

Substitution of Dietary Sulfur Amino Acids by DL-2-hydroxy-4-Methylthiobutyric Acid Increases Remethylation and Decreases Transsulfuration in Weaned Piglets

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ABSTRACT

Background: DL-2-hydroxy-4-methylthiobutyric acid (DL-HMTBA), an L-methionine (L-Met) hydroxyl analogue, has been suggested to be a dietary L-Met source. How dietary DL-HMTBA compared with L-Met affects whole-body L-Met kinetics in growing individuals is unknown.

Objective: We determined to what extent DL-HMTBA supplementation of an L-Met-deficient diet affects whole-body L-Met and L-cysteine (L-Cys) kinetics, protein synthesis (PS), and the L-Met incorporation rate in liver protein (L-MetInc) compared with L-Met and DL-Met supplementation in a piglet model.

Methods: Forty-five, 28-d-old weaned piglets (male, German Landrace) were allocated to 4 dietary groups: L-Met-deficient diet [Control: 69% of recommended L-Met plus L-Cys supply; 0.22% standardized ileal digestible (SID) L-Met; 0.27% SID L-Cys; $n = 12$] and Control diet supplemented equimolarly to 100% of recommended intake with either L-Met ($n = 12$; LMET), DL-Met ($n = 11$; DLMET), or DL-HMTBA ($n = 10$; DLHMTBA). At 47 d of age, the piglets were infused with L-[1-¹³C; methyl-²H₃]-Met and [3,3-²H₂]-Cys to determine the kinetics and PS rates. Plasma amino acid (AA) concentrations, hepatic mRNA abundances of L-Met cycle and transsulfuration (TS) enzymes, and L-MetInc were measured.

Results: During feed deprivation, L-Met kinetics did not differ between groups, and were ≤ 3 times higher in the fed state ($P < 0.01$). Remethylation (RM) was 31% and 45% higher in DLHMTBA than in DLMET and Control pigs, respectively, and the RM:transmethylation (TM) ratio was 50% higher in DLHMTBA than in LMET ($P < 0.05$). Furthermore, TS and the TS:TM ratio were 32% lower in DLHMTBA than in LMET ($P < 0.05$). L-MetInc was 42% lower in DLMET and DLHMTBA than in L-Met-deficient Control pigs, whereas plasma AA and hepatic mRNA abundances were similar among DL-HMTBA-, L-Met-, and DL-Met-supplemented pigs.

Conclusions: In piglets, DL-HMTBA compared with L-Met and DL-Met supplementation increases RM and reduces the TS rate to conserve L-Met, but all 3 Met isomers support growth at a comparable rate. *J Nutr* 2019;149:432–440.

Keywords: methionine, DL-2-hydroxy-4-methylthiobutyric acid, remethylation, transmethylation, transsulfuration, human infants, piglet model

Introduction

Methionine (Met) is an indispensable amino acid (AA) and originates mainly from dietary sources, but it is also provided by endogenous protein breakdown and remethylation (RM) of homocysteine (Hcys) (1, 2). The Met requirement for infants is much greater than that of adults (3). Children with protein malnutrition have a decreased availability of protein and thus L-Met, resulting in lower plasma L-Met concentrations (1). Protein, and thus AA, malnutrition leads to several diseases and developmental retardation and causes high mortality in

infants (4). To counteract a Met deficiency, Met can be added in 3 different ways: 1) as L-Met, 2) as DL-Met, or 3) as the keto acid DL-2-hydroxy-4-methylthiobutyric acid (DL-HMTBA), an L-Met hydroxyl analogue. In intermediary metabolism, DL-HMTBA and D-Met are converted to L-Met in 2 consecutive enzymatic steps (Supplemental Figure 1). Initially, L- and D-HMTBA are oxidized and transaminated to yield L-Met (5). In the transmethylation (TM) pathway, Met is converted to Hcys, which can be remethylated to form Met via methionine synthase (MTR) or betaine-homocysteine methyltransferase (BHMT) (Supplemental Figure 1). In the

transsulfuration (TS) pathway, Hcys is irreversibly eliminated via cystathionine β -synthase (CBS) and cystathionine γ -lyase (CGL) to form cysteine (Cys) (6). Low CBS activity may cause hyperhomocysteinemia (7), which is associated with greater risk of cardiovascular and cognitive diseases (8, 9). Previous studies speculated that dietary DL-HMTBA supplementation favors the TS over the RM pathway (10, 11). Currently, whether and to what extent dietary supplementation with DL-HMTBA, which is less expensive than L-Met and DL-Met, compared with L- or DL-Met affects the L-Met cycle is unknown. We selected piglets as animal models because they are considered a superior translational model for human infants and children to examine early-life Met metabolism (12). The first objective of this study was to determine whether dietary DL-HMTBA supplementation of a Met-deficient diet is as effective as DL-Met and L-Met at restoring the quantitative intermediary Met and Cys metabolism in young piglets. The second objective was to determine if the partitioning between TM and protein synthesis (PS) differs between DL-HMTBA, DL-Met, and L-Met supplementations. Finally, the third objective was to analyze the abundance of hepatic RNA of enzymes involved in the Met cycle to identify enzymatic steps underlying potential differences in whole-body Met partitioning.

Methods

Animals and diets

The study protocol was approved by the Animal Care Committee of the Ministry of Nutrition, Agriculture, Forestry and Fishery, Rostock, State Mecklenburg-Vorpommern, Germany (permission No. LALLF 7221.3-1-037/14) and conformed to the Directive 2010/63/EU on the protection of animals used for scientific purposes. Forty-eight male German Landrace piglets, bred at the Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, were weaned at 28 d of age [mean \pm SEM body weight (BW): 9.3 \pm 0.4 kg]. On the day after weaning, the piglets were allocated to 4 different isoenergetic and isonitrogenous diets. To minimize the sow effect, 4 littermates with comparable birth and weaning BWs were randomly assigned to the 4 dietary groups. A Met-deficient basal diet (Tables 1 and 2), providing 69% of the recommended Met plus Cys (13), was given to the control group [Control, standardized ileal digestible L-Met content 0.22% (as fed)]. Using the same basal diet, Met was supplemented by L-Met (+0.15%, LMET), DL-Met (+0.15%, DLMET), or DL-HMTBA (0.17%, DLHMTBA) on an equimolar basis to meet the recommendations for growing pigs (Tables 1 and 2) (13). The dietary supply of preformed dietary methyl groups (choline, betaine, folate) was not limiting and was the same in all dietary groups to preclude differences in RM. All diets were adequately supplemented with

