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NON RUMINANT NUTRITION

Effects of supplemental D-methionine in comparison to L-methionine on nitrogen retention, gut morphology, antioxidant status, and mRNA abundance of amino acid transporters in weanling pigs

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Abstract

An N-balance experiment was conducted to test the hypothesis that D-Methionine (D-Met) has the same bioavailability and efficacy as L-Methionine (L-Met) when fed to weanling pigs. A Met-deficient basal diet containing 0.24% standardized ileal digestible (SID) Met was formulated. Six additional diets were formulated by adding 0.036%, 0.072%, or 0.108% D-Met or L-Met to the basal diet, and these diets, therefore, contained 77%, 87%, or 97% of the requirement for SID Met. Fifty-six barrows (10.53 ± 1.17 kg) were housed in metabolism crates and allotted to the seven diets with eight replicate pigs per diet. Feces and urine were collected quantitatively with 7-d adaptation and 5-d collection periods. Blood and tissue samples from pigs fed the basal diet and pigs fed diets containing 0.108% supplemental Met were collected on the last day. Results indicated that N retention (%) linearly increased ($P < 0.01$) as supplemental D-Met or L-Met increased in diets. Based on N retention (%) as a response, the linear slope-ratio regression estimated the bioavailability of D-Met relative to L-Met to be 101% (95% confidence interval: 57%–146%). The villus height and crypt depth in the jejunum were not affected by the Met level or Met source. Total antioxidant capacity or thiobarbituric acid reactive substance concentrations in plasma or tissue samples from pigs fed the control diet or diets containing 0.108% supplemental D-Met or L-Met were not different. Abundance of mRNA for some AA transporters analyzed in intestinal mucosa of pigs also did not differ. Therefore, it is concluded that D-Met and L-Met are equally bioavailable for weanling pigs.

Key words: bioavailability, D-methionine, L-methionine, methionine, nitrogen retention, pigs

Abbreviations

AA	amino acids
ATTD	apparent total tract digestibility
BW	body weight
DAAO	D-amino acid oxidase
D-Met	D-methionine
DL-Met	DL-methionine
L-Met	L-methionine
RBV	relative bioavailability
SID	standardized ileal digestible
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances

Introduction

Methionine is usually the second or third limiting amino acid (AA) in swine diets based on cereal grains and soybean meal (Dilger and Baker, 2007). Because of its high requirement by pigs and its relatively low concentration in many feed ingredients, supplementation with synthetic sources of Met is common in diets fed to pigs. One of the main synthetic Met sources used to balance the requirement of Met in pig and poultry diets is DL-methionine (DL-Met; a 50:50 mixture of D- and L-isomers; 99%), which is considered 100% bioavailable to pigs (Wang et al., 2020). Recently, L-methionine (L-Met; 99%) has become available to the feed industry and several experiments have been conducted to compare the relative bioavailability (RBV) by pigs of L-Met relative to DL-Met (Chen et al., 2013; van Milgen et al., 2013; Tian et al., 2016; Htoo and Morales, 2016; Yang et al., 2019; Zeitz et al., 2019). In general, it was demonstrated that the RBV for L-Met is not different from that of DL-Met when fed to growing pigs. This is most likely because the D-amino acid oxidase (DAAO) enzyme, which is needed for the conversion of D-methionine (D-Met) to L-Met, is present in high concentrations in the liver, kidney, stomach, small intestine (i.e., duodenum, jejunum, and ileum), and muscle of weanling pigs (Fang et al., 2010). Pigs only utilize L-Met for protein synthesis, but because of the DAAO, conversion of D-Met to L-Met can take place in body tissues after absorption of D-Met into the portal blood (Wu, 2013), which is the reason DL-Met is believed to be 100% bioavailable. Indeed, Zhou et al. (2021) demonstrated that the RBV of Met in DL-Met was not different from L-Met if N retention was used as the response criterion. Therefore, it is possible that pigs are equally efficient in utilizing D-Met and L-Met for protein synthesis and for synthesis of Met metabolites involved in oxidative responses. However, information demonstrating the RBV of pure D-Met (99%) against L-Met (99%) for pigs is limited. Therefore, the objective of this experiment was to test the hypothesis that the RBV by weanling pigs of Met in D-Met is not different from that in L-Met pigs if N retention is used as the response criterion. The second objective was to test the hypothesis that there are no differences between D-Met and L-Met in their ability to maintain intestinal morphology, antioxidant capacity, and expression of genes related to Met metabolism.

Materials and Methods

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois. The experiment was conducted at the swine research center at the University of Illinois.

Dietary treatments, animals, and N-balance study procedure

Seven diets were formulated based on corn, soybean meal, field peas, and whey powder (Tables 1 and 2). All diets were formulated to contain 1.30% standardized ileal digestible (SID) Lys, which was 106% of the estimated requirement of 1.23% SID Lys for 11- to 25-kg pigs (NRC, 2012). The basal diet was formulated to be deficient in Met with a concentration of 0.24% SID Met, which was 67% of the estimated requirement of 0.36% SID Met for 11- to 25-kg pigs (NRC, 2012). However, the basal diet was adequate in all other nutrients and energy (NRC, 2012). Six additional diets that were similar to the basal diet with the exception that 0.036%, 0.072%, or 0.108% D-Met (99% purity; Acros Organics, Thermo Fisher Scientific, Waltham, MA) or 0.036%, 0.072%, or 0.108% of L-Met (99% purity; CheilJedang, Seoul, South Korea) was added at the expense of corn and soybean meal were formulated. The six diets with added D- or L-Met were calculated to contain 77%, 87%, or 97% of the requirement for SID Met (NRC, 2012; Table 3). All AA-containing ingredients were analyzed for AA before diet mixing, and diets were formulated based on analyzed AA concentrations of ingredients. Values for SID of AA used in diet formulations were from NRC (2012).

