



## Original Research Article

## Increased proline intake during gestation alleviates obesity-related impaired fetal development and placental function in gilts

Shuangbo Huang<sup>a,†</sup>, Jinfeng Li<sup>a,†</sup>, Hongxuan Ye<sup>a</sup>, Zihao Huang<sup>a</sup>, Junyi Wu<sup>a</sup>, Liudan Liu<sup>a</sup>, Shuo Ma<sup>a</sup>, Hefeng Luo<sup>b</sup>, Tanghong Wei<sup>b</sup>, Kai Liu<sup>c</sup>, Jinping Deng<sup>a</sup>, Dingfa Liu<sup>c</sup>, Chengquan Tan<sup>a,\*</sup>

<sup>a</sup> State Key Laboratory of Swine and Poultry Breeding Industry, Guangdong Provincial Key Laboratory of Animal Nutrition Control, National Engineering Research Center for Breeding Swine Industry, College of Animal Science, South China Agricultural University, Guangzhou 510642, China

<sup>b</sup> Dekon Food and Agriculture Group, Chengdu 610225, China

<sup>c</sup> Guangdong Foodstuffs IMP&EXP (Group) Corp, Guangzhou 510642, China

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## ABSTRACT

Maternal proline (Pro) supplementation enhances fetal survival and placental development in mice. However, the effect of Pro on fetal and placental development in gilts remains to be investigated, particularly in the context of obesity-induced impaired pregnancy. Here, we investigated the effect of dietary Pro on fetal and placental development in obese gilts. Exp.1: On day 60 of gestation, 48 gilts with similar delivery times were selected and followed up until delivery to determine the relationship between maternal obesity, litter performance, and Pro abundance in term placentae. The results showed that impaired reproductive performance was associated with body condition parameters and inadequate placental Pro availability of gilts. Exp. 2: A total of 114 gilts were then used in a 2 × 3 factorial design to investigate the interaction between body condition (factor I: normal or obese gilts) and dietary Pro levels (factor II: low [0.89%, L-Pro], medium [1.39%, M-Pro], and high [1.89%, H-Pro]) on farrowing performance and placental angiogenesis. This resulted in six treatment combinations: normal-L-Pro, obese-L-Pro, normal-M-Pro, obese-M-Pro, normal-H-Pro, and obese-H-Pro. The effective number of replicates per group was 17, 21, 19, 21, 18, and 18, respectively (1 gilt per replicate). The results showed that increasing Pro intake increased piglet birth weight ( $P = 0.001$ ), litter weight ( $P < 0.001$ ), placental efficiency ( $P = 0.036$ ) and placental vascular density ( $P < 0.001$ ), and decreased the number of mummified fetuses ( $P = 0.001$ ), the rate of low-birth-weight piglets ( $P = 0.005$ ), and the rate of invalid piglets ( $P = 0.029$ ). Interaction effects were observed between body condition and dietary Pro levels on piglet birth weight ( $P = 0.046$ ), within-litter birth weight variation ( $P = 0.012$ ), and placental vascular density ( $P = 0.007$ ). Moreover, the beneficial effect of Pro on farrowing performance may be related to the improvement of sirtuin 1-superoxide dismutase 2-mitochondrial reactive oxygen species axis homeostasis and angiogenesis in the placenta. Our results suggest that gestation diets need to provide adequate Pro to meet the needs of fetal and placental development, particularly in obese gilts.

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\* Corresponding author.

E-mail address: [tanchengquan@scau.edu.cn](mailto:tanchengquan@scau.edu.cn) (C. Tan).

† These authors contributed equally to the work.

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## 1. Introduction

In pig production, approximately 15% to 25% of piglets suffer from intrauterine growth restriction (IUGR) (Farmer and Edwards, 2022). Reported data also show that the incidence of low-birth-weight (LBW) piglets in obese sows is almost twice that of normal sows (Zhou et al., 2018). The placenta plays a crucial role in the exchange of substances between the fetus and the mother during pregnancy; therefore, such tissue is closely related to the

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development of the fetus (Aplin et al., 2020). Previous studies have shown that maternal obesity hinders placental angiogenesis by promoting lipotoxicity and oxidative stress within the placental environment, which further impairs the reproductive performance of sows (Brombach et al., 2022; Zhou et al., 2018). Therefore, improving the placental environment may be an important approach to mitigate obesity-related impairments in sow farrowing performance. There is an urgent need to explore potential effective intrauterine interventions to prevent obesity-related adverse pregnancy outcomes.

Dietary amino acid supply is closely related with both placental function and farrowing performance of sows (Tan et al., 2022). For example, supplementation of arginine and citrulline can enhance placental vascular development and improve delivery outcomes when endogenous arginine is in short supply due to limited resources (Weckman et al., 2019). However, the latest edition of Nutritional Requirements of Swine suggests that gestating sows do not need supplemental proline (Pro) in diets to achieve their maximum production performance (Council, 2012). A study in mice reported that Pro enhanced placental nutrient transport, angiogenesis and fetal survival (Liu et al., 2019). Reduced fetal growth induced by maternal malnutrition has been shown to be associated with reduced placental Pro transport (Wu et al., 2011). However, it remains unclear how placental Pro abundance relates to poor pregnancy outcomes in obese sows. Furthermore, there are insufficient data to support the beneficial effects of dietary Pro levels on fetal and placental development in gilts, particularly in the context of obesity-related poor pregnancy outcomes.

Therefore, we hypothesized that the body condition of the gilt may influence placental Pro abundance and further investigated the potential effects of dietary Pro on placental function and farrowing performance in gilts, particularly in the context of obesity-induced impaired pregnancy.

## 2. Materials and methods

### 2.1. Animal ethics statement

This study was conducted in a commercial research facility at Zhaoqing Baoyuan Agriculture Co., Ltd. The gilts used in the experiments were humanely treated following the practices outlined in the Guide for the Animal Ethics Committee of South China Agricultural University (2023G017).

### 2.2. Experimental design, diets, and data collection

The experimental design is shown in Fig. S1 (Exp. 1 and 2).

Exp. 1: On day 60 of gestation (G60d), 48 pregnant gilts (Pig Improvement Company, USA) with similar delivery times were selected and followed up until delivery. Gilts were housed in individual stalls and fed once (08:00) a day with a constant amount of 2.5 kg during gestation. The composition and nutrient levels of the diets are shown in Table S1. Gilts were given free access to water throughout the experiment. On G60d, the body weight of gilts was weighed and their backfat thickness was measured at position P2 using ultrasonography. The crown-rump length (the length from the crown of the head to the base of the tail) of gilts on G60d was measured to calculate body mass index (BMI). After farrowing, the number and weight of the piglets born, born alive, stillbirths and mummies were recorded. The vitality of piglets was visually assessed according to the scores described in a previous study (König et al., 2021). Invalid piglets included stillbirths and mummies. Piglets with a mean - 2 standard deviation (SD) lower birth weight (-30%) were defined as LBW piglets, as previously

described (D'Inca et al., 2010; Wu et al., 2023a). In this farm, the mean birth weight of the 1074 piglets was  $1.31 \pm 0.20$  kg. Therefore, piglets with a birth weight of less than 0.91 kg were defined as LBW piglets, while piglets with a birth weight greater than 0.91 kg were defined as normal-birth-weight (NBW) piglets. Correlations between body condition parameters, litter performance and placental amino acids were then analyzed.

Exp. 2: This experimental treatment was designed as a  $2 \times 3$  factorial with main effects of body condition on G60d (normal or obese gilts) and the levels of dietary Pro (0.89% [L-Pro], 1.39% [M-Pro], 1.89% [H-Pro]) from G60d until farrowing. A total of 126 gilts (Pig Improvement Company, USA) were included in the experiment, divided into groups of 21 gilts each. Twelve gilts were culled before delivery due to serious lameness and death, and a total of 114 gilts completed the trial and were used for data analysis. Finally, the effective number of replicates in normal-L-Pro, obese-L-Pro, normal-M-Pro, obese-M-Pro, normal-H-Pro, and obese-H-Pro group was 17, 21, 19, 21, 18, and 18, respectively (1 gilt per replicate). Gilts in different body conditions were categorized according to BMI and backfat thickness on G60d. The body condition classification criteria based on previous reports were as follows (Hu et al., 2021b; Rangstrup-Christensen et al., 2017): 1) gilts with a BMI and backfat close to the mean average ( $\pm 0.5$  SD) were identified as normal gilts, and their backbone and hip bones could be seen or felt when firm pressure was applied; 2) gilts with a mean + 2 SD higher BMI and backfat (+30%) were defined as obese gilts, and their backbone and hip bones couldn't be seen or felt when firm pressure was applied, or fat deposits were clearly visible. On this farm, the average BMI and average backfat thickness of gilts on G60d were  $62.0 \pm 2.5$  kg/m<sup>2</sup> and  $12.7 \pm 0.5$  mm, respectively ( $n = 350$ ). Finally, gilts were divided into two body condition groups: normal gilts with an average BMI of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm; obese gilts with an average BMI of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm. The gilts were individually housed in stalls and fed a constant amount of 2.5 kg per day during the gestation period. The ingredients and nutrient levels of the three iso-nitrogen diets are shown in Table 1. On G60d, body weight, back fat thickness and BMI of gilts were recorded. After farrowing, the number and weight of the live-born piglets, NBW piglets, LBW piglets, stillbirth fetuses, malformed fetuses, mummified fetuses and invalid piglets were recorded.

All experimental diets were formulated to meet the nutrient requirements for sows (National Research Council, 2012). Chemical concentrations were calculated using the values for feed ingredients from the National Research Council (2012). The contents of crude protein, crude fiber and neutral detergent fiber in the experimental diet were analyzed according to GB/T 6432-2018, GB/T 6434-2006 and GB/T 20806-2006, respectively. Feed intake during pregnancy is shown in Table S2.

### 2.3. Sample collection

On parturition day, blood samples of gilts were collected in 10-mL centrifuge tubes from the ear vein of the fasted sows at farrowing, followed by centrifugation at  $3000 \times g$  and  $4^\circ\text{C}$  for 15 min to recover the serum. During farrowing, the umbilical cord of each piglet was tied by a silk cord with a tag, enabling the match of each piglet to their placenta (Wilson et al., 1999). After the placentae were expelled, placentae from NBW piglets were collected. Approximately 3 g of placental samples (3-5 cm from the cord insertion point) were collected (Bai et al., 2022; Wu et al., 2023b). A portion of them was quickly snap-frozen in liquid nitrogen, while the remaining fresh placental tissue was immediately fixed in 4% paraformaldehyde.

