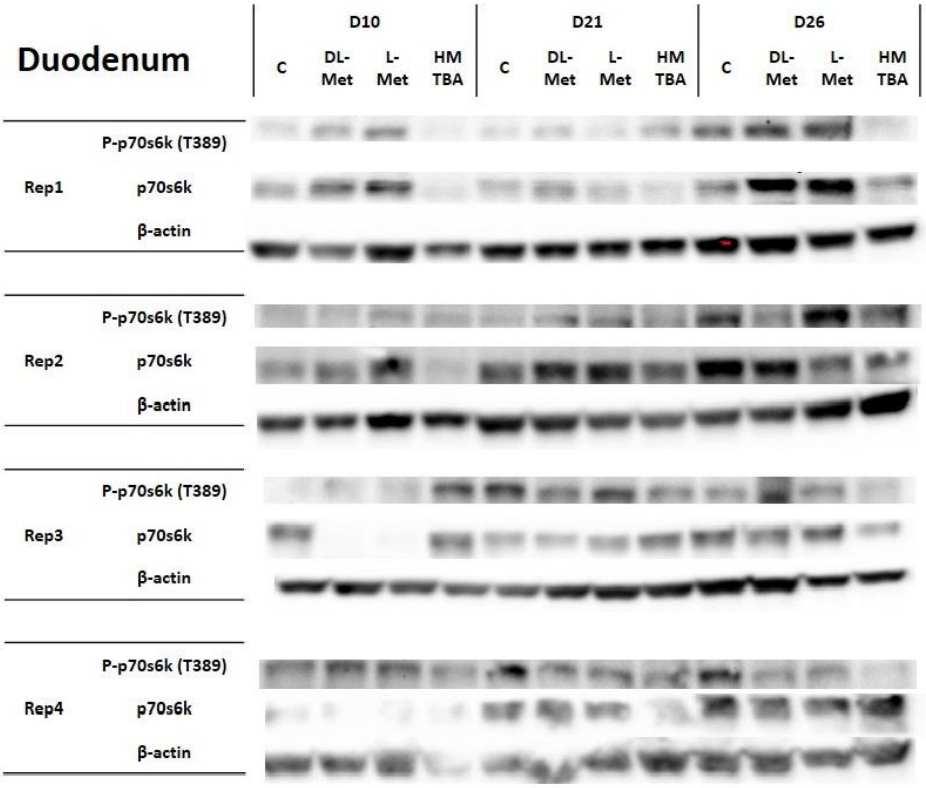
**(A)**


Liver
D10
D21
D26
C
DL-Met
L-Met
HM TBA
Rep1
Rep2
Rep3
Rep4
P-p70s6k (T389)
p70s6k
β-actin

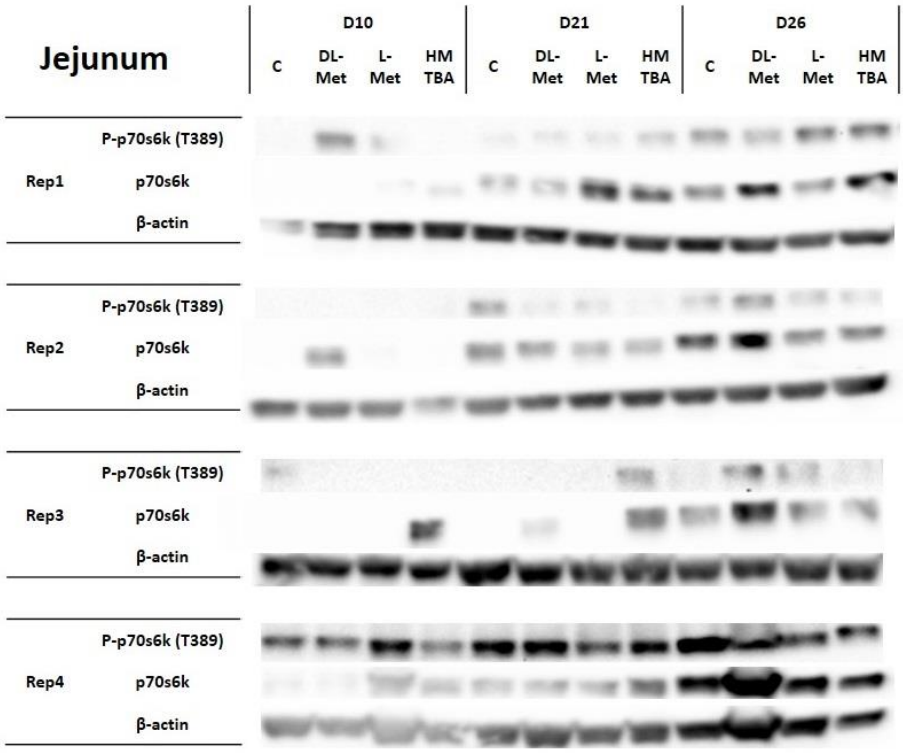
**(B)**


Breast Muscle
D10
D21
D26
C
DL-Met
L-Met
HM TBA
Rep1
Rep2
Rep3
Rep4
P-p70s6k (T389)
p70s6k
GAPDH

**(C)**


Duodenum
D10
D21
D26
C
DL-Met
L-Met
HM TBA
P-p70s6k (T389)
p70s6k
β-actin
Rep1
Rep2
Rep3
Rep4

**(D)**


Jejunum
D10
D21
D26
C
DL-Met
L-Met
HM TBA
P-p70s6k (T389)
p70s6k
β-actin
Rep1
Rep2
Rep3
Rep4

**(E)**

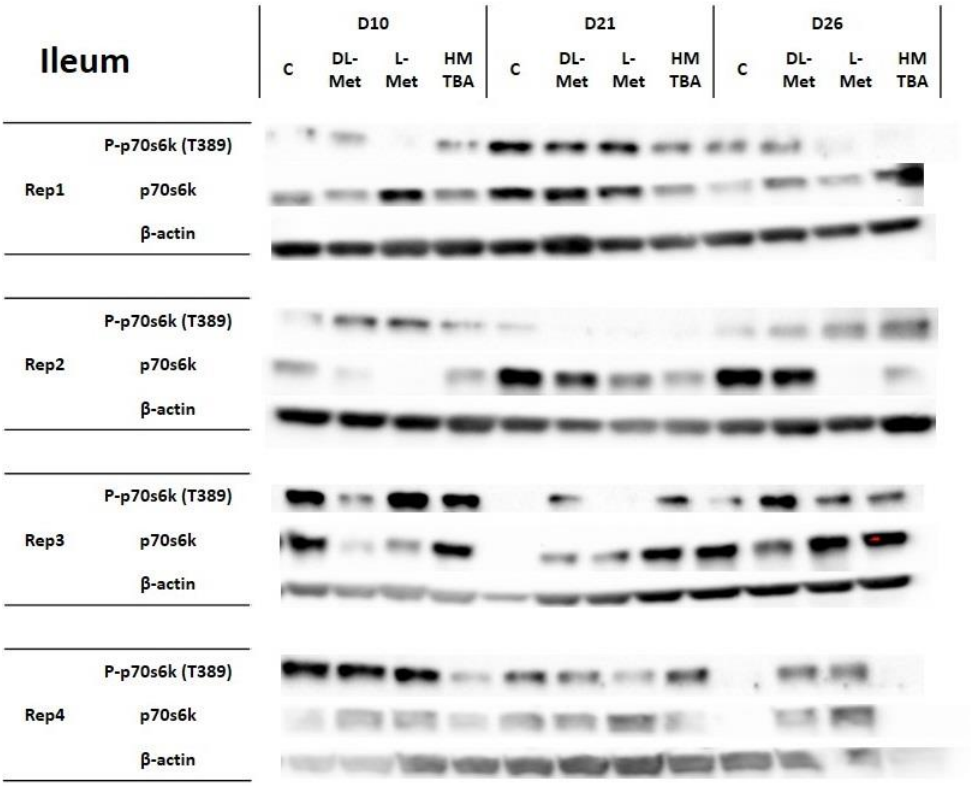



**Figure 4.11** Phospho-p70S6K (T389) and p70S6K protein expression in different tissues from chickens fed different methionine sources at post-hatch days 10, 21 and 26. Representative western blots of p70S6K and phospho-p70S6K (T389) in liver **(A)**, breast muscle **(B)**, duodenum **(C)**, jejunum **(D)** and ileum **(E)** in male broiler chickens fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-methylthio butanoic acid (DL-HMTBA) (n=4). Beta-actin was used as the internal control in liver and small intestine, while GAPDH was used as the internal control in breast muscle.

# CHAPTER 5 Expression of Intestinal Nutrient Transporters and Methionine Oxidases in In Vitro Cultured Chicken Tissues Supplemented with Methionine Isomers or Precursors

## 5.1 Abstract

The common dietary supplemental methionine (Met) sources include DL-Met and the analog DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). D-Met and DL-HMTBA must be converted to L-Met through oxidation for utilization. To determine the in vitro effect of dietary supplemental Met sources on intestinal amino acid transporter and oxidase gene expression, tissue explants (liver, breast muscle, jejunum and ileum) from day of hatch Cobb 500 broiler chickens were cultured in control growth media (C) and growth media without Met but supplemented with 2 mM or 10 mM L-Met, DL-Met or DL-HMTBA. Tissues were collected at 24h and 48h of culture (n=5). Lactate dehydrogenase (LDH) activity was monitored to estimate tissue viability. The mRNA abundance of 14 transporter genes $ATB^{0,+}$, $b^{0,+}AT$, $B^0AT$, 4F2hc, LAT1, MCT1, NHE3, PepT1, rBAT, SAT1, SAT2, SAT3, $y^+LAT1$, $y^+LAT2$ and three oxidase genes L-HMTBA oxidase (HAO1), D-HMTBA oxidase (LDHD) and D-Met oxidase (DAO) were assayed by real time PCR. The statistical analysis was conducted within each time point (24h and 48h) and the model included the main effects of treatment, tissue and their interactions. Data were analyzed by ANOVA using JMP 11.0. Significance was set at $P < 0.05$. Means were separated using Tukey's test. Only PepT1 showed greater mRNA expression in the jejunum than ileum; whereas the amino acid transporters, LAT1, $y^+LAT2$, SAT1, SAT3, NHE3, showed greater mRNA abundance in the ileum than

jejunum. Most changes in nutrient transporter gene expression occurred at 24h of culture. DL-HMTBA (10 mM) decreased mRNA expression of $b^{0,+}$AT, rBAT, and MCT1 relative to control. In contrast, 10 mM DL-HMTBA increased $ATB^{0,+}$ mRNA expression compared to C, L-Met (2 mM and 10 mM) and DL-Met (2 mM). At 48 h, 10 mM DL-HMTBA reduced rBAT mRNA expression compared to both C and 2 mM L-Met. For the oxidases, there were also significant tissue effects. At 48h, 10 mM DL-HMTBA reduced DAO mRNA expression compared to 2 mM L-Met. In conclusion, of the Met sources examined, 10 mM DL-HMTBA had the greatest effect on mRNA expression of specific amino acid transporters and oxidases after 24 hours of culture.

## 5.2 Introduction

DL-methionine (DL-Met) and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) are two commonly used dietary supplemental methionine (Met) sources in the poultry industry. DL-Met is a mixture of the natural Met isoform L-Met and its D-configuration D-Met; while DL-HMTBA is an organic acid but can act as a Met precursor. Carrier-mediated transport in the small intestine plays a major role in the uptake of these dietary Met sources (reviewed by Zhang, et al., 2015). After absorption, neither D-Met nor DL-HMTBA can be directly utilized by animals unless they are converted to L-Met. Oxidation is the key step in the conversion process, which mainly takes place in the liver and small intestine (Dibner, 1983; Martin-Venegas et al., 2006). There are different oxidases that are responsible for the specific substrates during this conversion, e.g., L-HMTBA is oxidized by L-2-hydroxy acid oxidase (EC 1.1.3.15, encoded by HAO1); D-HMTBA is oxidized by D-2-hydroxy acid dehydrogenase (EC 1.1.99.6, encoded by LDHD); and D-Met is oxidized by D-amino acid oxidase (EC 1.4.3.3, encoded by DAO) (Dibner and Knight, 1984).

In the previous chapters, some tissue-, age- and treatment-dependent expression patterns of nutrient transporter and oxidase genes were observed from chickens fed different dietary supplemental Met sources (DL-Met, L-Met and DL-HMTBA). To achieve a better understanding of how those genes are affected by different Met sources, we used an in vitro culture tissue model. The objective of this study was to determine the effects of different Met supplementation sources and concentration on gene expression of intestinal nutrient transporters and Met oxidases in vitro.

## 5.3 Materials and Methods

### 5.3.1 Animals and tissue culture

A total of 100 fertilized Cobb 500 chicken eggs were obtained from hatcheries in Siler City, NC and incubated at 37.5$^{o}$C for 21 days. On day of hatch (day 21 of incubation), newly hatched chicks were killed by cervical dislocation. Tissue samples from jejunum, ileum, breast muscle and liver were collected, minced and placed into 12-well cell culture plates. The protocol was approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Tissues were cultured in Dulbecco's Modified Eagle's Medium (with 4.5g/L glucose and sodium bicarbonate, without L-methionine, L-cystine and L-glutamine) (Sigma-Aldrich), and were supplemented with 2 mM L-glutamine (Sigma-Aldrich), 2 mM L-Cystine•2HCl (Sigma-Aldrich), 5% (v/v) fetal bovine serum (Sigma-Aldrich), 5% (v/v) chicken serum (Sigma-Aldrich), antibiotic antimycotic solution (Genesee Scientific, San Diego, CA) and gentamicin (50µg/mL, Thermo Scientific). Both the fetal bovine serum and the chicken serum were dialyzed using Fisherbrand™ Regenerated Cellulose Dialysis Tubing (3500 MWCO; Fisher Scientific, Waltham, MA) to remove amino acids before use. Different treatments were supplemented with different Met sources: Control (C, 0.2 mM L-Met), Treatment 1 (2 mM L-Met), Treatment 2 (10 mM L-Met), Treatment 3 (2 mM DL-Met), Treatment 4 (10 mM DL-Met), Treatment 5 (2 mM $Ca^{2+}$(DL-HMTBA)$_2$), Treatment 6 (10 mM $Ca^{2+}$(DL-HMTBA)$_2$). Culture plates were kept in a Modular Incubator Chamber (Billups-Rothenberg, San Diego, CA) at 37$^{o}$C in a modified atmosphere of 5% $CO_2$ and 95% $O_2$. Cultured tissue samples were collected at 24h and 48h (n=5).

