



Original Research Article

Effect of gestation dietary methionine-to-lysine ratio on methionine metabolism and antioxidant ability of high-prolific sows



Mao Xia ^{a,1}, Jie Peng ^{b,1}, Chenbin Cui ^a, Qiongyao Gu ^c, Linjie Zhou ^a, Chao Wang ^a, Haiqing Sun ^d, Jian Peng ^a, Hongkui Wei ^{a,*}

^a Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

^b Innovation Institute of Healthy Farming, College of Animal Sciences and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong 510225, China

^c Department of Chemistry, College of Science, Huazhong Agricultural University, Wuhan 430070, China

^d YangXiang Joint Stock Company, Guigang 537000, China

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ABSTRACT

The uptake and metabolism of methionine (Met) are critical for epigenetic regulation, redox homeostasis, and embryo development. Our previous study showed that appropriate supplementation of dietary Met promoted the birth weight and placental angiogenesis of high-prolific sows. To further explore the metabolic effect of Met on pregnant sows, we have evaluated the influence of dietary Met level on Met metabolism, and the relationship between metabolites of Met and reproductive performance, antioxidant ability, and placental angiogenesis throughout the gestation of high-prolific sows. Sixty sows (the 3rd parity, Large White) were randomly divided into 5 groups that were fed diets with standardized ileal digestible (SID) methionine-to-lysine (Met:Lys) ratios of 0.27 (control), 0.32, 0.37, 0.42, and 0.47 from the mating day (gestational d 0, G0d) until the farrowing day. HPLC-MS/MS analysis was used to simultaneously evaluate the metabolites related to Met, e.g. S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), homocysteine (Hcy), cysteine (Cys), and glutathione (GSH). The concentration of SAM and SAH in plasma had significant fluctuations, especially in late pregnancy. Increasing dietary Met supplementation significantly improved the plasma SAM and methylation potential (SAM-to-SAH ratio) at d 114 of pregnancy (G114d). Moreover, a positive association of the plasma SAM concentration at G114d was observed with the litter weight of born alive ($P < 0.05$; $R^2 = 0.58$). Furthermore, Hcy concentration in plasma was at the lowest level for 0.37 ratio group at G114d. However, it significantly increased during late pregnancy. Moreover, there were negative correlations between plasma Hcy concentration at G114d ($P < 0.05$) and the placental vascular density in the fold and stroma ($P < 0.05$). Compared with the control group, the expression of vascular endothelial growth factor-A (VEGF-A) in the placenta tissue of 0.37 ratio group increased significantly ($P < 0.05$). Collectively, these findings indicate that dietary Met:Lys ratio (0.37 to 0.57) in the pregnant diet dose not influence the antioxidant ability of the high-prolific sows; however, the improvement of fetal development and placental angiogenesis of high-prolific sows by supplementation of Met are closely associated to the key Met-related metabolite of SAM and Hcy, respectively.

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* Corresponding author.

E-mail address: weihongkui@mail.hzau.edu.cn (H. Wei).

¹ These authors contributed equally.

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1. Introduction

Recently, the genetic selection for the enhanced prolificacy of sows has received intensive attention, as the number of weaned piglets per sow per year is the main factor that affects the profitability of the pig industry. However, increased litter size from high-prolific sows results in a lower average piglet birth weight (Quiniou et al., 2002). Since the maternal transfer of nutrients to piglets has not matched the increased prolificacy during gestation, lower birth weights will suffer from higher neonatal morbidity and mortality rates (Bauer et al., 1998; Wu et al., 2006; Theil et al., 2014). Several mammalian investigations have determined that low birth weight or intrauterine growth retardation (IUGR) of the fetus is related to the restricted development of blood vessels in the placenta (Chen et al., 2002). Our recent study proved that piglets' birth weight and placental angiogenesis of high-prolific sows could be increased by optimal methionine-to-lysine (Met:Lys) ratio (0.37) in a gestation diet (Xia et al., 2019). However, the litter weight and placental angiogenesis decreased with excessive Met supplementation (dietary Met:Lys ratios elevated from 0.37 to 0.47) (Xia et al., 2019). Moreover, researchers found that dietary addition of 0.48% Met from 90 d of gestation to parturition significantly enhanced birth weights and survival rate of the piglets (Bin et al., 2018). However, the regulatory underlying mechanism of dietary supplementation with Met on the reproductive performance and placental angiogenesis of high-prolific sows remains unknown.

Met metabolism is involved in several cellular functions for coordinating the nucleotide and redox status, including remission of oxidative stress, methylated modification, and B vitamins metabolism (Sanderson et al., 2019). In humans, Met metabolism changes with ongoing pregnancy, with a higher efficiency of transsulfuration during early pregnancy and a higher efficiency of transmethylation of Met during late pregnancy (Dasarathy et al., 2010). Several nutritional strategies exist that promote embryonic and fetal development by supplying maternal nutrients at different phases of pregnancy (Council, 2012; Bin et al., 2018). Met is one of the major essential nutrients utilized for synthesizing protein. It functions as a precursor that is involved in fetal and placental growth and nutrients metabolism, such as S-adenosylmethionine (SAM), polyamines, cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), which are involved in biological events such as methylation and antioxidation (Rees et al., 2006; Bauchart-Thevret et al., 2009; Shiraki et al., 2014). During pregnancy, gene transcription is influenced by Met supply as SAM is not only the ubiquitous methyl donor for DNA methylation and the essential regulator of chromatin function, but also participates in the biosynthesis of vital components such as phospholipids (Rees et al., 2006; Bleich et al., 2014). Van Riet et al. (2019) observed a marked elevation of plasma SAM in sows during late pregnancy while the fetal growth rate was noticeably increased, indicating the importance of increased methyl supply during late pregnancy. Oxidative stress is a negative factor damaging the health of pregnant sows (Bin et al., 2018). Sulfur from Met is transferred to Cys, which is then utilized for the synthesis of GSH and is responsible for the elimination of free radicals and the clearance of lipid peroxides (Métayer et al., 2008; Zuo et al., 2019).

Met is the unique known source of Hcy, an intermediary sulfur AA, which is recognized as a prooxidant that intercedes the methylation and transsulfuration pathways, and excessive Met intake increases plasma Hcy levels (Selhub, 1999). However, Met is also considered to be the most toxic amino acid when an excessive amount is consumed because Hcy is highly toxic for humans and animals (Benevenga and Steele, 1984). Our previous study showed that litter size of the total born and total alive decreased at high-

levels of Met supplementation (Met:Lys ratio = 0.47) in high-prolific sows compared with the 0.37 ratio group (Xia et al., 2019), which may be associated with the abnormal Hcy level.

