

Impact of protein level and early-stage CDCA supplementation during gestation on maternal physiology in sows

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Abstract

Improving sow reproductive performance is an important goal for pig production, especially in China, where reproductive efficiency still lags behind that of some developed countries. In pigs, litter size is not determined only by ovulation rate, but is largely limited by embryonic and fetal survival during gestation. Because a large proportion of embryonic loss occurs during early gestation, especially around the peri-implantation period, this stage is considered an important window for nutritional regulation. Protein and amino acids are essential substrates for maternal tissue recovery, embryo development, placental growth, and fetal development. Therefore, increasing dietary protein supply during early gestation may be a potential strategy to improve reproductive performance in modern highly prolific sows. However, whether dietary protein levels within the commercial range can improve litter performance in multiparous sows, and how maternal dietary protein nutrition affects maternal metabolism, placental function, and fetal development, remain unclear.

Firstly, to evaluate whether dietary protein levels within the commercial range during early gestation could improve reproductive performance in multiparous sows, sows were fed diets containing 12%, 13.5%, or 15% crude protein (CP) from gestational day 0 (GD 0) to GD 30. Reproductive performance and body condition-related indicators were recorded, maternal blood samples were collected at GD 28, and placental samples were collected at farrowing. Serum hormones, serum biochemical indices, oxidative stress markers, inflammatory cytokines, and serum amino acid profiles were measured at GD 28. Placental oxidative stress markers, ATP content, and the expression of genes related to antioxidant defense, mitochondrial function, and nutrient transport were examined at farrowing. The results showed that increasing dietary protein levels from 12% to 15% during early gestation did not significantly improve litter size, live-born rate, or placental functional indicators at farrowing. However, the high protein (HP) diet altered maternal serum amino acid profiles and induced changes in serum alanine aminotransferase (ALT) activity and redox-related indicators at GD 28. These findings suggest that, in multiparous sows, moderate changes in dietary protein within the commercial range may not further improve litter performance when basal protein and amino acid requirements are already met, whereas maternal metabolic status is more sensitive to changes in protein supply.

Based on the observation that dietary protein variation in sows mainly affected maternal metabolic responses rather than litter performance, a mouse model was further used to explore the effects of stronger protein imbalance on early implantation-related regulation, fetal development, placental function, and maternal-fetal nutrient distribution. At GD 6.5, implantation number was not significantly affected by maternal dietary protein level. However, protein imbalance altered maternal serum hormones and lipid-related indicators, ovarian steroidogenesis-related genes, and hormone receptor expression at implantation sites, suggesting that early endocrine and local implantation-related responses were more sensitive than

implantation number itself. At GD 18.5, fetal number was not significantly changed, but the HP treatment reduced fetal weight and placental efficiency. These changes were accompanied by altered maternal serum amino acid profiles, non-synchronous changes in amino acid profiles among maternal serum, amniotic fluid, and fetal serum, increased blood urea nitrogen (BUN) in maternal serum, amniotic fluid, and fetal serum, reduced fetal serum glucose, placental oxidative stress, altered mitochondrial function, changes in nutrient transport-related genes, and altered fetal hepatic expression of genes related to glucose and amino acid metabolism. These results indicate that implantation number and fetal number may be relatively robust to maternal dietary protein variation, whereas fetal growth, maternal metabolic homeostasis, and placental function are more sensitive to stronger high-protein/low-carbohydrate nutritional imbalance.

Because the protein studies indicated that maternal metabolic status and placental functional adaptation were more sensitive to nutritional changes than litter size itself, Chenodeoxycholic acid (CDCA) was further used as a metabolism-targeted nutritional intervention during early gestation. This experiment aimed to evaluate whether improving maternal metabolic status during early gestation could exert sustained effects on maternal health and reproductive performance in late gestation. Sows were supplemented with CDCA during early gestation. At GD 110, maternal serum inflammatory cytokines, oxidative stress indicators, antioxidant capacity, glucose- and insulin-related metabolic indicators, serum metabolomic profiles, and fecal microbial composition were evaluated. Reproductive performance was recorded at farrowing. The results showed that early-gestation CDCA supplementation reduced maternal inflammatory responses and oxidative stress, enhanced antioxidant capacity, and improved insulin-related metabolic status at GD 110. Serum metabolomic analysis showed that CDCA affected metabolites related to lipid metabolism, amino acid metabolism, antioxidant responses, and inflammatory regulation. Fecal microbiota analysis showed that CDCA altered gut microbial composition, and correlation analyses suggested associations among specific microbial taxa, serum metabolites, inflammatory markers, oxidative stress indicators, insulin sensitivity, and reproductive performance. Importantly, CDCA supplementation increased total born and live-born piglets without reducing individual live-born piglet weight. These results suggest that early-gestation CDCA supplementation may improve late-gestation maternal physiological homeostasis and reproductive performance, possibly through the regulation of gut microbiota-host metabolic interactions, redox balance, inflammatory status, and insulin-related metabolism.