### 5.3.2 Tissue viability estimation and real-time PCR assay

Tissue viability was estimated at 0h, 24h and 48h of in vitro culture by monitoring lactate dehydrogenase (LDH) activity in the culture media using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific). Total RNA was isolated from collected tissue samples and purified and first-strand cDNA was synthesized, following the same protocol as described in section 3.3.5. Relative quantification real time PCR was used to measure the expression of 14 nutrient transporter genes $ATB^{0,+}$, $b^{0,+}AT$, $B^{0}AT$, 4F2hc, LAT1, MCT1, NHE3, PepT1, rBAT, SAT1, SAT2, SAT3, $y^{+}LAT1$ and $y^{+}LAT2$, three oxidase genes DAO, HAO1 and LDHD, and three reference genes PRL4, PRLP0 and PRLP1. The nutrient transporter genes were only measured in jejunum and ileum. Primers designed for real time PCR are listed in Table 3.1 and Table 4.1, and were validated for amplification efficiency before use (90-115% efficiency). Real-time PCR was performed and relative gene expression was calculated as described in section 3.3.5.

### 5.3.3 Data analysis

The LDH activity data were analyzed within each tissue (liver, muscle, jejunum and ileum): 1) within each time point (24h and 48h), the differences between 7 treatments were compared; 2) within each treatment, the differences between 3 time points (0h, 24h and 48h) were compared.

The gene expression data were analyzed within each time point (24h and 48h). The statistical model included the main effects of treatment, tissue and their interactions.

All data were checked for normality and homogeneity of variances and analyzed by ANOVA. Means were separated using Tukey's Test. JMP Pro version 11.0 (SAS

Institute, Cary, NC) was used for statistical analysis. Data are presented as least square means ± SEM and statistical significance assigned at $P < 0.05$.

**5.4 Results**

To estimate the viability of the cultured tissue, the LDH activity was monitored in each tissue (liver, breast muscle, jejunum and ileum) and compared among the seven treatment groups and among three time points (0h, 24h and 48h) (Table 5.1). The control group was cultured under normal culture condition and the other treatment groups were supplemented with different Met sources at different concentrations. Within each tissue, there was no significant difference between control and the other treatment groups within each time point (compared within each column in Table 5.1); and only one treatment group (control group in jejunum, $P = 0.010$) showed significantly increased LDH activity from 0h to 48h (compared within each row in Table 5.1). Those results indicated that the tissue viability and growth of most treatment groups were not affected by the composition of the culture medium.

For the nutrient transporter gene expression, most changes occurred at 24h of culture (Table 5.2). There were significant tissue distribution patterns for expression of the nutrient transporter gene. Only PepT1 at 24h culture showed greater mRNA expression in the jejunum than ileum explants; whereas the other amino acid transporters tested had either greater mRNA abundance in the ileum than jejunum (LAT1, $y^+$LAT2, SAT1, SAT3, NHE3 at 24h and $B^0$AT, rBAT, SAT3 at 48h, $P < 0.05$) or showed no significant difference (Table 5.2 and Table 5.3). At 24h, DL-HMTBA (10 mM) decreased mRNA expression of $b^{0,+}$AT ($P = 0.005$), rBAT ($P = 0.045$), and MCT1 ($P = 0.048$) relative to control (Figures 5.1A, 5.1B, 5.1D, respectively). In contrast, 10 mM DL-

HMTBA increased $ATB^{0,+}$ mRNA expression compared to C, L-Met (2 mM and 10 mM) and DL-Met (2 mM) (Figure 5.1E, $P = 0.005$). In addition, 2 mM DL-Met reduced $b^{0,+}AT$ mRNA expression relative to control (Figure 5.1A, $P = 0.005$), and reduced SAT3 mRNA expression relative to control and 10 mM DL-HMTBA (Figure 5.1C, $P = 0.012$). At 48h, 10 mM DL-HMTBA reduced rBAT mRNA expression compared to both C and 2 mM L-Met (Figure 5.1F, $P = 0.031$).

For the oxidases, there were also significant tissue effects (Table 5.4). Liver showed the greatest DAO (Figure 5.2A, $P < 0.001$), HAO1 (Figure 5.2C, $P < 0.001$) and LDHD (Figure 5.2B, $P < 0.001$) mRNA expression at 24h culture, and the greatest HAO1 mRNA expression at 48h culture (Figure 5.2C, $P < 0.001$). Jejunum showed the greatest DAO mRNA expression at 48h culture (Figure 5.2A, $P < 0.001$). Breast muscle had lowest DAO mRNA expression at both 24h and 48h culture (Figure 5.2A, both $P < 0.001$). Liver had greater DAO and LDHD mRNA abundance compared to breast muscle at 48h (Figure 5.2A and Figure 5.2B, $P < 0.001$ and $P < 0.001$, respectively). Few significant treatment effects were observed. At 48h, 10 mM DL-HMTBA reduced DAO mRNA expression compared to 2 mM L-Met (Figure 5.3, $P = 0.025$).

**5.5 Discussion**

To investigate the effects of Met supplementation on expression of nutrient transporters in vitro, tissue explants were incubated with media containing different Met sources. First it was necessary to monitor the viability of the tissue explants. LDH is a cytosolic enzyme present in many different cell types, and can be released into the culture media when the cell membrane is damaged (Decker and Lohmann-Matthes, 1988). Thus tissue viability can be estimated by measuring LDH concentration in the culture media.

Within each tissue, LDH-based cytotoxicity was compared between the seven treatment groups and between three culture time points (0h, 24h and 48h). In the control group, 0.2 mM L-Met was supplemented, making it equal to normal DMEM culture media. There was no significant difference between the control group with the other Met supplemented groups, indicating that none of these additives affected tissue viability. From 0h to 48h, the LDH activity in culture media would increase due to apoptosis. However, there was no significant increase in LDH level after 48h culture compared to initial state (0h) in almost all groups, illustrating the viable state of tissues at both time points (24h and 48h) for sampling. Even though there was one exception - the LDH activity was greater at 48h than at 24h and 0h in the control group in jejunum. It is reasonable to assume that the differences in mRNA abundance observed later should not be attributed to changes in tissue viability.

LDH activity in culture media is tightly related to the tissue amount added to the culture media. Thus the minced tissues were weighed and evenly distributed among each treatment group to ensure homogeneity. However, it is difficult to control the uniformity of initial LDH levels between different tissues. In addition, no one has reported standard LDH activity values in normal-growth chicken tissues, making it difficult to evaluate the tissue status in the control group. All of these are drawbacks using LDH activity for tissue viability estimation. There are other techniques that have been reported to measure tissue viability, e.g., Alamar Blue assay and morphological observation through histopathological analysis (Carranza-Torres et al., 2015). These assays may be more accurate in estimating viability, but are also more time-consuming and costly but are more suitable to assess viability of cells.

Real-time PCR was used to estimate the gene expression of 14 intestinal nutrient transporters and three Met oxidases in cultured tissue explants collected from day-of-hatch broiler chickens. The same gene profile was also measured in the in vivo animal trial, which was described in chapters 3 and 4. Similar to the in vivo results, there were significant tissue effects for expression of the transporter and oxidase genes. In the in vitro study, we only collected jejunum and ileum but not duodenum for culturing. However, the nutrient transporter mRNA abundance demonstrated similar patterns as that in the in vivo study: only the di- and tri- peptide transporter PepT1 showed significantly greater mRNA expression in jejunum than ileum. The other transporters tested had either greater mRNA abundance in ileum than jejunum or no significant difference. For the three oxidases, the tissue effect was similar to the in vivo pattern: liver is the tissue with the greatest HAO1 (encoding L-HMTBA oxidase) and LDHD (encoding D-HMTBA oxidase) gene expression; for DAO (encoding D-Met oxidase) especially at 24h culture, liver and jejunum had greater mRNA expression compared to breast muscle.

For treatment effects, there is little overlap between the in vitro and in vivo study. This may be explained by the age-dependent gene expression difference. The in vitro culture using tissues obtained from adult chickens is a great challenge due to microorganism contamination, particularly for trying to culture intestinal segments. In the in vitro study, most significant changes in nutrient transporter gene expression occurred at 24h of culture or with addition of 10 mM DL-HMTBA. The 10 mM DL-HMTBA supplementation decreased mRNA expression of neutral amino acid transporter $b^{0,+}AT$, its dimer rBAT, and the monocarboxylic acid transporter MCT1 relative to control. In contrast, 10 mM DL-HMTBA supplementation increased neutral amino acid transporter

$ATB^{0,+}$ mRNA expression compared to C, L-Met (2 mM and 10 mM) and DL-Met (2 mM). One possible explanation is that the toxicity of the high concentration of DL-HMTBA leads to down-regulation of MCT1, which was reported to be related to DL-HMTBA uptake in the small intestine (Martin-Venegas et al., 2007). Another reasonable interpretation is the effect of high concentration of $Ca^{2+}$. The calcium salt of HMTBA was the only commercial source that was available for this project. The regulation of amino acid transporters is very likely affected by a high concentration of $Ca^{2+}$ due to the high concentration of HMTBA calcium salt supplementation. The only significant treatment effect for Met oxidase gene expression is also related to 10 mM DL-HMTBA supplementation, with 2 mM L-Met showing greater DAO mRNA abundance compared to 10 mM DL-HMTBA. However, the in vitro studies conducted in human Caco-2 cells illustrated that 2 mM DL-HMTBA supplementation up-regulated the conversion of DL-HMTBA to L-Met (Martin-Venegas et al., 2011), as well as MCT1 mRNA expression (Martin-Venegas et al., 2014). This may be due to species difference, or difference between cells and tissue explants.

In conclusion, supplementation of different Met sources had limited effects on intestinal nutrient transporter and Met oxidase gene expression in vitro. Of the Met sources examined, 10 mM DL-HMTBA had the greatest effect on mRNA expression of specific amino acid transporters and oxidases after 24 hours of culture.

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**Table 5.1** Lactate dehydrogenase (LDH) activity in culture media of in vitro cultured liver, breast muscle, jejunum and ileum explants at 0h, 24h and 48h supplemented with different methionine sources.

| Treatment (n=5) | LDH Activity (A490nm-A680nm)[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Liver | | | Muscle | | | Jejunum | | | Ileum | | |
| | 0h | 24h | 48h | 0h | 24h | 48h | 0h | 24h | 48h | 0h | 24h | 48h |
| C | 2.90 | 3.57 | 3.58 | 1.75 | 2.39 | 2.61 | 1.75$^{b}$ | 1.96$^{b}$ | 2.52$^{a}$ | 1.75 | 2.05 | 2.47 |
| 2 mM L-Met | 3.01 | 3.61 | 3.59 | 1.90 | 2.25 | 2.41 | 1.82 | 2.08 | 2.47 | 1.88 | 2.14 | 2.46 |
| 10 mM L-Met | 3.09 | 3.57 | 3.55 | 1.87 | 2.35 | 2.35 | 1.80 | 1.97 | 2.42 | 1.74 | 1.99 | 2.40 |
| 2 mM DL-Met | 2.81 | 3.56 | 3.57 | 2.01 | 2.22 | 2.30 | 1.75 | 2.01 | 2.38 | 1.86 | 2.14 | 2.41 |
| 10 mM DL-Met | 2.93 | 3.53 | 3.49 | 1.90 | 2.30 | 2.27 | 1.83 | 1.87 | 2.35 | 1.87 | 1.99 | 2.41 |
| 2 mM DL-HMTBA | 2.58 | 3.60 | 3.50 | 1.82 | 2.16 | 2.20 | 1.79 | 1.87 | 2.07 | 1.88 | 2.02 | 2.28 |
| 10 mM DL-HMTBA | 2.65 | 3.60 | 3.54 | 1.86 | 1.99 | 2.21 | 1.77 | 1.85 | 2.04 | 1.90 | 1.90 | 2.22 |
| SEM | 0.32 | 0.04 | 0.06 | 0.14 | 0.21 | 0.26 | 0.10 | 0.10 | 0.29 | 0.13 | 0.16 | 0.35 |
| *P*-value | 0.89 | 0.87 | 0.84 | 0.90 | 0.86 | 0.92 | 1.00 | 0.69 | 0.83 | 0.96 | 0.93 | 1.00 |

[1]Data are shown as least squares means ± standard errors of the means. The statistical models include: 1) Analyzing within each tissue and each time point, compare the differences between 7 treatments. Under this model, means in a column without a common letter differ, $P < 0.05$. 2) Analyzing within each tissue and each treatment, compare the differences between 3 time points. Under this model, means in a row (in the same tissue) without a common letter differ, $P < 0.05$.