A total of 56 barrows (initial body weight [BW]: 10.53 ± 1.17 kg; L359 × Camborough, PIC, Hendersonville, TN) were allotted to a randomized complete block design with 7 diets and 2 blocks of 28 pigs. There were four replicate pigs per diet in each block for a total of eight replicate pigs per diet. Pigs were placed in individual metabolism crates that were equipped with a self-feeder, a nipple waterer, and slatted floors to allow for the total, but separate, collection of urine and fecal materials.

Pigs were weighed at the beginning of the adaptation period and at the end of collection. All diets were fed in meal form. Pigs were limit fed at 2.5 times the metabolizable energy requirement for maintenance (i.e., 197 kcal/kg × $BW^{0.60}$; NRC, 2012), which was provided each day in 2 equal meals at 0800 and 1600 h. Throughout the experiment, pigs had free access to water, but feed provisions were recorded daily. The initial 7 d were considered the adaptation period to the diet, whereas urine and fecal materials were collected during the following 5 d according to the marker-to-marker method (Adeola, 2001). On days 8 and 13, chromic oxide (approximately 3 g/kg) was

Table 1. Analyzed nutrient composition of ingredients, as-fed basis

Item, %	Corn	Soybean meal	Field peas	Whey powder	Spray-dried plasma
Dry matter	86.63	92.18	87.11	97.03	93.72
Crude protein	7.03	51.74	23.35	14.68	81.65
Indispensable amino acids					
Arg	0.35	3.75	2.08	0.34	4.70
His	0.19	1.33	0.57	0.25	2.41
Ile	0.24	2.34	0.94	0.82	2.47
Leu	0.80	3.95	1.67	1.42	7.72
Lys	0.24	3.20	1.61	1.19	7.42
Met	0.14	0.71	0.22	0.23	0.94
Phe	0.34	2.64	1.11	0.47	4.19
Thr	0.25	2.01	0.84	0.93	5.38
Val	0.32	2.46	1.06	0.78	5.52
Met + Cys	0.29	1.47	0.56	0.55	3.61

Analyses for dry matter and crude protein followed AOAC procedures (AOAC Int., 2007), and amino acids were analyzed following the procedure described by Llames and Fontaine (1994).

Table 2. Ingredient composition of experimental diets, as-fed basis

Item	Basal	D-Met			L-Met		
Supplemental Met, %	0.00	0.036	0.072	0.108	0.036	0.072	0.108
Ground corn	49.17	49.184	49.148	49.112	49.184	49.148	49.112
Soybean meal	20.00	19.95	19.95	19.95	19.95	19.95	19.95
Ground field peas	14.00	14.00	14.00	14.00	14.00	14.00	14.00
Whey powder	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Spray dried plasma	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Soybean oil	2.34	2.34	2.34	2.34	2.34	2.34	2.34
Ground limestone	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Dicalcium phosphate	1.15	1.15	1.15	1.15	1.15	1.15	1.15
L-Lys HCl	0.36	0.36	0.36	0.36	0.36	0.36	0.36
L-Thr	0.18	0.18	0.18	0.18	0.18	0.18	0.18
D-Met	–	0.036	0.072	0.108	–	–	–
L-Met	–	–	–	–	0.036	0.072	0.108
L-Trp	0.09	0.09	0.09	0.09	0.09	0.09	0.09
L-Val	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-His	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin-mineral premix[1,2]	0.30	0.30	0.30	0.30	0.30	0.30	0.30

[1]The vitamin-micromineral premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D_3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.
[2]United Animal Health, Sheridan, IN.

Table 3. Analyzed nutrient composition of experimental diets, as-fed basis

Item	Basal	D-Met			L-Met		
Supplemental Met, %	0.00	0.036	0.072	0.108	0.036	0.072	0.108
Dry matter, %	89.26	89.33	89.37	89.40	89.37	89.32	89.30
Net energy[1], kcal/kg	2.546	2.545	2.544	2.543	2.545	2.544	2.543
Metabolizable energy[1], kcal/kg	3.398	3.396	3.395	3.394	3.396	3.395	3.394
Ca[1], %	0.75	0.75	0.75	0.75	0.75	0.75	0.75
P[2], %	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Crude protein, %	20.09	20.20	20.08	20.08	20.48	19.93	19.44
Supplemental Met, %	–	0.036	0.066	0.104	0.039	0.069	0.103
Indispensable amino acids[3], %							
Arg	1.26	1.31	1.29	1.29	1.31	1.26	1.26
His	0.49	0.50	0.50	0.49	0.50	0.49	0.48
Ile	0.80	0.83	0.82	0.82	0.84	0.80	0.82
Leu	1.58	1.64	1.62	1.61	1.63	1.57	1.61
Lys	1.43	1.48	1.43	1.44	1.44	1.45	1.44
Met	0.26	0.30	0.32	0.37	0.30	0.33	0.36
Phe	0.90	0.94	0.92	0.92	0.93	0.89	0.90
Thr	0.95	0.96	0.95	0.94	0.93	0.96	0.93
Trp	0.32	0.32	0.33	0.32	0.32	0.32	0.32
Val	1.01	1.02	1.02	1.03	1.05	1.03	1.03
Met + Cys	0.58	0.63	0.65	0.69	0.63	0.66	0.69