Therefore, we hypothesized that dietary Met regulates the reproductive performance of the sows and placenta development through Met-related key metabolites of high-prolific sows in later gestation. The objectives of this study were to elucidate the responses of Met-related metabolites to the dietary Met:Lys ratio and the process of gestation. And the key Met-related metabolic effect was expected to be found by correlational analyses with reproductive performance and antioxidant ability of high-prolific sows, which would provide important insights for metabolic regulation mechanism of dietary Met on placental angiogenesis in high-prolific sows during pregnancy.

2. Materials and methods

2.1. Animals housing and experimental design

The overall design of experiments and dietary formulations used in the present study have been reported previously (Xia et al., 2019). Sixty sows were selected (the 3rd parity, Large White) from 325 sows and randomly divided them into 5 different groups that were fed diets with standardized ileal digestible (SID) Met:Lys ratios of 0.27 (the nutrient requirements of swine [NRC] 2012 level), 0.32, 0.37, 0.42, and 0.47 from the mating day (gestational d 0, G0d) until the farrowing day. Institutional animals' ethics committee of Huazhong Agricultural University approved all the animal studies described herein.

2.2. Sampling

Blood and placental samples were collected from study animals as outlined in a prior study (Xia et al., 2019). Briefly, blood samples were collected from 60 sows 2 h after feeding (7, 4, 9, 6 and 8 sows in each group had a litter size of over 13) on G0d, G40d, G90d, and G114d. Placental samples were collected from 8 sows per group on the farrowing day. Litter size, litter weight, placental weight, number of live births, and mean birth weight of individual litters were recorded within 24 h of farrowing. Sows with a litter size of ≥ 13 (high-prolific sows) were chosen for recording and sampling.

2.3. Placenta homogenate preparation

Random placental samples were collected and assessed immediately following farrowing. Plasma samples were stored at -80°C in 3-mL tubes (National Scientific) before analysis. For placental analyses, samples from high-prolific sows with an average piglet birth weight within one standard deviation above or below the mean were utilized. A 100-mg portion of each placental sample was isolated and ground via mortar and pestle using liquid nitrogen, followed by the addition of ice-cold KH_2PO_4 buffer (100 mmol/L, pH 7.4) to prepare a 10% tissue homogenate solution, and samples were spun at $13,000 \times g$ for 5 min. Supernatants were then collected to analyze antioxidant capacity and levels of Met-associated metabolites, while protein levels were assessed using the kit of Bicinchoninic Acid (BCA) Protein Assay (Beyotime, China).

2.4. Chemicals and reagents

SAM, Met, Hcy, SAH, Cys, LC-MS-grade acetonitrile, sodium hydroxide (NaOH, 0.1 mol/L), tris-(2-carboxyethyl) phosphine (TCEP), hydrochloric acid (HCl), GSH, formic acid, ascorbic acid, LC-grade methanol (MeOH), and ammonium acetate (NH_4OAc) were from

Sigma–Aldrich (MO, USA). Homocysteine-d8 was from CDN Isotopes (Pointe-Claire, Quebec, Canada). Ultra-pure grade water was prepared with a Millipore-Q instrument (Millipore, MA, USA).

2.5. HPLC-MS/MS

A high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) approach was employed to measure levels of plasma SAH, SAM, Cys, Hcy, and GSH and Met as previously described (Zuo et al., 2019). Briefly, after thawing, samples of plasma from appropriate sows were vortexed for 10 s, and 20 μ L of each sample was transferred to a fresh tube to which 80 μ L of TCEP solution was added. Samples were then allowed to rest for 10 min, after which this solution was added to 400 μ L of methanol containing 1% formic acid. Samples were then vortexed for 10 s before incubation for 2 h at -20°C . This was followed by the centrifugation of the samples at $13,000 \times g$ for 10 min and all the supernatants were passed through a filter with a 0.22- μ m pore size. Next, LC-MS/MS analyses were carried out with an LCMS-8050 triple quadrupole mass spectrometer connected to a LC-30AD system and a SIL-30AD autosampler (all from Shimadzu, Kyoto, Japan). Gradient elution was used to achieve chromatographic separation using a reversed-phase UPLC XSelect HSS T3 1.8 μ m, 100 mm \times 2.1 mm i.d. column (Waters, MA, USA). For this separation, the mobile phase was comprised of 10 mmol/L ammonium acetate (A) and methanol containing 20% (vol/vol) acetonitrile (B) in water. Gradient settings were as follows: 0.01 to 1.5 min, 5% B; 1.5 to 6 min, 5% to 50% B; 6 to 6.1 min, 50% to 95% B; 6.1 to 8 min, 95% B; 8 to 9 min, 95% to 5% B; 9 to 13 min, 5% B. The flow rate was maintained at 0.3 mL/min, and the total separation time was 13 min during which the autosampler was cooled to 4°C . A 1- μ L injection volume was used for all analyses.

2.6. Assessment of antioxidant activity in placental and plasma samples

The levels of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and malonaldehyde (MDA) in placental and plasma samples were measured in accordance with commercial detection kits. Samples were analyzed in duplicate, and after thawing all plasma samples were thoroughly mixed and centrifuged at $10,000 \times g$ for 10 min before analysis.

2.7. Placental vascular density analysis

Sections of placental tissue that had been fixed using buffered formalin were incubated overnight at 4°C in 70% ethanol, after

which they were incubated for 2 h in 95% ethanol, for 2 h in 100% ethanol, and then overnight in 100% ethanol, and incubated in xylene (2×2 h xylene, overnight xylene), and paraffin (2×2 h paraffin, overnight paraffin), as previously described (Song et al., 2018). The sections were trimmed and embedded in fresh paraffin for sectioning. The paraffin-embedded tissues were then sectioned (4 μ m), placed on coated glass slides, processed through a graded series of xylene and ethanol, stained with hematoxylin and eosin, processed through a graded series of ethanol and xylene, and coverslipped using Permount, as previously described (Song et al., 2018). A microscope (ECLIPSE Ti, Nikon, Japan) was then used to image 3 to 4 areas per tissue section, after which the placental vasculature was traced such that the total number of vessels and vessel area could be calculated. An image analysis approach was used to quantify the relative placental vascular area per unit of tissue area (Vonnahme et al., 2001).

2.8. Assessment of placental performance

Placental tissue sections were prepared and imaged as discussed in section 2.7 above (Vonnahme et al., 2001). Morphometric measurements (fold length per unit area of the placental fords and placental fold width) were calculated as described in previous studies (Vallet and Freking, 2007).