**Table 5.2** Relative intestinal nutrient transporter gene expression at 24h of in vitro tissue culture.

| Item | | Relative Nutrient Transporter Gene Expression[1] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *$B^0AT$* | *$b^{0,+}AT$* | *rBAT* | *PepT1* | *LAT1* | *$y^+LAT1$* | *$y^+LAT2$* | *SAT1* | *SAT2* | *SAT3* | *4F2hc* | *MCT1* | *NHE3* | *$ATB^{0,+}$* |
| Treatment (n=5) | C | 1.37 | $1.11^{a}$ | $0.84^{a}$ | 1.02 | 0.88 | 0.70 | 0.84 | 0.93 | 0.69 | $1.06^{a}$ | 1.09 | $0.83^{a}$ | 0.82 | $0.72^{b}$ |
| | 2 mM L-Met | 0.98 | $0.68^{ab}$ | $0.62^{ab}$ | 0.70 | 0.71 | 0.54 | 0.66 | 0.76 | 0.53 | $0.65^{ab}$ | 0.76 | $0.65^{ab}$ | 0.56 | $0.76^{b}$ |
| | 10 mM L-Met | 0.98 | $0.70^{ab}$ | $0.71^{ab}$ | 0.61 | 0.79 | 0.57 | 0.73 | 0.82 | 0.55 | $0.76^{ab}$ | 0.84 | $0.60^{ab}$ | 0.59 | $0.53^{b}$ |
| | 2 mM DL-Met | 0.99 | $0.57^{b}$ | $0.69^{ab}$ | 0.61 | 0.70 | 0.49 | 0.67 | 0.69 | 0.57 | $0.52^{b}$ | 0.77 | $0.61^{ab}$ | 0.62 | $0.50^{b}$ |
| | 10 mM DL-Met | 1.10 | $0.75^{ab}$ | $0.76^{ab}$ | 0.66 | 0.83 | 0.57 | 0.83 | 0.84 | 1.03 | $0.80^{ab}$ | 0.80 | $0.63^{ab}$ | 0.72 | $1.18^{ab}$ |
| | 2 mM DL-HMTBA | 1.00 | $0.65^{ab}$ | $0.69^{ab}$ | 0.52 | 0.79 | 0.50 | 0.80 | 0.75 | 0.86 | $0.83^{ab}$ | 0.90 | $0.64^{ab}$ | 0.75 | $1.06^{ab}$ |
| | 10 mM DL-HMTBA | 0.84 | $0.41^{b}$ | $0.50^{b}$ | 0.42 | 0.78 | 0.74 | 0.76 | 0.86 | 1.02 | $0.98^{a}$ | 0.38 | $0.59^{b}$ | 0.86 | $1.93^{a}$ |
| | SEM | 0.25 | 0.11 | 0.07 | 0.13 | 0.07 | 0.08 | 0.05 | 0.08 | 0.16 | 0.11 | 0.19 | 0.05 | 0.08 | 0.26 |
| | *P*-value | 0.87 | <0.01 | 0.04 | 0.07 | 0.62 | 0.26 | 0.13 | 0.44 | 0.12 | 0.01 | 0.27 | 0.04 | 0.06 | <0.01 |
| Tissue (n=5) | Jejunum | 0.90 | 0.67 | 0.67 | $0.77^{a}$ | $0.71^{b}$ | 0.55 | $0.72^{b}$ | $0.73^{b}$ | 0.66 | $0.61^{b}$ | 0.76 | 0.65 | $0.62^{b}$ | 0.85 |
| | Ileum | 1.18 | 0.72 | 0.70 | $0.53^{b}$ | $0.85^{a}$ | 0.62 | $0.80^{a}$ | $0.89^{a}$ | 0.84 | $0.99^{a}$ | 0.82 | 0.65 | $0.79^{a}$ | 1.05 |
| | SEM | 0.14 | 0.06 | 0.04 | 0.07 | 0.04 | 0.04 | 0.03 | 0.04 | 0.09 | 0.06 | 0.10 | 0.03 | 0.04 | 0.14 |
| | *P*-value | 0.15 | 0.59 | 0.60 | 0.02 | 0.01 | 0.25 | 0.04 | 0.01 | 0.16 | <0.01 | 0.68 | 0.94 | <0.01 | 0.32 |
| Interaction (n=5) | Treatment ×Tissue | 0.92 | 1.00 | 0.77 | 0.98 | 0.90 | 1.00 | 0.73 | 0.90 | 1.00 | 0.79 | 1.00 | 0.97 | 0.86 | 1.00 |

[1]Data are shown as least squares means ± standard errors of the means. The statistical model included main effects of treatment and tissue (jejunum, ileum) and their interaction. Means in a column without a common letter differ, $P < 0.05$.

**Table 5.3** Relative intestinal nutrient transporter gene expression at 48h of in vitro tissue culture.

| Item | | Relative Nutrient Transporter Gene Expression[1] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *$B^0AT$* | *$b^{0,+}AT$* | *rBAT* | *PepT1* | *LAT1* | *$y^+LAT1$* | *$y^+LAT2$* | *SAT1* | *SAT2* | *SAT3* | *4F2hc* | *MCT1* | *NHE3* | *$ATB^{0,+}$* |
| Treatment (n=5) | C | 0.65 | 0.95 | 1.09$^{a}$ | 1.17 | 0.89 | 1.16 | 0.82 | 0.71 | 1.29 | 0.71 | 0.77 | 0.84 | 1.09 | 1.07 |
| | 2 mM L-Met | 1.10 | 1.50 | 1.14$^{a}$ | 1.73 | 0.81 | 1.41 | 0.94 | 0.90 | 1.53 | 1.23 | 1.30 | 0.97 | 1.25 | 1.74 |
| | 10 mM L-Met | 0.88 | 1.30 | 1.04$^{ab}$ | 1.68 | 0.83 | 1.26 | 0.79 | 0.90 | 1.43 | 0.87 | 0.95 | 0.89 | 1.07 | 1.17 |
| | 2 mM DL-Met | 0.72 | 1.14 | 0.95$^{ab}$ | 1.39 | 0.74 | 1.07 | 0.76 | 0.72 | 1.06 | 0.71 | 1.21 | 1.00 | 0.75 | 1.06 |
| | 10 mM DL-Met | 0.83 | 1.23 | 1.01$^{ab}$ | 1.31 | 0.74 | 1.13 | 0.82 | 0.83 | 0.90 | 1.05 | 1.17 | 0.87 | 0.99 | 0.83 |
| | 2 mM DL-HMTBA | 0.91 | 1.21 | 0.98$^{ab}$ | 1.36 | 0.86 | 1.03 | 0.86 | 0.74 | 1.20 | 0.94 | 1.16 | 0.98 | 1.00 | 1.23 |
| | 10 mM DL-HMTBA | 1.04 | 0.75 | 0.67$^{b}$ | 1.21 | 0.79 | 1.27 | 0.72 | 0.80 | 1.15 | 1.02 | 0.89 | 0.92 | 1.12 | 1.29 |
| | SEM | 0.16 | 0.22 | 0.10 | 0.18 | 0.11 | 0.16 | 0.06 | 0.10 | 0.36 | 0.13 | 0.22 | 0.06 | 0.19 | 0.40 |
| | *P*-value | 0.45 | 0.29 | 0.03 | 0.21 | 0.96 | 0.65 | 0.26 | 0.67 | 0.90 | 0.09 | 0.62 | 0.42 | 0.68 | 0.80 |
| Tissue (n=5) | Jejunum | 0.69$^{b}$ | 1.00 | 0.87$^{b}$ | 1.51 | 0.76 | 1.10 | 0.80 | 0.74 | 1.16 | 0.75$^{b}$ | 1.01 | 0.91 | 0.99 | 1.14 |
| | Ileum | 1.06$^{a}$ | 1.31 | 1.10$^{a}$ | 1.30 | 0.86 | 1.28 | 0.83 | 0.85 | 1.29 | 1.12$^{a}$ | 1.11 | 0.94 | 1.09 | 1.26 |
| | SEM | 0.09 | 0.12 | 0.05 | 0.10 | 0.60 | 0.08 | 0.03 | 0.05 | 0.19 | 0.07 | 0.12 | 0.03 | 0.10 | 0.21 |
| | *P*-value | <0.01 | 0.06 | <0.01 | 0.13 | 0.23 | 0.13 | 0.55 | 0.14 | 0.64 | <0.01 | 0.56 | 0.58 | 0.46 | 0.70 |
| Interaction (n=5) | Treatment ×Tissue | 0.85 | 0.95 | 0.72 | 0.93 | 0.86 | 0.97 | 0.75 | 0.95 | 0.98 | 0.62 | 0.96 | 0.99 | 0.79 | 0.98 |

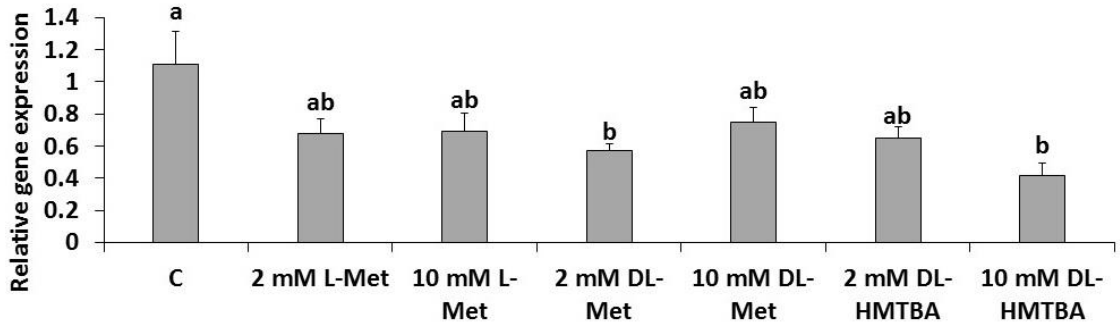
[1]Data are shown as least squares means ± standard errors of the means. The statistical model included main effects of treatment and tissue (jejunum, ileum) and their interaction. Means in a column without a common letter differ, $P < 0.05$.

**Table 5.4** Relative Met oxidase gene expression at 24h and 48h of in vitro tissue culture.

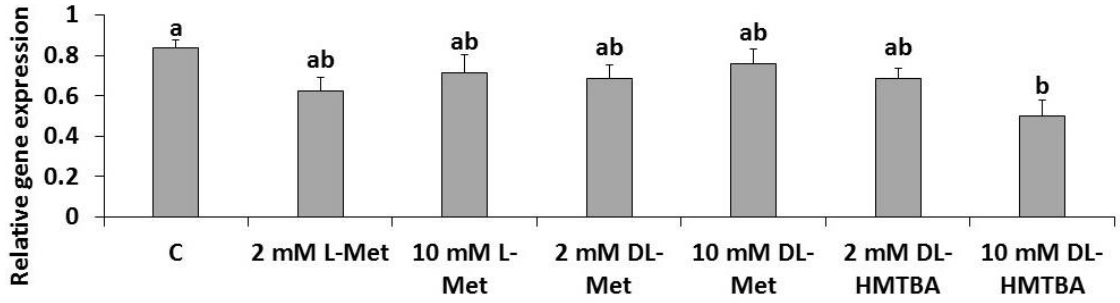
| Relative Oxidase Gene Expression[1] | | 24H | | | 48H | | |
|---|---|---|---|---|---|---|---|
| | | *DAO* | *LDHD* | *HAO1* | *DAO* | *LDHD* | *HAO1* |
| Treatment (n=5) | C | 1.68 | 1.78 | 4.60 | 1.72$^{ab}$ | 0.94 | 3.41 |
| | 2 mM L-Met | 1.93 | 1.69 | 4.53 | 1.91$^{a}$ | 1.35 | 3.80 |
| | 10 mM L-Met | 2.10 | 2.23 | 4.89 | 1.61$^{ab}$ | 1.51 | 2.51 |
| | 2 mM DL-Met | 1.64 | 1.21 | 3.87 | 1.76$^{ab}$ | 0.81 | 1.94 |
| | 10 mM DL-Met | 1.91 | 1.98 | 4.53 | 1.50$^{ab}$ | 0.94 | 1.15 |
| | 2 mM DL-HMTBA | 1.67 | 1.40 | 3.93 | 1.74$^{ab}$ | 0.81 | 2.48 |
| | 10 mM DL-HMTBA | 1.34 | 1.84 | 3.92 | 0.96$^{b}$ | 0.91 | 1.59 |
| | SEM | 0.29 | 0.39 | 1.05 | 0.19 | 0.26 | 0.68 |
| | *P*-value | 0.60 | 0.59 | 0.99 | 0.03 | 0.34 | 0.08 |
| Tissue (n=5) | Liver | 3.56$^{a}$ | 2.87$^{a}$ | 15.59$^{a}$ | 1.63$^{b}$ | 1.67$^{a}$ | 7.90$^{a}$ |
| | Muscle | 0.37$^{c}$ | 1.46$^{b}$ | 0.56$^{b}$ | 0.23$^{c}$ | 0.44$^{b}$ | 0.45$^{b}$ |
| | Jejunum | 1.84$^{b}$ | 1.24$^{b}$ | 0.53$^{b}$ | 2.55$^{a}$ | 0.97$^{ab}$ | 0.59$^{b}$ |
| | Ileum | 1.23$^{b}$ | 1.36$^{b}$ | 0.61$^{b}$ | 2.00$^{b}$ | 1.08$^{ab}$ | 0.71$^{b}$ |
| | SEM | 0.22 | 0.30 | 0.80 | 0.15 | 0.20 | 0.51 |
| | *P*-value | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Interaction (n=5) | Treatment ×Tissue | 0.40 | 0.99 | 1.00 | 0.21 | 0.72 | 0.08 |

[1]Data are shown as least squares means ± standard errors of the means. The statistical model included main effects of treatment and tissue (liver, breast muscle, jejunum, ileum) and their interaction. Means in a column without a common letter differ, $P < 0.05$.

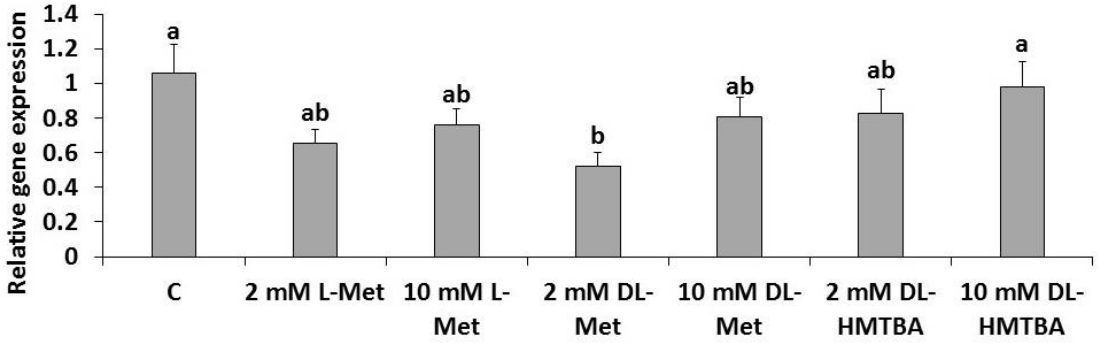
A. $b^{0,+}AT$ (24h)