Analyses for dry matter and crude protein followed AOAC procedures (AOAC Int., 2007), and amino acids were analyzed following the procedure described by Llames and Fontaine (1994).
[1]Values not analyzed, but determined via calculation (NRC, 2012).
[2]Calculated as standardized total tract digestible P (NRC, 2012).
[3]The basal diet was formulated to contain 1.30% SID Lys, 0.24% SID Met, 0.51% SID Met + Cys, 0.84% SID Thr, 0.29% SID Trp, 0.91% SID Val, 0.71% SID Ile, 1.37% SID Leu, 0.44% SID His, and 1.14% SID Arg. The basal diet (deficient in Met; 67% of requirement) with three graded levels of D-Met or L-Met (i.e., 0.036%, 0.072%, and 0.108% supplemental Met) was formulated to contain 77%, 87%, and 97% of SID Met requirement for 11- to 25-kg pigs, respectively (NRC, 2012).

included in the morning meal and urine collection started on day 8 in the morning and concluded on day 13 in the morning. During this period, urine was collected in buckets that were emptied daily and 50 mL of HCl was added to the buckets every day. Fecal samples and 20% of the collected urine were stored at –20 °C immediately after collection. At the conclusion of the

experiment, urine samples were thawed and mixed within animal and diet, and a urine sub-sample (i.e., approximately 10 mL) was lyophilized before analysis.

Collection of blood and tissue samples

At the completion of the N-balance study (i.e., day 13), pigs were fasted for 12 h and blood samples were collected from the jugular vein of pigs fed the basal diet and pigs fed diets containing 0.108% D-Met or 0.108% L-Met using 8-mL heparinized vacutainers (BD, Franklin Lakes, NJ). Plasma was collected after centrifugation at 1,500 × *g* at 4 °C for 15 min and plasma samples were stored at –80 °C until analyzed. After blood collection, pigs were euthanized. Tissue samples from the liver (i.e., right and left medial lobes) and muscle (*longissimus dorsi*) were collected. Combined samples of mucosa and submucosa from the duodenum (i.e., 0.15 m from the pylorus; Low et al., 1978) and jejunum (i.e., 2 m from the pylorus; Low et al., 1978) were also collected using a scraping technique. All tissue samples were snap frozen in liquid N and stored at –80 °C for later analyses. Three-centimeter segments from the middle of the duodenum and from the middle of the jejunum were also collected and fixed in 10% neutral buffered formalin. These samples were used to determine villus height and crypt depth.

Chemical analyses

At the conclusion of the experiment, fecal samples were thawed and mixed within pig and diet, and then dried at 50 °C using a forced-air drying oven. Fecal samples were finely ground through a 1-mm screen in a Wiley mill (Model 4; Thomas Scientific, Swedesboro, NJ). Ground fecal samples were homogenized, and a subsample (i.e., approximately 200 g) was collected for nutrient analysis. Ingredients, diets, and fecal samples were analyzed for crude protein (CP) using the combustion procedure (Method 990.03; AOAC Int., 2007) on an Elementar Rapid N-cube protein/nitrogen apparatus (Elementar Americas Inc., Mt. Laurel, NJ) and for dry matter (Method 930.15; AOAC Int., 2007). Lyophilized urine sub-samples were then analyzed for CP using Kjeltec 8400 (FOSS Inc., Eden Prairie, MN).

Amino acid concentrations in ingredients and diets were determined by ion-exchange chromatography with post-column derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with sodium metabisulfite (Llames and Fontaine, 1994; European Commission, 1998). Tryptophan concentration in diets was determined via high performance liquid chromatography with fluorescence detection (extinction 280 nm, emission 356 nm) after alkaline hydrolysis (European Commission, 2000). The concentration of free Met in diets was determined after extraction with 0.1 *N* HCL according to the procedure of Wang et al. (2020) and then quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm (European Commission, 1998).

Intestinal morphology

The fixed intestinal tissues were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Slides were scanned using a NanoZoomer Digital Pathology System (Hamamatsu Co., Bridgewater, NJ), and all measurements were conducted in the associated NDP.View2 software. At least 10 straight and integrated villi and their associated crypts were measured. The intestinal morphological measurements included villus height and crypt depth. Mean values of each criteria and villus height:crypt depth ratio within each segment were calculated.

Antioxidant capacity analysis

Plasma, liver, and muscle samples were analyzed in duplicate for the concentration of total glutathione, thiobarbituric acid reactive substances (TBARS), and total antioxidant capacity (TAC). Prior to glutathione analysis, 100 mg of muscle or liver were suspended in 1 mL of an ice-cold buffer containing 5% 5-sulfosalicylic acid. Samples were then homogenized with a tissue homogenizer and then centrifuged (10,000 × *g* at 4 °C for 10 min). The resulting supernatant was then used to determine concentrations of total glutathione using a commercial kit (Sigma-Aldrich, St. Louis, MO). For TBARS analysis, 25 mg of liver or muscle samples were suspended in 250 µL of radioimmunoprecipitation assay buffer (Pierce, Rockford, IL), homogenized with a tissue homogenizer, and then centrifuged (1,600 × *g* at 4 °C for 10 min). The resulting supernatant was used to determine concentrations of TBARS using a commercial kit (Cayman Chemical, Ann Arbor, MI). For TAC analysis, 25 mg of liver or muscle samples were suspended into a 400 µL of a protein mask solution (Sigma–Aldrich, St. Louis, MO) and homogenized with a tissue homogenizer and then centrifuged (1,600 × *g* at 4 °C for 10 min). The resulting supernatant was used to determine concentrations of TAC using a commercial kit (Sigma–Aldrich, St. Louis, MO).

mRNA abundance

Both duodenal and jejunal mucosa samples were used to determine mRNA abundance of AA transporters (Tables 4 and 5) via real-time polymerase chain reaction (Vandesompele et al., 2002; Bustin et al., 2009). Total RNA from mucosa samples was extracted using Qiagen RNeasy Mini kits according to the manufacturer's instructions (Qiagen, Hilden, Germany). The RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and an ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), respectively. All samples used for further analysis had an OD260/OD280 ratio of 1.9 to 2.1, an OD260/OD230 ratio of > 1.8, and an RNA integrity number of ≥ 8.