Overall, this thesis supports the view that maternal metabolic homeostasis is an important regulatory axis linking early-gestation nutritional input, placental functional adaptation, fetal development, and pregnancy outcomes. The sow protein trial showed that increasing dietary protein within a commercial range during early gestation did not necessarily improve litter performance in multiparous sows, but induced maternal metabolic responses. The mouse model further demonstrated that stronger and sustained high-protein/low-carbohydrate nutritional imbalance may

exceed placental adaptive capacity, disturb placental redox homeostasis, mitochondrial function, and nutrient transport, and impair fetal growth. The CDCA trial suggested that targeting maternal metabolic homeostasis during early gestation may improve maternal health and reproductive performance later in pregnancy. These findings indicate that future gestational nutrition strategies for sows should move beyond simply increasing CP supply and should instead focus on appropriate protein and amino acid balance, maintenance of maternal metabolic homeostasis, placental functional adaptation, and metabolism-targeted interventions. Such strategies may contribute to improving sow reproductive efficiency, maternal-fetal health, and sustainable pig production.

Keywords: sow; early gestation; dietary protein; maternal metabolic homeostasis; oxidative stress; nutrient transport; CDCA; reproductive performance

Résumé

L'amélioration des performances de reproduction des truies constitue un objectif important pour la production porcine, en particulier en Chine, où l'efficacité reproductive reste inférieure à celle observée dans certains pays développés. Chez le porc, la taille de portée n'est pas uniquement déterminée par le taux d'ovulation, mais dépend largement de la survie embryonnaire et fœtale au cours de la gestation. Une proportion importante des pertes embryonnaires survient au début de la gestation, en particulier autour de la période péri-implantatoire, ce qui fait de cette phase une fenêtre importante pour la modulation nutritionnelle. Les protéines et les acides aminés sont des substrats essentiels pour la récupération des tissus maternels, le développement embryonnaire, la croissance placentaire et le développement fœtal. Ainsi, l'augmentation de l'apport protéique alimentaire au début de la gestation pourrait constituer une stratégie potentielle pour améliorer les performances de reproduction des truies modernes hautement prolifiques. Cependant, il reste à déterminer si des niveaux de protéines alimentaires situés dans la gamme commerciale peuvent améliorer les performances de portée chez les truies multipares, et comment la nutrition protéique maternelle influence le métabolisme maternel, la fonction placentaire et le développement fœtal.

Premièrement, afin d'évaluer si les niveaux de protéines alimentaires dans la gamme commerciale au début de la gestation pouvaient améliorer les performances de reproduction des truies multipares, les truies ont reçu des régimes contenant 12 %, 13,5 % ou 15 % de protéines brutes (CP) du jour de gestation 0 (GD 0) à GD 30. Les performances de reproduction et les indicateurs liés à l'état corporel ont été enregistrés, les échantillons sanguins maternels ont été prélevés à GD 28, et les échantillons placentaires ont été collectés à la mise bas. Les hormones sériques, les paramètres biochimiques sériques, les marqueurs de stress oxydatif, les cytokines inflammatoires et les profils d'acides aminés sériques ont été mesurés à GD 28. Les marqueurs placentaires de stress oxydatif, la teneur en ATP, ainsi que l'expression de gènes liés à la défense antioxydante, à la fonction mitochondriale et au transport des nutriments ont été analysés à la mise bas. Les résultats ont montré que l'augmentation du niveau de protéines alimentaires de 12 % à 15 % au début de la gestation n'améliorait pas significativement la taille de portée, le taux de porcelets nés vivants ou les indicateurs fonctionnels placentaires à la mise bas. Cependant, le régime riche en protéines (HP) a modifié les profils d'acides aminés sériques maternels et a induit des changements de l'activité sérique de l'alanine aminotransférase (ALT) et des indicateurs liés à l'équilibre redox à GD 28. Ces résultats suggèrent que, chez les truies multipares, des modifications modérées de l'apport protéique dans la gamme commerciale ne permettent pas nécessairement d'améliorer davantage les performances de portée lorsque les besoins de base en protéines et en acides aminés sont déjà couverts, alors que l'état métabolique maternel est plus sensible aux variations de l'apport protéique.