**Table 1**  
Ingredients and nutrient levels of the three experimental diets in Exp. 2 (as-fed basis, %).

Item	Diets <sup>1</sup>		
	L-Pro	M-Pro	H-Pro
<b>Ingredients</b>			
Corn	29.08	29.04	29.01
Soybean meal (43%)	14.39	14.37	14.36
Barley	24.81	24.78	24.75
Sorghum	9.92	9.91	9.90
Wheat bran	9.92	9.91	9.90
Soybean husk	5.95	5.95	5.94
Soybean oil	0.99	0.99	0.99
Milddewcide (50%)	0.20	0.20	0.20
Premix <sup>2</sup>	3.96	3.96	3.96
Additional alanine	0.78	0.39	
Additional proline		0.50	0.99
Total	100.00	100.00	100.00
<b>Calculated nutrient levels<sup>3</sup></b>			
Dry matter	88.72	88.62	88.53
Crude protein	14.55	14.53	14.52
Crude fiber	5.40	5.40	5.39
Crude fat	3.55	3.55	3.55
Neutral detergent fiber	16.77	16.75	16.73
Net energy, kcal/kg	2259.54	2256.87	2254.59
Starch	40.17	40.12	40.07
Lysine	0.71	0.71	0.71
Methionine	0.24	0.24	0.24
Threonine	0.55	0.54	0.54
Tryptophan	0.16	0.16	0.16
Total proline	0.89	1.39	1.89
<b>Analyzed nutrient levels<sup>4</sup></b>			
Crude protein	14.44	14.41	14.52
Crude fiber	5.90	6.00	5.90
Neutral detergent fiber	16.50	15.90	16.20

<sup>1</sup> L-Pro = low proline level (0.89%) diet; M-Pro = medium proline level (1.39%) diet; H-Pro = high proline level (1.89%) diet. The Pro levels in diets are expressed as totals.

<sup>2</sup> Provided the following per kilogram of diets: VA 8000 IU, VD<sub>3</sub> 1200 IU, VE 60 IU, VK<sub>3</sub> 2 mg, VB<sub>1</sub> 1 mg, VB<sub>2</sub> 4 mg, VB<sub>6</sub> 1 mg, VB<sub>12</sub> 17 µg, folic acid 1.3 mg, biotin 0.2 mg, pantothenic acid 12 mg, choline 1 g, Cu 12 mg, Mn 25 mg, Zn 100 mg, Se 0.3 mg, I 0.4 mg.

<sup>3</sup> Chemical concentrations were calculated using the values for feed ingredients from the National Research Council (2012).

<sup>4</sup> Analyzed composition are shown as the mean values of two replicates.

## 2.4. Targeted metabolomics assays

Placental amino acids were measured by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Metabolites were extracted according to standard methods (Zhang et al., 2022). Briefly, 200 mg of placenta was mixed with 800 µL of acetonitrile. The samples were then homogenized, sonicated, centrifuged (12,500 × g, 4 °C for 15 min), dried and resolubilized, and 800 µL of the supernatant was used for HPLC-MS/MS analysis. Data analysis was performed using Xcalibur 4.7 (Thermo, USA).

## 2.5. Placental vascular density

Placental vascular density was measured by hematoxylin and eosin (H&E) staining as previously described (Hu et al., 2021a; Huang et al., 2023). Briefly, placental tissues fixed in 4% paraformaldehyde were paraffin-embedded and sectioned at 5 µm thickness, followed by staining with H&E. The area occupied by placental tissue was traced, and the placental vessels in these areas were also traced using a projection microscope (Olympus CX41, Japan). The placental vascular areas were then quantified by image analysis and evaluated for the relative number of placental vessels per unit tissue area.

## 2.6. Immunofluorescence

The fixed, permeabilized, and sliced (5 µm) placental samples were blocked with Quickblock blocking buffer (P0220, Beyotime, China), followed by incubation successively with primary (platelet endothelial cell adhesion molecule [CD31], ab28364, Abcam, USA) and secondary antibodies. Stained tissues were viewed using a confocal fluorescence microscope (Zeiss LSM510 META, Germany).

## 2.7. Reactive oxygen species (ROS) and mitochondrial ROS (mtROS)

Placental ROS and mtROS were determined using commercial kits (50101ES01, 40778ES50, Yeasen, China). Briefly, fresh placenta tissue was washed three times in pre-cooled PBS and then cut into small pieces of about 1 mm<sup>3</sup>. Subsequently, the pieces were placed on a 300-mesh nylon mesh net tied to a beaker, and the pieces were gently rubbed with a spatula and rinsed with PBS while rubbing. The obtained cell suspension was centrifuged at 500 × g for 10 min and the cells were washed once with PBS. Then, cell suspensions of uniform density (1 × 10<sup>7</sup> cells/mL) were incubated with 20 µmol/L 2,7-dichlorofluorescein diacetate or 5 mmol/L MitoSOX Red for 30 min at 37 °C. The cells were then washed twice with PBS to remove excess dye. Finally, the fluorescence intensity of the cells was measured using the fluorescence plate reader at excitation/emission = 488 nm/525 nm.

## 2.8. Measurement of mitochondrial DNA (mtDNA)

Total DNA was isolated from 200 mg of placenta using the Qiagen DNA Mini Kit (51306, Qiagen, Germany). Then, mtDNA was amplified using primers specific for the mitochondrial cytochrome *b* (forward: 5'-ATGAAACATTGAGTAGTCCTACTATTACC-3'; reverse: 5'-CTACGAGGTCTGTTCCGATATAAGG-3'), and normalized to genomic DNA by amplification of the 18S ribosomal RNA (forward: 5'-GGTAGT-GACGAAAATAACAATACAGGAC-3'; reverse: 5'-ATACGCTATTG-GAGCTGGAATTACC-3').

## 2.9. Biochemical analysis

Approximately 200 mg of placental samples were completely homogenized in saline and centrifuged at 12,000 × g for 5 min at 4 °C. The supernatant was then isolated for subsequent biochemical analysis. The levels of adenosine triphosphate (ATP; S0026, Beyotime, China), nicotinamide adenine dinucleotide reduced (NADH; S0175, Beyotime, China), nicotinamide adenine dinucleotide (NAD; S0175, Beyotime, China), malondialdehyde (MDA; S0131, Beyotime, China), Pro dehydrogenase (PRODH; BC4165, Solarbio, China), sirtuin 1 (Sirt1; MEIMIAN, China), and superoxide dismutase 2 (SOD2; A001-2-2, Nanjing Jiancheng Bioengineering Institute, China) were measured using commercial kits (Beyotime, China). Results were normalized to total protein (P0009, Beyotime, China) or weight.

## 2.10. Western blotting

Total proteins were extracted from 200 mg of placenta using a protein extraction kit (NCM, China). Then, protein concentration was detected using bicinchoninic acid protein assay kits (P0009, Beyotime, China). After being separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Merck Millipore) and blocked with bovine serum albumin Tris-Tween-buffered saline buffer. The membranes were then incubated with primary antibodies vascular endothelial growth factor-A (VEGF-A; 19003-1-AP, Proteintech, USA, 1:1000), basic fibroblast growth factor (bFGF; ab208687, Abcam, USA, 1:1500), signal transducer and activator of

transcription 3 (STAT3; ab76315, Abcam, USA, 1:1500), p-STAT3 (ab68153, Abcam, USA, 1:1500), NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFB8 (CI); A19732, ABclonal, China, 1:1000), succinate dehydrogenase complex flavoprotein subunit A (SDHA (CII); A13852, ABclonal, China, 1:1000), ubiquinol-cytochrome c reductase core protein 1 (UQCRC1 (CIII); A3339, ABclonal, China, 1:1000), cytochrome c oxidase IV subunit (COXIV (CIV); A6564, ABclonal, China, 1:1000), and ATPase beta chain (ATPB (CV); A5769, ABclonal, China, 1:1000), and  $\beta$ -actin (4970, CST, USA, 1:1000) and secondary antibodies (AS028, ABclonal, China), successively. Finally, images were captured using the ChemiDoc MP system (Bio-Rad, Hercules, CA, USA), and band densities were quantified using Image Lab software (Bio-Rad, Hercules, CA, USA) and then normalized to  $\beta$ -actin content.

### 2.11. Statistical analysis

All data were statistically analyzed using two-way analysis of variance (ANOVA). All data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests (with the significance level set at 5%) in SPSS 20.0 (SPSS, Inc, USA). The general linear model program of SPSS 22.0 statistics software was used for  $2 \times 3$  factor design analysis. Briefly, three diets with different Pro levels, two gilt body conditions, and their interactions were fixed factors. The linear model for analysis was:

$$Y_{ij} = \mu + d_i + b_j + R_{ij} + \varepsilon_{ij},$$

where  $Y_{ij}$  represents the observation of the dependent variable;  $\mu$  is the overall mean,  $d_i$  stands for the effect of the body condition ( $i$  = normal and obese gilts);  $b_j$  denotes the effect of the dietary Pro levels ( $j$  = L-Pro, M-Pro, and H-Pro diet);  $R_{ij}$  reflects the interactive effect between these two factors;  $\varepsilon_{ij}$  is the residual error associated with the observation.  $P_{(\text{body condition})}$  represents the main effect  $P$ -value of body condition. If the main effect of body condition was significant ( $P_{(\text{body condition})} < 0.05$ ), the  $t$ -tests were used to analyze the effect value of body condition under the three diets and significant differences ( $P < 0.05$ ), trend differences ( $0.05 \leq P < 0.01$ ) or no statistically differences ( $P \geq 0.10$ ) were uniformly expressed as \*, #, or NS, respectively, after the mean value of the obesity group.  $P_{(\text{Pro})}$  represents the  $P$ -value for the main effect of dietary Pro level, and if the main effect of the diet was significant ( $P_{(\text{Pro})} < 0.05$ ), it was subsequently subjected to multiple comparisons by the Bonferroni correction method and denoted by different lowercase letters (a, b, c).  $P_{(\text{body condition} \times \text{Pro})}$  represents the  $P$ -value for the interaction effect of diet and body condition. Rates of LBW piglets, stillbirths, and invalid piglets were analyzed using the Chi-square test with Bonferroni corrections. Pearson or Spearman correlation coefficient analysis was performed using the OmicStudio tools at <https://www.omicstudio.cn/tool>.