First-strand cDNA was produced from 800 ng of total RNA per sample using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) in a total volume of 20 µL. Total RNA was denatured at 65 °C for 5 min and immediately annealed on ice for at least 1 min. The reverse transcription reaction primed with oligo(dT) primers was carried out at 50 °C for 50 min, followed by heat inactivation at 85 °C for 5 min.

The mRNA expression levels of five genes involved in nutrient transport systems in duodenal and jejunal mucosa were analyzed by real-time polymerase chain reaction. Data normalization was accomplished using three internal control genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hydroxymethyl-bilane synthase (*HMBS*), and beta-actin (*ACTB*) (Yin et al., 2014; Vigors et al., 2014). The tested genes included solute carrier family 3 member 2 (***SLC3A2***; rBAT), solute carrier family 6 member 14 (***SLC6A14***; $ATB^{0,+}$), solute carrier family 6 member 19 (***SLC6A19***; B°AT), solute carrier family 7 member 6 (***SLC7A6***; y^+LAT2), and solute carrier family 7 member 7 (***SLC7A7***; y^+LAT1). These genes were chosen to determine if Met source affects regulation of AA absorption and transport in the small intestine. Primers were designed based on published sequences in pigs (Arredondo, 2011; Brunner et al., 2012) and synthesized by Integrated DNA Technologies (Coralville, IA). Each polymerase chain reaction of three replicates consisted

Table 4. Forward and reverse primer sequences used for quantitative reverse transcription polymerase chain reaction

Gene[1]	Accession no.	Primer sequence, 5′-3′	Reference
GAPDH	AF017079.1	F: CAGCAATGCCTCCTGTACCA R: ACGATGCCGAAGTTGTCATG	Vigors et al., 2014
HMBS	NM_001097412.1	F: CTGAACAAAGGTGCCAAGAACA R: GCCCCGCAGACCAGTTAGT	Vigors et al., 2014
ACTB	NM_001172909.1	F: CTGCGGCATCCACGAAACT R: AGGGCCGTGATCTCCTTCTG	Yin et al., 2014
SLC3A2	EU587016.1	F: GAGACCTAGCGAGCCTGAGC R: AGAGCAGCAGCTGGTAGAGC	Brunner et al., 2012
SLC6A14	NM_001166042.1	F: CCGTGGTAACTGGTCCAAAA R: CCAATCCCACTGCATATCCAA	Arredondo et al., 2011
SLC6A19	XM_003359855	F: GCCACCGTGGTCTACTCCAT R: GAAGTTCTCCTGCGTCACGTT	Vigors et al., 2014
SLC7A6	CX064558.1	F: CTGCCGCCTGCATGTGT R: TGTGCCCCACTTGACATAGG	Arredondo et al., 2011
SLC7A7	NM_001253680.1	F: TTTGTTATGCGGAACTGG R: AAAGGTGATGGCAATGAC	Yin et al., 2014

[1]*GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *HMBS*, hydroxymethyl-bilane synthase; *ACTB*, beta-actin; *SLC3A2*, solute carrier family 3 member 2; *SLC6A14*, solute carrier family 6 member 14; *SLC6A19*, solute carrier family 6 member 19; *SLC7A6*, solute carrier family 7 member 6; and *SLC7A7*, solute carrier family 7 member 7.

Table 5. List of genes involved in nutrient transport systems of porcine cells

Gene[1]	System	Protein	Location	Features
SLC3A2		rBAT		Dimerizes with $b^{0,+}$AT
SLC6A14	$B^{0,+}$	$ATB^{0,+}$	Brush border membrane	Na^+-dependent cationic amino acid transporter
SLC6A19	$B^{0,+}$	B^0AT	Brush border membrane	Na^+-dependent neutral amino acid transporter
SLC7A6	y^+L	y^+LAT2	Basolateral membrane	Na^+-dependent cationic/neutral amino acid exchanger, dimerizes with 4F2hc
SLC7A7	y^+L	y^+LAT1	Basolateral membrane	Na^+-dependent cationic/neutral amino acid exchanger; dimerizes with 4F2hc

[1]*SLC3A2*, solute carrier family 3 member 2; *SLC6A14*, solute carrier family 6 member 14; *SLC6A19*, solute carrier family 6 member 19; *SLC7A6*, solute carrier family 7 member 6; and *SLC7A7*, solute carrier family 7 member 7.

of 5 μL of SYBR Green polymerase chain reaction master mix (Applied Biosystems, Foster, CA), 0.4 μL of 10 μM forward primer, 0.4 μL of 10 μM reverse primer, 0.2 μL of DNase/RNase free water, and 4 μL of diluted cDNA. The reverse transcription quantitative polymerase chain reaction was conducted using the QuantStudio 7 Flex Real-Time polymerase chain reaction system (Applied Biosystems, Foster, CA) and was analyzed using QuantStudio Real-Time polymerase chain reaction software (Applied Biosystems, Foster, CA). Thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles with 15 s at 95 °C and 1 min at 60 °C. The dissociation cycle was 95 °C for 15 s plus 65 °C for 15 s. Standard curves were generated using serial dilutions of pooled cDNA from all samples. The arbitrary values were calculated based on the standard curve and normalized using the internal control genes. The fold changes were calculated based on the arbitrary values. To obtain the relative gene abundance, the average arbitrary value of the triplicate samples was divided by the geometric mean of the 3 internal control genes.