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Supplemental Figures 1 and 2, Supplemental Tables 1–5, and Supplemental Methods are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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Abbreviations used: AA, amino acid; ACN, acetonitrile; AHCY, S-adenosylhomocysteine hydrolase; AP, atom percent; BHMT, betaine-homocysteine methyltransferase; BW, body weight; CBS, cystathionine β -synthase; CGL, cystathionine γ -lyase; DLHMTBA, group fed DL-HMTBA-supplemented diet; DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; DLMET, group fed DL-Met-supplemented diet; Hcys, homocysteine; LMET, group fed L-Met-supplemented diet; L-MetInc, incorporation rate of L-Met in liver protein; MPE, mole percentage excess; MTR, methionine synthase; PS, protein synthesis; Q_c, carboxyl flux; Q_m, methyl flux; RM, remethylation; Tau, taurine; TM, transmethylation; TS, transsulfuration.

TABLE 1 Ingredients and nutrient composition of the basal control diet¹

	Contents, %
Ingredients	
Corn	42.7
Soybean meal (48% CP)	21.5
Peas	15.0
Whey powder	11.9
Soybean oil	4.9
Monocalcium phosphate	1.3
Calcium carbonate	1.0
Premix ²	0.5
L-Lys-HCl	0.4
NaCl	0.4
L-Thr ³	0.2
L-Val ⁴	0.1
L-Trp ⁵	0.1
Total	100.0
Calculated nutrient composition ⁶	
DM	89.6
Crude protein	20.5
Crude fiber	3.4
Ether extract	8.2
Ash	7.3
ME, MJ/kg	16.4
SID Met	0.25
SID Cys	0.27
SID Met + Cys	0.52

¹Control diet, Met plus Cys-deficient (69% of SID Met plus Cys recommendation). CP, crude protein; DM, dry matter; ME, metabolizable energy (MJ/kg); SID, standardized ileal digestible.

²Premix administered per kilogram of diet (0.5% dose): all-*trans* retinol equivalent 10,000 IU, cholecalciferol equivalent 2000 IU, RRR- α -tocopherol equivalent 40 mg, phyloquinone equivalent 1.5 mg, thiamine equivalent 1 mg, riboflavin equivalent 4 mg, pyridoxine equivalent 1.5 mg, cyanocobalamin equivalent 20 μ g, pantothenic acid equivalent 15 mg, nicotinamide equivalent 30 mg, biotin, 0.05 mg, folic acid equivalent 0.4 mg, choline chloride 150 mg, Fe (as FeSO₄ H₂O) 100 mg, Cu (as CuSO₄ 5H₂O) 20 mg, Zn (as ZnSO₄ H₂O) 70 mg, Mn (as MnO) 30 mg, I (as KI) 0.7 mg, Se (as Na₂SeO₃) 0.25 mg, and 125 mg antioxidant (Oxytrap PXN).

³L-Thr (ThreAMINO, Evonik Nutrition & Care GmbH; contains 98.5% L-threonine).

⁴L-Val (ValAMINO, Evonik Nutrition & Care GmbH; contains 98% L-valine).

⁵L-Trp (TrypAMINO, Evonik Nutrition & Care GmbH; contains 98% L-tryptophan).

⁶Calculated nutrient composition shows % of DM.

vitamins and minerals. To ensure comparable feed intake in all dietary groups, the daily assigned feed quantity was equivalent to 95% of the recommended energy intake. The daily feed allowance was provided in 2 equal meals (0600 and 1800), and the individual feed intake and BW were recorded. Pigs had free access to water at all times. At 33 d of age, piglets were housed individually in metabolic cages (0.5 m²) in a temperature-regulated room (24°C) with a standardized dark/light cycle (12 h:12 h). Data from 3 of the 48 piglets could not be used for tracer analysis owing to insufficient feed intake or catheter clogging. Sample size calculations were performed in CADEMO for Windows, ANOV-*Version 4.03* (2000; BioMath GmbH).

Catheter surgery

At 41 d of age, feed was withdrawn overnight, and the piglets were subjected to general anesthesia by a combination of ketamine (20 mg/kg BW; Serumwerke Bernburg), xylazine (2 mg/kg BW; Ecuphar), and diazepam (1–2 mg/kg BW; Arzneimittelfabrik Dresden) via intramuscular application. Carotid artery (*Arteria carotis communis*) and jugular vein (*Vena jugularis externa dextra*) catheters were implanted for arterial blood sampling and administration of tracers (14). During the first 3 d after surgery, the piglets received systemic analgesics (Metapyrin containing 500 mg/mL Metamizole-Na, Medistar Arzneimittelvertrieb;

TABLE 2 Nutrient composition of the basal diet (control), and control diet supplemented by L-Met, DL-Met, or DL-HMTBA based on analyzed values¹

Nutrient	Content (%) ²			
	Control	LMET	DLMET	DLHMTBA
Crude protein	18.6	18.5	18.6	18.8
Met	0.25	0.38	0.39	0.26
Cys	0.28	0.28	0.29	0.29
Met + Cys	0.53	0.67	0.67	0.54
Lys	1.33	1.32	1.33	1.35
Thr	0.88	0.88	0.88	0.90
Arg	1.18	1.16	1.18	1.21
Ile	0.78	0.77	0.77	0.79
Leu	1.51	1.49	1.50	1.53
Val	0.94	0.94	0.94	0.96
His	0.44	0.43	0.43	0.44
Phe	0.89	0.87	0.88	0.90
Gly	0.72	0.71	0.71	0.73
Ser	0.88	0.86	0.87	0.89
Pro	0.98	0.97	0.96	0.98
Ala	0.87	0.86	0.86	0.88
Asp	1.87	1.84	1.85	1.91
Glu	3.13	3.09	3.10	3.18
Ammonia	0.38	0.38	0.38	0.39
Total	17.30	17.24	17.30	17.56
Supplement				
Met	<0.01	0.14	0.14	<0.01
Lys	0.30	0.29	0.29	0.29
Thr	0.18	0.19	0.18	0.18
Val	0.11	0.11	0.11	0.11
DL-HMTBA ³	<0.02	<0.02	<0.02	0.17

¹Control diet, Met plus Cys-deficient (69% of standardized ileal digestible Met plus Cys recommendation); L-Met-supplemented diet (Control + 0.15% L-Met); DL-Met-supplemented diet (Control + 0.15% DL-Met); DL-HMTBA-supplemented diet (Control + 0.17% DL-HMTBA). Met sources were added on an equimolar basis (DL-HMTBA contains 12% water). DLHMTBA, group fed DL-HMTBA-supplemented diet; DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; DLMET, group fed DL-Met-supplemented diet; LMET, group fed L-Met-supplemented diet.