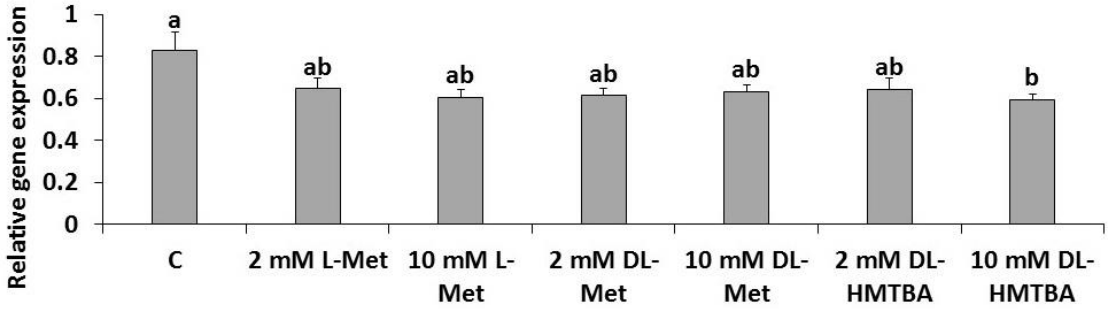

B. rBAT (24h)




C. SAT3 (24h)




D. MCT1 (24h)



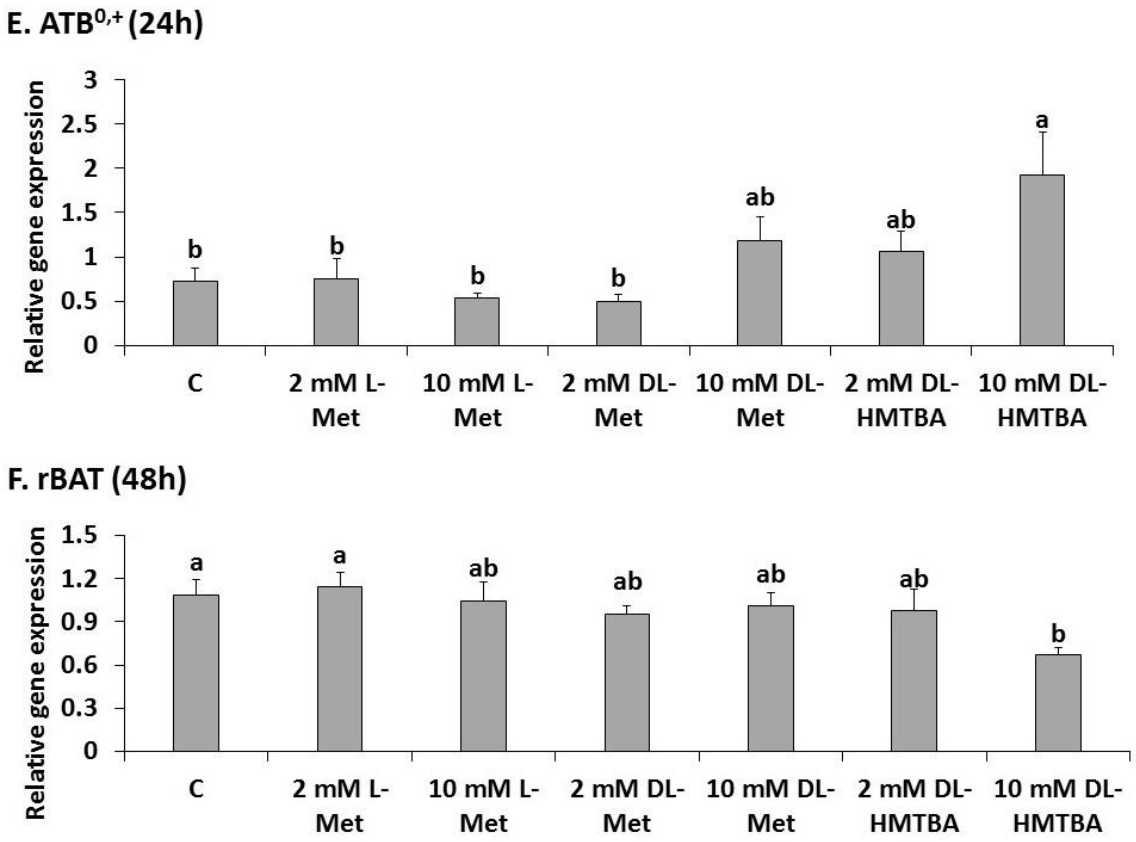



**Figure 5.1** Relative intestinal nutrient transporter gene expression at 24h and 48h of in vitro tissue culture. Relative $b^{0,+}AT$ **(A)**, rBAT **(B)**, SAT3 **(C)**, MCT1 **(D)** and $ATB^{0,+}$ **(E)** mRNA abundance at 24h and rBAT **(F)** mRNA abundance at 48h of in vitro cultured jejunum and ileum supplemented with different methionine sources (n=5). Figure shows main effect of treatment within each time point (24h and 48h) across jejunum and ileum. Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant differences, $P < 0.05$.

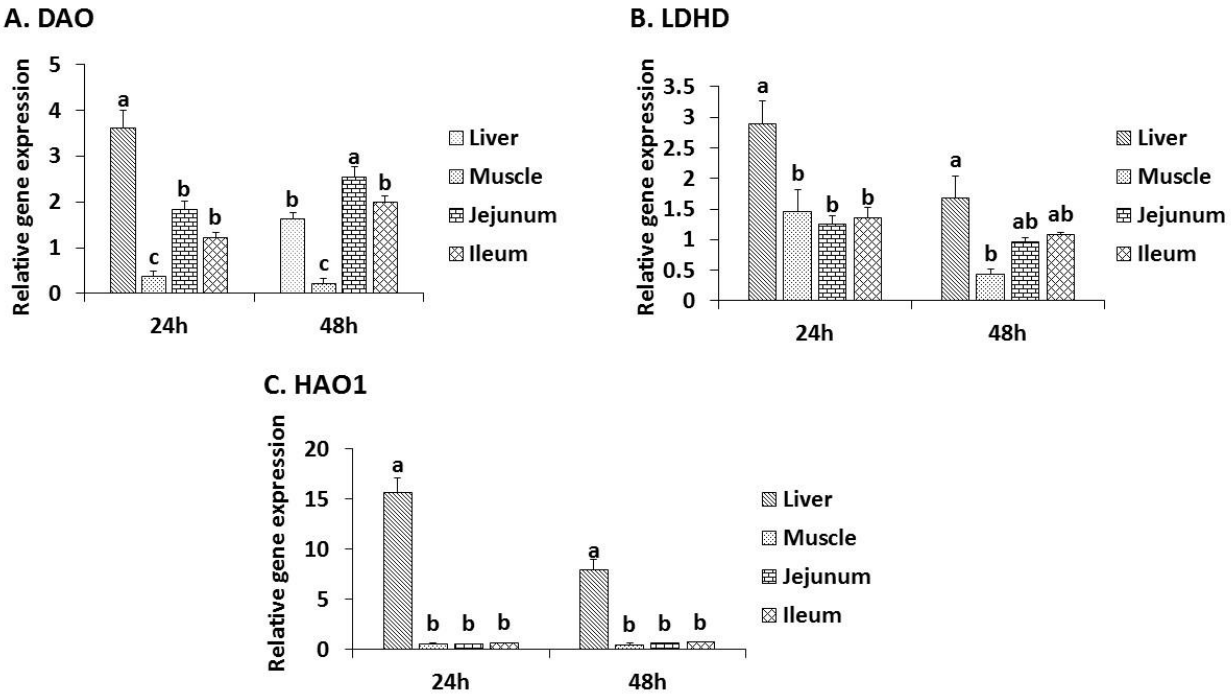



**Figure 5.2** Relative methionine oxidases gene expression at 24h and 48h of in vitro cultured liver, muscle and small intestine. **(A)** Relative D-amino acid oxidases (DAO), **(B)** D-HMTBA oxidase (D-2-hydroxy acid dehydrogenase, LDHD) and **(C)** L-HMTBA oxidase (L-2-hydroxy acid oxidase 1, HAO1) mRNA abundance at 24h and 48h of in vitro cultured liver, breast muscle, jejunum and ileum supplemented with different methionine sources (n=5). Figure shows main effect of tissue within each time point (24h and 48h) across all the treatments. Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant differences, $P < 0.05$.

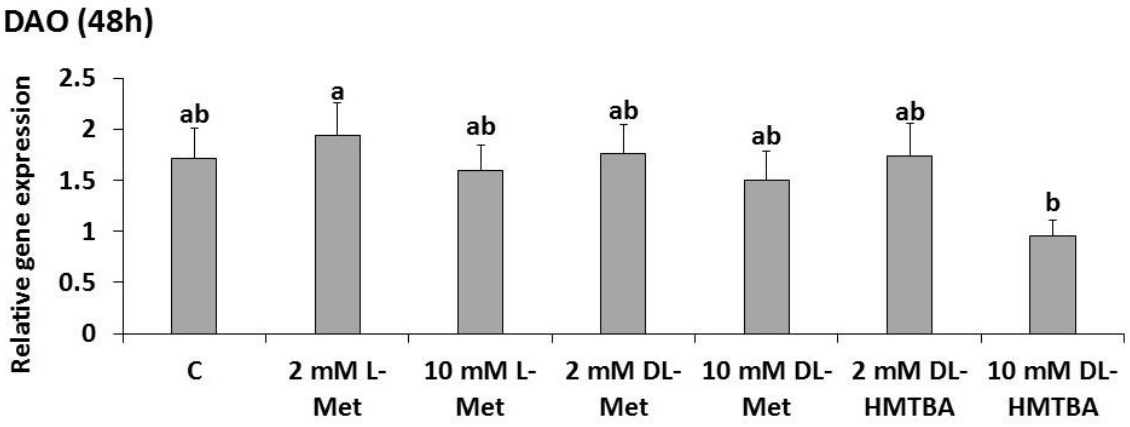



**Figure 5.3** Relative D-amino acid oxidases (DAO) mRNA abundance at 48h of in vitro tissue culture supplemented with different methionine sources. n=5. Figure shows main effect of treatment within each time point (24h and 48h) across all the tissue (liver, breast muscle, jejunum and ileum). Values represent least squares means ± SEM. Means were separated using Tukey's test. Bars with different letters represent significant differences, $P < 0.05$.

# CHAPTER 6 Hepatic Gene Expression via RNA Sequencing in Broiler Chickens Fed Methionine Isomers or Precursors

## 6.1 Abstract

The objective of this study was to identify differentially expressed genes (DEGs) in liver from chickens fed different dietary supplemental methionine (Met) sources including DL-methionine (DL-Met), L-methionine (L-Met) and DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) and Met deficient diet through next generation sequencing technologies. Liver samples from 16 male Cobb 500 broiler chickens fed different Met sources were collected at d26 (n=4). Total RNA was extracted and sequenced via the Illumina HiSeq platform. A total of 745 DEGs were identified. Among them, 107 DEGs were in common when one of the dietary Met supplemental groups (DL-Met, L-Met or DL-HMTBA) was compared with the control group (C, Met deficient). However, only two DEGs (FGD3 and TNNC1) were identified between DL-Met and L-Met and none between DL-Met and DL-HMTBA or L-Met and DL-HMTBA. The gene BHMT (betaine--homocysteine S-methyltransferase), which encodes a cytosolic enzyme that catalyzes the conversion of homocysteine to Met in the methionine degradation pathway, was down regulated in both DL-HMTBA and L-Met groups compared with C. These results indicate that Met deficiency could lead to many DEGs. However, different dietary supplemental Met sources minimally change the hepatic gene expression profile in broilers.

## 6.2 Introduction

Next-generation sequencing technology is being widely applied in molecular biology research for characterization and quantification of genomes, epigenomes and transcriptomes in the last few years. As a high-throughput technology, it can produce large and complex datasets at single nucleotide resolution, with continuously dropping cost (Han et al., 2015). RNA sequencing (RNA-seq) is a technique that generates cDNA sequences from the entire transcriptome, followed by library construction and massively parallel deep sequencing (Han et al., 2015). Since gene expression is usually time-, cell-type- and stimulus-dependent, many loci are only expressed under certain conditions. RNA-seq allows quantification of the abundance of each transcript under specific treatment conditions in a rather unbiased way with low background signals (Wang et al., 2009). The objective of this study was to characterize the differentially expressed genes (DEGs) in liver from chickens fed different dietary supplemental methionine (Met) sources: DL-methionine (DL-Met), L-methionine (L-Met) or DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA), and Met deficient diet, using RNA sequencing technology.

## 6.3 Materials and Methods

### 6.3.1 Animals

Details about the animals used in this study are the same as described in 3.3.1.

### 6.3.2 Total RNA extraction and sequencing

At d26, 16 liver samples from 4 replicates per treatment were collected for RNA sequencing (n=4). Total RNA was extracted from liver using Tri-Reagent (Molecular Research Center) and Tissue Lyser II (Qiagen). The RNeasy Mini Kit (Qiagen) and

RNase-free DNase I (Qiagen) were used for total RNA purification. The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a NanoDropTM 1000 (Thermo Scientific). The quality of the total RNA was evaluated with an Experion™ Automated Electrophoresis Station (Bio-rad) and Experion RNA StdSens analysis kit (Bio-rad). RNA-seq libraries were prepared by the poly A-selection method using the Illumina TruSeq$^{TM}$ DNA Library Preparation kit, followed by sequencing on a HiSeq 1000 using 101-cycle single reads, and performed at the Biocomplexity Institute of Virginia Tech (VTBI).

### 6.3.3 Mapping and data analysis

To generate predicted transcripts, RNA-seq reads were scrubbed for adaptor and quality using a combination of ea-utils (for adaptor) (Aronesty, 2011) and Btrim (for quality) (Kong, 2011). Following trimming, reads were mapped to the chicken genome, Gallus_ gallus-4.0 (released 2011), using Cufflinks/Cuffmerge and TopHat2 (Bowtie2) (Langmead and Salzberg, 2012; Ghosh and Chan, 2016). The assembled reads were counted via HTSeq (Anders et al., 2015). Differential expression was determined using contrasts between treatments and tested for significance using the Benjamini-Hochberg corrected Wald Test in the R-package DESeq2 (Love et al., 2014). Results were called differentially expressed if the adjusted *P*-value was less than 0.1.

To understand the biological systems involved, the identified DEGs were mapped and molecular networks generated using Ingenuity Pathways Analysis (IPA) software (Ingenuity® Systems) by VTBI. Pre-filtered DEG ($P < 0.05$) data were uploaded and the significant pathways were identified using Fisher's exact test with the cutoff *P*-value of

0.1. The *P*-value indicates the likelihood of the uploaded gene list in a given pathway or network being found together due to random chance (Zhang, 2014).

**6.4 Results and discussion**

The RNA sequencing data were single-end 130 nucleotide reads. Because there was a dark cycle at cycle 86, the data were hard clipped at cycle 85. However, analysis with and without the hard trim at 85 cycles revealed minimal difference in terms of reads mapping uniquely.

On average, 15.9M reads per sample (13.7M-18.7M) were received. After accounting for potential novel read islands, more than 85% of the reads mapped uniquely and to a read island. Of the 15% reads that did not get counted, about 10% were to a region without a defined feature (known or novel) and 5% did not map uniquely. A target of more than 75% was the goal. A majority of the reads not mapping is due to genomic contamination, bacterial/viral contamination, etc.

From RNA sequencing of liver at d26, there were 399 DEGs between control (C) and DL-Met, 298 DEGs between C and L-Met and 423 DEGs between C and DL-HMTBA. Among these genes, there were 107 DEGs in common between C and DL-Met, L-Met and DL-HMTBA. Only 2 genes were differentially expressed between DL-Met and L-Met. No DEGs were found between DL-HMTBA and DL-Met, and DL-HMTBA and L-Met (Figure 6.1).

The top up- and down-regulated genes comparing between C and the other treatment groups are listed in Table 6.1A-C. The common genes among them are shown in Table 6.1D. Three of the common top down-regulated genes were SLC1A6 (EAAT4), which is a transporter of glutamate and aspartate, OVCH2 (ovochymase 2 or oviductin),

which has serine-type endopeptidase activity and HMP19, which is similar to the HMP19 (a 19 kD encoding protein) in humans. A common top up-regulated gene was FKBP1B (FK506 binding protein 1B), which plays a role in basic cellular processes involving protein folding and trafficking. Even in mammals and humans, there is little report about the function or regulation of these genes. How these proteins relate to Met and Met analogue transport and utilization remain unknown.

