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ORIGINAL ARTICLE

L-Methionine supplementation maintains the integrity and barrier function of the small-intestinal mucosa in post-weaning piglets

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Abstract This study was conducted to test the hypothesis that different dietary Met levels affect small-intestinal mucosal integrity in post-weaning piglets. Two groups of piglets ($n = 6$/group) were weaned at 28 days of age and randomly allotted to a basal diet (without extra Met supplementation) or a Met-supplemented diet (with 0.12 % L-Met) for 14 days. The standardized ileal digestible (SID) Met levels were 0.24 and 0.35 %, respectively. At days 7 and 14 of the trial, venous blood samples were obtained from piglets, followed by their euthanasia for tissue collection. Piglets fed the diet supplemented with L-Met had a higher average daily gain during days 7–14 and improved feed efficiency during the entire period. Concentrations of sulfur amino acids (SAA), glutamate acid (Glu), glutamine (Gln), and taurine in the plasma and tissues were higher for the piglets in the Met-supplemented group. Met supplementation increased cysteine (Cys) and glutathione (GSH) concentrations in the plasma and tissues, leading to reductions in plasma Cys/CySS redox potential and tissue GSH/GSSH redox potential. The small-intestinal mucosa of Met-supplemented piglets exhibited improved villus architecture, compared with control piglets. Met supplementation increased transepithelial electrical resistance of the jejunal mucosa. Transport of Met, Gln and Cys across the jejunal mucosa did not differ between control and Met-supplemented piglets. The abundance occludin was higher, whereas the abundance of active caspase-3 was lower, in the jejunum of the Met-supplemented piglets. Collectively, adequate dietary Met is required for optimal protein synthesis and mucosal integrity in the small intestine of post-weaning piglets.

Keywords L-Methionine · Small intestinal mucosa · Piglets

Abbreviations

ADG	Average daily gain
ADFI	Average daily feed intake
EDTA	Ethylene diaminetetraacetic acid
FCR	Feed conversion ratio
GSH	Glutathione
Isc	Transepithelial short circuit current
KHB	Krebs–Ringer bicarbonate
MHA-FA	Methionine hydroxyl analog-free acid
PBS	Phosphate-buffered saline
SAA	Sulfur amino acids
SAM	*S*-adenosylmethionine
SID	Standardized ileal digestible
TEER	Transepithelial electrical resistance
TJ	Tight junctions

Introduction

The small intestine is the primary organ responsible for terminal digestion and absorption of nutrients including protein and amino acids (Wu 1998). Maintaining the integrity and function of the small intestine is crucial for all animals. This is particularly important for managing post-weaning piglets to relieve the post-weaning syndrome and decrease mortality. Post-weaning syndrome, manifested as anorexia, intestinal atrophy, diarrhea, and growth

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retardation in mammalian neonates, is a major problem in animal production industry, especially in developing countries (Wang et al. 2009a).

Sulfur amino acids (SAA), particularly methionine (Met) and cysteine (Cys), are critical for the intestine to maintain its function including the digestion, absorption and metabolism of nutrients, the immune surveillance of the intestinal epithelial layer and regulation of the mucosal response to foreign antigens (Fang et al. 2010). Methionine (Met) is a dietary essential amino acid that plays unique roles, both in protein structure and metabolism (Brosnan et al. 2007a). It has been considered as the second- or third-limiting amino acid in diets for nursery pigs and may have become the first-limiting amino acid in diets containing more than 6 % spray-dried porcine plasma (Owen et al. 1995; Gaines et al. 2005). Studies in piglets show that the gastrointestinal tract is a significant site of SAA metabolism in the body and metabolizes about 20 % of the dietary Met intake (Riedijk et al. 2007). Like other amino acids, Met affects protein metabolism in the small intestine. Meanwhile, Met is recognized to exert other significant functions since it is a precursor of essential molecules, such as Cys, S-adenosylmethionine (SAM), polyamines, and glutathione (GSH), and plays a role in the methylation process, participates in the regulation of intestinal epithelial oxidative status, and may contribute to intestinal mucosal growth and gut function (Shoveller et al. 2005, Tesseraud et al. 2009, Bauchart-Thevert et al. 2009a). Protein synthesis and accretion are affected when Met is provided at insufficient levels (Kino and Okumura 1986). Moreover, recently study showed that Met and Cys deficiency significantly suppresses the intestinal mucosal growth and reduces intestinal epithelial cell proliferation, and increases intestinal oxidant stress in piglets (Bauchart-Thevert et al. 2009b). Given the important effects of Met on intestinal physiology, dietary-adequate Met level is essential to maintain normal growth and basic function of the small intestine in post-weaning piglets (Wu 2010). However, the underlying mechanisms are largely unknown and need further investigation.

Dietary Met supplementation is usually provided using chemically synthesized dry DL-Met (purity ≥99 %) and liquid DL-methionine hydroxyl analog-free acid (MHA-FA, containing 88 % of active substance) in piglet diets (Hoehler et al. 2005). While L-Met is the only Met configuration for protein synthesis, cell activation, and metabolism, both D-Met (comprising 50 % of DL-Met) and MHA-FA must be converted to L-Met before they can be used by the intestine (Dibner and Ivey 1992). In this study, we hypothesized that given the high cellular turnover and metabolic activity of the gut, dietary-adequate Met level would preferentially play an important role in the small intestinal epithelial growth and barrier function in post-weaning piglets via meeting the nutritional requirement for protein synthesis and for supporting metabolism to generate the Met-related metabolites. To test this hypothesis, we evaluated the effects of dietary Met level using L-Met as the Met source on the intestinal mucosal morphology, permeability, and expression of tight junctions (TJ) proteins and apoptosis proteins of the jejunum in post-weaning piglets fed a basal diet or Met-supplemented diet for 14 days.

Materials and methods

Materials

L-Met was a product of CJ CheilJedang Co., Inc. (Seoul, Korea). The purity is ≥99 %, pH is 5.6–6.1, and loss on drying is no more than 0.5 %.