²Standardized to a dry matter content of 88%.

³Liquid supplement of DL-HMTBA, free acid, standardized to 88% purity.

50 mg/kg BW, intravenously) and antibiotics (Trimethosel, containing 200 mg/mL sulfadimidine-Na, and 40 mg/mL trimethoprim, Selectavet; 24 mg/kg BW, intravenously). Piglets resumed normal feed intake and had a normal body temperature 4 d after surgery and before the tracer infusion.

Tracer studies and analysis

At 47 and 50 d of age, after a 14-h overnight feed withdrawal, 2 basal arterial blood samples were drawn for background isotopic enrichment measurements at -20 and -10 min relative to the start of the tracer infusion (Figure 1). For the first 3 h of the tracer infusion, feed was withheld; subsequently, small meals were provided every half hour (7% of daily allowance each) until the end of infusion. Tracer solutions were infused via a 0.2- μ m sterile filter (Filtropur S 0.2, Sarstedt). Arterial blood samples were either drawn into lithium heparin-containing tubes and centrifuged (10 min at 3500 \times g and 4°C) to obtain plasma or drawn into K-EDTA-containing tubes to obtain whole blood. The samples were stored at -20°C.

Met and Cys kinetics.

Starting at 0800, 47-d-old piglets received a primed, continuous, intravenous 9-h tracer infusion of L-[1-¹³C, methyl-²H₃]-Met [1-¹³C-Met, 99 atom% (AP), methyl-²H₃-Met, 98 AP; Euriso-Top] (priming

dose 10 μ mol/kg BW, infusion rate 10 μ mol \cdot kg⁻¹ \cdot h⁻¹) and L-[3,3-²H₂]-Cys (98 AP; Euriso-Top) (priming dose 7.5 μ mol/kg BW, infusion rate 7.5 μ mol \cdot kg⁻¹ \cdot h⁻¹) following a published tracer protocol (15). Blood samples of 2.5 mL were collected every 30 min in feed-deprived (1–3 h of the tracer infusion) and fed (6–9 h) conditions (Figure 1).

Plasma Met and Cys were converted to *tert*-butyldimethylsilyl derivatives (15) after protein precipitation with acetonitrile (ACN; 3:1, vol:vol) using 3-mercaptopropionic acid to cleave the sulfur crosslinks of cystine. AA derivatives were separated by GC-MS (quadrupole; GC-MS QP 2010; Shimadzu). The Met derivative was separated on a 50 m \times 0.32 mm \times 0.52 μ m Ultra 2 column (Agilent Technologies) and the Cys derivative was separated on a 30 m \times 0.25 mm \times 0.25 μ m ZB5-HT column (Phenomenex). Positive chemical ionization was used for Met and electron impact ionization for Cys analysis. For [1-¹³C; methyl-²H₃]-Met, [1-¹³C]-Met, and [3,3-²H₂]-Cys, diagnostic ions of *m/z* 320 (M + 0), 321 (M + 1), and 324 (M + 4), and *m/z* 406 (M + 0) and 408 (M + 2) were used, respectively. Calculations of enrichments were based on tracer:tracee peak area ratios [expressed as mole percentage excess (MPE)]. The mean isotopic enrichments during feed deprivation were determined between 1.5 and 3 h, whereas fed state enrichments were averaged over 6–9 h relative to the start of the tracer infusions. In the DLMET, but not in the DLHMTBA group, the plasma Met peak consisted of L- and D-Met; D-Met accounted for 11.7% of total Met during the fed state (see below), and L-Met enrichment in the fed state was corrected for the plasma D-Met proportion. Quantitative Met kinetics, including Met methyl and carboxyl fluxes (Q_m and Q_c, respectively), TM, RM, TS, and PS rates, were calculated as described elsewhere (16).

Carbon dioxide production and Met oxidation.

Piglets at 50 d of age were administered 2 intravenous boluses of NaH¹³CO₃ (1 mg/kg BW, 98 AP, Campro Scientific), the first at 0800 and the second at 1300, to measure ¹³CO₂ enrichment in whole blood to calculate carbon dioxide production. Blood samples (2 mL) were collected at 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min after each bolus. Whole blood was acidified by 10% lactic acid, the ¹³C enrichment of released blood carbon dioxide was determined using an isotope ratio mass spectrometer (DELTAplus XL; Thermo Fisher Scientific), and carbon dioxide production was calculated for feed-deprived and fed conditions corrected for ¹³C background enrichments (14). The same method was applied for the measurement of ¹³CO₂ released from infused tracer L-Met to calculate L-Met oxidation (16).

L-¹³C-Met incorporation in liver protein.

To determine the incorporation rate of L-Met in liver protein (L-MetInc), piglets at age 62 d were feed-deprived overnight for 14 h and were intravenously administered a bolus dose of L-[1-¹³C]-Met (4 mg/kg BW, 99 AP, Sigma-Aldrich). After 2.5 h, the piglets were electrically stunned and killed by exsanguination. Liver tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Ground liver tissue (30 mg) was homogenized in 300 μ L of lysis buffer (17) and centrifuged at 3000 \times g and 4°C for 10 min. Proteins were precipitated by adding cold ACN, and the remaining supernatant lysate containing free AA was dried. Tissue free L-¹³C-Met enrichments were analyzed by GC-MS as described above. Protein pellets were washed with ACN/ultrapure water (3:1, vol:vol), dried, and hydrolyzed (18). GC-combustion isotope ratio MS (Delta V Advantage coupled with TriPlus RSH autosampler, GC Trace 1310, GC Isolink II, and Conflo IV; Thermo Fisher Scientific) was used to determine L-¹³C-Met enrichment after derivatization of Met to its N-pivaloyl-i-propyl ester (19). Separation of AAs was performed on a 30 m \times 0.25 mm \times 0.25 μ m TG-5MS column (Thermo Fisher Scientific). AA derivatives were combusted to carbon dioxide at 1000°C. The ¹³C enrichment of L-Met was corrected for background L-¹³C Met content in pig liver tissue, and the L-¹³C-Met content in labeled tissue was expressed in MPE. The L-MetInc rate was calculated as the MPE of L-¹³C-Met in liver protein, divided by the MPE of liver supernatant multiplied by the time elapsed after the L-¹³C-Met injection in days, and multiplied by 100%.