2.9. Real-time quantitative PCR (RT-qPCR)

Angiogenesis-related gene expression in placental samples from analyzed pigs was assessed through an RNase protection assay. VEGF-A, vascular endothelial growth factor-164 (VEGF164), and vascular endothelial growth factor-120 (VEGF120) primers were selected based on previous reports (Kaczmarek et al., 2009) (Table 1), and were produced by Sangon (China). An RNA extraction kit (GeneMark, Taiwan, China) was used to isolate total RNA from placental tissue samples, after which a first-strand cDNA synthesis kit (TOYOBO, Japan) was employed to prepare cDNA. This cDNA was in turn diluted 20 folds and used for RT-qPCR, with all reactions being conducted in a 10- μ L total volume using the SYBR GREEN qPCR mix (Bio-Rad, USA) and a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad). The cDNA products of expected size were confirmed by electrophoretic separation in a 1% agarose gel and staining with Sybrsafe (Appendix Fig. 1). Using the geometric mean expression of β -actin, glyceraldehyde-3phosphate dehydrogenase (GAPDH), and recombinant tyrosine 3/tryptophan 5 monooxygenase activation protein zeta (YWHAZ) via the $\Delta\Delta\text{CT}$ method, gene expression was normalized (Cleal et al., 2009). Briefly, mean triplicate values of CT for target genes were normalized to mean

Table 1
Primer sequences used for the real-time PCR analysis.

| Gene Symbol | PCR primer (5'-3') | Annealing temperature, $^{\circ}\text{C}$ | Product size, bp |
|----------------|---|---|------------------|
| β -actin | Forward: CCAGGTCATCACCATCGG Reverse: CCGTGTGGCGTAGAGGT | 56.1 | 135 |
| GAPDH | Forward: CCTCAAGGAGTAAGAGCC Reverse: GTCTGGGATGGAAGCTGG | 52.0 | 128 |
| YWHAZ | Forward: TGAAGCCATTGCTGAAGCTG Reverse: TTCATCTCCTGGGTATCCG | 53.3 | 90 |
| VEGF-A | Forward: GGCAGAAGGAGACCAGAA Reverse: CAGGACGGCTTGAAGATG | 53.5 | 109 |
| VEGF120 | Forward: AAGGCCAGCACATAGGAGAG Reverse: CCTCGCTTGTACATTTTT | 53.3 | 73 |
| VEGF164 | Forward: GAGGCAAGAAAATCCCTGTG Reverse: TCACATCTGCAAGTACGTTCC | 53.7 | 124 |

GAPDH = glyceraldehyde-3phosphate dehydrogenase; YWHAZ = recombinant tyrosine 3/tryptophan 5 monooxygenase activation protein zeta; VEGF-A = vascular endothelial growth factor-A; VEGF-120 = vascular endothelial growth factor-120; VEGF-164 = vascular endothelial growth factor-164.

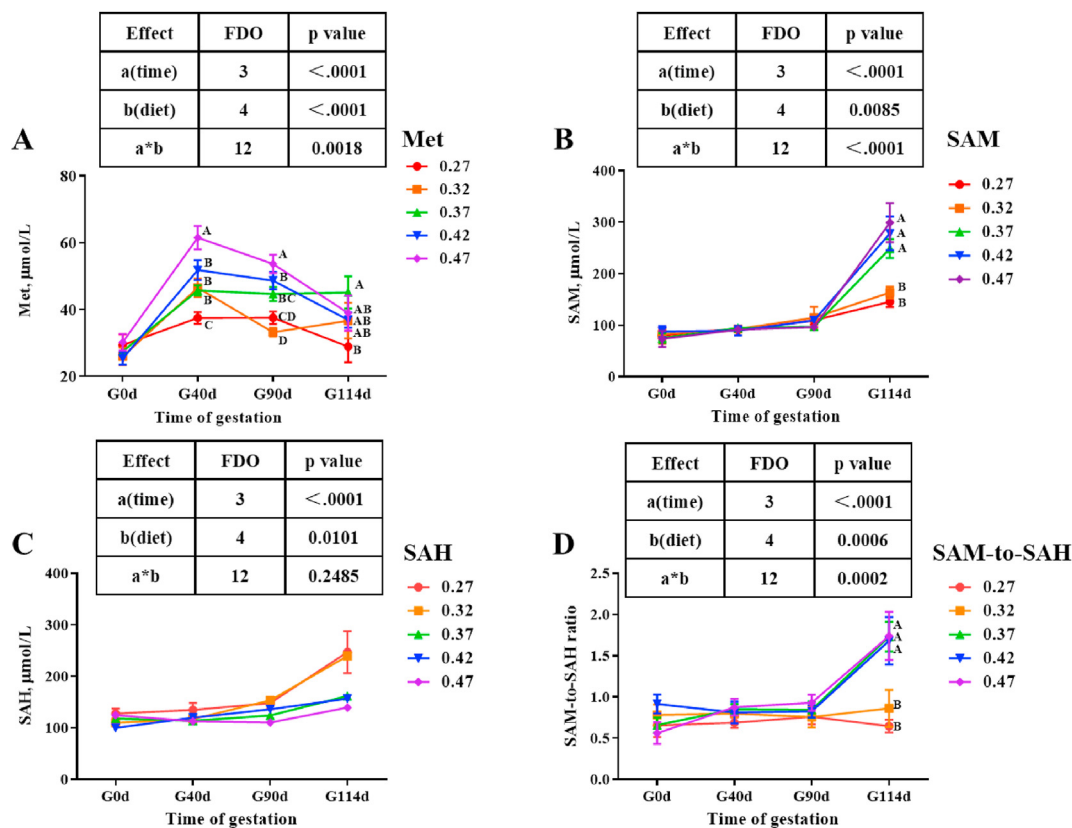


Fig. 1. The time response curve of the plasma transmethylation-related metabolites in high-prolific sows according to Met treatments. Data were reported as mean values with the standard error of the means (SEM) in MIXED procedures (factor a, time; factor b, diet; a*b, interaction; 4-time points in gestation; 5 supplementation levels of methionine [Met-to-Lys ratio]; 7, 4, 9, 6 and 8 sows in each group; $P < 0.05$ was considered to indicate a statistically significant difference). Multiple comparisons at the same time point. ^{A,B} Different letters denote significant differences ($P < 0.05$). (A). Effect of dietary Met level on plasma Met of high-prolific sows throughout gestation. (B). Effect of dietary Met on plasma SAM throughout gestation. (C). Effect of dietary Met on plasma SAH throughout gestation. (D). Effect of dietary Met on plasma S-adenosylmethionine-to-S-adenosylhomocysteine (SAM-to-SAM) ratio throughout gestation. FDO = freedom degree.

triplicate values of CT for appropriate housekeeping genes with the formula “2CT β -actin-CT target gene”, yielding relative target gene expression value. The geometric mean was used in these analyses to control for the potential effects of outliers on relative gene expression (Murthi et al., 2008).