Animals and diets

The animal use protocol for this study was approved by the Institutional Animal Care and Use Committee of the China Agricultural University (Beijing, China). Twelve crossbred (Duroc × Landrace × Yorkshire) barrows were breast-fed and then weaned at 28 days of age. All piglets had free access to a commercial diet between days 28 and 30 of age (days 0–3 post-weaning) for adapting to solid feed. After the adaptation period, piglets (average body weight of 7.1 ± 1.04 kg) were randomly allotted to two isocaloric and isonitrogenous diets (adjusted with L-alanine) formulated to meet or exceed National Research Council (1998)-recommended requirements of nutrients for 5–10 kg piglets, with the exception of Met (Table 1). The basal diet was without extra L-Met supplemented to generate a low dietary Met level and the Met-supplemented diet was with 0.12 % L-Met supplemented to meet the requirement for post-weaning piglets. The SID Met levels of the diets were 0.24 and 0.35 %, respectively. Both diets provided 190 g/kg of crude protein and 3.24 Mcal/kg of metabolic energy. The piglets were housed individually in stainless steel metabolic cages (1.25 × 0.55 × 0.8 m) and maintained in an environmentally controlled room with ambient temperature of 28 ± 2 °C. All Piglets had free access to drinking water and diets. Body weight gain and feed intake were recorded for the calculation of feed conversion ratio for the 14 days growth trial.

Chemical analysis

Before the growth trial, dietary crude protein, calcium, and total phosphorus were analyzed according to the methods of AOAC (2003). Dietary amino acids except SAA and tryptophan were determined by ion-exchange chromatography

Table 1 Ingredients composition and nutrient levels of the experimental diets (as-fed basis)

Items	Basal diet	Met-supplemented diet
Ingredients (%)		
Corn	52.91	52.87
Soybean meal	20.00	20.00
Extruded soybean meal	10.00	10.00
Dried whey	12.00	12.00
Soybean oil	1.00	1.00
Dicalcium phosphate	1.56	1.56
Limestone	0.50	0.50
NaCl	0.34	0.34
L-Lysine HCl	0.36	0.36
L-Methionine[a]	–	0.12
L-Threonine	0.20	0.20
L-Tryptophan	0.05	0.05
L-Alanine	0.08	0.00
Premix[b]	1.00	1.00
Total	100.00	100.00
Calculated nutrient levels (%)		
ME (Mcal/kg)	3.32	3.32
Crude protein	19.14	19.14
SID[c] lysine	1.20	1.20
SID methionine	0.24	0.35
SID Met + Cys	0.47	0.58
SID threonine	0.79	0.79
SID tryptophan	0.23	0.23
Calcium	0.81	0.81
Phosphorus	0.71	0.71
Analyzed nutrient levels (%)		
Crude protein	19.16	19.11
Total lysine	1.35	1.34
Total methionine	0.28	0.40
Total Met + Cys	0.55	0.69
Total threonine	0.97	0.98
Total tryptophan	0.26	0.27
Calcium	0.79	0.75
Total phosphorus	0.70	0.69

[a] L-Methionine, CJ CheilJedang Corporation, Seoul, Korea

[b] No amino acids added in vit. min. premix; Premix provided the following per kg of complete diet: vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 64 IU; vitamin K_3, 3 mg; vitamin B_{12}, 12 μg; riboflavin, 5.5 mg; pantothenic acid, 15 mg; niacin, 40 mg; choline chloride, 551 mg; folacin, 0.8 mg; thiamine, 1.5 mg; pyridoxine, 3 mg; biotin, 100 μg; Mn, 40 mg; Fe, 100 mg; Zn, 100 mg; Cu, 150 mg; I, 0.3 mg; Se, 0.3 mg

[c] SID means standardized ileal digestible value

using a Hitachi L-8800 AA Analyzer (Tokyo, Japan) after acid hydrolysis with 6 N HCl (reflux for 24 h at 110 °C). Met and Cys were measured after performic acid oxidation and subsequent hydrolysis with 6 N HCl (AOAC 2003). Tryptophan content was determined colorimetrically after alkaline hydrolysis at 120 °C for 16 h following the procedures described by Miller (1967).

Sample collection and processing

On days 7 and 14 of the trial, blood samples were collected from anterior vena cava of all piglets in vacuum tubes with ethylene diaminetetraacetic acid (EDTA). Plasma was obtained by centrifugation (3,000×*g* at 4 °C for 10 min) and frozen at −80 °C until analyzed. All piglets were killed by intracardial administration of sodium pentobarbital (50 mg/kg body weight) and jugular exsanguinations on the morning of day 15. The abdomen was opened, and the entire small intestine distal to the ligament of Treitz to the ileocecal junction was immediately flushed with ice-cold phosphate-buffered saline (PBS, pH 7.4). The 5 cm intestinal segments of the distal duodenum, mid-jejunum, and mid-ileum were flushed with ice-cold PBS and placed in 10 % neutral buffered formalin for histological analysis. About 12 cm of a piece of jejunum was removed and used to determine the intestinal permeability and transport of Met, Cys and glutamine (Gln) using the Ussing chambers technique. A piece of duodenum, jejunum, ileum and liver was flushed with ice-cold PBS and snap frozen in liquid nitrogen and stored at −80 °C until analysis. All samples were collected within 15 min after killing.

SAA, Glu, Gln and Met-related metabolites analysis by HPLC

Plasma and tissues (duodenum, jejunum, ileum and liver) Met, glutamic acid (Glu), Gln and taurine concentrations were determined by reverse-phase HPLC after derivatization with *o*-phthaldialdehyde using the method described by Wu et al. (1997). Plasma and tissues Cys, cystine (CySS), GSH and glutathione disulfide (GSSH) were quantified using the method described previously (Wu and Knabe 1994; Wu et al. 1997). Briefly, samples were acidified with 1.5 M $HClO_4$ and 12 mM iodoacetic acid (1:1 vol/vol) and then neutralized with 2 M K_2CO_3. The supernatant was reduced with 28 mM β-mercaptoethanol and then treated with 25 mM iodoacetic acid to form *S*-carboxymethyl derivatives. Polyamine concentrations of plasma and tissues were determined as described by Wu et al. (1998).