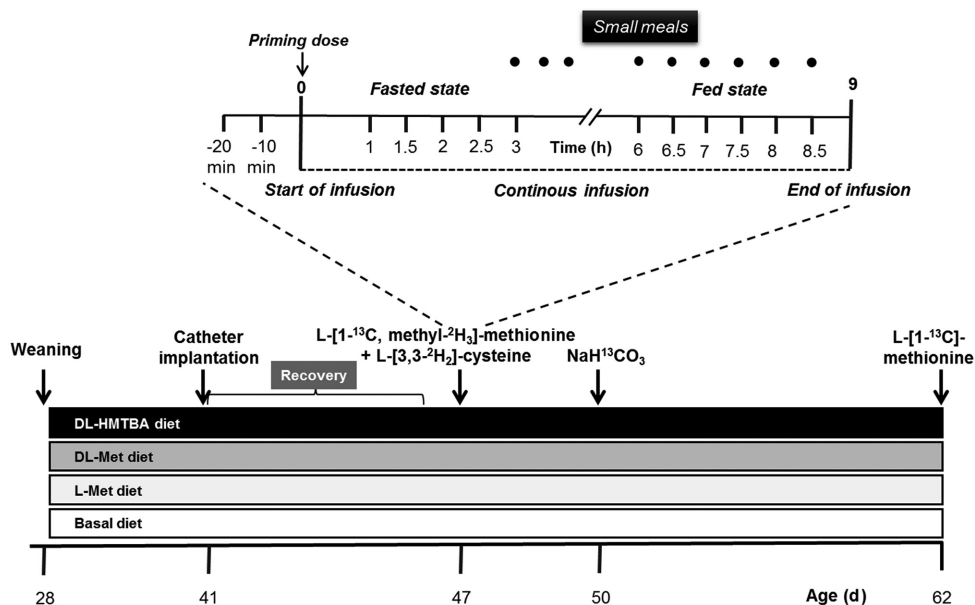


FIGURE 1 Schematic representation of the experimental design including the Met and Cys tracer infusion study. DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid.

Hepatic gene abundance

The mRNA abundance of hepatic genes involved in Met intermediary metabolism was determined by real-time PCR as described previously (20). Briefly, total RNA was extracted from liver tissue using an RNeasy kit (Qiagen). The RNA integrity factors were between 7.6 and 9.8. The cDNA was synthesized using RevertAid Reverse Transcriptase and random primers (Thermo Fisher Scientific). Primer sequences were designed with Primer3 software version 4.0 or adopted from previous studies (21–23) (Supplemental Table 1). The reaction, containing cDNA, 0.5 μ L of each primer, and 5 μ L of 2 \times buffer SensiFAST SYBR No-Rox Mix (Bioline), was performed in duplicate on a LightCycler96 (Roche Diagnostics). The efficiency of amplification amounted to 1.79–1.86. Data were quantified by qbasePlus software (Biogazelle) and normalized to the reference genes of ribosomal proteins L4, S18, and peptidylprolyl isomerase A (*M* value: 0.169; *V* value: 0.069) (24, 25).

Plasma and liver free AA concentrations

Blood was collected from the arterial catheter in a subset of 8 pigs at age 54 d. Concentrations of AA and taurine (Tau) in plasma and liver lysates (see above) were analyzed by HPLC (26). The same HPLC conditions were used to separate D- and L-Met, but the precolumn derivatization was performed with *ortho*-phthalaldehyde containing N-acetyl-L-cysteine (27) as a mercapto-component.

Folate, vitamin B-6, and plasma metabolite concentrations

The concentrations of folate and vitamin B-6 were determined in blood serum and in full blood by a commercial laboratory (Synlab.vet) using routine methods. The analysis of plasma metabolites (nonesterified fatty acids, cholesterol, urea, TGs, and glucose) is described in the Supplemental Methods.

Statistical analysis

Data were analyzed with SAS/STAT software for Windows, version 9.4 (SAS Institute Inc.). The test for normality was performed by the UNIVARIATE procedure of SAS. The data were analyzed by repeated-measures ANOVAs with the MIXED procedure. The ANOVA models contained the fixed effects of dietary group (Control, LMET, DLMET, DLHMTBA), status (fed, feed-deprived), the interaction group \times status, and a random sow effect. Repeated measures on the same animal were considered by the repeated statement of proc MIXED (repeated variable: status) using an unstructured type for the block diagonal

residual covariance matrix. Group and status (fed, feed-deprived) differences were analyzed using the Tukey–Kramer test. Statistical significance was considered at $P < 0.05$. The results are expressed as least square means (LSmeans) \pm SEMs. The primary outcomes of the study were Met and Cys quantitative kinetics, liver protein L-MetInc, and liver mRNA abundances. Plasma and liver free AA concentrations and vitamin and metabolite concentrations were secondary outcomes.

Results

The mean BW before the tracer studies was not different ($P > 0.10$) between the groups (age 45 d: 13.5 ± 0.5 kg), whereas at the age of 60 d, the BW of pigs supplemented with L-Met, DL-Met, and DL-HMTBA was similar and higher than the BW in pigs of the Met-deficient Control group (22.9 ± 0.7 kg compared with 19.9 ± 0.7 kg; $P < 0.05$). Feed intake was similar among the groups (data not shown).

Whole-body Met kinetics and Cys flux

The Met Q_m and Q_c flux rates and the TM, RM, PS, and TS rates were higher in fed than in feed-deprived conditions ($P < 0.01$) in all groups and were not different between groups in the feed-deprived state (Figures 2A–F). In the fed state, the Met Q_m and Q_c were higher in LMET, DLMET, and DLHMTBA pigs than in Control pigs ($P < 0.001$), and furthermore, Q_c was lower in DLHMTBA pigs than in DLMET pigs ($P < 0.05$). The fed state TM, TS, and PS rates were greater in LMET, DLMET, and DLHMTBA pigs than in Control pigs ($P < 0.05$). Notably, DLHMTBA pigs in the fed state had greater RM rates than Control and DLMET pigs ($P < 0.05$), whereas TS rates in fed DLHMTBA pigs were lower than those in L-Met- and DL-Met-supplemented pigs ($P < 0.05$). No differences in Cys flux were observed between the treatment groups (Supplemental Figure 2). Within group, Control and DLMET pigs showed higher Cys flux rates in the fed than in the feed-deprived state ($P < 0.05$; Supplemental Figure 2).