Calculations and statistical analysis

Retention of N for each pig was calculated based on analyzed N-concentrations using the following equation (NRC, 2012):

$$\text{Nr} = \{[\text{Ni} - (\text{Nf} + \text{Nu})] / \text{Ni}\} \times 100\%, \quad [1]$$

where Nr is the retention (%) of N, Ni is the intake (g) of N, Nf is the fecal output (g) of N, and Nu is the urinary output (g) of N of pigs during the collection period.

The apparent total tract digestibility (ATTD) of N was calculated using the following equation (NRC, 2012):

$$\text{ATTD} = [(\text{Fi} - \text{Ff}) / \text{Fi}] \times 100\%, \quad [2]$$

where ATTD is the apparent total tract digestibility of N (%), Fi is the total N intake (g), and Ff is the total fecal output of N (g).

Normality of data was verified and outliers were identified using the UNIVARIATE procedure (SAS Inst. Inc., Cary, NC). Outliers were identified using the BOXPLOT procedure (SAS Inst. Inc., Cary, NC) as values that deviated from the treatment mean by more than 1.5 times the interquartile range and removed (Devore and Peck 1993; Oliveira et al., 2020). Intestinal morphology, measures of antioxidant capacity, and gene abundance results were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with the pig as the experimental unit. The statistical models included diet as the main effect and block and replicate as random effects. When significant differences were detected, treatment means were separated using the Fisher's least significant difference test. Orthogonal polynomial contrasts were used to determine linear and quadratic effects of supplemental Met source (i.e., 0%, 0.036%, 0.072%, or 0.108%) on response variables related to N

balance. A contrast statement was also used to compare effects of the two supplemental sources of Met on response variables related to N balance.

Nitrogen retention data were subjected to the fundamental validity test of the slope-ratio assay described by Littell et al. (1997). A multivariate linear regression analysis was performed using the following equation:

$$y = a + b_1x_1 + b_2x_2 \quad [3]$$

where a is the common y-intercept of the 2 lines, b_1 is the slope of D-Met, b_2 is the slope of L-Met, x_1 is the percent supplemental D-Met, and x_2 is the percent supplemental L-Met.

Due to the shape of the response curve, N retention data were also fitted in a nonlinear exponential model with the following equation:

$$y = a + z \times \left[1\ e^{-(b_1x_1 + b_2x_2)}\right] \quad [4]$$

where a is the common y-intercept of the 2 lines, z is the common asymptotic response, b_1 is the slope of D-Met, b_2 is the slope of L-Met, x_1 is the percent supplemental D-Met, and x_2 is the percent supplemental L-Met.

The RBV of D-Met compared with L-Met was calculated as the ratio of their slopes [100 × (b_1/ b_2)] generated from both models (i.e., multivariate linear regression and nonlinear exponential models) as described by Littell et al. (1997). The pig was the experimental unit for all analyses. Statistical significance was considered at $P < 0.05$.

Results

The analyzed CP, total AA, and supplemental D-Met and L-Met concentrations of experimental diets were in good agreement with calculated values. All pigs remained healthy throughout the experiment and readily consumed their diets.

Nitrogen balance

The final BW of pigs was not affected by dietary treatments (Table 6). Addition of increasing concentrations of L-Met in the diet linearly increased ($P < 0.01$) feed intake; however, there were no linear or quadratic effects of treatments on N intake or fecal N output. However, there was a linear reduction ($P < 0.01$) in urinary N excretion as increasing levels of D-Met or L-Met were added to the Met-deficient diet. Quantities (g/5 d) of N retained increased (linear, $P < 0.01$; quadratic, $P < 0.05$) as D-Met or L-Met was added to the basal diet. Nitrogen retention (% of intake) increased linearly ($P < 0.01$) from 67.9% to 72.7%, 74.6%, and 75.4% by addition of increasing levels of D-Met in the diet, and from 67.9% to 72.3%, 75.4%, and 75.0% by addition of increasing levels of L-Met to the diets. There were no effects of increasing concentrations of dietary Met on ATTD of N regardless of Met source, but the ATTD of N was greater ($P < 0.05$) in diets containing D-Met compared with diets containing L-Met. Absorption of N increased (quadratic, $P < 0.05$) as increasing levels of L-Met was added to the diets. Using N retention (%) as the response variable, the multivariate linear regression estimated the RBV of D-Met to be 101% relative to L-Met with 95% confidence intervals being 57% to 146% (Figure 1). The bioavailability of Met in D-Met relative to L-Met based on N retention using the exponential model was 102% with 95% confidence intervals being 15 to 182% (Figure 2).

Morphology, antioxidant capacity, and mRNA abundance

In the duodenum, villus height and crypt depth were greater ($P < 0.01$) in samples from pigs fed the basal diet compared with pigs fed diets supplemented with 0.108% D-Met or L-Met (Table 7), but no differences were observed between pigs fed D-Met or L-Met. However, villus height:crypt depth ratio in the duodenal tissue was not influenced by dietary treatments. In the jejunum, there were no differences among dietary treatments for villus height, crypt depth, or villus height:crypt depth ratio.