Redox potential calculations

Plasma and tissue redox potential values (E_h) were calculated from the Cys/CySS and GSH/GSSH couples using the Nernst equation for pH 7.4 (Dahm and Jones 2000; Jones 2002): Cys/CySS, E_h (mV) = −250 + 30 log([CySS]/[Cys]^2), GSH/GSSH, E_h (mV) = −264 + 30 log([GSSH]/

$[GSH]^2$), where [CySS], [Cys], [GSSH] and [GSH] are the concentrations of cystine, cysteine, glutathione and glutathione disulfide, respectively, in the plasma and tissue.

Intestinal morphology

Morphological analysis was performed on formalin-fixed intestinal samples (duodenum, jejunum and ileum) that were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin basing on the method described by Burrin et al. (2000). The sections were visualized using Computer-Aided Video Microscopy (DXM1200C, Nikon). Villus height and crypts depth were measured and analyzed using the NIS-Elements BR software (version 2.20, Nikon). The 15–20 well-oriented villi and crypts were used for measuring the mean villus height and crypts depth. The villus height-to-crypt depth ratio was calculated.

Ussing chambers determination of transepithelial physiology of jejunum

Electrophysiological properties of the in vitro jejunum were measured in a set of six Ussing chambers (Physiologic Instruments, San Diego, CA, USA) as described previously (Sholly 2009). The chamber permits the separation and sampling of luminal vs. antiluminal fluid compartments, with the epithelial tissue itself serving as the barrier between the fluid compartments, mimicking in vivo barrier function, allowing precise study of transport and barrier function by the epithelium (He et al. 2013). About 12 cm of a piece of jejunum was removed from the euthanized animals, sliced longitudinally, and cleaned off by ice-cold Krebs–Ringer bicarbonate (KHB). Smooth muscle layers were stripped off and the jejunal mucosa was mounted onto Ussing chambers, with tissue bathed in 37 °C KHB stirred by gas-lift (95 % O_2, 5 % CO_2) oxygenation. The exposure area of the tissue was 0.5 cm^2. After 10 min of incubation, the mucosal tissue was physiologically stabilized and the maximal transepithelial electrical resistance (TEER) and the basal transepithelial short-circuit current (Isc) were measured using Ag/AgCl electrodes connected to the bath by 3 M KCl agar bridges. Met, Cys, and Gln transport were assessed by ΔIsc after addition of Met, Cys and Gln to the mucosal hemi chamber (final concentration 2 mM) of the six chambers within 30 min of incubation. Osmotic balance was maintained by the addition of mannitol to the serosal side.

Western blot for analysis of tight junctions and apoptosis-related proteins

Analysis of claudin-3, occludin, and caspase-3 proteins was performed by Western blot as described by Zhou et al. (2007). Jejunal tissue was pulverized in liquid nitrogen and homogenized in lysis buffer containing 20 mM Tris–HCl (pH 7.4), 50 mM NaF, 50 mM EDTA, 1 % Triton X-100, 1× protease inhibitor cocktail, and 1× phosphatase inhibitor cocktail. The homogenates were centrifuged at 12,000×*g* for 15 min at 4 °C. Proteins in the supernatant fluid were determined using the Pierce BCA Protein Assay Kit (Applygen Technology, Inc., Beijing, China). The extracted protein sample (40 μg) was boiled for 5 min, electrophoresed (Bio-Rad, Richmond, CA, USA) in 10 % sodium dodecylsulfate-polyacrylamide gel, and elelctroblotted (Bio-Rad) onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with buffer containing 5 % fat-free dry milk in TBST (0.05 % Tween 20, 100 mM Tris–HCl and 150 mM NaCl, pH 7.5) for 1 h, the membrane was washed in TBST three times and incubated overnight with a primary antibody (1:1,000 dilution) for claudin-3 (Invitrogen Technology, Inc., Danvers, MA, USA), occludin (Invitrogen Technology, Inc., Danvers, MA, USA) and caspase-3 (Cell Signaling Technology, Inc., Danvers, MA, USA), respectively. The membrane was then washed three times with TBST, and incubated with a secondary antibody (claudin-3, occludin and caspase-3: horseradish peroxidase-conjugated goat anti-rabbit IgG) (Applygen Technology, Inc., Beijing, China) at a 1:2,000 dilution for 1 h at room temperature. This was followed by three time washing for 5 min. Band densities were detected with the Enhanced Chemiluminescence Western blotting kit (ECL-plus, Amersham Biosciences, Sweden), visualized using the Image Quant LAS 4000 mini system (GE Healthcare Bio-sciences AB, Inc., Sweden), and quantified using a gel-imaging system with Image Quant TL software (GE Healthcare Life Science, Inc., USA).

Statistical analysis

Values are expressed as mean ± SE. Comparison of control group with Met-supplemented group values was performed with two-tailed Student's test for unpaired data with each animal as an experimental unit. All analyses were performed with SAS 8.0 software (SAS Institute Inc., Cary, NC, USA). P values <0.05 were taken to indicate statistical significance.

Results

Growth performance of piglets

The growth performance of pigs fed diets with or without the supplementation of L-Met is summarized in Table 2. For days 0–7 of the trial, average daily gain (ADG), average daily feed intake (ADFI) and the feed conversion ratio

Table 2 Effects of dietary Met level on the growth performance in post-weaning piglets

Items	Control group	Met-supplemented group	*P* value
Initial BW (kg)	7.2 ± 0.50	7.4 ± 0.36	0.79
Final BW (kg)	10.4 ± 0.64	11.2 ± 0.43	0.36
0–7 days			
ADG (g)	177 ± 16.08	188 ± 9.99	0.57
ADFI (g)	273 ± 20.66	270 ± 13.57	0.92
FCR	1.56 ± 0.06	1.44 ± 0.04	0.13
7–14 days			
ADG (g)	314 ± 8.26	352 ± 12.04	0.03
ADFI (g)	466 ± 10.08	477 ± 13.53	0.51
FCR	1.49 ± 0.05	1.36 ± 0.04	0.09
0–14 days			
ADG (g)	229 ± 20.04	270 ± 9.30	0.09
ADFI (g)	366 ± 24.24	388 ± 17.04	0.47
FCR	1.62 ± 0.05	1.44 ± 0.04	0.02

Values are mean ± SE, $n = 6$ piglets/group

Differences at $P < 0.05$ were considered significant

(FCR) of piglets did not differ between the control and Met-supplemented groups. Piglets fed the Met-supplemented diet had a higher ADG between days 7 and 14 of the trial and an improved FCR for the overall trial period ($P < 0.05$). ADFI was not different between the two groups of piglets.