Sur la base de l'observation selon laquelle la variation de l'apport protéique chez la truie affectait principalement les réponses métaboliques maternelles plutôt que les

performances de portée, un modèle murin a ensuite été utilisé afin d'explorer les effets d'un déséquilibre protéique plus marqué sur la régulation liée à l'implantation précoce, le développement fœtal, la fonction placentaire et la répartition des nutriments entre la mère et le fœtus. À GD 6.5, le nombre de sites d'implantation n'a pas été significativement affecté par le niveau protéique maternel. Cependant, le déséquilibre protéique a modifié les hormones sériques maternelles et les indicateurs liés au métabolisme lipidique, l'expression des gènes ovariens liés à la stéroïdogenèse, ainsi que l'expression des récepteurs hormonaux au niveau des sites d'implantation, suggérant que les réponses endocriniennes précoces et les réponses locales liées à l'implantation étaient plus sensibles que le nombre de sites d'implantation lui-même. À GD 18.5, le nombre de fœtus n'a pas été significativement modifié, mais le traitement HP a réduit le poids fœtal et l'efficacité placentaire. Ces changements étaient accompagnés d'une modification des profils d'acides aminés dans le sérum maternel, de variations non synchronisées des profils d'acides aminés entre le sérum maternel, le liquide amniotique et le sérum fœtal, d'une augmentation de l'azote uréique sanguin (BUN) dans le sérum maternel, le liquide amniotique et le sérum fœtal, d'une diminution du glucose sérique fœtal, d'un stress oxydatif placentaire, d'une altération de la fonction mitochondriale, de changements dans l'expression des gènes liés au transport des nutriments et d'une modification de l'expression hépatique fœtale des gènes impliqués dans le métabolisme du glucose et des acides aminés. Ces résultats indiquent que le nombre de sites d'implantation et le nombre de fœtus peuvent être relativement robustes face aux variations de l'apport protéique maternel, tandis que la croissance fœtale, l'homéostasie métabolique maternelle et la fonction placentaire sont plus sensibles à un déséquilibre nutritionnel plus marqué de type hyperprotéique/hypoglucidique.

Étant donné que les études sur les protéines indiquaient que l'état métabolique maternel et l'adaptation fonctionnelle du placenta étaient plus sensibles aux changements nutritionnels que la taille de portée elle-même, l'acide chénodésoxycholique (CDCA) a ensuite été utilisé comme intervention nutritionnelle ciblant le métabolisme au début de la gestation. Cette expérience visait à évaluer si l'amélioration de l'état métabolique maternel au début de la gestation pouvait exercer des effets durables sur la santé maternelle et les performances de reproduction en fin de gestation. Les truies ont reçu une supplémentation en CDCA au début de la gestation. À GD 110, les cytokines inflammatoires sériques maternelles, les indicateurs de stress oxydatif, la capacité antioxydante, les indicateurs liés au métabolisme du glucose et de l'insuline, les profils métabolomiques sériques et la composition du microbiote fécal ont été évalués. Les performances de reproduction ont été enregistrées à la mise bas. Les résultats ont montré que la supplémentation en CDCA au début de la gestation réduisait les réponses inflammatoires maternelles et le stress oxydatif, renforçait la capacité antioxydante et améliorait l'état métabolique lié à l'insuline à GD 110. L'analyse métabolomique sérique a montré que le CDCA affectait des métabolites liés au métabolisme lipidique, au métabolisme des acides aminés, aux réponses antioxydantes et à la régulation de l'inflammation. L'analyse du microbiote fécal a

montré que le CDCA modifiait la composition microbienne intestinale, et les analyses de corrélation ont suggéré des associations entre certains taxons microbiens, les métabolites sériques, les marqueurs inflammatoires, les indicateurs de stress oxydatif, la sensibilité à l'insuline et les performances de reproduction. Fait important, la supplémentation en CDCA a augmenté le nombre total de porcelets nés et de porcelets nés vivants sans réduire le poids individuel des porcelets nés vivants. Ces résultats suggèrent que la supplémentation en CDCA au début de la gestation pourrait améliorer l'homéostasie physiologique maternelle en fin de gestation et les performances de reproduction, possiblement par la régulation des interactions entre le microbiote intestinal et le métabolisme de l'hôte, de l'équilibre redox, de l'état inflammatoire et du métabolisme lié à l'insuline.