The proportions of Q_m utilized for TM and TS in L-Met-, DL-Met-, and DL-HMTBA-supplemented pigs were

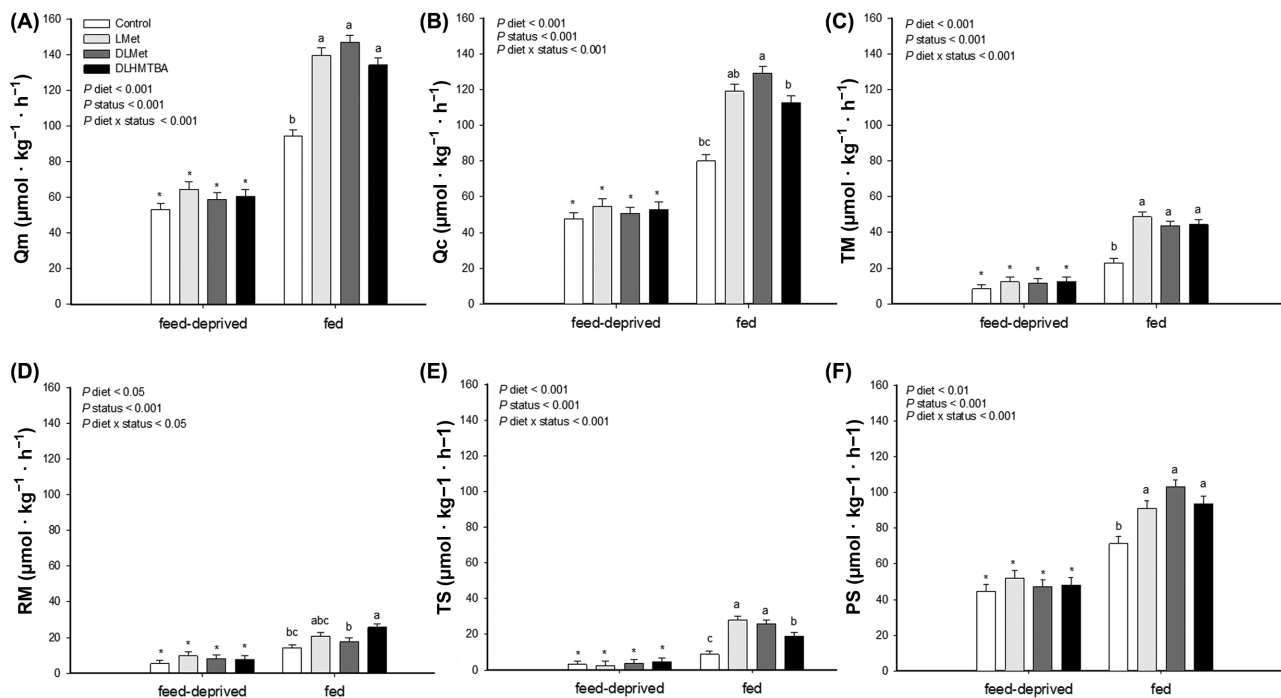


FIGURE 2 Quantitative Met cycle rates in piglets fed 4 diets differing in Met isomer type starting at weaning (age 28 d). (A) Whole-body Met Q_m , (B) Met Q_c , (C) TM, (D) RM, (E) TS, and (F) PS rates. Bars are LSmeans \pm SEMs. The Met kinetics were calculated using plasma Met isotopic enrichments derived from a primed 9-h intravenous L-[1- ^{13}C , methyl- $^2\text{H}_3$]-Met infusion at a rate of $10 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ with 3 h without feed followed by small meals provided every half hour over 6 h (providing 7% of the daily feed allowance each). Control diet, Met plus Cys-deficient (69% of standardized ileal digestible Met plus Cys recommendation) ($n = 12$); L-Met-supplemented diet (Control + 0.15% L-Met) ($n = 12$); DL-Met-supplemented diet (Control + 0.15% DL-Met) ($n = 11$); DL-2-hydroxy-4-methylthiobutyric acid-supplemented diet (Control + 0.17% DL-HMTBA) ($n = 10$). Met sources were added on an equimolar basis (DL-HMTBA contains 12% water). Labeled bars without a common letter differ, $P < 0.05$. *Different from fed, $P < 0.05$. ANOVA F test. DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; PS, protein synthesis; Q_c , carboxyl flux; Q_m , methyl flux; RM, remethylation; TM, transmethylation; TS, transsulfuration.

higher in the fed than in the feed-deprived state, whereas the PS: Q_m ratio was lower ($P < 0.01$; Table 3). In L-Met-deficient Control pigs, the ratios of PS: Q_m and TS: Q_m were not different between the feed-deprived and fed states. Whereas fed pigs in the Control and DL-HMTBA-supplemented groups utilized a greater fraction of Q_m for RM compared with the feed-deprived state ($P < 0.01$), the proportions of RM of whole body Q_m in L-Met- and DL-Met-supplemented pigs were independent of the feeding state (Table 3). In all groups of fed pigs, the highest proportion of Q_m was used for PS (65–76%) and lower proportions were used for TM (24–35%) and RM (12–19%). Pigs supplemented with L-Met used a proportionally lower share of Q_m for PS but higher proportions of Q_m for TM than Control pigs ($P < 0.05$). The latter was also true for DL-HMTBA-supplemented pigs, which utilized a higher proportion of Q_m for TM than Control pigs ($P < 0.05$). No differences in PS: Q_m and TM: Q_m ratios were observed between the 3 supplemented groups (LMET, DLMET, or DLHMTBA).