Concentrations of plasma and tissues SAA, Glu, Gln and Met-related metabolites

Plasma and tissues concentrations of SAA, Glu, Gln and Met-related metabolites were increased by dietary Met supplementation (Tables 3, 4). At the day 14 of the trial, plasma Met concentration was higher ($P < 0.05$) in Met-supplemented piglets and a significant increase in the plasma concentration of Gln ($P < 0.05$) was observed in this group. Dietary Met supplementation caused a marked increase in Cys concentrations ($P < 0.05$) in the plasma for the whole trail period, while no differences in CySS concentrations were observed in the plasma between the two groups. The GSH concentration was higher and GSSH concentration was lower in the plasma of the Met-supplemented piglets compared with control group, but only significantly at day 14. The taurine concentrations were higher (21 and 33 % at days 7 and 14, respectively, of the trial) in the plasma of piglets fed Met-supplemented diet than control piglets, though the differences were not statistically significant. As for the polyamines, the plasma spermine and spermidine concentrations were higher and putrescine concentration was lower ($P < 0.05$) in Met-supplemented piglets than in the control group at day 14 of the trial.

Table 3 Effects of dietary Met level on the plasma concentrations of SAAs, Glu, Gln, and Met-related metabolites in post-weaning piglets at 7 and 14 days of the experiment (nmol/ml plasma)

Items	Control group	Met-supplemented group	*P* value
7 days			
Methionine	29.97 ± 2.51	36.17 ± 2.69	0.12
Glutamic acid	188.09 ± 31.65	227.48 ± 24.60	0.35
Glutamine	272.86 ± 31.37	316.50 ± 32.09	0.35
Cysteine	13.22 ± 0.84	17.34 ± 0.71	<0.01
Cystine	109.21 ± 3.52	105.91 ± 4.87	0.60
Glutathione	2.51 ± 0.27	3.49 ± 0.39	0.07
Glutathione disulfide	0.65 ± 0.08	0.49 ± 0.05	0.13
Taurine	43.64 ± 14.02	53.02 ± 15.05	0.66
Polyamines			
Spermine	44.91 ± 1.18	48.41 ± 2.20	0.20
Spermidine	12.80 ± 0.54	10.58 ± 1.36	0.18
Putrescine	8.11 ± 0.45	9.19 ± 0.43	0.12
14 days			
Methionine	25.46 ± 3.36	45.00 ± 5.28	0.01
Glutamic acid	179.56 ± 17.70	225.83 ± 22.27	0.14
Glutamine	365.09 ± 13.45	455.34 ± 28.45	0.02
Cysteine	11.85 ± 0.31	20.18 ± 2.18	0.01
Cystine	72.48 ± 3.64	74.59 ± 1.82	0.62
Glutathione	4.65 ± 0.49	6.54 ± 0.56	0.03
Glutathione disulfide	0.47 ± 0.05	0.26 ± 0.04	0.02
Taurine	53.69 ± 3.93	71.53 ± 8.20	0.09
Polyamines			
Spermine	35.47 ± 1.23	44.44 ± 3.01	0.02
Spermidine	9.33 ± 1.70	16.41 ± 0.96	0.01
Putrescine	9.81 ± 0.30	7.72 ± 0.39	<0.01

Values are mean ± SE, $n = 6$ piglets/group

Differences at $P < 0.05$ were considered significant

In the tissues, higher concentrations of Met ($P < 0.05$) were found in the duodenum, jejunum, ileum, and liver of the Met-supplemented piglets, compared with the control group. Glu and Gln concentrations were increased in the small intestinal and liver tissues, especially in the jejunum ($P < 0.05$). Cys concentration in piglets fed Met-supplemented diet was higher ($P < 0.05$) in the duodenum and jejunum, respectively, while no difference was observed in the ileum and liver. CySS concentrations were not different in the intestines or liver between the two groups of piglets. GSH concentrations were significantly higher and GSSH concentrations were lower in the duodenum and jejunum ($P < 0.05$), respectively, but were not different in the ileum and liver, of Met-supplemented than control piglets. The taurine concentrations were higher in the four tissues, but only significantly in the jejunum and liver ($P < 0.05$).

Table 4 Effects of dietary Met level on the intestine and liver tissue concentrations of SAAs, Glu, Gln, and Met-related metabolites in post-weaning piglets (nmol/g tissue)