Dans l'ensemble, cette thèse soutient l'idée que l'homéostasie métabolique maternelle constitue un axe régulateur important reliant les apports nutritionnels en début de gestation, l'adaptation fonctionnelle du placenta, le développement fœtal et les issues de gestation. L'essai sur les niveaux protéiques chez la truie a montré que l'augmentation de l'apport protéique dans une gamme commerciale au début de la gestation n'améliorait pas nécessairement les performances de portée chez les truies multipares, mais induisait des réponses métaboliques maternelles. Le modèle murin a également montré qu'un déséquilibre nutritionnel plus marqué et prolongé, de type hyperprotéique/hypoglucidique, pouvait dépasser la capacité d'adaptation placentaire, perturber l'homéostasie redox placentaire, la fonction mitochondriale et le transport des nutriments, et altérer la croissance fœtale. L'essai CDCA a suggéré que cibler l'homéostasie métabolique maternelle au début de la gestation pourrait contribuer à améliorer la santé maternelle et les performances de reproduction plus tard au cours de la gestation. Ces résultats indiquent que les futures stratégies nutritionnelles pour les truies gestantes ne devraient pas reposer uniquement sur l'augmentation de l'apport en protéines brutes, mais devraient plutôt privilégier un équilibre approprié entre protéines et acides aminés, le maintien de l'homéostasie métabolique maternelle, l'adaptation fonctionnelle du placenta et les interventions nutritionnelles ciblant le métabolisme. De telles stratégies pourraient contribuer à améliorer l'efficacité reproductive des truies, la santé materno-fœtale et la durabilité de la production porcine.

Mots-clés: truie; début de gestation; protéines alimentaires; homéostasie métabolique maternelle; stress oxydatif; transport des nutriments; CDCA; performances de reproduction

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List of acronyms

ACTB: Actin beta

ADFI: Average daily feed intake

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

B0AT1: Broad neutral amino acid transporter 1

BAX: BCL2-associated X protein

Bcat2: Branched-chain amino acid transaminase 2

BCL2: B cell leukemia/lymphoma 2

BUN: Blood urea nitrogen

CAT: Catalase

Cat1: Cationic amino acid transporter 1

CD31: Cluster of differentiation 31

CDCA: Chenodeoxycholic acid

CON: Control group

Cox4i1: Cytochrome c oxidase subunit 4I1

CP: Crude protein

Cyp11a1: Cytochrome P450 family 11 subfamily A member 1

Cyp19a1: Cytochrome P450 family 19 subfamily A member 1

DEC: Decidua

DM: Dry matter

E2: Estradiol

ETC: Electron transport chain

FGF7: Fibroblast growth factor 7

FGR: Fetal growth restriction

FIS1: Mitochondrial fission 1

FXR: Farnesoid X receptor

G6pc: Glucose-6-phosphatase catalytic subunit

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GD: Gestational day

GLU: Glucose

GLUT1: Glucose transporter 1
Got1: Glutamic-oxaloacetic transaminase 1
Gpt1: Glutamic-pyruvic transaminase 1
GPx: Glutathione peroxidase
GSH: Glutathione
HDL: High-density lipoprotein
HE: Hematoxylin and eosin
HOMA-IR: Homeostasis model assessment of insulin resistance
HP: High protein
IFN- γ : Interferon- γ
IGF-1: Insulin-like growth factor 1
IL-1 β : Interleukin-1 β
IL-6: Interleukin-6
INS: Insulin
IUGR: Intrauterine growth restriction
JZ: Junctional zone
KEAP1: Kelch-like ECH-associated protein 1
Lat: L-type amino acid transporter
LDL: Low-density lipoprotein
LP: Low protein
LPCs: Lysophosphatidylcholines
LZ: Labyrinth zone
MDA: Malondialdehyde
ME: Metabolizable energy
MFN2: Mitofusin 2
MP: Medium protein
mRNA: Messenger RNA
mt-Co2: Mitochondrially encoded cytochrome c oxidase II
mtDNA: Mitochondrial DNA
mTOR: Mechanistic target of rapamycin
mTORC1: Mechanistic target of rapamycin complex 1