## 3. Results

### 3.1. Exp. 1: Impaired reproductive performance in obese gilts associated with inadequate placental Pro availability

Spearman correlation coefficients between litter performance, and body condition on G60d and placental amino acid abundance at term are presented in Table 2. BMI of G60d gilts was negatively correlated with piglet birth weight, piglet vitality, and number of NBW piglets ( $r = -0.62$ ,  $r = -0.31$ , and  $r = -0.32$ , respectively;  $P < 0.001$ ,  $P = 0.034$ , and  $P = 0.027$ , respectively), and positively correlated with number of LBW piglets ( $r = 0.46$ ;  $P = 0.001$ ). Backfat thickness of G60d gilts was negatively correlated with piglet birth

weight ( $r = -0.30$ ;  $P = 0.049$ ), and positively correlated with the number of LBW piglets ( $r = 0.30$ ;  $P = 0.044$ ). The correlation between body weight of G60d gilts and these parameters was not significant ( $P > 0.05$ ). Notably, Pro levels in the full-term placenta were positively correlated with piglet birth weight, piglet vitality and the number of NBW piglets ( $r = 0.87$ ,  $r = 0.32$ , and  $r = 0.43$ , respectively;  $P < 0.001$ ,  $P = 0.026$ , and  $P = 0.002$ , respectively), and negatively correlated with the number of LBW piglets ( $r = -0.49$ ;  $P < 0.001$ ). In addition, placental Pro levels at term were negatively correlated with BMI and backfat thickness of G60d gilts ( $r = -0.45$  and  $r = -0.34$ , respectively;  $P = 0.001$  and  $P = 0.019$ , respectively). These results suggest that impaired reproductive performance in obese gilts was associated with insufficient Pro availability in the placenta.

### 3.2. Exp. 2: Interaction between body condition and dietary Pro levels on farrowing performance and placental angiogenesis

We next investigated the potential effects of dietary Pro levels on placental function and farrowing performance in gilts, particularly in the context of obesity-related impaired pregnancy. The results in Table 2 showed that BMI and backfat thickness, but not body weight, were strongly correlated with impaired reproductive performance. Therefore, we used BMI and backfat thickness as body condition parameters for the gilts.

#### 3.2.1. Increasing dietary Pro levels reverses the impaired reproductive performance of obese gilts

The effect of body condition and dietary Pro level during gestation on the reproductive performance of gilts was first investigated (Table 3). For the body condition factor, obese gilts had significantly lower piglet birth weight ( $P_{(\text{body condition})} = 0.048$ ) and higher rates of LBW piglets ( $P_{(\text{body condition})} = 0.031$ ) compared to normal gilts. For the dietary Pro factor, the H-Pro group significantly increased piglet birth weight ( $P_{(\text{Pro})} = 0.001$ ), litter weight ( $P_{(\text{Pro})} < 0.001$ ), the number of NBW piglets ( $P_{(\text{Pro})} = 0.046$ ), and reduced the number of mummified fetuses ( $P_{(\text{Pro})} = 0.001$ ), the rates of LBW piglets ( $P_{(\text{Pro})} = 0.005$ ) and the rates of invalid piglets ( $P_{(\text{Pro})} = 0.029$ ) compared to the L-Pro group. Notably, an interaction effect was observed between body condition and dietary Pro levels in their effect on piglet birth weight ( $P_{(\text{body condition} \times \text{Pro})} = 0.046$ ) and within-litter birth weight variation ( $P_{(\text{body condition} \times \text{Pro})} = 0.012$ ).

#### 3.2.2. Increasing dietary Pro levels improves placental amino acid availability of obese gilts

The results of free amino acid concentration in serum and placenta of farrowing gilts are presented in Table 4. For the body condition factor, obese gilts had significantly lower Pro levels in serum ( $P_{(\text{body condition})} < 0.001$ ) compared to normal gilts. For the dietary Pro factor, increasing dietary Pro levels increased Pro levels in serum ( $P_{(\text{Pro})} < 0.001$ ).

For the body condition factor, obese gilts had significantly lower levels of lysine ( $P_{(\text{body condition})} = 0.014$ ), histidine ( $P_{(\text{body condition})} = 0.010$ ), glutamine ( $P_{(\text{body condition})} = 0.023$ ), threonine ( $P_{(\text{body condition})} = 0.010$ ), Pro ( $P_{(\text{body condition})} < 0.001$ ), valine ( $P_{(\text{body condition})} = 0.027$ ), methionine ( $P_{(\text{body condition})} = 0.029$ ), isoleucine ( $P_{(\text{body condition})} = 0.026$ ), leucine ( $P_{(\text{body condition})} = 0.025$ ) and phenylalanine ( $P_{(\text{body condition})} = 0.017$ ) in the placenta compared to normal gilts. For the dietary Pro factor, compared with the L-Pro group, the H-Pro group significantly increased the levels of arginine ( $P_{(\text{Pro})} < 0.001$ ), lysine ( $P_{(\text{Pro})} < 0.001$ ), histidine ( $P_{(\text{Pro})} < 0.001$ ), serine ( $P_{(\text{Pro})} = 0.046$ ), glutamine ( $P_{(\text{Pro})} < 0.001$ ), threonine ( $P_{(\text{Pro})} = 0.009$ ), Pro ( $P_{(\text{Pro})} = 0.006$ ), valine ( $P_{(\text{Pro})} < 0.001$ ), methionine ( $P_{(\text{Pro})} < 0.001$ ), tyrosine ( $P_{(\text{Pro})} < 0.001$ ), isoleucine

**Table 2**

Pearson correlations coefficient analysis of body condition of gilts on day 60 of gestation (G60d) and amino acid levels of full-term placentae with litter performance<sup>1</sup>.

Item	Piglet birth weight	Piglet vitality	Number of LBW piglets	Number of NBW piglets
BMI of G60d gilts	−0.62 (<0.001)	−0.31 (0.034)	0.46 (0.001)	−0.32 (0.027)
Backfat thickness of G60d gilts	−0.30 (0.049)	−0.09	0.30 (0.044)	−0.25
Body weight of G60d gilts	0.15	−0.09	−0.12	0.04
<b>Placental amino acid levels</b>				
Pro	0.87 (<0.001)	0.32 (0.026)	−0.49 (<0.001)	0.43 (0.002)
Valine	0.43 (0.002)	0.63 (<0.001)	−0.57 (<0.001)	0.15
Leucine	0.17	0.31 (0.034)	−0.32 (0.027)	−0.21
Asparagine	0.26	0.34 (0.019)	−0.35 (0.015)	−0.15
Threonine	0.25	0.31 (0.034)	−0.38 (0.008)	−0.16
Glutamate	0.41 (0.001)	0.18	−0.29	0.22
Glutamine	0.52 (<0.001)	0.09	−0.25	0.21
Arginine	0.49 (<0.001)	0.25	−0.23	0.10
Serine	0.43 (0.002)	0.02	−0.18	0.13
Glycine	0.12	0.19	−0.20	−0.18
Tryptophan	−0.04	0.30 (0.043)	−0.11	−0.24
Aspartate	0.27	0.32 (0.027)	−0.27 (0.037)	−0.18
Lysine	0.25	0.31 (0.031)	−0.23	−0.13
Methionine	0.14	0.28	−0.21	−0.23
Histidine	0.19	0.28	−0.15	−0.17
Alanine	0.10	0.21	−0.22	−0.20
Cysteine	0.11	0.01	0.01	−0.02
Isoleucine	0.02	0.29	−0.18	−0.24
Tyrosine	0.11	0.28	−0.24	−0.11
Item	BMI of G60d gilts	Backfat thickness of G60d gilts	Body weight of G60d gilts	
Placental Pro levels	−0.45 (0.001)	−0.34 (0.019)	0.26	

Pro = proline; BMI = body mass index; LBW = low-birth-weight; NBW = normal-birth-weight.

<sup>1</sup> n = 48 gilts or 48 placentae (1 placenta per gilt). The results in the table are shown as the Spearman correlation coefficient (P-value).

**Table 3**

Effects of body condition and dietary Pro levels during gestation on reproductive performance of gilts<sup>1</sup>.

Item	L-Pro		M-Pro		H-Pro		SD	P-value <sup>2</sup>		
	Normal	Obese	Normal	Obese	Normal	Obese		P <sub>(body condition)</sub>	P <sub>(Pro)</sub>	P <sub>(body condition × Pro)</sub>
Number of gilts	17	21	19	21	18	18				
<b>On G60d</b>										
BMI, kg/m <sup>2</sup>	62.7	67.9*	62.4	67.6*	63.0	67.1*	3.14	<0.001	0.737	0.356
Backfat thickness, mm	12.9	14.5*	13.0	14.3 <sup>#</sup>	12.9	14.3*	2.17	<0.001	0.982	0.922
Body weight, kg	162.7	164.0	160.8	163.4	160.0	162.9	11.22	0.292	0.781	0.950
Piglet vitality	2.6	2.5	2.8	2.6	2.6	2.6	0.27	0.302	0.117	0.335
Piglet birth weight, kg	1.3 <sup>a</sup>	1.2* <sup>a</sup>	1.4 <sup>b</sup>	1.3* <sup>b</sup>	1.4 <sup>c</sup>	1.5 <sup>NS c</sup>	0.21	0.048	0.001	0.046
Litter weight, kg	17.2 <sup>a</sup>	14.7 <sup>a</sup>	17.8 <sup>b</sup>	17.3 <sup>b</sup>	20.0 <sup>c</sup>	18.2 <sup>c</sup>	3.48	0.174	<0.001	0.190
Within-litter birth weight variation, %	17.8	20.1	15.6	19.6	19.3	15.8	5.68	0.384	0.459	0.012
<b>Number of piglets per litter</b>										
Total litter size	14.7	13.9	13.4	14.6	14.7	14.3	2.28	0.975	0.687	0.121
Live-born piglets	13.1	12.5	12.7	13.6	14.0	12.8	2.30	0.481	0.475	0.150
NBW piglets	11.8 <sup>a</sup>	10.6 <sup>a</sup>	12.1 <sup>ab</sup>	11.9 <sup>ab</sup>	12.9 <sup>b</sup>	12.3 <sup>b</sup>	2.45	0.116	0.046	0.675
LBW piglets	1.2	1.9	0.6	1.7	1.1	0.6	1.98	0.195	0.157	0.104
Stillbirth fetus	0.9	0.9	0.5	1.0	0.7	1.2	1.13	0.162	0.706	0.438
Malformed fetus	0.0	0.1	0.0	0.0	0.0	0.1	0.16	0.097	0.421	0.421
Mummified fetus	0.7 <sup>b</sup>	0.4 <sup>b</sup>	0.2 <sup>a</sup>	0.1 <sup>a</sup>	0.0 <sup>a</sup>	0.2 <sup>a</sup>	0.65	0.621	0.001	0.312
Invalid piglets	1.7	1.4	0.7	1.1	0.7	1.4	1.38	0.257	0.106	0.257
Rate of LBW piglets, %	9.5 <sup>b</sup>	14.9 <sup># b</sup>	5.0 <sup>ab</sup>	12.6* <sup>ab</sup>	7.5 <sup>a</sup>	4.3 <sup>NS a</sup>		0.031	0.005	
Rate of stillbirth fetus piglets, %	6.7	6.4	4.0	6.6	4.6	8.7		0.118	0.610	
Rate of invalid piglets, %	11.2 <sup>b</sup>	10.0 <sup>b</sup>	5.1 <sup>a</sup>	7.2 <sup>a</sup>	4.6 <sup>ab</sup>	10.1 <sup>ab</sup>		0.119	0.029	

Pro = proline; G60d = day 60 of gestation; SD = standard deviation; BMI = body mass index; NBW = normal-birth-weight; LBW = low-birth-weight.