2.10. Immunohistochemistry

Immunohistochemical (IHC) staining was used to evaluate VEGF localization and staining density in placental tissue sections. A polyclonal anti-rabbit antibody recognizing the VEGF121, VEGF165, and VEGF189 variants (Santa Cruz Biotechnologies, CA, USA) was used for IHC staining. Samples were incubated with this antibody (1:200) overnight at 4 °C, after which a Vectastain Elite ABC kit (Vector Laboratories, CA, USA) was used for the detection of staining for the primary antibody, and all samples were subjected to hematoxylin counterstaining. For preliminary characterization, we utilized a primary antibody that had been preabsorbed with a limited amount of antigen, while in subsequent analyses we determined that primary antibody omission was a sufficient negative control.

2.11. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (version 9.4; SAS Institute, Cary, NC, USA). Data were

analyzed using the MIXED procedures of SAS according to the following equation:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + t_l + \epsilon_{ijl}$$

where Y_{ij} is the response variable, μ is the overall mean, α_i is the fixed effect of dietary treatment (SID Met:Lys dietary ratios) ($i = 0.27, 0.32, 0.37, 0.42$ and 0.47), β_j is the fixed effect of pregnant stages ($j = G0d, G60d, G90d$ and $G114d$), $(\alpha\beta)_{ij}$ is the interaction effect between dietary treatment and pregnant stage, t_l is the random effect of sows used to explain repeated measurements within individual sow, and ϵ_{ijl} is the residual error. $P < 0.05$ was used to indicate significance between different means. One-way ANOVAs were used for multiple comparisons assessing the impact of Met supplementation on placental gene expression. Relationships between levels of plasma Met-related metabolites, plasma antioxidant capacity, and reproductive performance were assessed via Spearman's correlation analyses. Data were presented as means \pm standard error of the mean (SEM). P -values less than 0.05 were the considered as significant.

3. Results

3.1. Transmethylation-related metabolites in plasma of high-prolific sows during gestation

Transmethylation-related metabolites mainly consist of Met, SAM, and SAH. The SAM-to-SAH ratio was used to characterize the

methylation potential or the methylation index (Waterland, 2006; Zhang, 2018). Plasma concentration of the Met, SAM, and SAM-to-SAH ratio was affected by the interaction of time of gestation and Met:Lys ratio ($P < 0.05$; Fig. 1). However, no interaction was observed for the time and level of dietary Met on the plasma concentration of SAH. Plasma concentration of Met, SAM, and SAM-to-SAH ratio was affected either by the time of gestation or the level of dietary Met ($P < 0.05$; Fig. 1).

As shown in Fig. 1A, the plasma concentration of Met was lower during the beginning of gestation (G0d) which then increased markedly to the highest level at G40d and then decreased gradually during the middle (G90d) and late pregnancy (G114d). At G40d, the concentration of plasma Met for the 0.32, 0.37, 0.42, and 0.47 ratio groups was significantly higher than that of the 0.27 ratio group ($P < 0.05$). Further, the concentration of plasma Met for the 0.47 ratio group was significantly higher than that of the 0.32, 0.37, and 0.42 ratio groups ($P < 0.05$). The plasma Met concentration for 0.47 ratio group was higher than other groups at d 90 gestation. The concentration of plasma Met of the 0.42 and 0.47 ratio groups were significantly higher than that of the 0.27 and 0.32 ratio groups ($P < 0.05$), whereas the concentration of plasma Met of the 0.37 ratio group, was significantly higher than that of the 0.32 ratio group at d 90 gestation ($P < 0.05$). At G114d, the concentration of plasma Met of the 0.37 ratio group was significantly higher than that of the 0.27 ratio group ($P < 0.05$), but not different from the other treatments.

As shown in Fig. 1B–D, the concentration of SAM, SAH, and SAM-to-SAH ratio was maintained at a relatively low level from the beginning of gestation period to G90d, which then increased markedly during the late pregnancy. The dietary supplementation of Met did not affect the concentration of plasma SAM from the beginning of gestation period to G90d, but markedly increased the concentration of plasma SAM at G114d ($P < 0.05$). The dietary supplementation of Met did not affect the concentration of plasma SAH at the different gestation time points (G0d, G40d, G90d, and G114d). However, the effect of dietary supplementation of Met on SAM-to-SAH ratio in plasma of sows exhibited a significant increase at G114d, similar to that of SAM. This indicates that methylation potential is mainly determined by the levels of SAM.

3.2. Transsulfuration-related metabolites in plasma of high-prolific sows during gestation

Transsulfuration-related metabolites included Hcy (a central product in the metabolic pathway of Met metabolism), Cys (a sulfur amino acid), and GSH (a major intracellular antioxidant). According to Fig. 2 results, there were significant time \times diet interactions on the plasma concentration of Hcy and Cys ($P < 0.05$, $P = 0.0117$; Fig. 2). However, there was no time \times diet interaction on the plasma concentration of GSH. Plasma concentration of Hcy, Cys, and GSH was affected by the time factor (a process of gestation) ($P < 0.05$; Fig. 2) whereas only the plasma concentration of Hcy was affected by the diet factor (level of dietary Met) ($P < 0.05$; Fig. 2). Therefore, plasma Hcy was the only metabolite that was stable in response to both dietary Met and the process of gestation, and the dietary effect of Met resulted in a difference in plasma Hcy concentration during the mid and late pregnancy.

In Fig. 2A, the diet factor demonstrated no effect on the Hcy concentration at G40d but increased the Hcy concentration at G90d. However, on G114d, the concentration of Hcy increased significantly in the 0.42 and 0.47 ratio groups compared with the 0.37 ratio group, where the concentration of Hcy was maintained at a stable level.

As shown in Fig. 2B, the concentration of Cys did not fluctuate markedly throughout the pregnancy. Diet factor exhibited no effect

on the concentration of Cys at G40d and G114d of the pregnancy but decreased the concentration of Cys at G90d, similar to that of Hcy. Furthermore, Hcy is the only precursor that transsulfurated to Cys (Kurpad et al., 2014).

In Fig. 2C, the concentration of GSH was relatively stable from G0d to G90d of pregnancy and showed a tendency to decrease from G90d to G114d. It is possible that the increased oxidative stress during the perinatal period increased the depletion of GSH. Increasing dietary Met did not affect the concentration of GSH on G40d and G90d but increased the concentration of GSH at G114d.