NAD: Nicotinamide adenine dinucleotide
 NADH: Reduced nicotinamide adenine dinucleotide
 NCG: N-carbamylglutamate
 Ndufs8: NADH: ubiquinone oxidoreductase core subunit S8
 NEFA: Non-esterified fatty acid
 NFE2L2: Nuclear factor erythroid 2 like 2
 NQO1: NAD(P)H quinone dehydrogenase 1
 NRC: National Research Council
 OTUs: Operational taxonomic units
 P4: Progesterone
 PAS: Periodic acid-Schiff
 PGF_{2α}: Prostaglandin F_{2α}
 ROS: Reactive oxygen species
 RPS18: Ribosomal protein S18
 SID: Standardized ileal digestible
 SNAT: Sodium-coupled neutral amino acid transporter
 SOD: Superoxide dismutase
 T-AOC: Total antioxidant capacity
 TC: Total cholesterol
 TFAM: Transcription factor A mitochondrial
 TGC: Trophoblast giant cell
 TGF-β: Transforming growth factor-β
 TGR5: Takeda G protein-coupled receptor 5
 TNF-α: Tumor necrosis factor-α
 TP: Total protein
 y+Lat1: y+L amino acid transporter 1
 3β-HSD: 3β-hydroxysteroid dehydrogenase
 4F2HC: 4F2 cell-surface antigen heavy chain
 8-OHdG: 8-hydroxy-2'-deoxyguanosine

Chapter 1

General introduction

1. Global perspective on sow productivity and the performance gap in China

1.1 Global production status and efficiency gap

China is the world's largest producer and consumer of pork, accounting for approximately 45-50% of global pork production and consumption (FAO, 2023; USDA, 2023). However, there is still a major gap in reproductive efficiency between Chinese sow herds and those in developed Western countries.

As an example, we can take the number of weaned piglets per sow per year (PSY), widely recognized as a key indicator of a sow's productivity (Guan et al., 2022). In Denmark, the average PSY is around 34, whereas in China the average remains around 20 (Guan et al., 2021; Guan et al., 2022; Theil et al., 2023). Despite the widespread use of imported or genetically improved breeds, there is still a gap of more than 10 piglets per sow per year. Beyond genetic potential, nutritional management and other farm-level practices represent important areas for improving sow productivity. These include feeding and body condition management, housing conditions, and herd health management through biosecurity, vaccination and timely diagnosis and treatment of disease. Improving PSY is of major economic importance, as it allows the fixed costs of sow maintenance and replacement to be distributed across a larger number of piglets, thereby enhancing overall production efficiency and farm profitability.

The European context also highlights the need to consider productivity alongside environmental sustainability. Pig production systems differ across European countries in their management practices and environmental impacts (Dourmad et al., 2014). Assessments of European pig farms therefore consider economic performance together with environmental outcomes and animal health and welfare (Ruckli et al., 2022). Nutritional management provides one approach to addressing these multiple objectives. For example, European recommendations include reducing dietary crude protein while meeting energy and digestible amino acid requirements to limit nitrogen excretion (European Commission, 2017). This broader context highlights the importance of feeding strategies that support sow reproductive performance while avoiding unnecessary nutrient supply.

1.2 Determinants of litter size: ovulation versus prenatal mortality

Litter size at birth is one of the primary determinants of PSY. In pigs, however, litter size is not primarily limited by ovulation rate, but rather by the survival of embryos and fetuses during gestation. As a typical polytocous species, sows ovulate approximately 20 to 30 oocytes per estrous cycle (Geisert et al., 2020), with a fertilization rate approaching 90% (Gonzalez-Añover et al., 2011). Theoretically, this would result in 18 to 27 viable embryos in early pregnancy. However, actual litter sizes are consistently lower than this biological potential in both commercial and non-commercial production systems.

Pigs exhibit one of the highest rates of prenatal loss among domestic mammals, with total gestational mortality estimated at 20-45% (Pope, 1988). This substantial loss highlights that embryonic and fetal survival is the primary limiting factor for reproductive efficiency.

1.3 The dual challenges of gestation: early embryonic loss and late metabolic stress

The gestation period in modern sows is characterized by two major physiological challenges: significant embryonic loss during early pregnancy and severe metabolic stress during late gestation.

Early gestation represents a critical window for embryo implantation and the successful establishment of pregnancy. It is estimated that 20-30% of embryos are lost during the peri-implantation period (Geisert and Schmitt, 2002), accounting for approximately 75% of total embryonic mortality throughout the entire gestation (Ford et al., 2002). The primary causes of this loss are implantation failure and impaired post-implantation embryonic development. In addition, embryonic loss may continue after implantation due to uterine capacity limitations, particularly in highly prolific sows (Langendijk et al., 2016). Consequently, this early window represents the critical phase for determining the maximum potential litter size.