The two genes differentially expressed between DL-Met and L-Met were FYVE, RhoGEF and PH domain containing 3 (FGD3) and Troponin C type 1 (slow) (TNNC1). FGD3 was expressed greater in DL-Met fed chickens than L-Met fed chickens ($P$ = 0.079). It is a guanyl nucleotide exchange factor that exchanges bound GDP for free GTP and plays a role in regulating the actin cytoskeleton and cell shape. TNNC1 was also expressed greater in DL-Met fed chickens than L-Met fed chickens ($P$ = 0.079). Troponin C type 1 is encoded by the TNNC1 gene. Troponin C is the central regulatory protein of striated muscle contraction.

All the DEGs were subjected to pathway analysis and the top associated pathways related to diseases and disorders, molecular and cellular functions, and physiological system development and functions containing the DEGs in each comparison group are shown in Table 6.2.

Through IPA analysis, the top regulated canonical pathways were identified in each comparison group, with the threshold of -log (p-value) ≥ 1.5, shown in Figure 6.2A-C. The canonical pathways revealed that are directly related to Met metabolism are the superpathway of methionine degradation and the methionine salvage pathway. When comparing L-Met and C, there were 3 genes in the superpathway of methionine

degradation that met the cutoff criteria: BHMT (betaine--homocysteine S-methyltransferase), CTH (cystathionase, cystathionine gamma-lyase) and PCCA (propionyl CoA carboxylase, alpha polypeptide) (Table 6.3A). When comparing DL-HMTBA and C, there were 2 genes in the methionine salvage pathway that had differential expression: BHMT and MTR (5-methyl-tetrahydrofolate-homocysteine methyltransferase) (Table 6.3B). All of these genes encode a cytoplasmic enzyme. BHMT catalyzes the conversion of betaine and homocysteine to dimethylglycine and Met, respectively. CTH catalyzes the conversion of cystathione derived from Met into cysteine in the transsulfuration pathway. PCCA catalyzes the carboxylation reaction of propionyl CoA. MTR catalyzes the final step in Met biosynthesis. Their location in the corresponding pathways are shown in Figure 6.3A-B, highlighted with colored diamonds.

In summary, the RNA sequencing data provided a global analysis of hepatic gene expression. A total of 745 DGEs between DL-Met, L-Met, or DL-HMTBA and C were identified. Interestingly there were only two genes (FGD3 and TNNC1) that were differentially expressed between DL-Met and L-Met and none between DL-Met and DL-HMTBA or L-Met and DL-HMTBA. A total of 107 genes were differentially expressed in common between one of the dietary Met supplemental groups (DL-Met, L-Met or DL-HMTBA) and C. The gene BHMT in the methionine degradation pathway, which encodes a cytosolic enzyme that catalyzes the conversion of homocysteine to Met, was down regulated in both DL-HMTBA and L-Met compared with C. Down regulation of this gene would be expected to decrease the endogenous production of Met, which makes sense since there is sufficient Met available from the diet in the Met supplementation groups. In conclusion, Met deficiency greatly altered the hepatic gene expression profile.

However, different dietary supplemental Met sources had only minimal impact on overall hepatic gene expression in broiler chickens.

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**Table 6.1** Top differentially expressed hepatic genes in chickens fed different supplemental methionine sources compared to methionine deficient group.

**(A)** DL-methionine (DL-Met) fed chickens vs. control group.

| Up-regulated Genes | | | Down-regulated Genes | | |
|---|---|---|---|---|---|
| Genes[1] | Genebank ID | Fold change | Genes[2] | Genebank ID | Fold change |
| DPP10 | XM_422126.5 | 2.353 | OVCH2 | XM_015286412.1 | -3.787 |
| SHROOM3 | XM_004941163.2 | 2.277 | SLC1A6 | XM_015299799.1 | -2.820 |
| COL4A4 | XM_015276987.1 | 2.061 | MYOM1 | XM_015277934.1 | -2.621 |
| FKBP1B | XM_015284949.1 | 2.028 | HMP19 | BM491299.1 | -2.589 |
| CDKN2B | NM_204433.1 | 1.960 | ATP13A5 | XM_015291588.1 | -2.450 |
| ADAMTSL1 | XM_004949124.2 | 1.957 | ATP2B2 | XM_015293477.1 | -2.404 |
| CYR61 | NM_001031563.1 | 1.899 | THSD7B | XM_015289897.1 | -2.319 |
| OSBPL3 | XM_015281839.1 | 1.868 | IL22RA2 | XM_001233761.4 | -1.995 |
| PKP2 | XM_416362.5 | 1.780 | KIAA1107 | XM_015290685.1 | -1.832 |
| CDH17 | XM_015282915.1 | 1.755 | CA4 | XM_415893.5 | -1.760 |

[1] DPP10: dipeptidyl-peptidase 10; SHROOM3: shroom family member 3; COL4A4: collagen, type IV, alpha 4; FKBP1B: FK506 binding protein 1B; CDKN2B: cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4); ADAMTSL1: ADAMTS-like 1; CYR61: cysteine rich angiogenic inducer 61; OSBPL3: oxysterol binding protein-like 3; PKP2: plakophilin 2; CDH17: cadherin 17, LI cadherin (liver-intestine).

[2] OVCH2: ovochymase 2; SLC1A6: solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6, EAAT4; MYOM1: myomesin 1; HMP19: similar to human HMP19 protein; ATP13A5: ATPase type 13A5; ATP2B2: ATPase, $Ca^{++}$ transporting, plasma membrane 2; THSD7B: thrombospondin, type I, domain containing 7B; IL22RA2: interleukin 22 receptor, alpha 2; KIAA1107: coding protein, also known as BTBD8; CA4: carbonic anhydrase IV.

**(B)** L-methionine (L-Met) fed chickens vs. control group.

| Up-regulated Genes | | | Down-regulated Genes | | |
|---|---|---|---|---|---|
| Genes[1] | Genebank ID | Fold change | Genes[2] | Genebank ID | Fold change |
| SHROOM3 | XM_004941163.2 | 1.935 | OVCH2 | XM_015286412.1 | -3.148 |
| DPP10 | XM_422126.5 | 1.672 | MYOM1 | XM_015277934.1 | -2.664 |
| DHDH | XM_015273067.1 | 1.671 | SLC1A6 | XM_015299799.1 | -2.406 |
| FKBP1B | XM_015284949.1 | 1.660 | HMP19 | BM491299.1 | -1.983 |
| GUCY1A2 | XM_001233953.4 | 1.632 | ERMAP | Not accessible | -1.963 |
| CPE | XM_420392.5 | 1.593 | IL22RA2 | XM_001233761.4 | -1.945 |
| HLA-A | NM_001245061.1 | 1.561 | GUCY2C | XM_416207.5 | -1.835 |
| PITX2 | NM_205010.1 | 1.509 | RBM11 | XM_416676.5 | -1.752 |
| ETV7 | XM_004934884.2 | 1.507 | GLOD5 | XM_001234978.3 | -1.728 |
| DOC2B | XM_004946708.2 | 1.457 | FBP2 | XM_425039.5 | -1.716 |

[1] SHROOM3: shroom family member 3; DPP10: dipeptidyl-peptidase 10; DHDH: dihydrodiol dehydrogenase; FKBP1B: FK506 binding protein 1B; GUCY1A2: guanylate cyclase 1, soluble, alpha 2; CPE: carboxypeptidase E; HLA-A: major histocompatibility complex, class II, alpha; PITX2: paired-like homeodomain 2; ETV7: ets variant 7; DOC2B: double C2-like domains, beta.

[2] OVCH2: ovochymase 2; MYOM1: myomesin 1; SLC1A6: solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6, EAAT4; HMP19: similar to human HMP19 protein; ERMAP: similar to human erythroblast membrane associated protein; IL22RA2: interleukin 22 receptor, alpha 2; GUCY2C: guanylate cyclase 2C; RBM11: RNA binding motif protein 11; GLOD5: glyoxalase domain containing 5; FBP2: fructose-1,6-bisphosphatase 2.

**(C)** DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) fed chickens vs. control group.

| Up-regulated Genes | | | Down-regulated Genes | | |
|---|---|---|---|---|---|
| Genes[1] | Genebank ID | Fold change | Genes[2] | Genebank ID | Fold change |
| OSBPL3 | XM_015281839.1 | 2.669 | OVCH2 | XM_015286412.1 | -3.204 |
| ZFHX3 | NM_001293088.1 | 2.594 | MYOM1 | XM_015277934.1 | -2.800 |
| ANXA13 | NM_001199501.1 | 2.320 | HEMGN | XM_430508.5 | -2.404 |
| DAB1 | NM_204238.1 | 2.002 | HMP19 | BM491299.1 | -2.359 |
| RERG | XM_416404.5 | 1.920 | GLOD5 | XM_001234978.3 | -2.333 |
| GUCA2A | NM_001197038.1 | 1.890 | NAAA | XM_015276425.1 | -2.142 |
| FKBP1B | XM_015284949.1 | 1.826 | ATP13A5 | XM_015291588.1 | -2.141 |
| KRT20 | NM_001277981.1 | 1.785 | GUCY2C | XM_416207.5 | -2.097 |
| ALDH1A3 | NM_204669.1 | 1.772 | SLC1A6 | XM_015299799.1 | -2.088 |
| GUCY1A2 | XM_001233953.4 | 1.756 | ACTN2 | NM_205323.1 | -2.003 |

[1] OSBPL3: oxysterol binding protein-like 3; ZFHX3: zinc finger homeobox 3; ANXA13: annexin A13; DAB1: Dab, reelin signal transducer, homolog 1 (Drosophila); RERG: RAS-like, estrogen-regulated, growth inhibitor; GUCA2A: guanylate cyclase activator 2B (uroguanylin); FKBP1B: FK506 binding protein 1B; KRT20: keratin 20; ALDH1A3: aldehyde dehydrogenase 1 family, member A3; GUCY1A2: guanylate cyclase 1, soluble, alpha 2.

[2] OVCH2: ovochymase 2; MYOM1: myomesin 1; HEMGN: hemogen; HMP19: similar to human HMP19 protein; GLOD5: glyoxalase domain containing 5; NAAA: N-acylethanolamine acid amidase; ATP13A5: ATPase type 13A5; GUCY2C: guanylate cyclase 2C; SLC1A6: solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6, EAAT4; ACTN2: actinin, alpha 2.

**(D)** Summary of the common 10 most up- and down-regulated genes.

| **Common Up-regulated Genes[1]** | | | |
|---|---|---|---|
| Genes | DL-Met vs. control | L-Met vs. control | DL-HMTBA vs. control |
| DPP10 | + | + | |
| SHROOM3 | + | + | |
| FKBP1B | + | + | + |
| GUCY1A2 | | + | + |
| **Common Down-regulated Genes[2]** | | | |
| Genes | DL-Met vs. control | L-Met vs. control | DL-HMTBA vs. control |
| OVCH2 | + | + | + |
| SLC1A6 | + | + | + |
| MYOM1 | + | + | + |
| HMP19 | + | + | + |
| GUCY2C | | + | + |
| IL22RA2 | + | + | |
| ATP13A5 | + | | + |

[1] DPP10: dipeptidyl-peptidase 10; SHROOM3: shroom family member 3; FKBP1B: FK506 binding protein 1B; GUCY1A2: guanylate cyclase 1, soluble, alpha 2.
Control: 0.78% Met+Cys for starter diet, 0.70% Met+Cys for grower diet and 0.62% Met+Cys for finisher diet; DL-Met: basal diet supplemented with 0.22% DL-methionine; L-Met: basal diet supplemented with 0.22% L-methionine; DL-HMTBA: basal diet supplemented with 0.31% DL-2-hydroxy-4-methylthio butanoic acid.

[2] OVCH2: ovochymase 2; SLC1A6: solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6, EAAT4; MYOM1: myomesin 1; HMP19: similar to human HMP19 protein; GUCY2C: guanylate cyclase 2C; IL22RA2: interleukin 22 receptor, alpha 2; ATP13A5: ATPase type 13A5.

**Table 6.2.** Top diseases and Bio Functions in liver from chickens fed different supplemental methionine sources compared to methionine deficient group based on prediction analysis of differentially-expressed genes through RNA sequencing.