Items	Control group	Met-supplemented group	*P* value
Duodenum			
Methionine	102 ± 18	247 ± 18	<0.01
Glutamic acid	4,902 ± 164	6,289 ± 416	0.02
Glutamine	715 ± 95	925 ± 120	0.20
Cysteine	286 ± 28	412 ± 37	0.02
Cystine	190 ± 33	247 ± 24	0.19
Glutathione	1,478 ± 72	1,734 ± 72	0.03
Glutathione disulfide	80.83 ± 6.45	62.42 ± 5.34	0.05
Taurine	1,473 ± 173	1,985 ± 279	0.15
Polyamines			
Spermine	344 ± 34	463 ± 35	0.03
Spermidine	1,176 ± 107	1,494 ± 70	0.04
Putrescine	173 ± 16	213 ± 14	0.09
Jejunum			
Methionine	110 ± 6.15	162 ± 8.38	<0.01
Glutamic acid	5,889 ± 359	7,331 ± 144	0.01
Glutamine	636 ± 95	982 ± 34	0.01
Cysteine	308 ± 30	423 ± 35	0.03
Cystine	196 ± 17	234 ± 13	0.11
Glutathione	1,361 ± 72	1,685 ± 60	0.01
Glutathione disulfide	91.30 ± 8.93	68.34 ± 4.40	0.04
Taurine	1,212 ± 286	1,964 ± 99	0.04
Polyamines			
Spermine	318 ± 22	368 ± 19	0.12
Spermidine	966 ± 72	947 ± 112	0.89
Putrescine	171 ± 15	198 ± 18	0.27
Ileum			
Methionine	87 ± 6.27	111 ± 7.14	0.03
Glutamic acid	7,524 ± 519	8,448 ± 280	0.16
Glutamine	550 ± 61	842 ± 77	0.01
Cysteine	428 ± 18	498 ± 57	0.26
Cystine	235 ± 26	244 ± 19	0.78
Glutathione	1,334 ± 54	1,492 ± 66	0.09
Glutathione disulfide	75.81 ± 11.54	60.89 ± 5.84	0.28
Taurine	896 ± 186	1,395 ± 136	0.06
Polyamines			
Spermine	302 ± 12	331 ± 18	0.21
Spermidine	1,177 ± 93	1,232 ± 68	0.65
Putrescine	211 ± 12	215 ± 11	0.78
Liver			
Methionine	83.03 ± 4.81	103.08 ± 6.13	0.03
Glutamic acid	3,802 ± 257	4,378 ± 226	0.12
Glutamine	1,858 ± 131	2,572 ± 269	0.05
Cysteine	570 ± 76	643 ± 37	0.41
Cystine	185 ± 10	221 ± 23	0.19
Glutathione	2,165 ± 211	2,499 ± 228	0.31
Glutathione disulfide	106.34 ± 14.96	92.16 ± 13.11	0.49
Taurine	539 ± 57	1,104 ± 158	0.01
Polyamines			
Spermine	348 ± 18	436 ± 24	0.02
Spermidine	1,244 ± 133	1,914 ± 158	0.01
Putrescine	164 ± 10	189 ± 16	0.22

Values are mean ± SE, $n = 6$ piglets/group

Differences at $P < 0.05$ were considered significant

Spermine and spermidine concentrations of the duodenum and liver were significantly higher ($P < 0.05$) in the Met-supplemented group. However, no difference was observed for putrescine concentrations in the four tissues between the two groups.

Plasma and tissues redox potential

During the 14 day period, plasma E_h, determined from the Cys/CySS couple, was decreased in Met-supplemented compared with control piglets (Fig. 1a). The redox potential, determined from GSH/GSSH couples, was lower in the duodenum and jejunum, but not different in the ileum and liver, of Met-supplemented group compared with control group (Fig. 1b).

Intestinal morphology

Morphological analysis of the intestine (duodenum, jejunum, and ileum) showed a higher ratio of villus height-to-crypt depth ($P < 0.05$) for the three segments of the small intestine in the Met-supplemented piglets than in the control group (Table 5). The crypt depth was lower in the duodenum and the villus height was higher ($P < 0.05$) in the jejunum of Met-supplemented than control piglets (Table 5).

Transepithelial physiology of jejunum

Measurement of electrical properties of piglet mid-jejunum mucosal tissue revealed an increase in TEER from piglets fed the Met-supplemented diet for 14 days ($P < 0.05$) (Fig. 2). The basal Isc was not different between the two groups (Table 6). Compared with the control group piglets, Met, Cys and Gln transport across the jejunal mucosa, assessed by ΔIsc, were increased in the piglets fed the Met-supplemented diet but not statistically significant (Table 6).

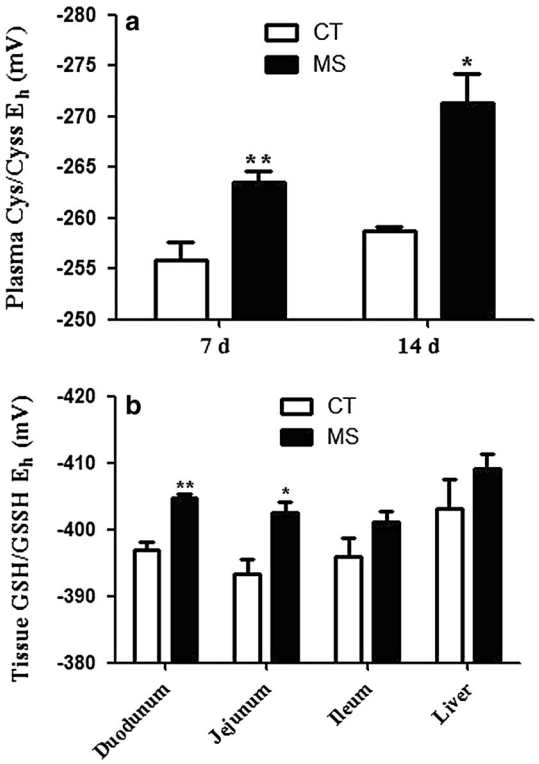

Fig. 1 Redox potential values (Eh) from cysteine–cystine (Cys/CySS) couple in plasma (**a**) and glutathione–glutathione disulfide (GSH/GSSH) couple in tissues (**b**) in post-weaning piglets fed a control diet or Met-supplemented diet for 14 days. CT means control group, MS means Met-supplemented group. Values shown represent mean ± SE, $n = 6$ piglets/group. $^{**}P < 0.01$ versus control; $^{*}P < 0.05$ versus control

Table 5 Effects of dietary Met level on the intestinal morphology in post-weaning piglets

Items	Control group	Met-supplemented group	*P* value
Duodenum			
Villus height (μm)	400 ± 13.79	419 ± 12.04	0.33
Crypt depth (μm)	154 ± 1.65	146 ± 2.48	0.03
Villus height/crypt depth	2.60 ± 0.09	2.86 ± 0.05	0.04
Jejunum			
Villus height (μm)	337 ± 5.07	361 ± 7.55	0.03
Crypt depth (μm)	147 ± 3.82	143 ± 2.32	0.36
Villus height/crypt depth	2.29 ± 0.04	2.53 ± 0.06	0.01
Ileum			
Villus height (μm)	330 ± 8.21	354 ± 7.50	0.06
Crypt depth (μm)	147 ± 2.51	148 ± 2.01	0.74
Villus height/crypt depth	2.25 ± 0.05	2.40 ± 0.04	0.04