There was an increased ($P < 0.05$) total glutathione concentration in liver tissue from pigs fed the 0.108% D-Met supplemented diet when compared with pigs fed the basal diet, but no differences were observed between pigs fed the 0.108% D-Met supplemented diet and pigs fed the 0.108% L-Met supplemented diet (Table 8). However, no differences were observed in TAC or TBARS concentrations in liver, muscle, or plasma samples between pigs fed the control diet and pigs fed diets containing 0.108% supplemental D-Met or L-Met. Likewise, there was no difference in the total glutathione concentration in muscle tissue from pigs fed the three experimental diets. The relative mRNA abundance of all AA transporter genes (i.e., *SLC6A19*, *SLC3A2*, *SLC6A14*, *SLC7A6*,

Table 6. Nitrogen balance of pigs fed a Met-deficient basal diet or diets containing increasing levels of D-Met or L-Met

Item	Basal	D-Met			L-Met			SEM	Contrast P-values				
									Linear[1]		Quadratic[1]		
Supplemental Met, %	0.00	0.036	0.072	0.108	0.036	0.072	0.108		D-Met	L-Met	D-Met	L-Met	D-Met vs. L-Met
Initial BW[2], kg	10.5	10.6	10.6	10.7	10.4	10.5	10.4	0.70	0.715	0.999	0.889	0.976	0.590
Final BW, kg	14.9	15.0	15.3	14.8	14.7	15.1	15.3	0.92	0.977	0.312	0.384	0.534	0.889
Feed intake, g/5 d	2,757	2,839	2,834	2,813	2,819	2,863	2,876	126	0.412	0.049	0.249	0.581	0.516
N intake, g/5 d	88.6	91.8	91.1	90.4	92.4	91.3	89.5	4.05	0.473	0.821	0.185	0.055	0.980
Fecal N, g/5 d	11.1	10.7	10.1	9.9	11.0	11.1	11.6	0.62	0.126	0.524	0.809	0.659	0.036
Urinary N, g/5 d	17.2	14.3	13.6	12.6	14.6	11.4	10.7	1.02	0.001	<0.001	0.289	0.297	0.103
ATTD[2] of N, %	88.2	88.3	88.8	89.1	88.1	87.8	87.0	0.54	0.225	0.130	0.825	0.491	0.022
N retention, %	67.9	72.7	74.6	75.4	72.3	75.4	75.0	1.29	<0.001	<0.001	0.105	0.052	0.998
N retained, g/5 d	60.3	66.8	68.8	67.2	66.7	68.8	67.1	3.73	0.006	0.004	0.023	0.021	0.954
N absorbed, g/5 d	77.5	81.1	80.8	80.4	81.3	80.1	77.8	3.82	0.203	0.973	0.184	0.044	0.400

Data are least squares means of eight observations per treatment.
[1]Linear and quadratic effects of increasing levels of supplemental Met (i.e., 0%, 0.036%, 0.072%, or 0.108%).
[2]BW, body weight; ATTD, apparent total tract digestibility.

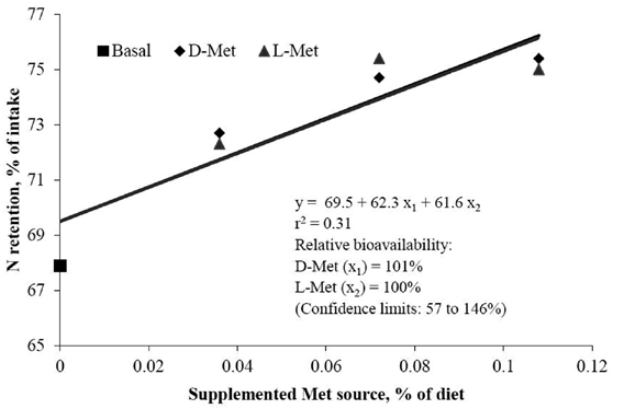

Figure 1. Bioavailability of Met in D-Met relative to Met in L-Met using N retention (%) as the response criteria (linear regression model).

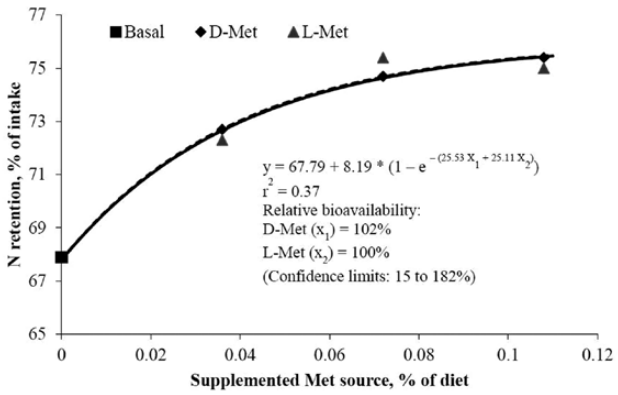

Figure 2. Bioavailability of Met in D-Met relative to Met in L-Met using N retention (%) as the response criteria (exponential regression model).

SLC7A7) measured in duodenal or jejunal mucosa of pigs was not affected by dietary treatments (Table 9).

Discussion

An N-balance experiment was conducted to determine the RBV of D-Met relative to L-Met because N-balance usually results in less variation among pigs in terms of feed intake compared with pigs used in growth performance experiments. Therefore, N retention, as an indication of body protein deposition, was used as the primary response criterion in the experiment. Feed intake was restricted based on the initial BW of pigs, but because of greater amounts of orts from pigs fed the basal diet, feed intake was less for pigs fed the basal diet than for pigs fed the other diets. When given a choice, pigs prefer a diet with a balanced concentration of Met to a Met-deficient diet (Roth et al., 2006), and it is believed that pigs are able to detect nutrient deficiencies and subsequently reduce feed intake to partly avoid nutritional deficiency (Bradford and Gous, 1991). Despite the lack of a linear increase in feed intake for pigs fed diets with increasing concentrations of D-Met, feed intake of pigs fed diets containing D-Met did not differ from that of pigs fed diets containing L-Met.