Nevertheless, successful embryo implantation does not guarantee a favorable pregnancy outcome. As gestation progresses, the rapid fetal growth imposes a substantial metabolic burden on the sow, particularly in modern hyperprolific genotypes. Late gestation is thus considered a high-risk period characterized by increased metabolic stress, including elevated oxidative stress, insulin resistance, and systemic inflammation (Vivas et al., 2016; Yang et al., 2023). Such maternal metabolic disorders directly compromise placental function and nutrient transfer, leading to late fetal loss, stillbirths, and piglets with reduced birth weight (Sultana et al., 2023; Yang et al., 2023). Therefore, improving reproductive efficiency in modern sows requires a dual strategy: enhancing embryo survival during early gestation and improving maternal metabolic resilience during late pregnancy.

2. Early gestation as a critical window for embryo development and maternal metabolic programming

Early pregnancy is not only a critical stage that determines successful embryo implantation, but it also serves as a "programming window" that sets the metabolic foundation for the entire gestational period. During this stage, multiple processes, including embryo implantation, the establishment of the uterine microenvironment, early placental development, and maternal metabolic adaptation, are highly synchronized to dictate the success or failure of the pregnancy outcome.

2.1 Embryo implantation and establishment of uterine receptivity

Embryo implantation refers to the process in which the blastocyst establishes close contact with the uterine endometrium and forms a stable adhesion, thereby enabling

a functional connection at the maternal-fetal interface, which ultimately leads to placental formation and successful pregnancy (Carson et al., 2000). It is one of the key steps determining reproductive success. In pigs, the successful establishment of pregnancy depends on highly coordinated spatiotemporal communication between the rapidly developing embryo and the hormonally regulated uterine endometrium.

2.1.1 Blastocyst formation and conceptus elongation

After fertilization in the sow, the zygote undergoes sequential cleavage into 2-cell and 4-cell embryos within the oviduct, and enters the uterus around gestational day 3 (GD3), reaching the blastocyst stage by GD 5. The blastocyst hatches from the zona pellucida on GD 6-7. Subsequently, embryos actively migrate and space themselves uniformly throughout the uterine horns, thereby creating spatial conditions for subsequent attachment. During this process, the embryo differentiates into the inner cell mass (ICM), primitive endoderm (PE), and trophoblast (Tr). Among these, the trophoblast, as the precursor of the future placenta, is the key structure responsible for establishing contact with the uterine endometrium (Bazer and Johnson, 2014). Between GD 10 and GD12, porcine embryos undergo a dramatic morphological transformation, changing from 10mm spherical blastocysts through a tubular intermediate stage, to rapidly elongating filamentous forms that can reach several hundred millimeters in length (Ka et al., 2018). This process markedly increases the surface area of contact between the embryo and the uterine endometrium, thereby enhancing physical interaction and signal exchange at the maternal-fetal interface, and improving the utilization of uterine histotroph (Geisert et al., 1990; Bazer, 2013). By GD 13, the trophoblast begins to progressively adhere to the uterine luminal epithelium (LE), and interdigitating microvilli are formed between the trophoblast and uterine epithelium. The entire implantation process is generally completed around GD 18 (Bazer and Johnson, 2014) (Figure 1-1).

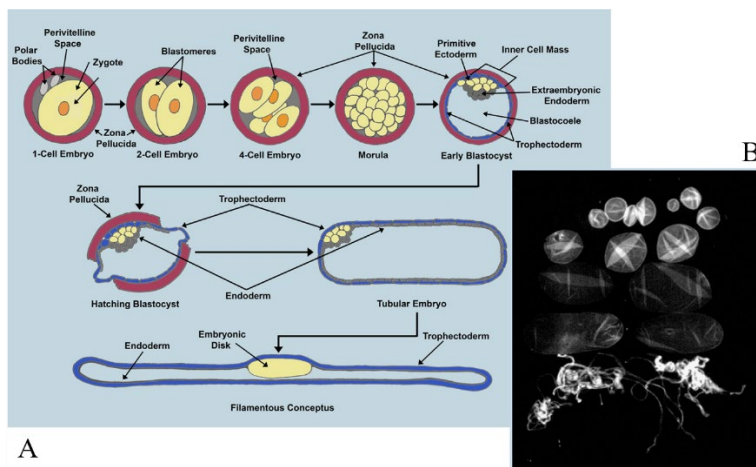


Figure 1-1: Pre-implantation development and conceptus elongation in pigs. From Fuller W. Bazer et al., 2014.