Values are mean ± SE, $n = 6$ piglets/group

Differences at $P < 0.05$ were considered significant

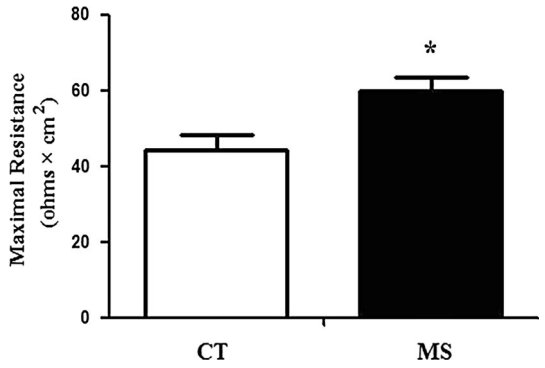

Fig. 2 Transepithelial electrical resistance (TEER) (ohms × cm^2) of the jejunum in post-weaning piglets fed a control diet or Met-supplemented diet for 14 days. Maximal resistance recorded during incubation period, CT means control group, MS means Met-supplemented group. Values shown represent mean ± SE, $n = 6$ piglets/group. $^{*}P < 0.05$ versus control

Table 6 Effects of dietary Met level on the amino acids transport in the jejunum of post-weaning piglets (μA/cm^2)

Items	Control group	Met-supplemented group	*P* value
Basal Isc	88.56 ± 8.16	120.92 ± 14.28	0.08
Δ Isc			
Methionine	17.00 ± 1.15	20.12 ± 0.94	0.06
Cysteine	12.22 ± 0.98	14.71 ± 1.76	0.24
Glutamine	11.52 ± 0.90	14.66 ± 1.39	0.09

Values are mean ± SE, $n = 6$ piglets/group

Expression of tight junctions proteins and apoptotic proteins in the jejunum

Western blot analysis revealed a markedly increase in the abundance of TJ protein occludin in the jejunum from Met-supplemented piglets compared with control group; however, there was no change in the protein abundance of claudin-3 (Fig. 3a). Protein level of apoptotic protein active caspase-3 was reduced in the jejunum of Met-supplemented piglets, compared with control group piglets (Fig. 3b).

Discussion

Met is a dietary essential amino acid that plays unique roles, both in protein structure and metabolism (Brosnan et al. 2007a, b). Adequate amount of Met must be supplied in pig diets to optimize performance (Opapeju et al. 2012). Results of the current study indicate that piglets fed the basal diet which was with a low level of Met had a lower ADG on days 7–14 of the trial and had a poorer FCR in the overall trial period (Table 2). Kino and Okumura (1986) reported that Met and Cys deficiency induced a decrease in

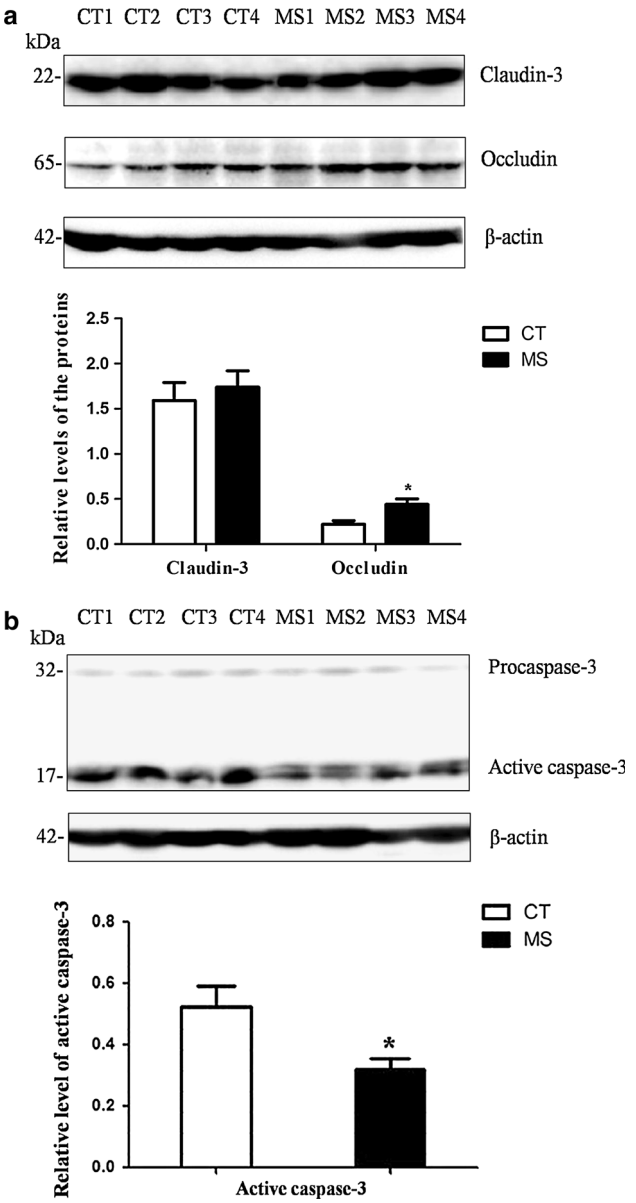

Fig. 3 Western blot analysis of claudin-3, occludin and caspase-3 in the jejunum of post-weaning piglets fed a control diet or Met-supplemented diet for 14 days. β-Actin was used as the internal reference protein. CT means control group, MS means Met-supplemented group. Values shown represent mean ± SE, $n = 4$ piglets/group. *$P < 0.05$ versus control

rates of body weight gain and protein accretion in growing chickens and that these adverse effects were more pronounced than those in birds fed diets deficient in lysine and histidine. Conde-Aguilera et al. (2010) found that feeding piglets with a diet deficient in total SAA (a reduction of 36 %) resulted in a decrease in protein retention and daily gain. These results suggest that decreased growth of chickens or piglets fed Met- or SAA-deficient diets was probably caused by the lower rates of whole-body protein synthesis.