Met entering TM can further be partitioned into the RM or TS pathways. In the fed state, L-Met- and DL-Met-supplemented pigs showed a higher fraction of TM passing through TS than in Met-deficient Control pigs (Table 3). In contrast, the fraction of TM being remethylated was relatively lower ($P < 0.001$) in L-Met- and DL-Met-supplemented pigs than in Control and DL-HMTBA-supplemented pigs, respectively. In fed Control and DL-HMTBA-supplemented pigs, the fraction of Q_m used for TS was lower than that in fed L-Met- and DL-Met-supplemented pigs ($P < 0.05$;

Table 3). The Met-deficient Control pigs showed a greater proportion of RM and a lesser proportion of TS relative to TM than L-Met- and DL-Met-supplemented pigs ($P < 0.05$). Comparing DL-HMTBA- to L-Met-supplemented pigs, DL-HMTBA-supplemented pigs utilized a higher fraction of TM for RM but a lower fraction for TS ($P < 0.05$; Table 3). In L-Met-, DL-Met-, and DL-HMTBA-supplemented pigs, similar fractions of Met were utilized for TM and PS ($P > 0.05$), whereas in Met-deficient Control pigs, TM as a ratio of PS was lower than that in L-Met-supplemented pigs ($P < 0.05$; Table 3). Whole-body carbon dioxide production did not differ between groups but was lower in the feed-deprived ($35.5 \pm 1.2 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) than in the fed state ($48.5 \pm 1.8 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; $P < 0.001$).

Liver L-MetInc and transcript abundance

The liver protein L-MetInc was lower in the DLMET and DLHMTBA groups than in the Control group in the feed-deprived state, whereas in L-Met-supplemented pigs, it was intermediate ($P < 0.05$; Figure 3). In feed-deprived pigs, the hepatic mRNA abundance of S-adenosylhomocysteine hydrolase (*AHCY*) was lower in DL-Met- and DL-HMTBA-supplemented pigs than in Control pigs, and *MTR* abundance was lower in the L-Met-supplemented pigs than in the Control group ($P < 0.05$; Supplemental Table 2). The abundance of the L-2-hydroxyacid oxidase transcript was higher in Control pigs than in L-Met-supplemented pigs ($P < 0.05$).

TABLE 3 Ratios of whole-body Met kinetic parameters in 47-d-old feed-deprived and fed-state piglets fed 4 diets differing in Met isomer type starting at weaning (age 28 d)¹

Item	Status	Control (n = 12)	LMET (n = 12)	DLMET (n = 11)	DLHMTBA (n = 10)	SEM	P value ²		
							Diet	Status	Diet × status
PS:Q _m	Feed-deprived	0.84	0.81*	0.81*	0.80*	0.02	0.08	<0.001	0.27
	Fed	0.76 ^a	0.65 ^b	0.70 ^{a,b}	0.70 ^{a,b}	0.02			
TM:Q _m	Feed-deprived	0.16*	0.19*	0.20*	0.20*	0.02	<0.05	<0.001	0.13
	Fed	0.24 ^b	0.35 ^a	0.30 ^{a,b}	0.33 ^a	0.02			
RM:Q _m	Feed-deprived	0.10*	0.15	0.14	0.13*	0.02	0.25	<0.05	<0.001
	Fed	0.15 ^{a,b}	0.14 ^b	0.12 ^b	0.19 ^a	0.02			
TS:Q _m	Feed-deprived	0.06	0.04*	0.06*	0.08*	0.02	<0.05	<0.001	<0.001
	Fed	0.09 ^c	0.21 ^a	0.18 ^{a,b}	0.14 ^{b,c}	0.02			
TS:TM	Feed-deprived	0.38	0.29*	0.32*	0.38	0.05	0.41	<0.001	<0.001
	Fed	0.36 ^c	0.62 ^a	0.59 ^{a,b}	0.42 ^{b,c}	0.05			
RM:TM	Feed-deprived	0.62	0.77*	0.72*	0.62	0.05	0.62	<0.001	<0.001
	Fed	0.64 ^a	0.38 ^c	0.41 ^{b,c}	0.58 ^{a,b}	0.05			
TM:PS	Feed-deprived	0.19*	0.24*	0.25*	0.26*	0.05	0.09	<0.001	0.20
	Fed	0.33 ^b	0.56 ^a	0.44 ^{a,b}	0.46 ^{a,b}	0.05			

¹Values are LSmeans and SEMs; the largest SEM is shown; *n* refers to the number of pigs per dietary group. Control diet, Met plus Cys-deficient (69% of standardized ileal digestible Met plus Cys recommendation); L-Met-supplemented diet (Control + 0.15% L-Met); DL-Met-supplemented diet (Control + 0.15% DL-Met); DL-HMTBA-supplemented diet (Control + 0.17% DL-HMTBA). Met sources were added on an equimolar basis (DL-HMTBA contains 12% water). Labeled LSmeans in a row without a common letter differ, *P* < 0.05. DLHMTBA, group fed DL-HMTBA-supplemented diet; DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; DLMET, group fed DL-Met-supplemented diet; LMET, group fed L-Met-supplemented diet; PS, protein synthesis; Q_m, methyl flux; RM, remethylation; TM, transmethylation; TS, transsulfuration.

²ANOVA *F* test.

*Different from fed, *P* < 0.05.

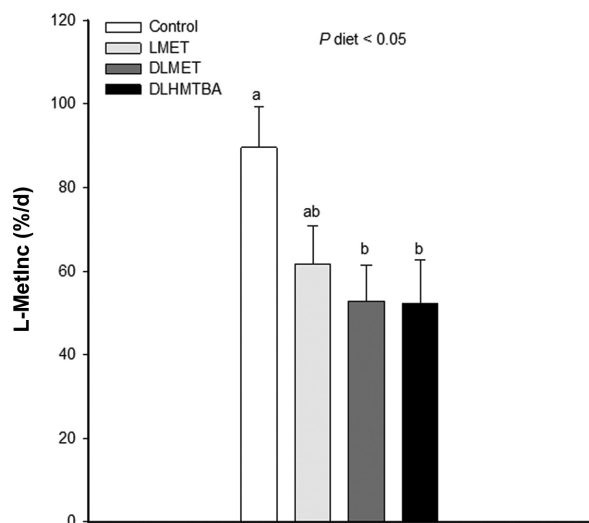


FIGURE 3 L-MetInc (percentage per day) in feed-deprived piglets fed 4 diets differing in Met isomer type starting at weaning (age 28 d). Bars are LSmeans ± SEMs. Hepatic L-MetInc was calculated using ¹³C-Met isotopic enrichments in liver protein and liver tissue supernatant derived from an intravenous L-[1-¹³C]-Met bolus administration. Control diet, Met plus Cys-deficient (69% of standardized ileal digestible Met plus Cys recommendation) (*n* = 9); L-Met-supplemented diet (Control + 0.15% L-Met) (*n* = 9); DL-Met-supplemented diet (Control + 0.15% DL-Met) (*n* = 10); DL-2-hydroxy-4-methylthiobutyric acid-supplemented diet (Control + 0.17% DL-HMTBA) (*n* = 7). Met sources were added on an equimolar basis (DL-HMTBA contains 12% water). Labeled bars without a common letter differ, *P* < 0.05. DLHMTBA, group fed DL-HMTBA-supplemented diet; DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; DLMET, group fed DL-Met-supplemented diet; LMET, group fed L-Met-supplemented diet; L-MetInc, L-Met incorporation rate in liver protein.