**(A)** DL-methionine (DL-Met) fed chickens vs. control group:

| **Diseases and Disorders** | | |
|---|---|---|
| Name | p-value | # Genes |
| Cardiovascular Disease | 4.01E-05 - 2.92E-02 | 28 |
| Organismal Injury and Abnormalities | 4.01E-05 - 2.92E-02 | 117 |
| Endocrine System Disorders | 4.54E-05 - 2.67E-02 | 30 |
| Gastrointestinal Disease | 4.54E-05 - 2.92E-02 | 46 |
| Cancer | 1.31E-04 - 2.92E-02 | 181 |
| **Molecular and Cellular Functions** | | |
| Name | p-value | # Genes |
| Cellular Growth and Proliferation | 4.54E-05 - 2.92E-02 | 39 |
| Cell-To-Cell Signaling and Interaction | 1.30E-04 - 2.92E-02 | 35 |
| Cellular Movement | 5.10E-04 - 2.92E-02 | 32 |
| Cell Morphology | 6.88E-04 - 2.92E-02 | 46 |
| Cellular Assembly and Organization | 6.88E-04 - 2.92E-02 | 43 |
| **Physiological System Development and Function** | | |
| Name | p-value | # Genes |
| Embryonic Development | 4.54E-05 - 2.92E-02 | 50 |
| Skeletal and Muscular System Development and Function | 1.30E-04 - 2.92E-02 | 35 |
| Tissue Development | 1.30E-04 - 2.92E-02 | 68 |
| Hematological System Development and Function | 2.36E-04 - 2.92E-02 | 24 |
| Organismal Development | 2.36E-04 - 2.92E-02 | 60 |

**(B)** L-methionine (L-Met) fed chickens vs. control group.

| **Diseases and Disorders** | | |
|---|---|---|
| Name | p-value | # Genes |
| Gastrointestinal Disease | 6.05E-04 - 4.53E-02 | 96 |
| Cancer | 7.00E-04 - 4.06E-02 | 173 |
| Metabolic Disease | 1.08E-03 - 3.68E-02 | 21 |
| Organismal Injury and Abnormalities | 1.08E-03 - 4.53E-02 | 69 |
| Renal and Urological Disease | 1.08E-03 - 2.87E-02 | 15 |
| **Molecular and Cellular Functions** | | |
| Name | p-value | # Genes |
| Cellular Development | 6.05E-04 - 3.95E-02 | 17 |
| Carbohydrate Metabolism | 1.79E-03 - 3.39E-02 | 14 |
| Cell-To-Cell Signaling and Interaction | 1.79E-03 - 4.61E-02 | 24 |
| Cellular Growth and Proliferation | 3.00E-03 - 2.47E-02 | 14 |
| Lipid Metabolism | 3.51E-03 - 3.91E-02 | 14 |
| **Physiological System Development and Function** | | |
| Name | p-value | # Genes |
| Digestive System Development and Function | 6.05E-04 - 4.69E-02 | 13 |
| Embryonic Development | 6.05E-04 - 4.69E-02 | 24 |
| Organ Development | 6.05E-04 - 4.69E-02 | 26 |
| Organismal Development | 6.05E-04 - 4.69E-02 | 32 |
| Tissue Development | 6.05E-04 - 4.69E-02 | 37 |

**(C)** DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) fed chickens vs. control group:

| **Diseases and Disorders** | | |
|---|---|---|
| Name | p-value | # Genes |
| Cancer | 4.56E-09 - 3.28E-02 | 238 |
| Organismal Injury and Abnormalities | 4.56E-09 - 3.31E-02 | 151 |
| Reproductive System Disease | 4.56E-09 - 3.31E-02 | 137 |
| Gastrointestinal Disease | 2.51E-05 - 3.31E-02 | 125 |
| Hereditary Disorder | 3.58E-05 - 3.31E-02 | 52 |
| **Molecular and Cellular Functions** | | |
| Name | p-value | # Genes |
| Cell Cycle | 2.06E-07 - 3.31E-02 | 52 |
| Cellular Assembly and Organization | 3.36E-07 - 3.31E-02 | 75 |
| DNA Replication, Recombination, and Repair | 3.36E-07 - 3.31E-02 | 23 |
| Cellular Movement | 1.46E-05 - 3.07E-02 | 58 |
| Cellular Function and Maintenance | 3.49E-04 - 3.04E-02 | 62 |
| **Physiological System Development and Function** | | |
| Name | p-value | # Genes |
| Tissue Morphology | 3.25E-05 - 3.31E-02 | 38 |
| Endocrine System Development and Function | 1.40E-04 - 1.82E-02 | 4 |
| Tissue Development | 1.40E-04 - 3.31E-02 | 85 |
| Organ Morphology | 2.56E-04 - 3.31E-02 | 36 |
| Skeletal and Muscular System Development and Function | 2.56E-04 - 3.31E-02 | 31 |

**Table 6.3** Hepatic genes that met the cutoff criteria in chickens fed different supplemental methionine sources compared to methionine deficient group.

**(A)** Genes that met the cutoff criteria in the superpathway of methionine degradation, comparing L-methionine (L-Met) fed chickens to the control group.

| Symbol | Entrez Gene Name | Genebank ID | Log Ratio | False Discovery Rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| BHMT | betaine--homocysteine S-methyltransferase | XM_414685.4 | -1.513 | 3.52E-03 | Cytoplasm | enzyme (EC 2.1.1.5) |
| CTH | cystathionase (cystathionine gamma-lyase) | XM_422542.5 | -0.755 | 2.90E-02 | Cytoplasm | enzyme (EC 4.4.1.1) |
| PCCA | propionyl CoA carboxylase, alpha polypeptide | XM_416970.5 | -1.188 | 1.24E-06 | Cytoplasm | enzyme (EC 6.4.1.3) |

**(B)** Genes that met the cutoff criteria in the methionine salvage pathway, comparing DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) fed chickens to the control group.

| Symbol | Entrez Gene Name | Genebank ID | Log Ratio | False Discovery Rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| BHMT | betaine--homocysteine S-methyltransferase | XM_414685.4 | -1.513 | 3.52E-03 | Cytoplasm | enzyme (EC 2.1.1.5) |
| MTR | 5-methyl-tetrahydrofolate-homocysteine methyltransferase | NM_001031104.1 | -0.675 | 2.72E-03 | Cytoplasm | enzyme (EC 2.1.1.13) |

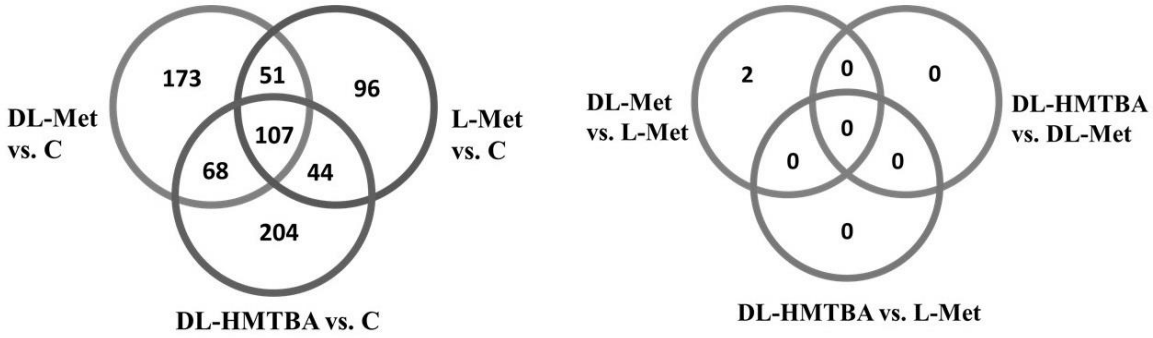
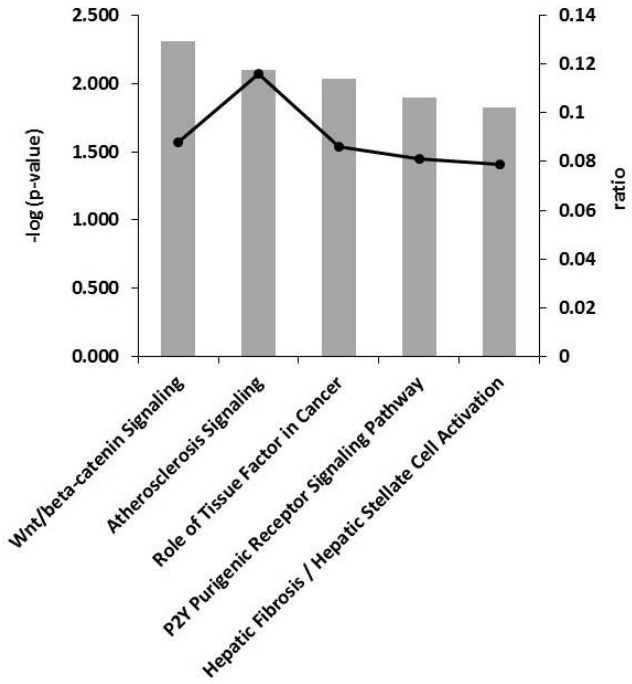
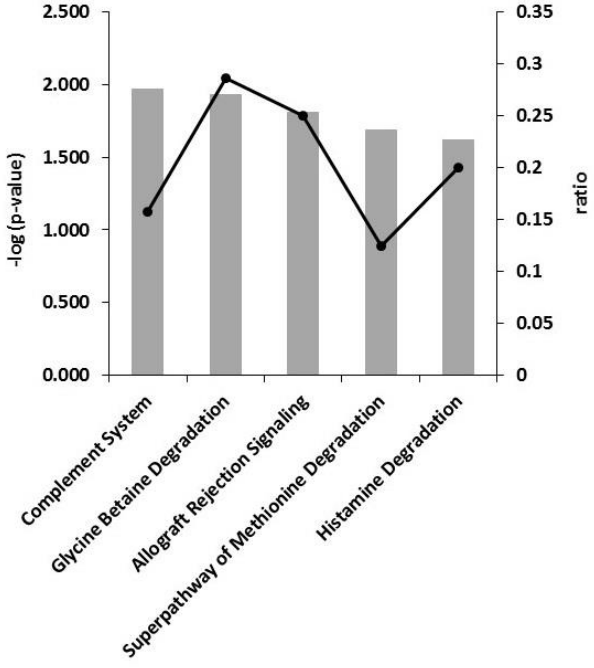



**Figure 6.1** Venn diagrams for differentially expressed hepatic genes in chickens fed different supplemental methionine sources compared to methionine deficient group and their overlapping genes. Male broiler chickens were fed the diet deficient in Met+Cys (C), or the same diet supplemented with 0.22% DL-methionine (DL-Met), 0.22% L-methionine (L-Met) or 0.31% DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) and liver samples were collect at day 26 (n=4). Numbers of differentially expressed hepatic genes are shown for each group.

A. DL-Met vs. control




B. L-Met vs. control




C. DL-HMTBA vs. control

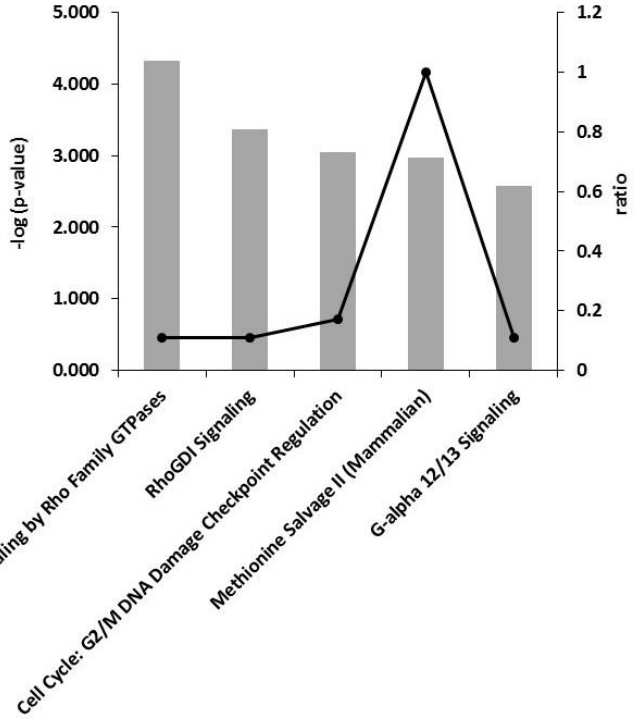
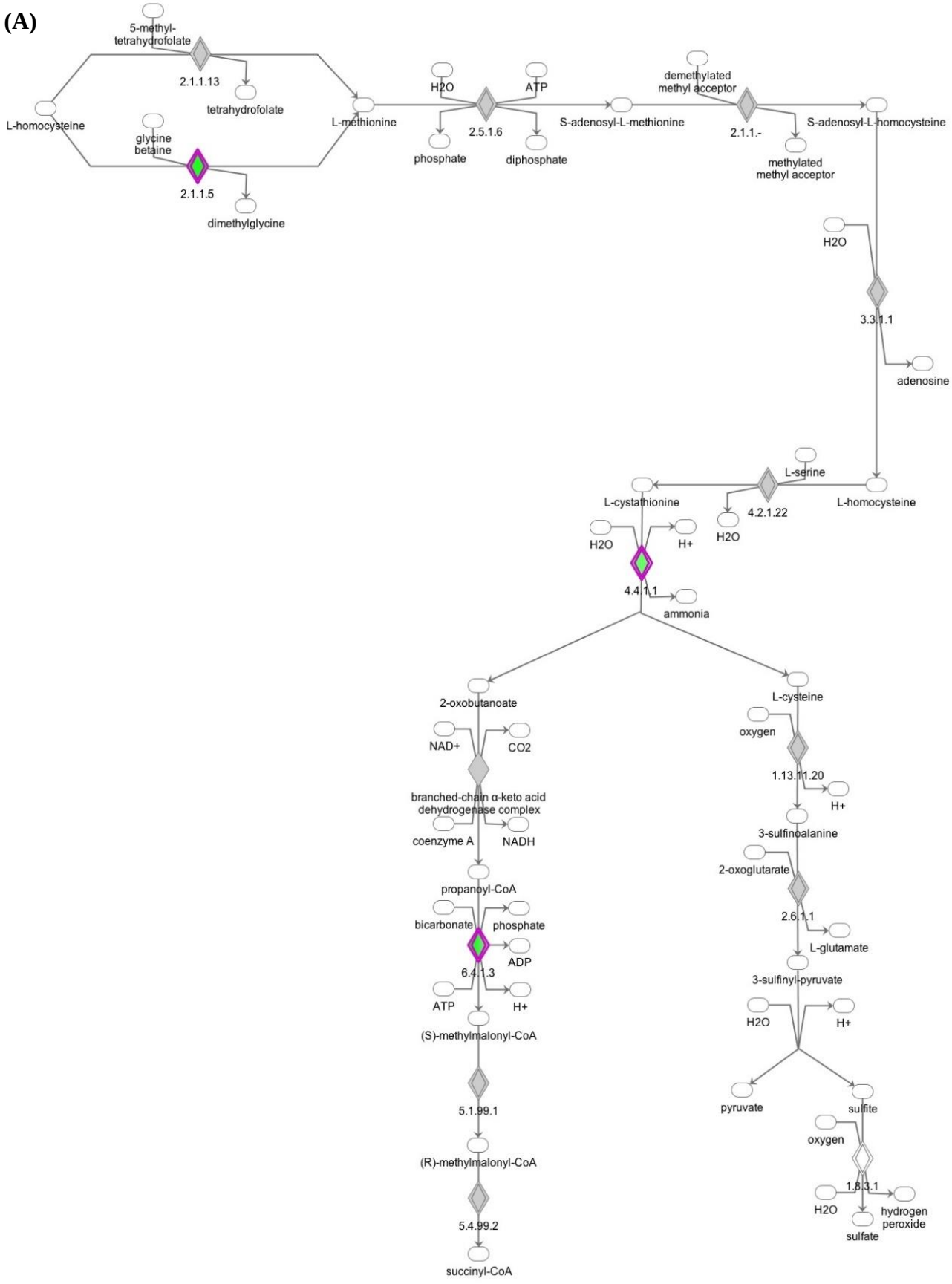


**Figure 6.2.** Top canonical pathways in liver from chickens fed different supplemental methionine sources compared to methionine deficient group. (-log (p-value) ≥ 1.5 as the threshold). **(A)** DL-methionine (DL-Met) fed chickens vs. control group. **(B)** L-methionine (L-Met) fed chickens vs. control group. **(C)** DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) fed chickens vs. control group. The length of the bar indicates the significance level of the pathway. The line indicates the ratio of numbers of genes that met the cutoff criteria / the total number of genes that make up that pathway.