3.3. Antioxidant ability in plasma of high-prolific sows during gestation

The antioxidant abilities in the plasma of high-prolific sows are shown in Fig. 3. The gestation time factor had a significant effect on the SOD and GSH-Px activity, and MDA concentration in the plasma of high-prolific sows ($P < 0.05$; Fig. 3). The SOD and GSH-Px activity decreased gradually from G0d to G114d, which indicated that the antioxidant abilities decreased as the pregnancy proceeded. However, the MDA level in the plasma of high-prolific sows increased significantly from G0d to G40d and from G90d to G114d, respectively but maintained a relatively stable level from G40d to G90d.

At G40d, the SOD activity of high-prolific sows in the 0.42 ratio group was significantly higher than those of the 0.32, 0.37, and 0.47 ratio groups ($P < 0.05$). However, dietary supplementation of Met showed no significant effect of the SOD and GSH-Px activity, and the MDA content in plasma of high-prolific sows compared with that of the control group during gestation.

3.4. Correlation between met-related metabolites and reproductive performance

To further investigate the key Met-related metabolites that respond to the effect of dietary Met on the reproductive performance of high-prolific sows, a total of 5 reproductive traits (number of total born, number of born alive, litter weight of born alive, average pig birth weight, and birth weight variable coefficient) were measured for their association with 5 Met-related metabolites and SAM-to-SAH ratio in plasma at G40d, G90d and G114d.

We observed a non-significant association between the reproductive traits and Met-related metabolites at G40d and G90d (Tables 2 and 3). At G114d, SAM concentration in plasma had a positive association with the litter weight of the born alive and the birth weight variable coefficient ($P < 0.05$; $R^2 = 0.50$; $P < 0.05$, $R^2 = 0.40$; Table 4). However, Hcy concentration in plasma at d 114 of gestation had an inverse association with the number of born alive and the average pig birth weight ($P < 0.05$; $R^2 = -0.49$; $P < 0.05$; $R^2 = -0.50$; Table 4). The significant correlations between the reproductive traits and key Met-related metabolites of SAM and Hcy were observed at late pregnancy, during which the fetus is rapidly growth and demanding higher nutrients exchanged efficiency in placenta.

3.5. Relationship between Met metabolism in plasma and placental traits

The heat map in Fig. 4 shows that the maternal plasma level of SAM at G114d was correlated positively to the placental fold vascular density ($P < 0.05$). Meanwhile, the GSH in plasma correlated negatively with the fold width of the placenta ($P < 0.01$). The Cys in plasma at G114d correlated positively with the SOD of the placenta ($P < 0.01$). However, the level of Hcy in maternal plasma at G114d correlated negatively with the placental fold vascular density, placental stroma vascular density, and the placental SOD

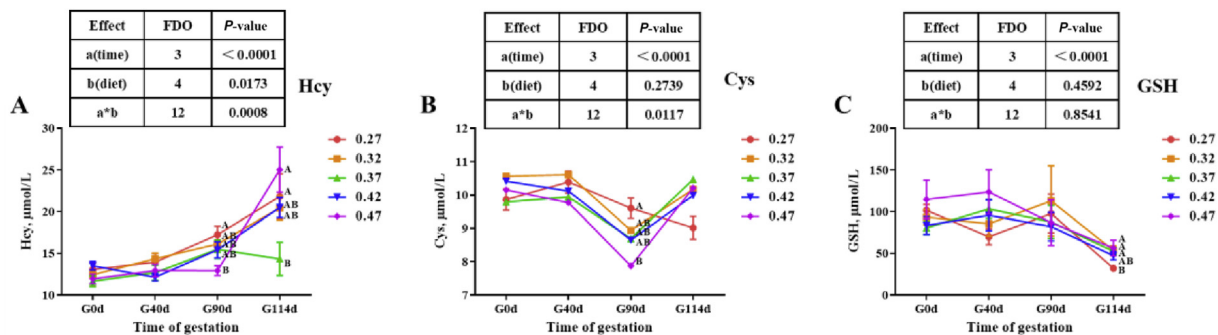


Fig. 2. Effect of dietary Met on plasma transsulfuration-related metabolites throughout gestation. Data were reported as mean values with the standard error of the means (SEM) in MIXED procedures (factor a, time; factor b, diet; a*b, interaction; 5 supplementation levels of Met; 7, 4, 9, 6 and 8 sows in each group; $P < 0.05$ was considered to indicate a statistically significant difference). Multiple comparisons at the same time point; different letters denote significant differences; $P < 0.05$, FDO = freedom degree. (A). Effect of dietary Met on plasma Hcy throughout gestation. (B). Effect of dietary Met on plasma Cys throughout gestation. (C). Effect of dietary Met on plasma GSH throughout gestation.

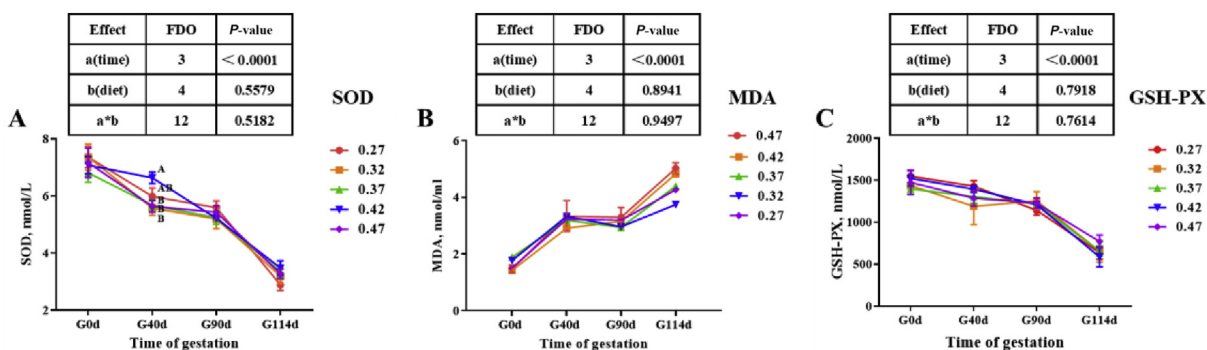


Fig. 3. Effect of dietary Met on plasma antioxidant capacity throughout gestation. Data were reported as mean values with the standard error of the means (SEM) in MIXED procedures (4-time points in gestation; 5 supplementation levels of Met; 7, 4, 9, 6 and 8 sows in each group) and multiple comparisons at the same time point, FDO = freedom degree. (A). Effect of dietary Met on plasma glutathione peroxidase (GSH-Px) activity throughout gestation. (B). Effect of dietary Met on plasma superoxide dismutase (SOD) throughout gestation. (C). Effect of dietary Met on plasma malonaldehyde (MDA) throughout gestation.