The reason pigs fed the basal diet had increased urine excretion of N and lower N retention compared with pigs fed diets supplemented with D-Met or L-Met is that the basal diet was deficient in Met, which is a prerequisite for using the slope-ratio procedure to determine the RBV of one nutrient relative to another (Littell et al., 1997). The observation that the greatest N retention was observed for the greatest inclusion level (0.108%) of both Met sources further demonstrates that addition of D-Met or L-Met to the basal diet resulted in more balanced diets. The observed reduction in urinary excretion of N, as well as the increased N retention as D-Met or L-Met increased in the diet, is also indicative of reduced deamination of AA and a more efficient utilization of dietary N. These observations are in agreement with data indicating a linear reduction in urinary N excretion and increased N retention in growing pigs when a Met-deficient diet was supplemented with increased levels of D-Met or L-Met (Kong et al., 2016a).

Pigs deposit and utilize Met in the levorotatory form; thus, D-Met is absorbed by pigs and then converted into L-Met via transamination in the liver or kidneys (Shen et al., 2014). Therefore, it is not clear why diets containing D-Met had greater ATTD of N than diets containing L-Met; nevertheless, it appears that pigs retained N similarly regardless of Met source. Because the RBV of Met in D-Met was 101% relative to L-Met and because the confidence interval overlapped 100%, it is concluded that D-Met was as effective as L-Met in supporting N retention in pigs. This conclusion is in agreement with data indicating that the RBV of D-Met was 90% relative to L-Met based on N retention, but did not differ as the 95% confidence interval overlapping 100% as in the current experiment (Kong et al., 2016b). These results are also in agreement with data based on growth performance responses by Chung and Baker (1992) that indicated that the RBV of D-Met relative to L-Met is 100% in weanling pigs. Based on results from an N-balance experiment, it was concluded that DL-Met and L-Met are equally bioavailable for pigs (Tian et al., 2016), further indicating that D-Met and L-Met are equally bioavailable. These data are also in agreement with results of experiments demonstrating that weanling pigs fed diets supplemented with DL-Met had growth performance that was not different from that of pigs fed diets supplemented with L-Met (Chung and Baker, 1992; Chen et al., 2013; van Milgen et al., 2013; Kong et al., 2016b). Using slope ratio regression analyses, it was demonstrated that there is no difference in growth performance of weanling pigs fed diets supplemented with L-Met or DL-Met, and it was, therefore, estimated that the RBV of Met in DL-Met was 100% relative to Met in L-Met (Htoo and Morales, 2016; Yang et al., 2019; Zeitz et al., 2019). However, a tendency for an increased average

Table 7. Intestinal morphology in the duodenum and jejunum of pigs fed a Met-deficient basal diet or diet supplemented with D-Met or L-Met

Item	Basal	D-Met	L-Met	SEM	P-value
Supplemental Met, %	0.00	0.108	0.108		
Duodenum					
Villus height, µm	182[a]	131[b]	149[b]	10.0	0.001
Crypt depth, µm	71[a]	61[b]	60[b]	3.0	0.001
Villus height:crypt depth	2.3	2.1	2.4	0.3	0.255
Jejunum					
Villus height, µm	335	358	354	16.0	0.133
Crypt depth, µm	166	175	173	9.0	0.838
Villus height:crypt depth	2.0	2.0	2.1	0.6	0.856

Data are least squares means of eight observations per treatment.
[a,b]Means within a row lacking a common letter are different ($P < 0.05$).

Table 8. Various measures of oxidative status in plasma, liver, and muscle tissue of pigs fed a Met-deficient basal diet or diet supplemented with D-Met or L-Met

Item	Basal	D-Met	L-Met	SEM	P-value
Supplemental Met, %	0.00	0.108	0.108		
Total antioxidant capacity					
Plasma, nmol/μL	2.44	2.65	2.92	0.41	0.309
Liver, nmol/μL	0.94	1.19	0.93	0.12	0.245
Muscle, nmol/μL	0.84	0.80	0.94	0.08	0.500
Thiobarbituric acid reactive substances					
Plasma, μM	1.63	4.13	2.62	3.09	0.560
Liver, μM	18.21	19.69	18.05	3.27	0.812
Muscle, μM	2.93	2.56	3.84	0.55	0.238
Total glutathione[1]					
Liver, μM	1.51[b]	4.92[a]	4.03[a,b]	1.83	0.049
Muscle, μM	27.37	30.37	32.04	1.87	0.227

Data are least squares means of eight observations per treatment.
[1]Total glutathione levels in plasma samples were below detectable levels, and therefore, were not reported.
[a,b]Means within a row lacking a common letter are different ($P < 0.05$).