Free hepatic and plasma AA, vitamin, and plasma metabolite concentrations

Plasma concentrations of Met, Ser, and Tau were increased in response to feed intake relative to the feed-deprived state in all groups (*P* < 0.05; Table 4). In contrast, plasma Cys concentrations in Control pigs were higher in the fed than in the feed-deprived state (*P* < 0.05; Table 3). However, plasma concentrations of Gly decreased in L-Met- and DL-Met-supplemented animals in response to feed intake (*P* < 0.05). Feed-deprived concentrations of Met and Tau were higher in L-Met- (*P* < 0.01), DL-Met-, and DL-HMTBA-supplemented pigs (*P* < 0.05) than in Control pigs. Plasma Cys concentrations during feed deprivation were higher in L-Met-supplemented pigs (*P* < 0.05). The fed state concentrations of Met were higher in L-Met- (*P* < 0.001), DL-Met-, and DL-HMTBA-supplemented pigs than in Control pigs (*P* < 0.05). The fed-state concentrations of Ser were lower in L-Met-supplemented pigs than in Control pigs, whereas Tau concentrations were higher in L-Met-supplemented pigs than in Control animals (*P* < 0.05). Plasma threonine and lysine concentrations were lower in feed-deprived pigs supplemented with L-Met, DL-Met, and DL-HMTBA than in Met-deficient Control pigs (*P* < 0.05; Supplemental Table 3). In the fed state, plasma threonine concentrations were lower in L-Met-, DL-Met-, and DL-HMTBA-supplemented pigs than in the Control pigs, whereas plasma lysine was lower than in Control pigs only in DL-Met-supplemented pigs (*P* < 0.05) (Supplemental Table 3). Free hepatic Cys concentrations were higher in L-Met-, DL-Met-, and DL-HMTBA-supplemented pigs than in Control pigs (*P* < 0.01; Supplemental Table 4). Concentrations of Gly were higher in L-Met- and DL-HMTBA-supplemented pigs than in Control pigs (*P* < 0.05) (Supplemental Table 4). The plasma concentrations of folate and vitamin B-6, cofactors of enzymes involved in RM and TS, were not different between the groups (20.9 ± 1.6 ng/mL and 15.8 ± 3.4 µg/L, respectively).

The plasma metabolites cholesterol, urea, and nonesterified fatty acids increased with age (*P* < 0.05), whereas

TABLE 4 Plasma free amino acid and Tau concentrations in 54-d-old feed-deprived and fed-state piglets fed 4 diets differing in Met isomer type starting at weaning (age 28 d)¹

Amino acid	Status	Amino acid concentration (mM)					P value ²		
		Control (n = 8)	LMET (n = 8)	DLMET (n = 8)	DLHMTBA (n = 8)	SEM	Diet	Status	Diet × status
Met	Feed-deprived	0.01 ^{b,*}	0.02 ^{a,*}	0.02 ^{a,*}	0.02 ^{a,*}	0.002	<0.001	<0.001	<0.001
	Fed	0.02 ^b	0.08 ^a	0.09 ^a	0.08 ^a	0.006			
Cys	Feed-deprived	0.07 ^{b,*}	0.09 ^a	0.09 ^a	0.09 ^a	0.004	<0.05	<0.01	0.92
	Fed	0.08	0.10	0.09	0.10	0.005			
Tau	Feed-deprived	0.03 ^{b,*}	0.04 ^{a,*}	0.04 ^{a,*}	0.04 ^{a,*}	0.003	<0.01	<0.001	0.46
	Fed	0.03 ^b	0.05 ^a	0.04 ^{a,b}	0.05 ^{a,b}	0.003			
Ser	Feed-deprived	0.26 [*]	0.22 [*]	0.22 [*]	0.22 [*]	0.019	0.07	<0.001	0.13
	Fed	0.40 ^a	0.30 ^b	0.32 ^{a,b}	0.34 ^{a,b}	0.022			
Gly	Feed-deprived	0.88	1.11 [*]	1.13 [*]	1.04	0.092	0.77	<0.05	0.96
	Fed	0.91	0.98	0.99	1.02	0.085			

¹Values are LSmeans and SEMs; the largest SEM is shown; n refers to the number of pigs analyzed per dietary group. Control diet, Met plus Cys-deficient (69% of standardized ileal digestible Met plus Cys recommendation); L-Met-supplemented diet (Control + 0.15% L-Met); DL-Met-supplemented diet (Control + 0.15% DL-Met); DL-HMTBA-supplemented diet (Control + 0.17% DL-HMTBA). Met sources were added on an equimolar basis (DL-HMTBA contains 12% water). Labeled LSmeans in a row without a common letter differ, $P < 0.05$. DLHMTBA, group fed DL-HMTBA-supplemented diet; DL-HMTBA, DL-2-hydroxy-4-methylthiobutyric acid; DLMET, group fed DL-Met-supplemented diet; LMET, group fed L-Met-supplemented diet; Tau, taurine.

²ANOVA F test.

*Different from fed, $P < 0.05$.

glucose and TG concentrations remained unchanged (Supplemental Table 5). The cholesterol concentrations were higher in the Met-supplemented groups at 62 d of age ($P < 0.05$). Urea concentrations were lower in L-Met- and DL-Met-supplemented pigs and were intermediate in DL-HMTBA-supplemented pigs compared with those in Control pigs ($P < 0.001$), and TG concentrations were partially lower in L-Met-, DL-Met-, and DL-HMTBA-supplemented pigs than in Control animals ($P < 0.01$; Supplemental Table 5).