Table 2
Correlation (*R*) between Met-related metabolites and reproductive performance at d 40 of pregnancy.¹

| Item | SAM | SAH | SAM-to-SAH ratio | Hcy | Cys | GSH |
|-----------------------------------|-------|-------|------------------|-------|-------|-------|
| Number of total born | 0.30 | 0.06 | 0.02 | 0.20 | 0.18 | 0.24 |
| Number of born alive | 0.26 | 0.05 | -0.06 | 0.20 | 0.36 | 0.02 |
| Litter weight of born alive, kg | -0.01 | 0.19 | -0.18 | 0.09 | 0.12 | -0.06 |
| Average pig birth weight, kg | 0.09 | 0.12 | -0.12 | 0.04 | -0.04 | -0.15 |
| Birth weight variable coefficient | 0.25 | -0.35 | 0.39* | -0.21 | -0.23 | 0.28 |

SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; Hcy = homocysteine; Cys = cysteine; GSH = glutathione.

*Indicates correlations with $P < 0.05$.

¹ Spearman correlation values (*R*) are stated.

Table 3
Correlation (*R*) between Met-related metabolites and reproductive performance at d 90 of pregnancy.¹

| Item | SAM | SAH | SAM-to-SAH ratio | Hcy | Cys | GSH |
|-----------------------------------|-------|-------|------------------|-------|-------|-------|
| Number of total born | -0.27 | 0.14 | 0.14 | -0.04 | -0.09 | -0.17 |
| Number of born alive | -0.16 | 0.30 | 0.30 | -0.04 | 0.20 | -0.06 |
| Litter weight of born alive, kg | 0.06 | 0.15 | 0.15 | -0.20 | 0.28 | 0.03 |
| Average pig birth weight, kg | -0.06 | -0.01 | -0.01 | -0.12 | 0.31 | -0.04 |
| Birth weight variable coefficient | -0.07 | -0.11 | -0.11 | 0.17 | -0.23 | -0.12 |

SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; Hcy = homocysteine; Cys = cysteine; GSH = glutathione.

*Indicates correlations with $P < 0.05$.

¹ Spearman correlation values (*R*) are stated.

Table 4
Correlation (R) between Met-related metabolites and reproductive performance at d 114 of pregnancy.¹

| Item | SAM | SAH | SAM-to-SAH ratio | Hcy | Cys | GSH |
|-----------------------------------|------|-------|------------------|--------|-------|-------|
| Number of total born | 0.15 | -0.04 | -0.01 | -0.24 | 0.07 | -0.25 |
| Number of born alive | 0.22 | -0.38 | 0.1 | -0.49* | -0.39 | -0.31 |
| Litter weight of born alive, kg | 0.5* | 0.04 | 0.1 | -0.17 | -0.21 | 0.09 |
| Average pig birth weight, kg | 0.15 | 0.08 | 0.14 | -0.5* | 0.09 | 0.04 |
| Birth weight variable coefficient | 0.4* | -0.08 | 0.27 | 0.11 | 0.27 | 0.03 |

SAM = S-Adenosylmethionine; SAH = S-Adenosylhomocysteine; Hcy = Homocysteine.

Cys = cysteine; GSH = glutathione.

*Indicates correlations with $P < 0.05$.

¹ Spearman correlation values (R) are stated.

($P < 0.05$; $P < 0.01$; $P < 0.05$). Similarly, a recent in vitro study demonstrated the negative effects of Hcy on the angiogenesis of human umbilical vein endothelial cells (Pan et al., 2017).

3.6. Effect of dietary Met on the expression of gene related to placental angiogenesis of sows

Our previous study demonstrated a quadratic relationship between the dietary Met:Lys ratio and the placental vascular density, which presented a trend of an initial increase and a latter decrease, with the optimal level of placental vascular density being observed in the 0.37 ratio group (Xia et al., 2019). Expression of key angiogenic genes in the placenta including *VEGF-A*, *VEGF164*, and *VEGF120* are presented in Fig. 5A–C. Compared with the control group (Met:Lys ratio = 0.27), the expression levels of *VEGF-A* and *VEGF164* in the placenta tissue of the 0.37 ratio group were significantly increased ($P < 0.05$). Immunohistochemically analysis showed that the *VEGF-A* levels in placentae of the 0.37 ratio group were significantly higher than that of the other groups (Fig. 5D to E; $P < 0.05$).

4. Discussion

We evaluated the Met metabolism in high-prolific sows supplemented with dietary Met throughout their pregnancy. Using HPLC-MS/MS, we analyzed the impact of maternal dietary Met level and time of gestation on Met metabolism (Figs. 1 and 2). A total of 6 metabolites were tentatively characterized, including Met, SAM, SAH, Hcy, Cys, and GSH, which are involved in transmethylation and transsulfuration of Met metabolism.

In Fig. 1, we observed an increase in the plasma levels of SAM and SAM-to-SAH ratio during the last phase of gestation. The high transformation rate of SAM into SAH indicate more frequent methylation reactions during this phase (Bonilla et al., 2010). A similar result was observed in van Riet's work, which showed that the methylation potential (SAM-to-SAH ratio in plasma) of the highly prolific sows increased throughout gestation but fluctuated strongly in the period around parturition (Van Riet et al., 2019). Moreover, the rate of transmethylation in pregnant women during the third trimester was higher than that of the first trimester (Dasarathy et al., 2010). These studies indicate a higher demand for methyl supplementation during late pregnancy. Increasing diet Met from 0.36% to 0.48% administered during the late gestation period can improve the piglet birth weight of sows and maintain their health (Bin et al., 2018). Our previous study found that the 0.37 ratio group increased the vascular density in placental fold (Xia et al., 2019), in which the microvessels developed rapidly during late pregnancy (Thornburg and Louey, 2013). Based on these previous findings, increasing the one-carbon donors and cofactors in maternal circulation during late pregnancy, when fetal growth and nutrient demand are rapidly increasing, can partially benefit the fetal development and protein synthesis in the neonatal piglets (O'Neill et al., 2014; Robinson et al., 2016). Methyl donor deficiency may occur in high-prolific sows during their late pregnancy due to the continuous selection for larger litters. Therefore, it is necessary to further investigate the underlying mechanism of SAM regulating the development of the fetus in late pregnancy by evaluating the methylation potential of high-prolific sows by increasing dietary Met supply.