Mean values with \*, #, and NS indicate significant ( $P_{(body\ condition)} < 0.05$ ), trend ( $0.05 \leq P_{(body\ condition)} < 0.10$ ), and no statistically difference ( $P_{(body\ condition)} \geq 0.10$ ) of body conditions under the same diet, respectively; mean values sharing no common letter (a to c) indicate a significant effect ( $P_{(Pro)} < 0.05$ ) of dietary Pro levels.

<sup>1</sup> Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet, Normal = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm; Obese = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm.

<sup>2</sup>  $P_{(body\ condition)}$  represents the main effect P-value of body condition;  $P_{(Pro)}$  represents the P-value for the main effect of dietary Pro level;  $P_{(body\ condition \times Pro)}$  represents the P-value for the interaction effect of diet and body condition.

( $P_{(Pro)} < 0.001$ ), leucine ( $P_{(Pro)} < 0.001$ ), phenylalanine ( $P_{(Pro)} = 0.001$ ), and tryptophan ( $P_{(Pro)} = 0.002$ ) in the placenta. Notably, an interaction effect was observed between body condition and dietary Pro levels in their effect on placental Pro levels ( $P_{(body\ condition \times Pro)} = 0.040$ ).

### 3.2.3. Increasing dietary Pro levels improves placental angiogenesis of obese gilts

A previous study in mice has reported that dietary Pro may improve reproductive performance by increasing placental angiogenesis (Liu et al., 2019). In addition, our previous study

**Table 4**  
Effects of body condition and dietary Pro level during gestation on serum and placental amino acid abundance of gilts<sup>1</sup>.

Item	L-Pro		M-Pro		H-Pro		SD	P-value <sup>2</sup>		
	Normal	Obese	Normal	Obese	Normal	Obese		$P_{(\text{body condition})}$	$P_{(\text{Pro})}$	$P_{(\text{body condition} \times \text{Pro})}$
Number of placentae per group	8	8	8	8	8	8				
Serum Pro levels, µg/mL	4.4 <sup>a</sup>	3.0* <sup>a</sup>	5.4 <sup>b</sup>	4.0* <sup>b</sup>	5.6 <sup>c</sup>	5.0 <sup>NS c</sup>	1.19	<0.001	<0.001	0.298
<b>Placental amino acid levels, mg/g wet tissue</b>										
Arginine	3.0 <sup>a</sup>	2.5 <sup># a</sup>	3.4 <sup>b</sup>	3.0 <sup>NS b</sup>	4.1 <sup>c</sup>	3.8 <sup>NS c</sup>	0.80	0.120	<0.001	0.873
Asparagine	0.3	0.2	0.3	0.3	0.4	0.3	0.10	0.055	0.064	0.717
Aspartate	0.1	0.1	0.2	0.1	0.2	0.2	0.10	0.410	0.276	0.895
Alanine	0.3	2.6	3.6	4.0	4.6	4.3	0.90	0.278	0.110	0.061
Glycine	0.6	0.8	0.7	0.7	1.0	0.9	0.50	0.941	0.353	0.633
Glutamine	1.2 <sup>a</sup>	0.9 <sup># a</sup>	1.2 <sup>a</sup>	1.1 <sup>NS a</sup>	1.7 <sup>b</sup>	1.4 <sup>NS b</sup>	0.40	0.023	<0.001	0.665
Glutamate	10.5	9.9	10.8	1.7	12.2	12.7	2.70	0.765	0.075	0.692
Histidine	1.1 <sup>a</sup>	0.9* <sup>a</sup>	1.1 <sup>a</sup>	1.0 <sup>NS a</sup>	1.3 <sup>b</sup>	1.2 <sup>NS b</sup>	0.20	0.010	<0.001	0.325
Isoleucine	25.3 <sup>a</sup>	16.2* <sup>a</sup>	27.9 <sup>b</sup>	25.1 <sup>NS b</sup>	35.0 <sup>c</sup>	33.5 <sup>NS c</sup>	8.90	0.026	<0.001	0.243
Leucine	27.3 <sup>a</sup>	17.5* <sup>a</sup>	30.1 <sup>b</sup>	27.1 <sup>NS b</sup>	37.8 <sup>c</sup>	36.1 <sup>NS c</sup>	9.60	0.025	<0.001	0.240
Lysine	2.5 <sup>a</sup>	1.8* <sup>a</sup>	2.8 <sup>b</sup>	2.0 <sup># a</sup>	3.9 <sup>b</sup>	3.2 <sup>NS b</sup>	1.10	0.014	<0.001	0.990
Methionine	1.7 <sup>a</sup>	1.2 <sup># a</sup>	1.9 <sup>a</sup>	1.4* <sup>a</sup>	2.4 <sup>b</sup>	2.3 <sup>NS b</sup>	0.70	0.029	<0.001	0.630
Serine	0.4 <sup>a</sup>	0.4 <sup>a</sup>	0.4 <sup>ab</sup>	0.4 <sup>ab</sup>	0.5 <sup>b</sup>	0.5 <sup>b</sup>	0.10	0.120	0.046	0.627
Threonine	0.7 <sup>a</sup>	0.5 <sup>NS a</sup>	0.8 <sup>a</sup>	0.6* <sup>a</sup>	0.8 <sup>b</sup>	0.8 <sup>NS b</sup>	0.20	0.010	0.009	0.314
Tyrosine	4.8 <sup>a</sup>	3.3 <sup>a</sup>	5.1 <sup>a</sup>	4.7 <sup>a</sup>	7.0 <sup>b</sup>	7.1 <sup>b</sup>	1.80	0.090	<0.001	0.221
Tryptophan	17.4 <sup>a</sup>	11.2 <sup>a</sup>	16.6 <sup>a</sup>	16.6 <sup>a</sup>	21.1 <sup>b</sup>	20.4 <sup>b</sup>	5.70	0.115	0.002	0.154
Pro	5.1 <sup>a</sup>	3.7* <sup>a</sup>	6.4 <sup>b</sup>	5.9 <sup># b</sup>	8.4 <sup>c</sup>	8.3 <sup>NS c</sup>	1.80	<0.001	0.006	0.040
Phenylalanine	15.5 <sup>a</sup>	9.6* <sup>a</sup>	15.7 <sup>a</sup>	13.5 <sup>NS a</sup>	19.6 <sup>b</sup>	18.3 <sup>NS b</sup>	5.20	0.017	0.001	0.308
Valine	20.5 <sup>a</sup>	16.5* <sup>a</sup>	20.6 <sup>a</sup>	19.2 <sup>NS a</sup>	24.0 <sup>b</sup>	23.2 <sup>NS b</sup>	3.90	0.027	<0.001	0.329

Pro = proline; SD = standard deviation.

Mean values with \*, #, and NS indicate significant ( $P_{(\text{body condition})} < 0.05$ ), trend ( $0.05 \leq P_{(\text{body condition})} < 0.10$ ), and no statistically difference ( $P_{(\text{body condition})} \geq 0.10$ ) of body conditions under the same diet, respectively; mean values sharing no common letter (a to c) indicate a significant effect ( $P_{(\text{Pro})} < 0.05$ ) of dietary Pro levels.

<sup>1</sup> Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet, Normal = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm; Obese = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm.

<sup>2</sup>  $P_{(\text{body condition})}$  represents the main effect P-value of body condition;  $P_{(\text{Pro})}$  represents the P-value for the main effect of dietary Pro level;  $P_{(\text{body condition} \times \text{Pro})}$  represents the P-value for the interaction effect of diet and body condition.

demonstrated the importance of STAT3 activation and downstream angiogenic factors (such as bFGF and VEGF-A) activation in porcine placental angiogenesis (Hu et al., 2021a; Wu et al., 2023b). Therefore, we analyzed the effect of body condition and dietary Pro levels during gestation on placental angiogenesis and the STAT3-bFGF/VEGF-A signaling pathway (Table 5 and Fig. 1).

For the body condition factor, compared with placentae from normal gilts, placentae from obese gilts had lower placental vascular density ( $P_{(\text{body condition})} = 0.008$ ), relative CD31 fluorescence intensity ( $P_{(\text{body condition})} < 0.001$ ), and the STAT3-bFGF/VEGF-A signaling pathway activity ( $P_{(\text{body condition})} = 0.010$ ,  $P_{(\text{body condition})} = 0.028$ , and  $P_{(\text{body condition})} < 0.001$ , respectively). For the dietary Pro factor, compared with the L-Pro group, the H-Pro group significantly increased the average placental weight ( $P_{(\text{Pro})} < 0.001$ ), placental efficiency ( $P_{(\text{Pro})} = 0.036$ ), placental vascular density

( $P_{(\text{Pro})} < 0.001$ ), relative CD31 fluorescence intensity ( $P_{(\text{Pro})} < 0.001$ ), and the STAT3-bFGF/VEGF-A signaling pathway activity ( $P_{(\text{Pro})} < 0.001$ ). There were also significant interactions between body condition and dietary Pro levels on placental vascular density ( $P_{(\text{body condition} \times \text{Pro})} = 0.007$ ) and relative CD31 fluorescence intensity ( $P_{(\text{body condition} \times \text{Pro})} = 0.042$ ).

### 3.2.4. Increasing dietary Pro levels alleviates placental mitochondrial oxidative stress and impaired mitochondrial function of obese gilts

Placental oxidative stress is closely related to angiogenesis (Huang et al., 2021). Next, mitochondrial oxidative stress and mitochondrial function of gilts were assessed (Table 6 and Fig. 2).

For the body condition factor, compared with placentae from normal gilts, placentae from obese gilts had higher relative ROS

**Table 5**  
Effects of body condition and dietary Pro level during gestation on placental angiogenesis of gilts<sup>1</sup>.