**(A)**


5-methyl-
tetrahydrofolate
2.1.1.13
tetrahydrofolate
L-homocysteine
glycine
betaine
2.1.1.5
dimethylglycine
L-methionine
H2O
ATP
2.5.1.6
phosphate
diphosphate
S-adenosyl-L-methionine
demethylated
methyl acceptor
2.1.1.-
methylated
methyl acceptor
S-adenosyl-L-homocysteine
H2O
3.3.1.1
adenosine
L-serine
L-cystathionine
4.2.1.22
L-homocysteine
H2O
H2O
H+
4.4.1.1
ammonia
2-oxobutanoate
NAD+
CO2
branched-chain α-keto acid
dehydrogenase complex
coenzyme A
NADH
propanoyl-CoA
bicarbonate
phosphate
ADP
6.4.1.3
ATP
H+
(S)-methylmalonyl-CoA
5.1.99.1
(R)-methylmalonyl-CoA
5.4.99.2
succinyl-CoA
L-cysteine
oxygen
1.13.11.20
H+
3-sulfinoalanine
2-oxoglutarate
2.6.1.1
L-glutamate
3-sulfinyl-pyruvate
H2O
H+
pyruvate
sulfite
oxygen
1.8.3.1
H2O
hydrogen
peroxide
sulfate

**(B)**

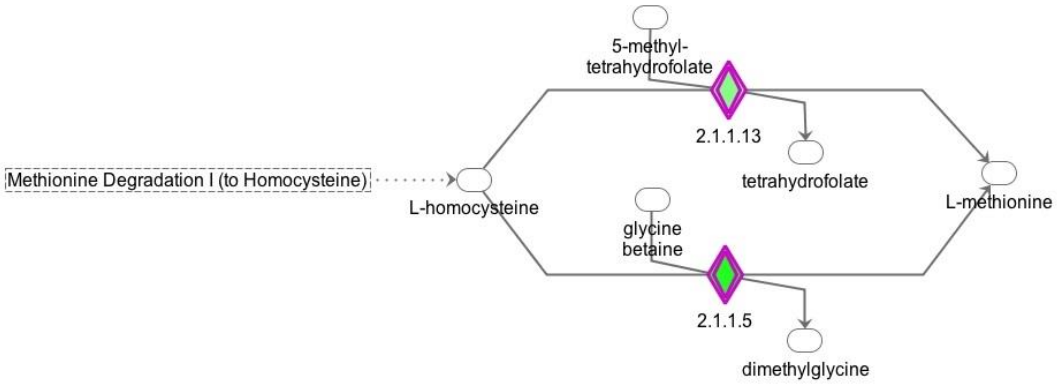



**Figure 6.3** Superpathway of methionine degradation (A) and methionine salvage pathway (B) with genes highlighted that met the cutoff criteria. Genes with differential expression are marked with colored diamonds when comparing L-methionine (L-Met) fed chickens to the control group (C), and comparing DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) fed chickens to the control group (B).

# CHAPTER 7 Epilogue

The research on methionine (Met) isomers and its analogue DL-2-hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) in poultry nutrition has increased in the last 50 years. Most of the studies focused on the relative efficacy between the common supplementation forms DL-Met and DL-HMTBA in poultry. In recent years, an increasing number of studies are investigating the metabolism of these Met sources and the related underlying mechanisms. In this dissertation, three treatment diets L-Met, DL-Met or DL-HMTBA were fed in a broiler chicken trial. A Met deficient group was set as control. The recommended minimum methionine plus cysteine (Met + Cys) levels for Cobb-500 broilers in starter, grower and finisher diets are 0.98%, 0.89% and 0.82%, respectively. In our basal diet, the Met + Cys levels are 0.78%, 0.70% and 0.62% for starter, grower and finisher diets respectively (Table A), with an approximate difference of 0.2%. This amount of deficiency caused an obvious growth restriction. Based on the diet formula, these three dietary Met sources were expected to be equally efficacious. The growth performance data showed that these supplementations were equivalent, with no significant differences in body weight gain, feed conversion ratio and relative breast muscle weight between the three treatments. The objective of this dissertation was to explore the underlying physiological and biochemical changes affected by these four treatment groups.

Our primary interest was on the intestinal uptake of these dietary Met sources, which is the prerequisite before they can be utilized by cells (Figure 7.1). As an organic acid, a large proportion of DL-HMTBA is absorbed through diffusion. Even though, carrier dependent uptake is still an important mechanism to transport these three Met

sources. Therefore, the transporter gene expression was expected to be influenced by dietary Met supplementation. However, only a few amino acid transporter genes were affected, e.g., the neutral amino acid transporter $ATB^{0,+}$ was upregulated by both DL-Met and L-Met supplementation compared to control in duodenum. The monocarboxylic acid transporter MCT1 was upregulated by supplementation with all three Met sources compared to control in jejunum. Because gene expression of intestinal nutrient transporters is usually segment- and age- dependent, there were complex regulation effects of nutrient transporter gene expression by the dietary supplemental Met sources.

The conversion of L-HMTBA, D-HMTBA and D-Met to the L-Met isoform was the next process we were interested in (Figure 7.1). Because L-Met is the metabolically active Met form animals can use, either DL-HMTBA or D-Met must undergo oxidation and transamination steps to be converted into L-Met. Oxidation is the limiting step and different substrates are catalyzed by their corresponding oxidases. Even though DL-HMTBA and L-Met supplementation showed greater hepatic HAO1 (gene encodes L-HMTBA oxidase) gene expression, the treatment effects for all oxidases and transaminases were complex. Similar to the nutrient transporter gene expression, this was due to the interaction with tissue effects and age effects. For the three oxidases that convert D-Met, L-HMTBA and D-HMTBA, the in situ hybridization assay demonstrated similar tissue distribution pattern for mRNA as that shown by real-time PCR assay, but no significant results among treatments. The enzymatic activity assay also exhibited non-significant treatment effects on protein expression. The tissue distribution patterns of protein expression were different from those of mRNA expression. Nevertheless, the in situ hybridization for L-HMTBA oxidase gene HAO1 and D-HMTBA oxidase gene

LDHD showed some interesting findings. In the small intestine, both mRNAs are more abundant in epithelia and in crypts, with decreasing expression towards the villus tips. Based on the mRNA localization in different tissues, we can also conclude that liver is the major tissue for mRNA expression of both D- and L-HMTBA oxidases, while the small intestine can express high levels of D-HMTBA oxidase mRNA. Theoretically, D-Met accounts for 50% of the components in DL-Met. It was reported that D-Met has 90% relative bioavailability compared to the L-isomer in chickens (Data from Baker, D.H. (1994). Utilization of precursors of L-amino acids. In: Amino Acids in Farm Animal Nutrition (J. P. F. D' Mello, Ed.), CAB Int'l, London, pp. 37-63.). Thus the D-isomer in DL-Met can be greatly converted, leading to the roughly equivalent bio-efficacy between DL-Met and L-Met.

An in vitro study was conducted to further identify the expression of the intestinal nutrient transporter and Met oxidase gene. The gene expression pattern observed in the animal trial was not replicated in the in vitro cultured tissue explants. Thus in vitro cultured explants may not be a good model for our in vivo chicken trial.

As the product of D-Met and DL-HMTBA conversion, L-Met can go into either the transmethylation cycle or transsulfuration pathway for metabolism (Figure 7.1). S-adenosylmethionine (SAM) is an intermediate from the transmethylation cycle and is the major methyl donor in cells. Therefore, the hepatic DNA methylation and methyltransferase activity in different treatments were measured, revealing that dietary supplemental Met sources had no significant effects on DNA methylation in liver of broiler chickens.

In regulation of cell function and metabolism, L-Met mainly plays three roles: 1) precursors for protein synthesis, 2) antioxidant function (the reactive oxygen species (ROS) scavengers glutathione (GSH) and cysteine are all intermediates in the transsulfuration pathway, see Figure 1.1), and 3) nutrient signaling to affect intracellular kinases. Thus the goal was to explore whether different dietary supplemental Met sources and Met deficiency can also affect these roles.

To check the effect of dietary Met sources and Met deficiency on protein synthesis, a potential idea was to examine the first step of mRNA translation in which Met is mainly involved. More specifically, we can measure the expression of translation initiator methionyl-tRNA (Met-tRNAi) under different treatment groups. Due to technical limitations, this has not been conducted yet and can act as a future research direction.

To check the effect of dietary Met sources and Met deficiency on antioxidant function, some oxidative stress markers were examined. There were no significant treatment effects for those markers, which includes the thiol redox status (ratio of reduced GSH to total GSH), protein oxidation (protein carbonyl level), lipid peroxidation (thiobarbituric acid reactive substances level) and ferric antioxidant power. However, in breast muscle, both L-Met and DL-HMTBA supplementation to the Met deficient diet increased total GSH level, which indicates an improvement in intracellular antioxidant capacity to some extent. Moreover, some blood/plasma parameters related to oxidative stress were evaluated. Although neither white blood cell differential count nor red blood cell packed cell volume was affected by Met sources, L-Met and DL-Met supplementation reduced acute phase protein serum amyloid A level compared to control,

but DL-HMTBA did not. In summary, dietary Met supplementation especially L-Met supplementation can cause some changes in the antioxidant function of Met.

To check the effect of dietary Met sources and Met deficiency on nutrient signaling, our target intracellular kinase is the 70-kDa ribosomal protein S6 kinase (p70S6K), which is an effector of the extensively explored mammalian target of rapamycin (mTOR) pathway (Figure 7.2). p70S6K was chosen also because there was a slight change in duodenum for the amino acid sensor SAT2 gene expression, and SAT2 most likely functions through the mTOR/p70S6K pathway. DL-HMTBA supplementation decreased the expression of phosphorylated p70S6K in jejunum compared to control, indicating reduced protein synthesis. This result was not expected. Since there exist other downstream effectors of mTOR, such as eukaryotic initiation factor 4E binding protein (4E-BP1), and the mTOR-independent effectors, such as eukaryotic initiation factor 2α (eIF2α), the co-function of these factors and the influence between these multiple pathways may lead to a final explanation (Figure 7.2). The phosphatidylinositide 3-kinases (PI3K) that participate in the amino acid signaling pathway to affect protein degradation can also interact with mTOR (Figure 7.2). As a result, a reasonable follow-up study for this project would be checking the protein expression of these potential factors (4E-BP1, eIF2α, PI3K, mTOR, etc.) in treatments with different dietary Met sources compared to the Met deficient group. This would be beneficial to better understand the role of different Met sources as nutrient signals to affect intracellular kinases and protein turnover.

To summarize the above results, the different dietary supplemental Met sources L-Met, DL-Met and DL-HMTBA exhibited great similarity in impacting intestinal amino

acid/peptide/monocarboxylic acid transporter gene expression and Met converting enzyme activity in male broiler chickens. The regulation roles of Met as antioxidant and nutrient signaling in cell metabolism were not affected by different dietary supplemental Met sources (Figure 7.1). These conclusions were further validated by our RNA sequencing results in liver. Few differentially expressed hepatic genes were found between L-Met, DL-Met and DL-HMTBA fed chickens. However, Met deficiency of ~0.2% altered the mRNA expression of distinct nutrient transporters (e.g., MCT1) and Met converting enzymes (e.g., DAO) compared to the Met sufficient groups. The RNA sequencing results also identified more than 700 differentially expressed genes in liver when comparing the control group with the three Met supplemental groups.

Sulfur amino acids especially Met play a vital role in animal growth and development. Understanding the underlying mechanisms of how different supplemental Met sources affect animals can greatly benefit the animal nutrition industry, particularly the poultry industry. Our results demonstrated that both DL-Met and DL-HMTBA can act as effective supplemental Met sources in broiler chicken diets, considering that they showed very similar effects on many physiological and biochemical aspects compared to the metabolically active Met form L-Met. Moreover, some recent studies are focusing on the clinical utilization of amino acids as dietary supplements in humans, aiming to minimize the loss of muscle mass and impaired immune function. Our study on mechanisms have the potential to be extended into the biomedical research area, and may contribute to future studies using other animal models to promote the health of humans.

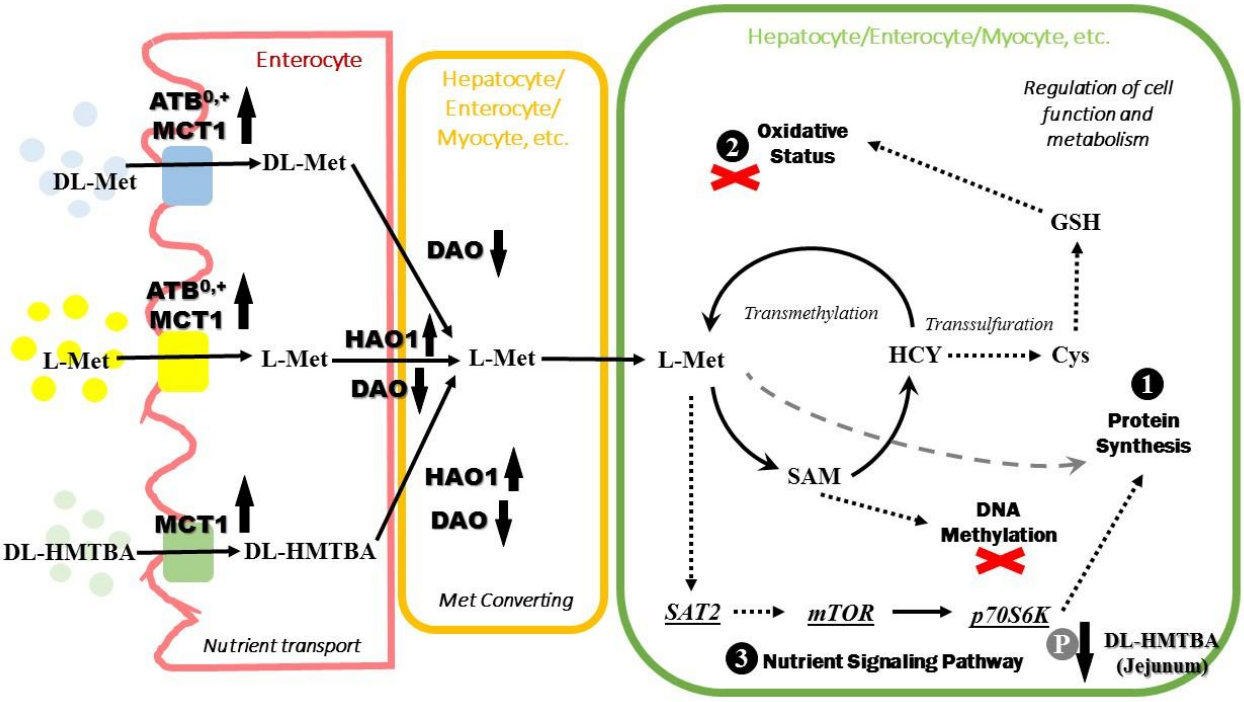



**Figure 7.1** Effect of common dietary supplemental methionine (Met) sources on nutrient uptake, methionine metabolism and regulation function in cells. Different dietary supplemental Met sources L-Met, DL-Met and DL-HMTBA upregulated some intestinal nutrient transporter and Met converting enzyme gene expression in male broiler chickens. DL-HMTBA decreased protein expression of phospho-p70S6K in jejunum. Dietary Met sources did not change oxidative status and hepatic DNA methylation. DL-Met: DL-methionine; L-Met: L-methionine; DL-HMTBA: DL-2-hydroxy-4 (methylthio) butanoic acid; SAM: S-adenosylmethionine; HCY: homocysteine; Cys: cysteine; GSH: glutathione; $ATB^{0,+}$: SLC6A14, neutral and cationic amino acid transporter; MCT1: monocarboxylic acid transporter 1; HAO1: L-2-hydroxy acid oxidase; DAO: D-aspartate oxidase; SAT2: SLC38A2, neutral amino acid transporter; mTOR: mammalian target of rapamycin; p70S6K: 70-kDa ribosomal protein S6 kinase.