In our study, the transmethylation-related metabolites in gestation were affected by the interactive effect of time of gestation and dietary Met level, which suggests that different stages of pregnancy have a significant influence on the dietary Met effect on the metabolism of methyl supplementation. According to our result, dietary Met significantly increased the plasma SAM and SAM-to-SAH ratio as the Met:Lys ratio increased in the late pregnancy. Further investigation on the correlation analysis showed that the birth weight and placental fold vascular density of piglets were positively related to SAM at later pregnancy. Consistent with these results, our previous study also found that the birth weight of piglets increased significantly as the dietary Met:Lys ratio increased from 0.27 to 0.37 (Xia et al., 2019). Increasing the dietary Met:Lys ratio from 0.37 to 0.47 had no influence on the plasma SAM,

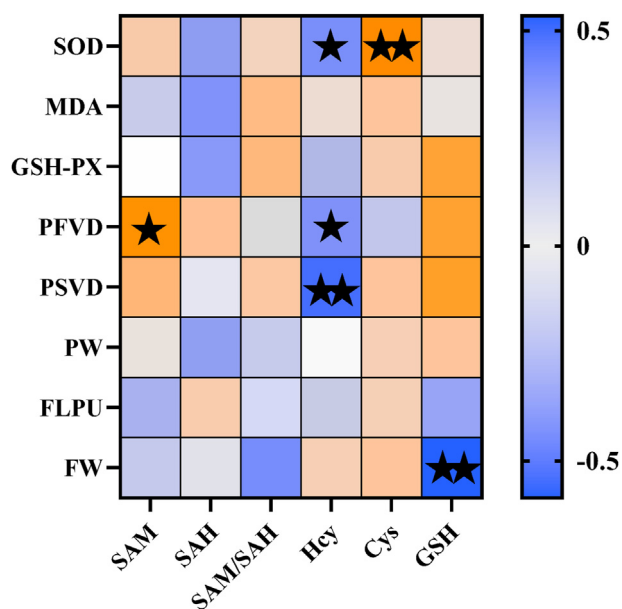


Fig. 4. Relationship between the Met metabolism in plasma at d 114 of pregnancy and placental traits. Heat map for the correlation analysis between placental traits associated and Met metabolism in plasma at d 114 of pregnancy; SOD = superoxide dismutase; MDA = malondialdehyde; GSH-PX = glutathione peroxidase; PFVD = placental fold vascular density; PSVD = placental stroma vascular density; PW = placental weight; FLPU = folds length per unit area of the placental folds; FW = folds width of the placenta; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; SAM-to-SAH ratio = S-adenosylmethionine-to-adenosylhomocysteine ratio; Hcy = homocysteine; Cys = cysteine; GSH = glutathione. ★★ $P < 0.01$, ★ $P < 0.05$.

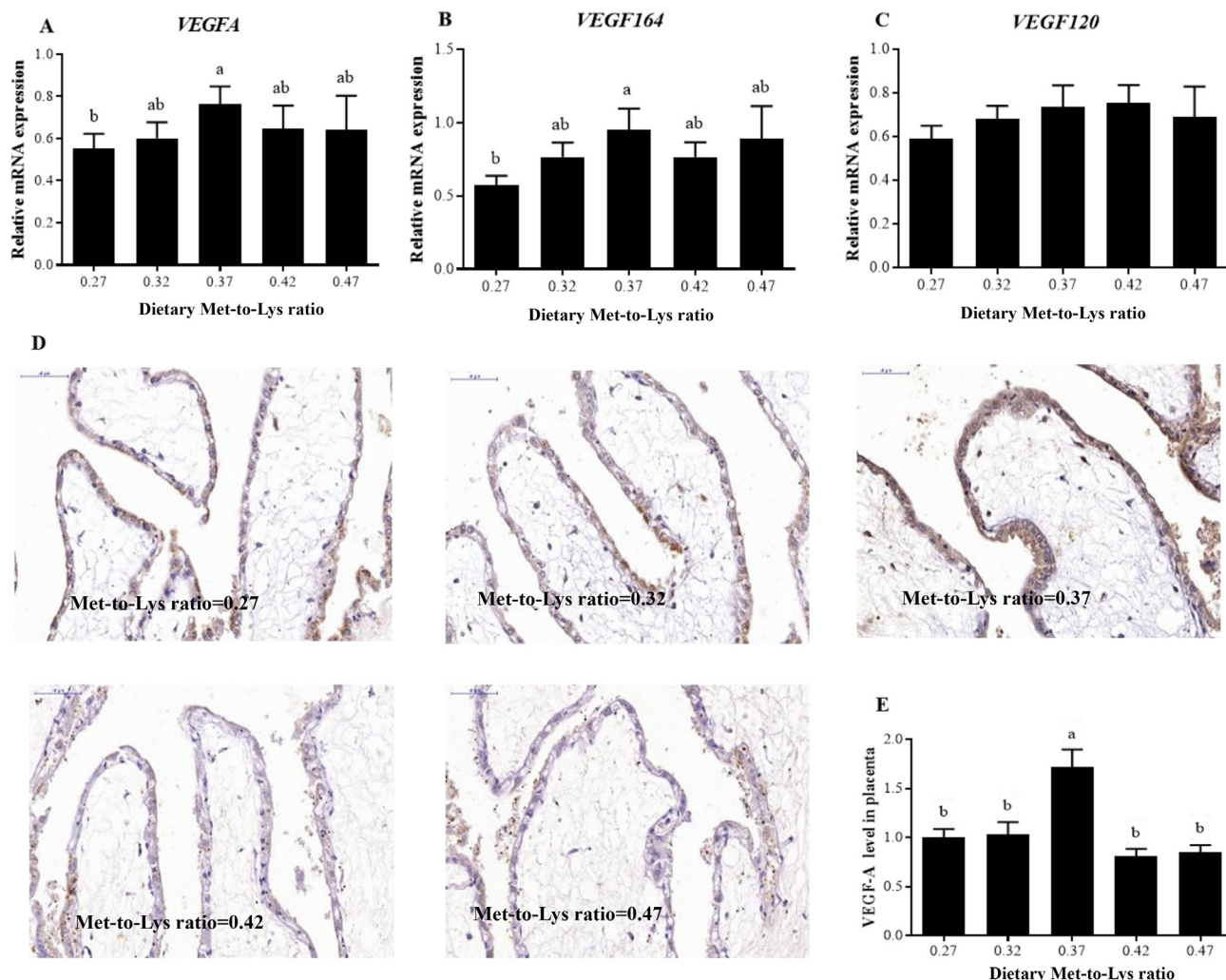


Fig. 5. Effect of the dietary Met-to-Lys ratio in gestation on the mRNA expression of gene related to placental angiogenesis of sows (A to C). Immunohistochemically analyses of VEGF-A at the placenta after parturition in sows with different dietary Met-to-Lys ratio (D to E). Values are means \pm SEM, ^{a,b} Means without a common letter differ significantly ($P < 0.05$).

indicating that SAM was not the key metabolite related to the reduction of litter weight of the piglets. Nevertheless, among the metabolites in Met metabolism, plasma SAM level had a positive correlation with the development and growth of the fetus during later pregnancy.