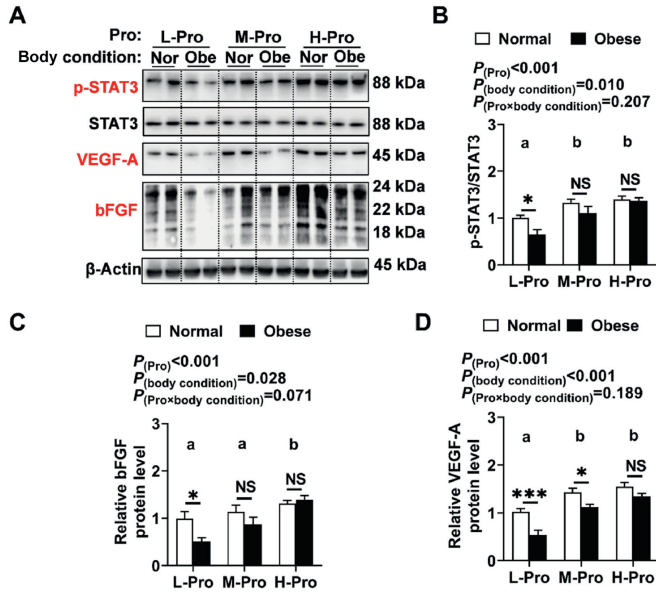
Item	L-Pro		M-Pro		H-Pro		SD	P-value <sup>2</sup>		
	Normal	Obese	Normal	Obese	Normal	Obese		$P_{(\text{body condition})}$	$P_{(\text{Pro})}$	$P_{(\text{body condition} \times \text{Pro})}$
Number of gilts per group	17	21	19	21	18	18				
Average placental weight, kg	0.21 <sup>a</sup>	0.20 <sup>a</sup>	0.23 <sup>b</sup>	0.22 <sup>b</sup>	0.23 <sup>b</sup>	0.22 <sup>b</sup>	0.102	0.176	<0.001	0.848
Placental efficiency	5.46 <sup>a</sup>	5.43 <sup>a</sup>	5.76 <sup>ab</sup>	5.39 <sup>ab</sup>	6.13 <sup>b</sup>	5.75 <sup>b</sup>	0.253	0.099	0.036	0.584
Number of placentae per group	6	6	6	6	6	6				
Placental vascular density, %	4.06 <sup>a</sup>	2.75* <sup>a</sup>	5.27 <sup>ab</sup>	3.47* <sup>ab</sup>	6.47 <sup>b</sup>	7.14 <sup>NS b</sup>	0.815	0.008	<0.001	0.007
Relative CD31 fluorescence intensity	1.00 <sup>a</sup>	0.65* <sup>a</sup>	1.52 <sup>b</sup>	0.91* <sup>b</sup>	1.82 <sup>c</sup>	1.81 <sup>NS c</sup>	0.382	<0.001	<0.001	0.042

Pro = proline; CD31 = platelet endothelial cell adhesion molecule; SD = standard deviation.

Mean values with \*, #, and NS indicate significant ( $P_{(\text{body condition})} < 0.05$ ), trend ( $0.05 \leq P_{(\text{body condition})} < 0.10$ ), and no statistically difference ( $P_{(\text{body condition})} \geq 0.10$ ) of body conditions under the same diet, respectively; mean values sharing no common letter (a to c) indicate a significant effect ( $P_{(\text{Pro})} < 0.05$ ) of dietary Pro levels.

<sup>1</sup> Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet, Normal = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm; Obese = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm.

<sup>2</sup>  $P_{(\text{body condition})}$  represents the main effect P-value of body condition;  $P_{(\text{Pro})}$  represents the P-value for the main effect of dietary Pro level;  $P_{(\text{body condition} \times \text{Pro})}$  represents the P-value for the interaction effect of diet and body condition.



**Fig. 1.** Effects of body condition and dietary Pro level during gestation on the STAT3-bFGF/VEGF-A signaling pathway of gilts. (A) Immunoblots of p-STAT3, STAT3, bFGF, and VEGF-A in the placentae of gilts. (B) Quantification of p-STAT3/STAT3 (CI). (C) Quantification of bFGF. (D) Quantification of VEGF-A. Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. Nor = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm, Obe = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm, L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet. Pro = proline; STAT3 = signal transducer and activator of transcription 3; VEGF-A = vascular endothelial growth factor-A, bFGF = basic fibroblast growth factor. n = 6 placentae from 6 litters per group. Mean values with \*, #, and NS indicate significant (\* $P_{(body\ condition)} < 0.05$ , \*\* $P_{(body\ condition)} < 0.01$ , \*\*\* $P_{(body\ condition)} < 0.001$ ), trend ( $0.05 \leq P_{(body\ condition)} < 0.10$ ), and no statistically difference ( $P_{(body\ condition)} \geq 0.10$ ) of body condition factor under the same diet, respectively; mean values sharing no common letter (a and b) indicate a significant effect ( $P_{(Pro)} < 0.05$ ) of dietary Pro levels factor.

level ( $P_{(body\ condition)} < 0.001$ ), relative mtROS level ( $P_{(body\ condition)} < 0.001$ ), and MDA levels ( $P_{(body\ condition)} < 0.001$ ), but lower ATP level ( $P_{(body\ condition)} < 0.001$ ), relative NDUFB8 (CI) protein level ( $P_{(body\ condition)} = 0.003$ ) and relative COXIV (CIV) protein level ( $P_{(body\ condition)} < 0.001$ ). For the dietary Pro factor, placentae from the H-Pro group had lower relative ROS level ( $P_{(Pro)} < 0.001$ ) and relative mtROS level ( $P_{(Pro)} = 0.009$ ), but high levels of ATP ( $P_{(Pro)} = 0.018$ ), relative NDUFB8 (CI) protein level ( $P_{(Pro)} < 0.001$ ) and relative COXIV (CIV) protein level ( $P_{(Pro)} < 0.001$ ) compared to placentae from the L-Pro group. Notably, there was a significant

interaction between body condition and dietary Pro levels on the relative levels of ROS ( $P_{(body\ condition \times Pro)} = 0.043$ ), mtROS ( $P_{(body\ condition \times Pro)} = 0.012$ ), and relative COXIV (CIV) protein level ( $P_{(body\ condition \times Pro)} = 0.027$ ).

**3.2.5. Benefits of dietary Pro may be related to the placental NAD-Sirt1-SOD2-ROS axis**

The NAD-Sirt1-SOD2 axis is strongly associated with placental oxidative stress, especially with mtROS imbalance (Silveira et al., 2019; Wątroba et al., 2023). Therefore, we determined the levels of the placental NAD-Sirt1-SOD2-ROS axis (Table 7). For the body condition factor, compared with placentae from normal gilts, placentae from obese gilts had lower SOD2 activity ( $P_{(body\ condition)} = 0.004$ ), relative NAD/NADH ( $P_{(body\ condition)} < 0.001$ ), and PRODH activity ( $P_{(body\ condition)} = 0.035$ ). For the dietary Pro factor, placentae from the H-Pro group had higher SOD2 activity ( $P_{(Pro)} = 0.003$ ), relative NAD/NADH ( $P_{(Pro)} < 0.001$ ), PRODH activity ( $P_{(Pro)} < 0.001$ ) and Sirt1 activity ( $P_{(Pro)} = 0.003$ ) compared to placentae from the L-Pro group. Notably, there was a significant interaction between body condition and dietary Pro levels on placental relative NAD/NADH ( $P_{(body\ condition \times Pro)} = 0.026$ ) and PRODH activity ( $P_{(body\ condition \times Pro)} = 0.028$ ).

Furthermore, Pearson correlation coefficient analysis (Table 8) showed that piglet birth weight was negatively correlated with placental relative ROS levels ( $r = -0.29$ ;  $P = 0.045$ ) and positively correlated with placental vascular density ( $r = 0.43$ ;  $P = 0.002$ ), placental relative NAD/NADH ( $r = 0.32$ ;  $P = 0.025$ ), placental Sirt1 activity ( $r = 0.39$ ;  $P = 0.020$ ) and placental SOD2 activity ( $r = 0.39$ ;  $P = 0.006$ ). Relative placental ROS level was also negatively correlated with placental relative NAD/NADH ( $r = -0.47$ ;  $P = 0.001$ ), placental SOD2 activity ( $r = -0.40$ ;  $P = 0.005$ ) and placental vascular density ( $r = -0.52$ ;  $P < 0.001$ ). In addition, placental vascular density was negatively correlated with placental relative mtROS level ( $r = -0.37$ ;  $P = 0.010$ ), and positively correlated with placental relative NAD/NADH ( $r = 0.54$ ;  $P < 0.001$ ) and placental SOD2 activity ( $r = 0.64$ ;  $P < 0.001$ ). These results suggest that the effect of dietary Pro on birth weight may be related to the improvement of placental angiogenesis and the NAD-Sirt1-SOD2-ROS axis.

**4. Discussion**

During mid-gestation, dietary energy is supplied primarily for the maintenance of the gilts' body components and the growth of the fetuses. Particular attention must be paid to avoid excessive fat gain during this period as this could lead to obesity-related

**Table 6**  
Effects of body condition and dietary Pro level during gestation on placental mitochondrial oxidative stress and mitochondrial function of gilts.<sup>1</sup>

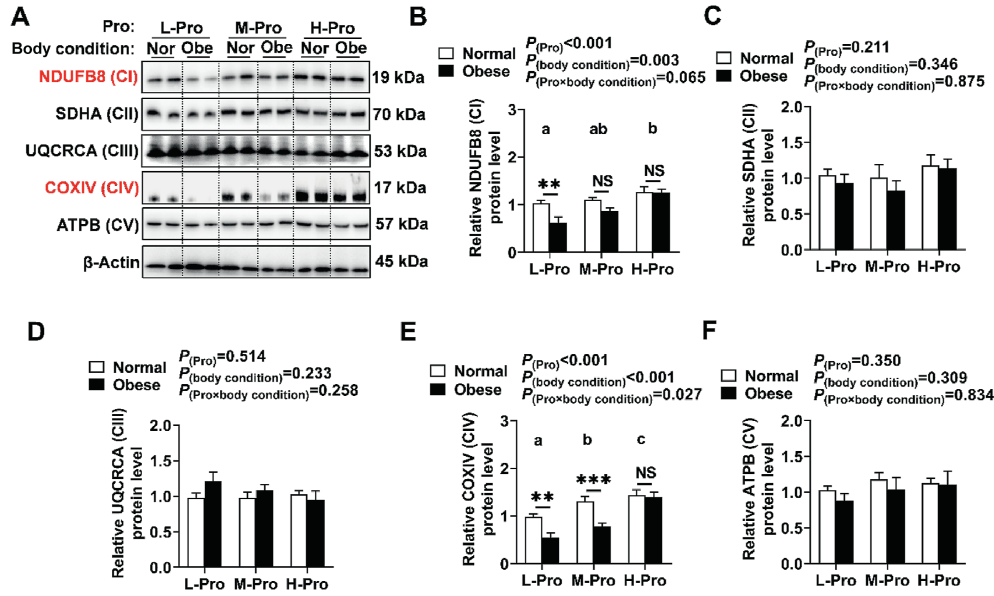
Item	L-Pro		M-Pro		H-Pro		SD	$P$ -value <sup>2</sup>		
	Normal	Obese	Normal	Obese	Normal	Obese		$P_{(body\ condition)}$	$P_{(Pro)}$	$P_{(body\ condition \times Pro)}$
Number of placentae per group	6	6	6	6	6	6				
Relative ROS level	1.00 <sup>b</sup>	1.62* <sup>b</sup>	0.93 <sup>ab</sup>	1.21 <sup>NS</sup> <sup>ab</sup>	0.78 <sup>a</sup>	0.84 <sup>NS</sup> <sup>a</sup>	0.310	<0.001	<0.001	0.043
Relative mtROS level	1.00 <sup>b</sup>	1.85* <sup>b</sup>	0.93 <sup>ab</sup>	1.40* <sup>ab</sup>	0.99 <sup>a</sup>	1.19 <sup>NS</sup> <sup>a</sup>	0.251	<0.001	0.009	0.012
MDA level, nmol/mg prot	0.52	0.70 <sup>#</sup>	0.38	0.67* <sup>#</sup>	0.42	0.61 <sup>#</sup>	0.198	<0.001	0.258	0.629
Relative mtDNA level	1.00	1.05	1.35	1.17	1.41	1.20	0.300	0.583	0.362	0.852
ATP level, nmol/mg prot	2.37 <sup>a</sup>	1.38* <sup>a</sup>	2.61 <sup>ab</sup>	1.76 <sup>#</sup> <sup>ab</sup>	2.83 <sup>b</sup>	2.43 <sup>NS</sup> <sup>b</sup>	0.689	<0.001	0.018	0.500