This figure summarizes the pre-implantation development and morphological transition of

porcine embryos. (A) Schematic representation of porcine preimplantation development. After fertilization, the embryo undergoes cleavage divisions and develops into the morula and blastocyst stages. In pigs, embryos usually enter the uterus at around the 4-cell stage and hatch from the zona pellucida by approximately GD 7. Around GD 10, the conceptus becomes an expanded blastocyst and then rapidly elongates, changing from a spherical to a tubular, and finally a filamentous structure. This elongation increases the contact surface between the trophoblast and the uterine luminal epithelium. (B) Representative photographs of porcine conceptuses at different stages of elongation. Conceptuses are small and spherical on GD 10, become larger and spherical by GD 10.5, develop into tubular forms on GD 11, and appear as long filamentous structures by GD 12.

2.1.2 Maternal recognition and regulation of the uterine microenvironment

The peri-implantation period in pigs is a highly coordinated maternal-conceptus interaction process driven by hormones, cytokines, and growth factors. These factors collectively regulate endometrial function, the immune environment, and adhesion capacity, ultimately ensuring successful conceptus attachment and the establishment of pregnancy.

During early gestation, progesterone secreted by the corpus luteum is the key hormone responsible for establishing uterine receptivity and maintaining pregnancy. Progesterone regulates the function of endometrial epithelial and glandular cells, thereby stimulating the secretion of histotroph, a complex mixture rich in growth factors (e.g., EGF, IGF-1, FGF7, VEGF), cytokines (e.g., IL-6, LIF, TGF- β), and various nutrients (Steinhauser et al., 2017). Before the establishment of a functional placental circulation, histotroph serves as the primary source of amino acids, carbohydrates, lipids, and vitamins for the developing conceptus. Therefore, its composition and availability critically determine the microenvironment for early embryonic development.

Maternal recognition of pregnancy in pigs primarily depends on conceptus-derived estrogen. Around GD 11-12, conceptus-secreted estrogen alters the secretion pattern of endometrial prostaglandins (PGs), redirecting luteolytic prostaglandin F₂ α (PGF₂ α) from an endocrine to an exocrine pathway into the uterine lumen. This prevents PGF₂ α from entering systemic circulation and inducing luteolysis, thereby maintaining progesterone secretion and supporting pregnancy establishment (Bazer and Thatcher, 1977; Bazer and Johnson, 2014). This process exhibits a threshold effect: when the number of conceptuses is insufficient, such as when the number is fewer than four, the total estrogen production may be inadequate to effectively redirect PGF₂ α secretion, resulting in corpus luteum regression and pregnancy failure (Lambert et al., 1991). However, emerging evidence suggests that estrogen may not be strictly essential for pre-implantation development, conceptus elongation, or early luteal maintenance, but remains critical for sustaining pregnancy beyond approximately GD 30 (Meyer et al., 2019). In addition, conceptus-derived prostaglandin E₂ (PGE₂) may act synergistically with estrogen during maternal recognition of pregnancy (Pfeiffer, 2019).

Beyond hormonal regulation, successful implantation requires bidirectional molecular communication between the conceptus and the endometrium. In addition

to estrogen, the conceptus secretes various cytokines, including interleukin-1 β 2 (IL-1 β 2), interferon- δ (IFND), and interferon- γ (IFNG). These conceptus-derived signals act in concert with endometrial factors (such as TGF- β , FGF7, and CXCL12), as well as steroid hormones, to regulate gene expression in both the endometrium and the conceptus (Almeida and Dias, 2022). This coordinated regulation promotes the transformation of the uterine luminal epithelium from a non-adhesive to an adhesive state, thereby providing a structural basis for stable conceptus attachment (Almeida and Dias, 2022).

Moreover, these signaling interactions induce a controlled pro-inflammatory microenvironment within the endometrium, maintaining a dynamic balance between maternal immune tolerance and localized inflammatory responses (Mathew et al., 2015; Yoo et al., 2019). This finely tuned immune state is essential for successful conceptus attachment and pregnancy establishment. In summary, embryo implantation in pigs is a highly integrated process involving endocrine regulation, immune adaptation, and nutrient supply. Among these factors, the availability of nutrients within the uterine lumen, which is directly shaped by maternal nutritional status, is a critical determinant of early conceptus survival, positioning maternal nutrition as a tractable target for improving embryo implantation outcomes.