Discussion

Our main objectives were to investigate whether dietary DL-HMTBA supplementation of a Met-deficient diet affects whole-body Met and Cys kinetics compared with L-Met or DL-Met supplementation in a weaned piglet model. Piglets fed the Met-deficient diet showed lower PS and growth performance than pigs with supplementation of L-Met, DL-Met, or DL-HMTBA, which was similar among the supplemented groups, and confirms earlier results (28). In neonatal pigs consuming a completely sulfur AA-free diet, growth performance and whole-body PS, as well as Met and Cys flux rates, were lower than in their adequately fed counterparts (16). The stimulation of the Met cycle rates by feed intake is attributed to the increase in available Met, which agrees with previous findings in humans, in which meal ingestion stimulated whole-body PS, TM, RM, and TS (29). The proportion of TM entering RM was reduced with feed intake in L-Met- and DL-Met-supplemented pigs but not in Met-deficient Control and DL-HMTBA-supplemented pigs, suggesting a similarity between DL-HMTBA-supplemented and Met-deficient pigs. Our 47-d-old pigs showed similar flux values as well as TM, TS, RM, and PS rates to those reported for 27-d-old piglets (30), but the TM, TS, and RM values were lower by ~12%, 9%, and 42%, respectively, than in 10-d-old piglets (16). However, the whole-body PS rate was comparable among all age groups. The Q_m , TM, TS, RM, and PS rates of weaned piglets were several times higher than for adult humans (31, 32), which may be

due to an age- but also species-dependent effect. Furthermore, we observed a higher L-Met incorporation rate in liver protein of Met-deficient pigs compared with that of Met-supplemented pigs, which might indicate that Met-deficient pigs partition relatively more of the dietary available Met to PS in the liver, whereas less Met is transsulfurated. This assumption is supported by our finding of a higher PS: Q_m ratio in the Control group suggesting that a larger share of the available Met enters PS because PS is prioritized. Nonetheless, the magnitude of the L-Met incorporation rate in our 62-d-old L-Met-, DL-Met-, and DL-HMTBA-supplemented pigs was similar to the fractional PS rate observed for 36-d-old fed pigs (33).

Higher RM and lower TS rates in DLHMTBA pigs than in L-Met- and DL-Met-supplemented pigs were observed, whereas TM, PS, and the Cys flux did not differ between groups, indicating that Hcys is primarily used for RM but less for TS in DL-HMTBA-supplemented pigs. In rats, under a low dietary Met intake, RM is favored over TS (34), again suggesting that DL-HMTBA supplementation resembles a Met-deficient state to some extent. The higher TS rate for L- and DL-Met-supplemented pigs than for DL-HMTBA-supplemented pigs indicates that more Met is oxidized and converted to L-Cys, which is utilized for the synthesis of proteins and other essential metabolites, including glutathione. Our results are in contrast to earlier reports in pigs and chickens that speculated that dietary DL-HMTBA, compared with other Met isomers, would favor the TS rate (10, 11, 35). However, these earlier conclusions are probably not valid because they were drawn only based on greater plasma Tau, Cys, and glutathione concentrations in DL-HMTBA- than in Met-treated animals. As the concentration of vitamin B-6, the co-factor of CBS and CGL, did not differ between groups in the present study, the higher RM and lower TS rates in DLHMTBA pigs than in LMET pigs may likely be caused by increased activity of enzymes catalyzing the RM pathway (MTR, BHMT), inhibition of enzymes involved in the TS pathway (CBS, CGL), or a combination thereof. Several mechanisms regulating the RM to TS balance that compete for the utilization of Hcys exist (36–38). Acute dietary supplementation of betaine, a methyl donor for RM, increased hepatic S-adenosylmethionine, S-adenosylhomocysteine, and Met concentrations in mice, whereas the

CBS activity remained surprisingly unaltered (39). Based on the concomitantly depleted hepatic cystathionine and Hcys concentrations, the authors concluded that betaine enhances both TS and RM (39, 40); the latter is likely caused by a betaine-induced stimulation of BHMT. Nonetheless, in the present study we showed preferential RM with DL-HMTBA supplementation; thus, DL-HMTBA might act similarly to betaine and stimulate MTR or BHMT activity, but this hypothesis needs to be tested. Higher RM might also be a compensatory response to lower bioavailability of L-Met from DL-HMTBA and thus may be a way to maintain L-Met concentrations in fast-growing piglets. It is unlikely that the upregulation of RM and downregulation of TS in response to DL-HMTBA supplementation in the present study can be attributed to differences in the availability of enzyme cofactors or cosubstrates because all diets contained comparable amounts of micronutrients and vitamins, and we did not observe group differences in plasma Ser, Gly, folate, or vitamin B-6 concentrations. The lower TS rate in DL-HMTBA-supplemented pigs might be due to an inhibition of CBS, the rate-limiting enzyme of the TS pathway, or due to the shift of Hcys to RM, resulting in Hcys not being available for TS.

In the fed state, plasma Met, Tau, and Ser concentrations were higher in all piglet groups. Lower plasma Met concentrations in Met-deficient pigs than in supplemented pigs were expected because of lower intake. Higher plasma Ser concentrations in Met-deficient pigs than in L-Met-supplemented pigs agree with a lower TS rate because less Ser is used for TS and, thus, may accumulate (41). Owing to the lower TS rate, it is plausible that Met-deficient piglets had lower plasma Tau concentrations than L-Met pigs because Tau originates from Cys.

Hepatic gene regulation serves as an important regulatory point for controlling Met metabolism (41). Overall, only small or no differences in the mRNA abundances of hepatic enzymes involved in Met cycling and TS between Met-deficient and Met-supplemented pigs were observed, which might be because tissues were collected from feed-deprived but not fed pigs. This interpretation agrees with our findings that under feed-deprived conditions, the Met cycle rates did not differ between groups. The collection of liver samples in the feed-deprived state only is a limitation of the study because the stage of the tissue at collection time might have affected gene expression. The expression of *AHCY* was lower with DL-Met and DL-HMTBA supplementation than with Met-deficient feed-deprived pigs. This result might be explained by increased concentrations of available Met stimulating Met metabolism via TM which, in turn, is inhibited by its product (42). These results partly agree with findings in bovine primary hepatocytes treated with DL-Met and DL-HMTBA (42), in which a reduction in *AHCY* expression by DL-HMTBA, but no effect of DL-Met, was found. Likewise, the expression of *MTR* in L-Met pigs was downregulated when compared with Control pigs, possibly through product inhibition.

In conclusion, the effects of DL-HMTBA, DL-Met, and L-Met supplementation in piglets are similar in terms of growth performance. However, DL-HMTBA compared with L-Met and DL-Met supplementation favors RM and reduces the TS rate, thereby conserving more Met, resembling a Met-deficient condition. Our results argue against earlier speculations (10, 11, 35) that DL-HMTBA compared with other Met sources would increase TS.

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