Pro = proline; ROS = reactive oxygen species, mtROS = mitochondrial reactive oxygen species, MDA = malondialdehyde, mtDNA = mitochondrial DNA, ATP = adenosine triphosphate; SD = standard deviation.

Mean values with \*, #, and NS indicate significant ( $P_{(body\ condition)} < 0.05$ ), trend ( $0.05 \leq P_{(body\ condition)} < 0.10$ ), and no statistically difference ( $P_{(body\ condition)} \geq 0.10$ ) of body conditions under the same diet, respectively; mean values sharing no common letter (a and b) indicate a significant effect ( $P_{(Pro)} < 0.05$ ) of dietary Pro levels.

<sup>1</sup> Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet, Normal = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm; Obese = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm.

<sup>2</sup>  $P_{(body\ condition)}$  represents the main effect  $P$ -value of body condition;  $P_{(Pro)}$  represents the  $P$ -value for the main effect of dietary Pro level;  $P_{(body\ condition \times Pro)}$  represents the  $P$ -value for the interaction effect of diet and body condition.



**Fig. 2.** Effects of body condition and dietary Pro level during gestation on placental mitochondrial complexes of gilts. (A) Immunoblots of NDUFB8 (CI), SDHA (CII), UQCRC1 (CIII), COXIV (CIV), and ATPB (CV) in the placentae of gilts. (B) Quantification of NDUFB8 (CI). (C) Quantification of SDHA (CII). (D) Quantification of UQCRC1 (CIII). (E) Quantification of COXIV (CIV). (F) Quantification of ATPB (CV). Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. Nor = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm, Obe = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm, L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet. Pro = proline; NDUFB8 (CI) = NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8; SDHA (CII) = succinate dehydrogenase complex flavoprotein subunit A; UQCRC1 (CIII) = ubiquinol-cytochrome c reductase core protein 1; COXIV (CIV) = cytochrome c oxidase IV subunit; ATPB (CV) = ATPase beta chain. n = 6 placentae from 6 litters per group. Mean values with \*, #, and NS indicate significant (\* $P_{(body\ condition)} < 0.05$ , \*\* $P_{(body\ condition)} < 0.01$ , \*\*\* $P_{(body\ condition)} < 0.001$ ), trend ( $0.05 \leq P_{(body\ condition)} < 0.10$ ), and no statistically difference ( $P_{(body\ condition)} \geq 0.10$ ) of significant body condition factor under the same diet, respectively; Mean values sharing no common letter (a to c) indicate a significant effect ( $P_{(Pro)} < 0.05$ ) of dietary Pro levels factor.

**Table 7**

Effects of body condition and dietary Pro level during gestation on levels of SOD2, NAD/NADH ratio, PRODH and Sirt1 in placenta<sup>1</sup>.

Item	L-Pro		M-Pro		H-Pro		SD	P-value <sup>2</sup>	$P_{(body\ condition)}$	$P_{(Pro)}$	$P_{(body\ condition \times Pro)}$
	Normal	Obese	Normal	Obese	Normal	Obese					
Number of placentae per group	6	6	6	6	6	6	0.884	0.004	0.003	0.318	
Placental SOD2 activity, U/mg prot	3.31 <sup>a</sup>	1.73* <sup>a</sup>	4.16 <sup>b</sup>	2.85* <sup>b</sup>	4.22 <sup>b</sup>	4.45 <sup>NS b</sup>	0.226	<0.001	<0.001	0.026	
Placental relative NAD/NADH	1.00 <sup>a</sup>	0.66* <sup>a</sup>	1.05 <sup>a</sup>	0.84* <sup>a</sup>	1.26 <sup>b</sup>	1.20 <sup>NS b</sup>	33.535	0.035	<0.001	0.028	
Placental PRODH activity, μmol/g tissue per min	283.76 <sup>a</sup>	193.75* <sup>a</sup>	312.97 <sup>b</sup>	287.97 <sup>NS b</sup>	329.12 <sup>b</sup>	343.63 <sup>NS b</sup>	0.282	0.340	0.003	0.060	
Placental Sirt1 activity, ng/mg prot	5.89 <sup>a</sup>	4.59* <sup>a</sup>	5.27 <sup>a</sup>	5.63 <sup>NS a</sup>	6.45 <sup>b</sup>	6.54 <sup>NS b</sup>					

Pro = proline; SOD2 = superoxide dismutase 2; NAD/NADH = nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide reduced; PRODH = proline dehydrogenase; Sirt1 = sirtuin 1; SD = standard deviation.

Mean values with \*, #, and NS indicate significant ( $P_{(body\ condition)} < 0.05$ ), trend ( $0.05 \leq P_{(body\ condition)} < 0.10$ ), and no statistically difference ( $P_{(body\ condition)} \geq 0.10$ ) of body conditions under the same diet, respectively; mean values sharing no common letter (a and b) indicate a significant effect ( $P_{(Pro)} < 0.05$ ) of dietary Pro levels.

<sup>1</sup> Statistical significance was determined by 2 × 3 factorial treatment arrangement, factor 1 = body conditions, factor 2 = dietary Pro levels. L-Pro = low Pro level (0.89%) diet, M-Pro = medium Pro level (1.39%) diet, H-Pro = high Pro level (1.89%) diet, Normal = gilts with an average body mass index of 62.7 kg/m<sup>2</sup> and an average backfat thickness of 12.9 mm; Obese = gilts with an average body mass index of 67.6 kg/m<sup>2</sup> and an average backfat thickness of 14.4 mm.

<sup>2</sup>  $P_{(body\ condition)}$  represents the main effect P-value of body condition;  $P_{(Pro)}$  represents the P-value for the main effect of dietary Pro level;  $P_{(body\ condition \times Pro)}$  represents the P-value for the interaction effect of diet and body condition.

complications that could affect gilts' metabolism, litter performance and, in particular, placental function. Overfeeding gilts causes obesity, induces disturbances in glucolipid metabolism, and increases the levels of triglyceride and non-esterified fatty acids in the gilts' placenta. It also reduces placental vascularization by decreasing the expression of proteins associated with angiogenesis, vasculogenesis and endothelial permeability (Hu et al., 2021a, 2019). However, in actual sow production, inadequate maternal body condition due to inappropriate feeding strategies is one of the major causes of poor reproductive health (Muro et al., 2023). In the present study, the body condition of G60d gilts showed a significant negative correlation with litter performance (piglet birth weight and number of NBW piglets). This result was consistent with

previous observations, which reported a negative correlation between backfat thickness of gilts at the end of gestation and piglet birth weight (Zhou et al., 2018). Thus, in the present study, obese gilt on G60d was used as an animal model for impaired pregnancy outcomes.

Amino acid supplementation during pregnancy may be a promising strategy for diminishing the incidence of LBW piglets. For example, a lack of arginine and glutamine in the diet could damage the development of the placenta and embryo (Huang et al., 2021; Tan et al., 2022). Consistently, arginine or glutamine supplementation during gestation can improve reproductive performance and enhance embryonic growth and survival, indicating a functional role of amino acids in pregnant mammals (Li et al., 2023;

**Table 8**

Pearson correlation coefficient analysis between piglet birth weight, placental vascular density, placental ROS and placental PRODH, placental SOD2, placental Sirt1, and placental NAD/NADH levels<sup>1</sup>.

Item	Placental relative ROS levels	Placental relative mtROS levels	Placental relative NAD/NADH	Piglet birth weight	Placental SOD2 activity	Placental vascular density	Placental PRODH activity	Placental Sirt1 activity
Placental relative ROS levels	1.00	–	–	–	–	–	–	–
Placental relative mtROS levels	0.45 (0.001)	1.00	–	–	–	–	–	–
Placental relative NAD/NADH	–0.47 (0.001)	–0.54 (<0.001)	1.00	–	–	–	–	–
Piglet birth weight	–0.29 (0.045)	–0.22	0.32 (0.025)	1.00	–	–	–	–
Placental SOD2 activity	–0.40 (0.005)	–0.37 (0.011)	0.32 (0.025)	0.39 (0.006)	1.00	–	–	–
Placental vascular density	–0.52 (<0.001)	–0.37 (0.010)	0.54 (<0.001)	0.43 (0.002)	0.64 (<0.001)	1.00	–	–
Placental PRODH activity	–0.19	–0.05	0.19	0.02	0.27	0.07	1.00	–
Placental Sirt1 activity	–0.04	–0.14	0.10	0.39 (0.020)	0.47 (0.004)	0.27	0.30	1.00

ROS = reactive oxygen species; SOD2 = superoxide dismutase 2; NAD/NADH = nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide reduced; PRODH = proline dehydrogenase; Sirt1 = sirtuin 1.

<sup>1</sup> The results in the table are shown as the Pearson correlation coefficient (*P*-value).