Homocysteine is widely recognized as a known teratogen (Matte et al., 2006; Van Mil et al., 2010). High levels of Hcy in pregnant females always has a close connection to abnormal embryo development and elevated prenatal death (Vollset et al., 2000; Guay et al., 2002; Dasarathy et al., 2010). High Hcy levels have also been observed in sows experiencing early pregnancy failure compared with the plasma concentration in normal pregnant sows (Van Wettere et al., 2013). These negative effects of Hcy may have inhibited the attempts to increase the Met availability by increasing Met consumption. Homocysteine elimination is under the strict regulation of enzymes responsible for transsulfuration and remethylation, which are regulated by cofactors, including vitamin B₁₂, vitamin B₆, and SAM (allosteric regulation) in the liver (Finkelstein, 1990). According to the correlation analysis, Hcy had a negative correlation with the number of born alive. The maternal metabolic function of Hcy is partly influenced by the demands of Met metabolism for the fetus as it rapidly grows and restricts Hcy elimination in late pregnancy. Moreover, the plasma Hcy in gestation was

affected by the interactive effect of the time of gestation and dietary Met level. The metabolic changes of Hcy under the effect of dietary Met also occur during late pregnancy. In response to the different dietary intakes of Met, plasma Hcy is regulated by changing the activity of enzymes and the availability of coenzyme that are involved in Hcy metabolism (Finkelstein and Martin, 1986). We found that the Hcy levels in plasma at G114d increased significantly during the pregnancy, which can be attributed to the lower activity of enzymes participating in homocysteine disposal. It has been reported that the activity of key enzymes (cystathionine β synthase and cystathionine γ -lyase) for Hcy's irreversible oxidation are low or absent in the fetal liver of pigs (Mcbreairty, 2014). Cystathionine β synthase (CBS) activities have been demonstrated to be controlled by Met concentration in rat or human liver slices, where SAM is reportedly an allosteric activator of CBS (Finkelstein et al., 1971; Janošik et al., 2001; Robinson et al., 2018). Therefore, plasma Hcy decreased as the Met:Lys ratio increased from 0.27 to 0.47 at G90d. However, Met metabolism is hard to characterize because there are multiple enzymes and coenzymes participating in both circulatory metabolism and degradation metabolism (Rees et al., 2006). Hcy elimination is also under the regulation of 5, 10-methylene-tetrahydrofolate reductase (MTHFR) which is a key enzyme responsible for remethylation and is considered to be inhibited by SAM (Zheng

et al., 2019). The decrease of Hcy as the Met:Lys ratio increased from 0.27 to 0.37 at G114d may be connected to the regulation of SAM on MTHFR. Nevertheless, the research of dietary nutrients on enzyme activity changes in the liver is still deficient, especially in pregnant females. The relationship between Met intake and advantageous or adverse consequences are further complicated by the metabolic interactions of the Met cycle.

Met is regarded as an antioxidant, thereby eliminating reactive oxygen species by Met residues or through GSH synthesis (Zeitz et al., 2017; Zeitz et al., 2018). Azad found that a diet including 0.48% Met during late gestation and lactation can increase the antioxidant capacity of piglets (Azad et al., 2018), however, they did not show the result of antioxidant capacity of sows. Met supplemented diets can increase muscular antioxidant capacity in low birth weight pigs (Li et al., 2017). However, our result showed that dietary Met had no significant effect on antioxidant capacity of sows during the entire gestation, and the plasma GSH level also showed no significant change in different dietary Met:Lys ratio groups. As we know, redox balance in mammals is important and involves various regulatory mechanisms to maintain the stability of redox levels. There was no significant change of antioxidant capacity of sows, and the Cys and Hcy in plasma at G114d correlated positively and negatively with placental SOD respectively. Cys was the rate-limiting factor in the synthesis of glutathione, however, high levels of Hcy were reported to increase oxidative stress and stimulate ROS production during the autoxidation process (Gaiday et al., 2018; Ding et al., 2019).

Angiogenesis of the placenta and vascular endothelial cell is connected with several metabolites involved in Met metabolism such as polyamine, Hcy, and H₂S (Wu et al., 2005; Pan et al., 2017; Longchamp et al., 2018; Peng et al., 2020). Our previous studies found a quadratic relationship between dietary Met and placental vascular density (Xia et al., 2019). Interestingly, the plasma Hcy at G114d exhibited a down and up altered trend as dietary Met:Lys ratio increased from 0.27 to 0.47, which was also observed at the extreme point in the 0.37 ratio group. A report studied on chick suggested that Hcy inhibited the angiogenesis by decreasing the VEGF protein level and *VEGFR1/2* mRNA in vitro and in vivo through the cytoskeleton remodeling (Pan et al., 2017). Furthermore, the negative correlation between plasma Hcy at G114d and placental vascular density enriches the potential regulatory mechanism of the effect of Met metabolism on the reproduction performance of the high-prolific sows. Therefore, it is important to avoid the accumulation of Hcy to further improve the efficiency of dietary Met in pregnant sows.

In contrast, compared with the control group (Met:Lys ratio = 0.27; 0.32% Met), the supplementation of Met significantly increased the plasma GSH at G114d. However, it has been reported that the plasma GSH content in both the 0.48% Met and the 0.60% Met groups were significantly lower than that in the control group (0.36% Met) (Bin et al., 2018). Besides the 0.32 ratio group, other dietary Met groups showed no significant effect of the SOD and GSH-Px activity, and MDA content in plasma of high-prolific sows compared with that of the control group during gestation, indicating that effect of dietary Met on reproductive performance is independent of the antioxidant capacity despite the elevation of GSH at G114d.

5. Conclusions

In conclusion, late gestation was the main phase that Met metabolites presented significant change, and SAM and Hcy were the key metabolites that respectively had a positive association with the litter weight of born alive and negative association with the placental vascular density at G114d. Moreover, optimal SID Met:Lys

ratio in gestation diets was 0.37, as plasma Hcy concentration was lowest in 0.37 ratio group at G114d, and the expression of VEGFA in placenta showed the highest level in 0.37 ratio group. In future research we will focus on the role of Met metabolites and later stage of pregnancy when investigating the regulation of Met on reproductive performance of high-prolific sows.

Author contribution

Hongkui Wei, Jie Peng and Jian Peng conceptualized and designed the study, **Chao Wang and Haiqing Sun** provided field test guidance, **Mao Xia, Chenbin Cui, Qiongyao Gu and Linjie Zhou** acquired the data, **Mao Xia and Chenbin Cui** analyzed and interpreted the data, **Mao Xia, Jie Peng and Hongkui Wei** drafted and revised the article. All authors critically reviewed the manuscript for intellectual content and gave final approval for the version to be published.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2021.02.006>.

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