Besides its role in protein synthesis, Met, as a crucial precursor of the essential molecules, plays unique roles in the regulation of numerous biological functions. Results of this study indicated that dietary supplementation with 0.12 % L-Met (as 0.35 % dietary SID Met) increased the plasma and tissue Met concentrations in the piglets compared with the control group. The gastrointestinal tract is a metabolically significant site of SAA metabolism in the body and consumes 20 % of the dietary Met (Riedijk et al. 2007). Much of dietary Met metabolized by the portal-drained visceral tissues, mainly the small intestine, is transmethylated to form homocysteine via SAM, which is the major methyl group donor for a variety of methyltransferases and plays a singular role in epigenetic regulation of gene expression via modulation of DNA methylation (Brosnan and Brosnan 2006; Bauchart-Thevert et al. 2009a). Disorders of methyl balance lead to several diseases (liver disease, CVD, closure of the neural tube, synthesis of creatine, cancer), suggesting the important roles of the methylation reactions (Brosnan et al. 2007a, b; Williams and Schalinske 2007). Another major role of SAM is to donate an aminopropyl group for the synthesis of the higher polyamines (Shoveller et al. 2005). In the process of polyamines synthesis, SAM decarboxylated to form decarboxylated 5-adenosylmethionine, and this compound donates its propylamine group to putrescine to form spermidine, and to spermidine to form spermine (Wu et al. 2000; Mato et al. 2013). In the current study, the concentration of putrescine was decreased while spermine and spermidine concentrations were increased in the plasma of Met-supplemented piglets at day 14 of the trial. Meanwhile, both spermidine and spermine concentrations were higher in the duodenum and liver of piglets fed the Met-supplemented diet. These results indicate that dietary Met supplementation stimulate transmethylation metabolism and synthesis of polyamines in the small intestine and other tissues. Polyamines, either synthesized endogenously or supplied luminally, stimulate normal intestinal epithelial cell proliferation, enhance differentiation during early stage of mucosal development and are required for the maintenance of mucosal integrity (Wang 2007). Polyamine depletion has been shown to both enhance and protect against apoptosis in several cell lines (Seiler and Raul 2005). Thus, dietary-adequate Met intake is critical for normal intestinal growth of the post-weaning piglets via regulating intestinal SAM pool and hence polyamines synthesis.

Another important metabolic function of Met is to provide sulfur for Cys synthesis, which then be degraded into taurine or be utilized for GSH synthesis (Stipanuk 2004; Wang et al. 2013). Cys will become conditionally indispensable in particular situations such as stress conditions or inflammatory states and play important roles in

antioxidant defense, nutrient metabolism, and regulation of cell function (Tesseraud et al. 2009). Cys and its disulfide, CySS, are the predominant low molecular weight thiol/disulfide pool found in the plasma and are central to the maintenance of the redox status of the plasma proteins (Jones et al. 2000). GSH is the most abundant intracellular antioxidant, and GSH/GSSH system plays an important role in determining intracellular redox balance and antioxidant function (Wu et al. 2004; Nkabyo et al. 2006). Maintaining normal GSH concentration is essential to most tissue, especially the intestine, to protect the intestine form damage by luminal toxins and oxidants derived from the diet as well as endogenous generated reactive oxygen species (Bauchart-Thevert et al. 2009a). The current study showed that supplementation with L-Met markedly increased plasma Cys concentrations compared with the control group, leading to a further reduction in the Cys/CySS E_h. A higher GSH and lower GSSH concentrations were observed in the tissues of Met-supplemented piglets, especially in the duodenum and jejunum, associated with reduced GSH/GSSH E_h. Nkabyo et al. (2006) reported that SAA supplementation resulted in a more reducing plasma Cys/CySS redox potential. The present data was consistent with the previous report. Bauchart-Thevert et al. (2009b) reported that Met, Cys, taurine and total erythrocyte GSH concentrations were markedly decreased and a more oxidized status of the Cys/CySS E_h was observed in plasma of neonatal piglets fed a SAA-free diet. Also, intestinal tissue concentrations of Met and Cys were markedly reduced in SAA-free compared with control pigs. All these findings strongly suggest that sulfur amino acids Met and Cys play a important role in the control of intestinal growth and function associated with a regulated redox status.

The gastrointestinal tract of post-weaning piglets is immature, the transition from sow's milk to solid feed during weaning results in reduced growth performance and the subsequent negative impact on intestinal structure and function (Gu et al. 2002; Wang et al. 2008). The latter is primarily a consequence of high rates of mucosal cell turnover and apoptosis in the young piglets that did not receive sufficient nutrients to meet the requirement for mucosal protein synthesis and growth (Rezaei et al. 2013a, b). In the current study, a higher villus height of the jejunum and a lower crypt depth of the duodenum, as well as an increased ratio of villus height-to-crypt depth in the small intestine were observed in Met-supplemented than control group piglets. Previous report demonstrated that a SAA-free diet administered enterally to piglets for 7 days led to a reduced intestinal mucosal growth associated with villus atrophy, a reduced epithelial cell proliferation, and a lower goblet cell number (Bauchart-Thevert et al. 2009b). Thus, dietary Met supplementation ameliorated Met insufficiency, and adequate Met level is nutritionally essential to maintain normal mucosal growth in post-weaning piglets. The intestinal epithelium is one of the most rapidly proliferating tissues in the body, and extensive studies indicate that polyamines stimulate normal intestinal epithelial cell proliferation; thus, a decrease in cellular polyamines inhibits cell proliferation and disrupts epithelial integrity (McCormack and Johnson 1991; Wang 2007). In the current study, the intestinal mucosal growth was improved in Met-supplemented piglets, in association with higher concentrations of spermine and spermidine in the small intestines, especially in the duodenum. It can be surmised that dietary supplementation with L-Met could stimulate the synthesis of polyamines which is essential for intestinal mucosal growth and integrity, besides its role for intestinal protein synthesis. Meanwhile, Glu and Gln concentrations were increased in the plasma and tissues of the piglets fed a Met-supplemented diet. Glu is a carbon precursor for proline and arginine, which are essential for young piglets (Wu et al. 2011a, 2013). Gln, considered as a conditionally essential amino acid for piglets (Wu et al. 2011b), is a major fuel for absorptive epithelial cells of the small intestine, and plays an important role in maintaining gut integrity and preventing the entry of luminal pathogenic microorganism into the systemic circulation (Wu 1998, Wang et al. 2009b, Haynes et al. 2009).