Wu et al., 2017, 2013a). In recent years, there has been increasing interest in the study of Pro metabolism and nutrition. For example, previous studies in mice have shown that dietary supplementation with Pro improves fetal survival at embryonic stage and reproductive performance at term (Liu et al., 2019). This is one of the few *in vivo* studies demonstrating a functional role for Pro in fetal survival and placental development in animals. However, National Research Council (2012) suggests that supplemental Pro (the physiological isomer) is not required in the diet of pregnant sows to achieve their maximum production performance. This reflects a lack of knowledge about Pro biochemistry and nutrition in mammals. Importantly, gilts are fed plant-based diets during gestation, which generally contain a low level of Pro (Hou et al., 2019; Li et al., 2011). The Pro levels in wheat-based diets for gestating sows, which are widely used by several large pig producers in China (actual measured value: 0.65% to 0.90%, unpublished data), were much lower than the recommended amounts reported by Wu et al. (recommended value: 1.03% to 1.53%) (Wu et al., 2013b). Furthermore, a previous study in Huanjiang pigs has reported that a diet containing 1.67% Pro during pregnancy could promote fetal and placental growth and development and reduce the incidence of IUGR (Zha et al., 2016). Therefore, based on the above reports, we configured three diets with three levels of Pro (basal level-0.89% [0.65% to 0.90%]), recommended level-1.39% [1.03% to 1.53%], and high dose –1.89% [>1.53%], respectively) to test their intervention effects on litter performance and placental function.

It is worth noting that the effect of Pro supplementation on sow reproductive performance may be strongly modulated by maternal characteristics. A previous study on Landrace × Yorkshire cross-breed inseminated sows demonstrated that the effect of Pro supplementation on litter size and birth weight was strongly modulated by parity and prolificacy; Pro improved litter size, piglet birth weight and litter uniformity in second-parity sows and highly-prolific primiparous gilts, but had no significant effect on the reproductive performance of mature three-parity sows (Gonzalez-Añover and Gonzalez-Bulnes, 2017). Highly-prolific first-parity gilts have a higher requirement for protein deposition than older sows (Young et al., 2005). Thus, the necessity of adequate availability of Pro in protein synthesis would explain the beneficial effect of Pro supplementation in highly prolific first-parity gilts (Gonzalez-Añover and Gonzalez-Bulnes, 2017). In the present study, we showed that increasing dietary Pro levels reversed the reduced piglet birth weight of obese gilts, suggesting that Pro is a key regulator for maximizing reproductive performance in gilts. However, in the present study, we did not assess the effect of Pro on nitrogen metabolism and protein deposition in gilts of different body conditions, which needs to be included in future studies.

Reduced Pro availability of conceptus may contribute to reduced placental and fetal growth both in natural IUGR and in response to dietary protein deficiency (Schoknecht et al., 1994; Wu et al., 2006). Therefore, we first investigated the effect of sow obesity on Pro availability and found that both circulating and placental Pro levels were significantly reduced in obese gilts. Circulating Pro concentrations are mainly influenced by dietary Pro, intestinal Pro degradation and endogenous Pro synthesis (Wu et al., 2011). On the one hand, the gut flora of obese mothers has a high Pro-degrading capacity (e.g., high abundance of *Prevotella*), which may lead to lower circulating Pro levels (Mayneris-Perxachs et al., 2022). On the other hand, we suspected that the decrease in placental Pro abundance in obese gilts may also be related to placental Pro transport. Previous studies have reported decreased Pro transport (e.g., solute carrier family 6 member 20 and proline transporter) activity in malnutrition-induced LBW placentae compared to NBW placentae (Wu et al., 2008). Although previous studies have shown that there is a mechanistic relationship between placental amino acid transport and maternal obesity (Gallo et al., 2017), there are few reports on placental Pro transporters in obese mothers. Therefore, these inferences need further validation.

Interestingly, we noted that increasing dietary Pro levels not only increased placental Pro levels, but also increased the abundance of other amino acids such as arginine, glutamine, histidine, isoleucine, leucine, and lysine in the placenta. Changes in placental Pro levels are easy to understand because placental Pro can be regulated by circulating Pro, which is dynamically regulated by dietary Pro. However, for the other amino acids whose levels are up-regulated, on the one hand, dietary Pro improves placental angiogenesis and increases blood flow, which naturally increases the concentration of other amino acids in the placenta. On the other hand, Pro supplementation may regulate the expression of non-Pro transporters in the placenta (Liu et al., 2019). Previous studies have reported that the mRNA levels of solute carrier family 36 member 4 (a Pro transporter protein) and other neutral amino acid transporters (solute carrier family 6 member 14, solute carrier family 38 member 2, and solute carrier family 38 member 4) were significantly increased in the placentae of Pro-supplemented mice (Liu et al., 2019).

Increased oxidative stress during mid and late gestation could lead to a higher percentage of stillbirths and LBW piglets, which is a substantial cause of economic loss in livestock production (Jonker, 2004). The placenta is particularly sensitive to ROS, and oxidative stress could lead to poor angiogenesis and barrier damage. In the present study, maternal obesity increased the level of placental oxidative stress and decreased placental angiogenesis, which is consistent with the previous findings (Mele et al., 2014). Emerging

evidence suggests that increased Pro availability reduces oxidative stress (Kaul et al., 2008; Wang et al., 2020). Our results further demonstrate that dietary Pro reversed obesity-induced oxidative stress and impaired angiogenesis, suggesting that Pro may play a protective role by alleviating oxidative stress. A large number of previous studies have confirmed the importance of oxidative stress-STAT3 signaling and the expression of downstream angiogenesis-related factors in placental and fetal development. For instance, STAT3, a key regulator of the adaptive response to oxidative stress (particularly mtROS) in the placenta, modulates the expression of VEGF-A and bFGF, two of the most important factors promoting placental angiogenesis (Hu et al., 2021a; Pereira et al., 2015). Changes in STAT3 activity may be related to the increased oxidative stress and the altered trophoblast invasion and placental angiogenesis. In our previous study, placental NADPH oxidase 2 (a major generator of ROS) inhibited nuclear p-STAT3 and the expression of downstream angiogenesis-related factors (VEGF-A) by inducing the production of mtROS. Therefore, the above signaling pathways were further determined in the present study. We provide evidence that the effect of increasing dietary Pro levels to improve placental angiogenesis in obese gilts may be related to activation of the STAT3-bFGF/VEGF-A pathway and a reduction of ROS. Inconsistent with our results, studies in mice have shown that the regulatory effects of Pro on farrowing performance and placental vascular development were associated with activation of the mechanistic target of rapamycin complex 1 (a critical signaling pathway for protein synthesis) and suppression of the general control nonderepressible 2-eukaryotic translation initiation factor 2 subunit alpha-activating transcription factor 4 signaling pathway in the placental tissue (Liu et al., 2019). However, the mechanism needs further investigation.

The molecular mechanism by which Pro alleviates oxidative stress is controversial. On one hand, the *in vitro* graft copolymerization assay system shows that Pro has a similar ability to directly scavenge ROS as glutathione (Kaul et al., 2008) and on the other hand, Pro can alleviate oxidative stress by regulating the SOD levels. Previous studies have reported that dietary Pro supplementation can increase SOD activity and decrease plasma MDA levels of piglets (Kang et al., 2014). In particular, SOD2, which is targeted to the mitochondrial matrix, plays a crucial role in the removal of mtROS (Wu et al., 2022; Zhu et al., 2012). Furthermore, Sirt1 regulates the enzymatic activity of SOD2 through NAD substrate-dependent mediated transcriptional expression of *SOD2* (Wu et al., 2022; Zhu et al., 2012). In the present study, Pro reversed the obesity-induced increase in mtROS. Therefore, to better understand the molecular mechanism of Pro in the regulation of oxidative stress homeostasis in the porcine placenta, we determined the activity of the Sirt1-SOD2 axis in the placenta. In the present study, increased dietary Pro levels reversed the reduced placental SOD2 activity and relative NAD/NADH in obese gilts. Furthermore, correlation coefficient analysis showed that dietary Pro on birth weight may be related to the improvement of placental angiogenesis and NAD-Sirt1-SOD2-ROS axis. Thus, we suspected that the role of Pro in improving placental oxidative stress and angiogenesis, and litter performance could be explained, at least in part, by the NAD-Sirt1-SOD2-ROS axis.

The porcine placenta has a high capacity for Pro catabolism (Wu et al., 2008). Reduced placental and fetal growth is associated with a reduction in placental PRODH (a key Pro-degrading enzyme) activity in pregnant dams with either naturally occurring or malnutrition-induced growth retardation (Wu et al., 2008). The PRODH activity in fetal fluids of gilts fed a 0.5% protein diet was reduced by 80% compared to gilts fed a protein adequate diet (Wu et al., 2008). In the present study, increased dietary Pro levels reversed the reduced placental PRODH activity in obese gilts,

suggesting that Pro-PRODH catabolism may play a key role in regulating piglet birth weight. In addition, our unpublished *in vitro* data suggest that Pro-PRODH catabolism may act as a metabolic switch for the NAD-Sirt1 pathway. However, further studies are required to elucidate the underlying mechanisms responsible for this regulation in the future.

There are still some limitations about Pro nutrition that need to be addressed in future studies. Firstly, the effect of dietary supplementation with any amino acid depends on its supplemental dose and the amount of other amino acids in the diet. For example, Pro supplementation has no effect on piglet growth when glutamine is deficient in the basal diet (Chung and Baker, 2010). Therefore, in order to maximize the potential of Pro to improve the reproductive performance of sows, the effects of diet type, dietary amino acid composition, and the ideal amino acid pattern must be fully considered. Secondly, in the present study, the gradient of dietary Pro levels was insufficient and the highest dose of Pro (1.89%) had the best effect. Future studies need to focus on whether very high doses of dietary Pro (>1.89%) may have deleterious effects on reproductive performance and other phenotypes in gilts. Thirdly, placental vascular development is also influenced by other maternal factors, such as parity, litter size, breed and gestational season (Groothuis et al., 2005). Therefore, future studies need to focus on Pro requirements under these specific maternal factors. Finally, this study examined the interaction between Pro and obesity-related pregnancy outcomes. However, the potential role of Pro metabolism in other gestational metabolic disorders, such as gestational glucose intolerance and insulin resistance in sows, deserves further investigation.

## 5. Conclusions

In the present study, impaired reproductive performance in obese gilts was associated with inadequate Pro availability. Importantly, dietary inclusion of an adequate amount of Pro (1.39% to 1.89%) from G60d to parturition could increase average piglet birth weight, litter weight, number of NBW piglets, and reduce the number of mummified fetuses, LBW rates and invalid piglet rates. Interaction effects were observed between body condition and dietary Pro levels in their effect on piglet birth weight, within-litter birth weight variation, and placental vascular density. Overall, increasing dietary Pro levels was shown to reverse the reduced piglet birth weight of obese gilts. The positive effect of Pro on litter performance may be related to the improvement of Sirt1-SOD2-ROS axis homeostasis and angiogenesis in the placenta.