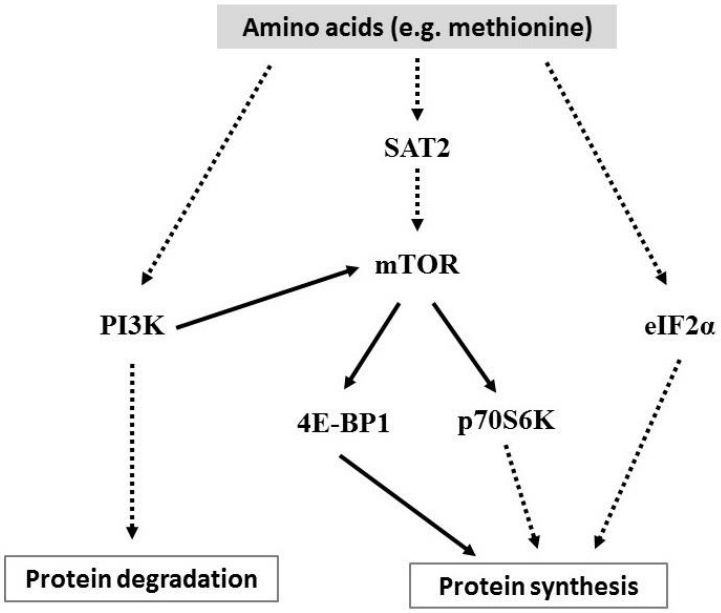



**Figure 7.2** Pathways for amino acids as nutrient signals to affect protein turnover. SAT2: SLC38A2, neutral amino acid transporter; mTOR: mammalian target of rapamycin; p70S6K: 70-kDa ribosomal protein S6 kinase; 4E-BP1: eukaryotic initiation factor 4E binding protein; eIF2α: eukaryotic initiation factor 2α; PI3K: phosphatidylinositide 3-kinases.

## APPENDIX A

**Table A.** Chemical composition and amino acid profile of the trial diets of the different growth phases.

| Basal diet | Starter (d0 to d10) | Grower (d11 to d21) | Finisher (d22 to d35) |
|---|---|---|---|
| Ingredients (%) | | | |
| Soybean meal, 48% CP | 30.2 | 26.3 | 24.4 |
| Wheat | 32.9 | 23.8 | 28.0 |
| Corn | 20.0 | 20.0 | 20.0 |
| Peas | / | 17.0 | 17.0 |
| Corn gluten meal, 60% CP | 6.65 | 2.62 | / |
| Soybean oil | 4.43 | 5.86 | 6.27 |
| Dicalcium phosphate | 1.92 | 1.89 | 1.91 |
| Vitamin-mineral premix[1] | 1.00 | 1.00 | 1.00 |
| Limestone ($CaCO_3$) | 1.00 | 0.93 | 0.94 |
| L-lysine | 0.61 | 0.13 | 0.03 |
| Salt (NaCl) | 0.27 | 0.27 | 0.27 |
| Sodium bicarbonate | 0.83 | 0.14 | 0.15 |
| L-threonine | 0.09 | 0.03 | 0.03 |
| L-valine | 0.05 | / | / |
| Coccidiostat | 0.05 | 0.05 | 0.05 |
| Calculated content (%) | | | |
| ME (kcal/kg) | 3035 | 3083 | 3107 |
| DM | 89.1 | 89.1 | 89.2 |
| CP | 24.6 | 22.2 | 20.1 |
| Ca | 1.04 | 1.00 | 1.00 |
| Available P | 0.50 | 0.50 | 0.50 |

| Analytical Value (%) | Starter (d0 to d10) | | | | Grower (d11 to d21) | | | | Finisher (d22 to d35) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treat / Nutrient | C | DL-Met | L-Met | DL-HMTBA | C | DL-Met | L-Met | DL-HMTBA | C | DL-Met | L-Met | DL-HMTBA |
| DM | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 | 88.0 |
| CP | 25.4 | 24.8 | 25.4 | 24.9 | 22.9 | 22.8 | 22.7 | 22.5 | 21.0 | 21.2 | 21.1 | 20.8 |
| MET | 0.38 | 0.60 | 0.61 | 0.37 | 0.33 | 0.55 | 0.55 | 0.32 | 0.28 | 0.51 | 0.52 | 0.28 |
| CYS | 0.40 | 0.40 | 0.40 | 0.40 | 0.37 | 0.36 | 0.36 | 0.37 | 0.34 | 0.34 | 0.33 | 0.34 |
| M+C | 0.78 | 1.01 | 1.01 | 0.78(0.98)[2] | 0.70 | 0.91 | 0.91 | 0.69(0.89)[2] | 0.62 | 0.85 | 0.85 | 0.62(0.82)[2] |
| LYS | 1.45 | 1.47 | 1.46 | 1.44 | 1.27 | 1.26 | 1.27 | 1.25 | 1.13 | 1.15 | 1.13 | 1.11 |
| THR | 0.94 | 0.95 | 0.95 | 0.95 | 0.86 | 0.83 | 0.83 | 0.85 | 0.78 | 0.77 | 0.76 | 0.78 |
| TRP | | - | - | - | - | - | - | - | - | - | - | - |
| ARG | 1.51 | 1.50 | 1.50 | 1.47 | 1.51 | 1.47 | 1.49 | 1.46 | 1.40 | 1.40 | 1.38 | 1.37 |
| ILE | 1.09 | 1.07 | 1.09 | 1.03 | 1.01 | 0.99 | 1.01 | 0.98 | 0.90 | 0.93 | 0.92 | 0.88 |
| LEU | 2.28 | 2.26 | 2.29 | 2.20 | 1.92 | 1.87 | 1.92 | 1.90 | 1.61 | 1.63 | 1.61 | 1.59 |
| VAL | 1.24 | 1.21 | 1.23 | 1.17 | 1.10 | 1.08 | 1.11 | 1.06 | 0.98 | 1.01 | 1.02 | 0.96 |
| HIS | 0.61 | 0.60 | 0.61 | 0.59 | 0.57 | 0.56 | 0.57 | 0.56 | 0.52 | 0.53 | 0.52 | 0.51 |
| PHE | 1.31 | 1.29 | 1.31 | 1.27 | 1.19 | 1.16 | 1.18 | 1.18 | 1.05 | 1.07 | 1.05 | 1.05 |
| TYR | - | - | - | - | - | - | - | - | - | - | - | - |
| GLY | 0.97 | 0.96 | 0.97 | 0.95 | 0.93 | 0.92 | 0.93 | 0.92 | 0.86 | 0.87 | 0.86 | 0.85 |
| SER | 1.16 | 1.19 | 1.18 | 1.19 | 1.10 | 1.05 | 1.05 | 1.10 | 1.00 | 0.97 | 0.93 | 1.00 |
| PRO | 1.65 | 1.65 | 1.64 | 1.64 | 1.41 | 1.34 | 1.36 | 1.43 | 1.26 | 1.28 | 1.27 | 1.28 |
| ALA | 1.26 | 1.25 | 1.27 | 1.22 | 1.08 | 1.05 | 1.08 | 1.07 | 0.91 | 0.92 | 0.91 | 0.91 |
| ASP | 2.29 | 2.27 | 2.28 | 2.22 | 2.27 | 2.22 | 2.24 | 2.23 | 2.07 | 2.09 | 2.07 | 2.04 |
| GLU | 5.03 | 5.02 | 5.05 | 4.97 | 4.47 | 4.36 | 4.44 | 4.40 | 4.08 | 4.12 | 4.07 | 4.07 |

DM: dry matter; CP: crude protein; MET: methionine; CYS: cysteine; M+C: methionine + cysteine, the values in brackets also considering DL-2-hydroxy-4-(methylthio) butanoic acid content; LYS: lysine; THR: threonine; TRP: tryptophan; ARG: arginine; ILE: isoleucine; LEU: leucine; VAL: valine; HIS: histidine; PHE: phenylalanine; TYR: tyrosine; GLY: glycine; SER: serine; PRO: proline; ALA: alanine; ASP: aspartate; GLU: glutamate.

[1] The vitamin-mineral premix supplied the following per kilogram of diet: 12,000 IU vitamin A (retinyl acetate), 2,400 IU vitamin $D_3$ (cholecalciferol), 50 mg vitamin E (DL-α-tocopherol), 1.5 mg vitamin $K_3$ (menadione), 2.0 mg vitamin $B_1$ (thiamine), 7.5 mg vitamin $B_2$ (riboflavin), 3.5 mg vitamin $B_6$ (pyridoxine-HCl), 20 μg vitamin $B_{12}$ (cyanocobalamin), 35 mg niacin, 10 mg D-pantothenic acid, 460 mg choline chloride, 1.0 mg folic acid, 0.2 mg biotin, 267 mg ferrous sulfate, 48 mg copper sulfate, 142 mg manganese oxide, 169 mg zinc sulfate, 1.1 mg potassium iodide, 0.33 mg sodium selenite.

[2] Numbers in brackets shows results of a calculation to transform HMTBA content to the equivalent Met+Cys content by multiplying the HMTBA content by 0.65 (bioavailability of HMTBA).

## APPENDIX B

**Table B.** Experimental design for the animal trial, sampling and data collection. Arrangements of animals and treatments in floor pens were illustrated in (A), and time points to collect samples and perform assays were specified in (B).

**(A)**

| Floor Pen Number | Treatments | Number of Chickens | | Floor Pen Number | Treatments | Number of chickens |
|---|---|---|---|---|---|---|
| 9B2 | DL-HMTBA | 10 | | 10B2 | DL-HMTBA | 10 |
| 9B1 | L-Met | 10 | | 10B1 | L-Met | 10 |
| 9A2 | DL-Met | 11 | | 10A2 | DL-Met | 11 |
| 9A1 | C | 10 | | 10A1 | C | 10 |
| 7D2 | DL-HMTBA | 11 | | 8D2 | DL-HMTBA | 10 |
| 7D1 | L-Met | 9 | Passage | 8D1 | L-Met | 10 |
| 7C2 | DL-Met | 11 | | 8C2 | DL-Met | 10 |
| 7C1 | C | 10 | | 8C1 | C | 9 |
| 7B2 | DL-HMTBA | 10 | | 8B2 | DL-HMTBA | 10 |
| 7B1 | L-Met | 10 | | 8B1 | L-Met | 10 |
| 7A2 | DL-Met | 11 | | 8A2 | DL-Met | 11 |
| 7A1 | C | 9 | | 8A1 | C | 9 |

**(B)**

| Items | Day 0 | Day 3 | Day 5 | Day 10 | Day 21 | Day 26 | Day 35 |
|---|---|---|---|---|---|---|---|
| Number of chickens euthanized | 8 | 24 | 24 | 24 | 24 | 24 | 24 |
| Individual body weight recorded | × | × | × | × | × | × | × |
| Small intestine sampled | × | × | × | × | × | × | × |
| Liver sampled | × | × | × | × | × | × | × |
| Breast muscle sampled | | | | × | × | × | |
| Carcass weight recorded | | | | × | × | × | × |
| Abdominal fat weight recorded | | | | × | × | × | × |
| Kidney weight recorded | | | | × | × | × | × |
| Liver weight recorded | | | | × | × | × | × |
| Heart weight recorded | | | | × | × | × | × |
| Empty small intestine weight recorded | | | | | × | × | × |
| Dry matter digestibility measured | | | | | | × | |
| Blood parameter measured | | | | | × | × | × |
| Acute phase protein level | | | | | × | × | |
| Intestinal nutrient transporter gene expression | × | × | × | × | × | × | × |
| Methionine converting enzyme assay | | | | × | × | × | |
| In situ hybridization assay | | | | × | | | |
| Oxidative stress marker expression | | | | × | | × | |
| Western blot for p70S6K expression | | | | × | × | × | |
| RNA sequencing | | | | | | × | |

## APPENDIX C

**Table C.** Relative D-amino acid oxidase (DAO, GenBank ID: NC_006102.2) gene expression in chapter 4 considering the statistical model that included main effects of treatment (C, DL-Met, L-Met, DL-HMTBA), age (d10, 21, 26), and tissue (liver, muscle, duodenum, jejunum, ileum) and their interactions.

| | | Relative gene expression[1,2,3] |
|---|---|---|
| Treatment (n=5) | C | 1.77 |
| | DL-Met | 1.80 |
| | L-Met | 1.76 |
| | DL-HMTBA | 1.71 |
| | SEM | 0.12 |
| | *P*-value | 0.95 |
| Tissue (n=5) | Duodenum | 2.32 |
| | Jejunum | 2.35 |
| | Ileum | 0.56 |
| | Muscle | 0.28 |
| | Liver | 3.30 |
| | SEM | 0.11 |
| | *P*-value | **<0.0001** |
| Age (n=5) | Day 10 | 2.09 |
| | Day 21 | 1.43 |
| | Day 26 | 1.77 |
| | SEM | 0.10 |
| | *P*-value | **0.0001** |
| Interaction | Treatment × Tissue | 0.85 |
| | Tissue × Age | **<0.0001** |
| | Treatment × Age | 0.88 |
| | Treatment × Tissue × Age | 0.95 |

[1] Data are shown as least squares means ± standard errors of the means.

[2] The original record of D-amino acid oxidase (DAO, GenBank ID: NC_006102.2) in chicken (Gallus gallus) was removed from NCBI for unknown reasons.

[3] The primer sequences used for real-time PCR are:
CGGTTTTGGGAGGCATCTAC/GGTCCTGAGCACTGTTCTCTTCA (5' → 3', sense/antisense).