Table 9. Relative mRNA expression of genes in the duodenum and jejunum of pigs fed a Met-deficient basal diet or diet supplemented with D-Met or L-Met

Item	Basal	D-Met	L-Met	SEM	P-value
Supplemental Met, %	0.00	0.108	0.108		
Duodenum					
SLC6A19[1]	1.07	1.09	1.06	0.04	0.458
SLC3A2[1]	1.10	1.11	1.10	0.03	0.687
SLC6A14[1]	1.23	1.25	1.23	0.02	0.408
SLC7A6[1]	1.20	1.23	1.20	0.03	0.313
SLC7A7[1]	0.93	0.94	0.92	0.05	0.533
Jejunum					
SLC6A19	1.05	1.02	1.03	0.06	0.336
SLC3A2	1.17	1.18	1.14	0.03	0.584
SLC6A14	1.23	1.22	1.24	0.01	0.541
SLC7A6	1.24	1.21	1.22	0.06	0.222
SLC7A7	0.95	0.91	0.94	0.06	0.387

Data are least squares means of eight observations per treatment.
[1]*SLC6A19*, solute carrier family 6 member 19; *SLC3A2*, solute carrier family 3 member 2; *SLC6A14*, solute carrier family 6 member 14; *SLC7A6*, solute carrier family 7 member 6; *SLC7A7*, solute carrier family 7 member 7.

daily gain of weanling pigs fed a diet containing L-Met compared with pigs fed a diet containing DL-Met was also reported (Shen et al., 2014). Differences in the efficiency of utilization of Met isomers may be a result of differences in the age of the pigs used (Chung and Baker, 1992; Shen et al., 2014). However, based on all experiments conducted, it is concluded that there are no differences in N-balance or growth performance between pigs fed diets containing L-Met, D-Met, or DL-Met.

The greater duodenal villus height that was observed in pigs fed the basal diet compared with pigs fed diets with D-Met or L-Met was unexpected and the reason for this observation is not clear. However, because the crypt depth was also lower in pigs fed the basal diet, it is possible that these pigs had to synthesize more enterocytes and goblet cells to maintain the same duodenal villus height:crypt depth ratio as pigs fed the other diets. Synthetic AA are rapidly absorbed in the small intestine (Yen et al., 2004), and this may result in a reduced utilization of synthetic Met for intestinal epithelial growth and integrity. There was, however, no indication that the source of Met affected intestinal morphology in the duodenum because no differences for any measurements of intestinal morphology were observed in the jejunum. The lack of differences between Met sources in villus height:crypt depth ratio in the duodenum and jejunum of pigs also indicates that the Met from the intact protein (i.e., 0.24% Met) in the basal diet, which is assumed to be L-Met, appears to be sufficient to support villus integrity. A lack of differences in villus height, crypt depth, and the villus height:crypt depth ratio in the duodenum and jejunum between weanling pigs fed DL-Met or L-Met were also observed by Zeitz et al. (2019) who concluded that intestinal D-Met conversion to L-Met was sufficient and rapid enough to support all functions of Met in the gut. It was further concluded that L-Met availability in intestinal cells was sufficient in pigs fed diets containing DL-Met to support synthesis of enterocytes (Zeitz et al., 2019).

The observation that values for TAC, TBARS, and glutathione were not different between pigs fed D-Met and pigs fed L-Met indicates that the source of Met did not affect antioxidant activity in pigs. These data support results by Zeitz et al. (2019) who also observed that the concentrations of total glutathione, TAC, and TBARS in liver and jejunum of weanling pigs were not different between pigs fed diets with the same inclusion level of DL-Met or L-Met. The fact that the relative mRNA abundance of genes for AA transporters including the Na^+-independent exchanger rBAT and Na^+-dependent B^0AT1 and $ATB^{0,+}$ (i.e., *SLC3A2*, *SLC6A14*, *SLC6A19*, *SLC7A6*, and *SLC7A7*) in duodenal and jejunal mucosa was not different among treatments indicates that D-Met and L-Met impact expression of genes to the same degree. There is, therefore, no evidence that D-Met is less efficient than L-Met in terms of stimulating absorption of AA from the small intestine. Dietary supplementation of DL-Met increases the efficiency of both D-Met and L-Met absorption at physiologically relevant luminal Met concentrations along the small intestine of pig ex vivo, including increased efficiency of the Na+-dependent transport system (Romanet et al., 2020).

Based on the results from the current experiment and data from previous publications, it is concluded that in pigs, N retention, gut morphology, antioxidant status of plasma, liver, and muscle, and abundance of mRNA for AA transporters in the duodenum and jejunum of pigs are not affected by the source of Met consumed. The most likely reason the RBV of D-Met is close to 100% relative to L-Met is that the DAAO enzyme, which is needed for the conversion of D-Met to L-Met, is present in high concentrations in weanling pigs not only in the liver, but also in the kidney, stomach, duodenum, jejunum, ileum, and muscle (Fang et al., 2010). A similar observation was made in broiler chickens (Brachet and Puigserver, 1992). The conversion of D-Met to L-Met can take place in these tissues after absorption of D-Met into the portal blood (Wu, 2013). The existence of high intestinal DAAO activity indicates that the intestine may convert D-Met to L-Met, which can then be used in protein synthesis or in subsequent metabolism (Fang et al., 2010). Bacteria in the lumen of the small intestine possess DAAO, and therefore, conversion of D-Met to L-Met can also be facilitated by luminal bacteria of the small intestine in pigs and poultry (Wu, 2013).

Conclusion

Supplementing D-Met or L-Met to a Met-deficient basal diet improved N retention and decreased urinary N excretion of weanling pigs. Based on data for N retention (%), it was

concluded that the RBV for D-Met was 101% with L-Met used as the standard. Addition of D-Met to diets resulted in indicators for morphology in the duodenum and jejunum that were not different from those of pigs fed diets containing L-Met. Antioxidant-related parameters in liver, muscle, and plasma, as well as abundance of mRNA needed to express AA transporters involved in Met metabolism in duodenum and jejunum, were also not impacted by the source of Met that was added to the diet.

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Conflict of interest statement

J.K.H. and J.C.G. are employees at Evonik Operations GmbH, Hanau-Wolfgang, Germany, a global supplier of methionine to the feed industry. All other authors have no real or perceived conflicts of interest.

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