A novel finding from the current study is that supplementation with L-Met improved the jejunal epithelial barrier function and amino acid absorption in post-weaning piglets, as measured by Ussing chambers in vitro. TEER, which reflects the opening of the TJ between epithelial cells (also consider as the paracellular permeability of the intestinal mucosa), was determined at first. Then, the short circuit current (Isc) on the mucosa was measured as an indicator of active electrogenic ion transport and electrolyte-dependent amino acid absorption across the epithelium (Boudry 2005; Wijtten et al. 2011). The transport systems involved in intestinal Met absorption were described as Na^+-dependent transport systems B^0 and $B^{0,+}$, and Na^+-independent transport mechanisms $b^{0,+}$ and L, and system y^+, which is specific for cationic amino acids but is also able to transport neutral amino acids, including L-Met, in the presence of Na^+ (Martín-Venegas et al. 2009). In the current study, a significant increase in TEER was observed in the jejunal mucosa of the piglets fed a Met-supplemented diet. Na^+-dependent Met transport across the jejunal mucosa tended to increase for the piglets fed the Met-supplemented diet and numerical improvements were observed for Cys and Gln transport, although the statistical analysis was not significant. Gln is a major fuel for absorptive epithelial cells of the small intestine and plays an important role in maintaining gut integrity (Wang et al. 2008; Haynes et al. 2009). Improved absorption of Met, Cys and Gln may contribute to enhanced protein synthesis,

so as to sustain optimal nutrient requirements for the small intestine. Although active ion transport and amino acid absorption did not appear to be affected by dietary Met supplementation, the changes observed in TEER indicate a decrease in paracellular permeability, which may contribute to improved barrier function of the jejunal mucosa of the Met-supplemented piglets. Martín-Venegas et al. (2013) reported that MHA-FA, as a source of dietary Met, prevents the increase in paracellular permeability induced by H_2O_2 or tumor necrosis factor-α. This effect can be due to the capacity of MHA-FA to stimulate the transsulfuration pathway and the production of metabolites with antioxidant properties that can protect epithelial barrier function. In the current study, piglets fed Met-supplemented diet had higher concentrations of Cys and taurine, which are the metabolites of the Met transsulfuration in the liver (Wu 2013a), may be the underlying reason for the jejunal epithelial barrier function.

Tight junction is an intercellular junction complex found in epithelial and endothelial cells that are responsible for the formation of functional epithelial and endothelial barriers (Rhoads and Wu 2009). TJ plays an important role in regulating the passage of cells and solutes and preventing free interchange of nutrients, water, toxins, and antigens through the paracellular space (Ramalingam et al. 2010; Steed et al. 2010). Members of the claudin family play a critical role in TJ formation and determine permeability characteristics in a variety of tissue, including the gut (Tsukita and Furuse 2000). Occludin has been implicated in regulating the permeability properties of TJ and, in particular, has been linked to the regulation of size-selective diffusion (Steed et al. 2010). Here, in the jejunum, the abundance of occludin was significantly increased in the Met-supplemented diet while no obvious change in claudin-3 was observed. Hou et al. (2012, 2013) reported that dietary supplementation of *N*-acetylcysteine, a widely used precursor of L-cysteine, could increase the levels of claudin-1 and occludin in the small intestine of LPS-challenged piglets, which indirectly supports our current results. L-Met was also the essential precursor of L-cysteine. Higher Cys concentrations were observed in the plasma and the small intestinal tissues of the Met-supplemented piglets in the current study. Thus, dietary Met may affect the epithelial metabolism and function through the regulation of Cys synthesis. Recent evidence indicates that polyamines are implicated in regulation of the intestinal epithelial barrier function and depletion of cellular polyamines increases epithelial paracellular permeability (Guo et al. 2003). Furthermore, polyamines regulate expression of various TJ proteins through different mechanisms (Guo et al. 2005). These results suggest that the altered TJ expression may result from an increase of polyamines concentrations in the jejunum of Met-supplemented piglets.

It should also be noted that the TJ protein changes may underlie functional changes in the jejunum. Moreover, Met supplementation may induce many other changes in the epithelium besides TJ barrier changes. Caspase-3, a frequently activated death protease, catalyzes the specific cleavage of many key cellular proteins, and is one of the key components of the apoptotic pathway in the small intestine (Porte and Jänicke 1999; Tan et al. 2010). In the current study, a lower expression level of active caspase-3 was observed in the jejunum of Met-supplemented piglets, which provided a molecular basis to explain the finding that the intestinal morphology of the jejunum was improved by dietary supplementation with L-Met. L-Met is an effective precursor of Cys for tissue GSH synthesis, and participates in the control of oxidative status, which acts as one of the most important determinant of cell proliferation and apoptosis (Wu et al. 2004).

In conclusion, dietary-adequate Met level alleviated intestinal injury and improved jejunum epithelial barrier function in post-weaning piglets. These new findings implicate the functional importance of dietary Met for intestinal growth and function, beyond its role as a precursor for protein synthesis (Wu 2013b). The improved barrier function is associated with increased abundance of TJ proteins in the small intestine. Meanwhile, the mechanisms and cell signaling responsible for the beneficial effect of Met on oxidative stress, proliferation, and apoptosis of the intestinal epithelial warrant further investigation.

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Conflict of interest The authors declare that they have no conflict of interest.

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