## CRedit authorship contribution statement

**Shuangbo Huang:** Writing – original draft, Methodology, Investigation, Data curation. **Jinfeng Li:** Investigation, Data curation. **Hongxuan Ye:** Investigation. **Zihao Huang:** Investigation. **Junyi Wu:** Investigation. **Liudan Liu:** Investigation. **Shuo Ma:** Investigation. **Hefeng Luo:** Resources. **Tanghong Wei:** Resources. **Kai Liu:** Resources. **Jinping Deng:** Conceptualization. **Dingfa Liu:** Resources. **Chengquan Tan:** Writing – review & editing, Project administration, Methodology, Conceptualization.

## Declaration of competing 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

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## References

- Aplin JD, Myers JE, Timms K, Westwood M. Tracking placental development in health and disease. *Nat Rev Endocrinol* 2020;16:479–94.
- Bai G, Jiang X, Qin J, Zou Y, Zhang W, Teng T, et al. Perinatal exposure to glyphosate-based herbicides impairs progeny health and placental angiogenesis by disturbing mitochondrial function. *Environ Int* 2022;170:107579.
- Brombach C, Tong W, Giussani DA. Maternal obesity: new placental paradigms unfolded. *Trends Mol Med* 2022;28:823–35.
- Chung TK, Baker DH. A note on the dispensability of proline for weanling pigs. *Anim Prod* 2010;56:407–8.
- Council NR. Nutrient requirements of swine. 2012.
- D'Inca R, Kloareg M, Gras-Le Guen C, Le Huërou-Luron I. Intrauterine growth restriction modifies the developmental pattern of intestinal structure, transcriptomic profile, and bacterial colonization in neonatal pigs. *J Nutr* 2010;140:925–31.
- Farmer C, Edwards SA. Review: improving the performance of neonatal piglets. *Animal* 2022;16(Suppl 2):100350.
- Gallo LA, Barrett HL, Dekker Nitert M. Review: placental transport and metabolism of energy substrates in maternal obesity and diabetes. *Placenta* 2017;54:59–67.
- Gonzalez-Anover P, Gonzalez-Bulnes A. Maternal age modulates the effects of early-pregnancy L-proline supplementation on the birth-weight of piglets. *Anim Reprod Sci* 2017;181:63–8.
- Groothuis PG, Nap AW, Winterhager E, Grümmer R. Vascular development in endometriosis. *Angiogenesis* 2005;8:147–56.
- Hou Y, He W, Hu S, Wu G. Composition of polyamines and amino acids in plant-source foods for human consumption. *Amino Acids* 2019;51:1153–65.
- Hu C, Wu Z, Huang Z, Hao X, Wang S, Deng J, et al. Nox2 impairs VEGF-A-induced angiogenesis in placenta via mitochondrial ROS-STAT3 pathway. *Redox Biol* 2021a;45:102051.
- Hu C, Yan Y, Ji F, Zhou H. Maternal obesity increases oxidative stress in placenta and it is associated with intestinal microbiota. *Front Cell Infect Microbiol* 2021b;11:671347.
- Hu C, Yang Y, Li J, Wang H, Cheng C, Yang L, et al. Maternal diet-induced obesity compromises oxidative stress status and angiogenesis in the porcine placenta by upregulating Nox2 expression. *Oxid Med Cell Longev* 2019;2019:2481592.
- Huang S, Zhang L, Luo J, Wu D, Ma K, Chen Y, et al. Cysteamine and N-Acetyl-cysteine alleviate placental oxidative stress and barrier function damage induced by deoxynivalenol. *J Agric Food Chem* 2023;71:6846–58.
- Huang Z, Huang S, Song T, Yin Y, Tan C. Placental angiogenesis in mammals: a review of the regulatory effects of signaling pathways and functional nutrients. *Adv Nutr* 2021;12:2415–34.
- Jonker FH. Fetal death: comparative aspects in large domestic animals. *Anim Reprod Sci* 2004;82–83:415–30.
- König NL, Wähner M, Seeger J, Sigmarsson HL, Kauffold J. An investigation into uterine capacity based on litter and placental characteristics in two sow lines with different prolificacy (Danish Landrace x Danish Yorkshire versus German Saddleback). *Reprod Domest Anim* 2021;56:34–45.
- Kang P, Zhang L, Hou Y, Ding B, Yi D, Wang L, et al. Effects of L-proline on the growth performance, and blood parameters in weaned lipopolysaccharide (LPS)-challenged pigs. *Asian-Australas J Anim Sci* 2014;27:1150–6.
- Kaul S, Sharma SS, Mehta IK. Free radical scavenging potential of L-proline: evidence from in vitro assays. *Amino Acids* 2008;34:315–20.
- Li X, Bazer FW, Johnson GA, Burghardt RC, Wu G. Dietary supplementation with L-citrulline improves placental angiogenesis and embryonic survival in gilts. *Exp Biol Med (Maywood, NJ, U S)* 2023;248:702–11.
- Li X, Rezaei R, Li P, Wu G. Composition of amino acids in feed ingredients for animal diets. *Amino Acids* 2011;40:1159–68.
- Liu N, Dai Z, Zhang Y, Chen J, Yang Y, Wu G, et al. Maternal L-proline supplementation enhances fetal survival, placental development, and nutrient transport in mice. *Biol Reprod* 2019;100:1073–81.
- Mayneris-Perxachs J, Castells-Nobau A, Arnoriaga-Rodríguez M, Martín M, de la Vega-Correa L, Zapata C, et al. Microbiota alterations in proline metabolism impact depression. *Cell Metabol* 2022;34:681–701.e610.
- Mele J, Muralimanoharan S, Maloyan A, Myatt L. Impaired mitochondrial function in human placenta with increased maternal adiposity. *Am J Physiol Endocrinol Metab* 2014;307:E419–25.
- Muro BB, Carnevale RF, Leal DF, Almond GW, Monteiro MS, Poor AP, et al. The importance of optimal body condition to maximise reproductive health and perinatal outcomes in pigs. *Nutr Res Rev* 2023;36:351–71.
- Pereira RD, De Long NE, Wang RC, Yazdi FT, Holloway AC, Raha S. Angiogenesis in the placenta: the role of reactive oxygen species signaling. *BioMed Res Int* 2015;2015:814543.
- Rangstrup-Christensen L, Krogh MA, Pedersen LJ, Sørensen JT. Sow-level risk factors for stillbirth of piglets in organic sow herds. *Animal* 2017;11:1078–83.
- Schoknecht PA, Newton GR, Weise DE, Pond WG. Protein restriction in early pregnancy alters fetal and placental growth and allantoic fluid proteins in swine. *Theriogenology* 1994;42:217–26.
- Silveira MAD, Marcondes JPC, Lara JR, Scarano WR, Calderón IMP, Rudge MVC, et al. Mitochondrial-related gene associated to obesity can be modulated by in utero hyperglycemic environment. *Reprod Toxicol* 2019;85:59–64.
- Tan C, Huang Z, Xiong W, Ye H, Deng J, Yin Y. A review of the amino acid metabolism in placental function response to fetal loss and low birth weight in pigs. *J Anim Sci Biotechnol* 2022;13:28.
- Wang J, Xue Z, Lin J, Wang Y, Ying H, Lv Q, et al. Proline improves cardiac remodeling following myocardial infarction and attenuates cardiomyocyte apoptosis via redox regulation. *Biochem Pharmacol* 2020;178:114065.
- Wątroba M, Szewczyk G, Szukiewicz D. The role of sirtuin-1 (SIRT1) in the physiology and pathophysiology of the human placenta. *Int J Mol Sci* 2023;24.
- Weckman AM, McDonald CR, Baxter JB, Fawzi WW, Conroy AL, Kain KC. Perspective: L-arginine and L-citrulline supplementation in pregnancy: a potential strategy to improve birth outcomes in low-resource settings. *Adv Nutr* 2019;10:765–77.
- Wilson ME, Biensen NJ, Ford SP. Novel insight into the control of litter size in pigs, using placental efficiency as a selection tool. *J Anim Sci* 1999;77:1654–8.
- Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Knabe DA, et al. Proline and hydroxyproline metabolism: implications for animal and human nutrition. *Amino Acids* 2011;40:1053–63.
- Wu G, Bazer FW, Datta S, Johnson GA, Li P, Satterfield MC, et al. Proline metabolism in the conceptus: implications for fetal growth and development. *Amino Acids* 2008;35:691–702.
- Wu G, Bazer FW, Johnson GA, Herring C, Seo H, Dai Z, et al. Functional amino acids in the development of the pig placenta. *Mol Reprod Dev* 2017;84:870–82.
- Wu G, Bazer FW, Satterfield MC, Li X, Wang X, Johnson GA, et al. Impacts of arginine nutrition on embryonic and fetal development in mammals. *Amino Acids* 2013a;45:241–56.
- Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* 2006;84:2316–37.
- Wu G, Wu Z, Dai Z, Yang Y, Wang W, Liu C, et al. Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. *Amino Acids* 2013b;44:1107–13.
- Wu QJ, Zhang TN, Chen HH, Yu XF, Lv JL, Liu YY, et al. The sirtuin family in health and disease. *Signal Transduct Targeted Ther* 2022;7:402.
- Wu Z, Nie J, Wu D, Huang S, Chen J, Liang H, et al. Dietary adenosine supplementation improves placental angiogenesis in IUGR piglets by up-regulating adenosine A2a receptor. *Anim Nutr* 2023a;13:282–8.
- Wu Z, Xiang Q, Feng L, Wu D, Huang S, Zhang L, et al. Adenosine-ADORA2A promotes ang-induced angiogenesis in intrauterine growth restriction placenta via the stat3/Akt pathway. *Arterioscler Thromb Vasc Biol* 2023b;43:e190–209.
- Young MG, Tokach MD, Aherne FX, Main RG, Dritsch SS, Goodband RD, et al. Effect of sow parity and weight at service on target maternal weight and energy for gain in gestation. *J Anim Sci* 2005;83:255–61.
- Zha W, Kong X, Tan M, Geng M, Xu L, You J. Effects of dietary proline supplementation on reproductive performance and plasma biochemical parameters of pregnant Huanjiang mini-pigs. *Chinese Journal of Animal Nutrition* 2016;28:579–84.
- Zhang L, Tan C, Xin Z, Huang S, Ma J, Zhang M, et al. UPLC-Orbitrap-MS/MS combined with biochemical analysis to determine the growth and development of mothers and fetuses in different gestation periods on Tibetan sow model. *Front Nutr* 2022;9:836938.
- Zhou Y, Xu T, Cai A, Wu Y, Wei H, Jiang S, et al. Excessive backfat of sows at 109 d of gestation induces lipotoxic placental environment and is associated with declining reproductive performance. *J Anim Sci* 2018;96:250–7.
- Zhu Y, Park SH, Ozden O, Kim HS, Jiang H, Vassilopoulos A, et al. Exploring the electrostatic repulsion model in the role of Sirt3 in directing MnSOD acetylation status and enzymatic activity. *Free Radic Biol Med* 2012;53:828–33.