2.2 Early initiation of placental development and its functional significance

The porcine placenta is classified as an epitheliochorial type, characterized by a non-invasive mode of implantation (Aplin and Kimber, 2004). Placentation is initiated following embryo implantation. Around GD 14, embryonic trophoblast cells begin to adhere closely to the endometrial epithelium, forming initial microvilli structures by GD 15-16. Subsequently, a complex interdigitated microvilli system gradually forms at the maternal-fetal interface, significantly increasing the contact surface area, and facilitating the transition of nutrient supply pattern from predominantly histotrophic nutrition to hemotrophic nutrition (Dantzer, 1985). The period between GD 18 and GD 30 is a critical phase for the rapid expansion and development of the chorion and allantois, which fuse to form the chorioallantois (Aplin and Kimber, 2004). Furthermore, around GD 25, placental folds begin to form between the trophoblast and the endometrial epithelium, further expanding the surface area for maternal-fetal exchange (Johnson et al., 2021).

During early pregnancy, the structure and function of the placenta are still in the developmental phase, with its transport systems and associated metabolic pathways gradually taking shape. Research has indicated that the demand for nutritional substrates, such as amino acids, increases significantly during placental development, with the total concentration of amino acids in the placenta showing a continuous upward trend as gestation progresses (Wu et al., 2017). Therefore, the placenta is not merely a passive organ for nutrient exchange, but also serves as a critical regulatory hub linking maternal metabolic status with fetal development. Maternal nutritional status may influence embryonic development by modulating placental development and its transport capacity.

2.3 The highly sensitive window for nutritional intervention and uterine microenvironment remodeling

Before the establishment of a functional placental vascular network, the conceptus cannot directly obtain nutrients from the maternal circulation and instead relies primarily on histotroph for nutritional support (Spencer et al., 2007). Therefore, early gestation represents a highly sensitive window in the reproductive cycle of sows in response to nutritional changes. During this period, alterations in maternal nutritional status can influence endocrine signals and the composition of histotroph, thereby affecting uterine receptivity and the embryonic microenvironment, which ultimately impacts embryo attachment and survival.

A large body of evidence indicates that nutritional interventions can effectively modulate the uterine microenvironment during early pregnancy and improve embryo survival. For instance, folic acid supplementation during early gestation has been shown to reduce embryonic mortality by regulating the expression of folate metabolism-related genes in the endometrium (Matte et al., 1984; Vallée et al., 2002). Short-chain fatty acids, medium-chain fatty acids, and n-3 polyunsaturated fatty acids can enhance uterine phospholipid metabolism and promote the synthesis of prostaglandins and progesterone, thereby increasing the number of implantation sites (Gokuldas et al., 2018; Ye et al., 2019). In addition, supplementation with B vitamins or functional compounds such as glucosamine may positively affect reproductive performance by regulating the expression of key developmental genes or improving placental fold development (Vallet et al., 2017; Kaur et al., 2019). Collectively, these findings suggest that nutritional modulation of embryo implantation has become an important strategy to improve reproductive efficiency in sows.

2.4 Early gestation as a potential critical window for maternal metabolic regulation

While much of the foundational evidence derives from human and rodent studies, converging findings from swine models support the hypothesis that early gestational metabolic programming is equally operative in pigs, given their comparable reproductive physiology and gestational timeline. Pregnancy is a dynamic process of metabolic reprogramming. Even in early gestation, the maternal body begins to undergo metabolic adaptations, such as the regulation of insulin sensitivity and the remodeling of lipid metabolism (Sferruzzi-Perri et al., 2020). Therefore, early interventions may influence maternal metabolism and exert effects on metabolic status in later stages of pregnancy. Previous studies have demonstrated that nutritional interventions during early gestation can significantly affect embryo survival and subsequent pregnancy outcomes (Wu et al., 2004; Chen et al., 2019). Moreover, human clinical studies have shown that lifestyle or nutritional interventions during early pregnancy can reduce the risk of metabolic disorders in mid and late pregnancy, such as gestational diabetes mellitus (Quotah et al., 2024), suggesting that the early stage may represent a key window for regulating maternal metabolism. In addition, maternal metabolic profiles in early pregnancy not only reflect the current physiological state but can also partially predict subsequent

pregnancy outcomes and fetal development, indicating that the early metabolic state may influence the direction and capacity for later metabolic adaptation (Yang et al., 2026). Furthermore, studies have reported that supplementation with functional nutrients (e.g., ω -3 fatty acids, folic acid, and certain micronutrients) before or during early pregnancy can improve pregnancy outcomes by modulating maternal metabolic status (Ramakrishnan et al., 2012; Skoracka et al., 2021). Considering that early gestation is a critical period for placental development, establishment of the maternal-fetal interface, and activation of multiple metabolic signaling pathways, nutritional interventions during this stage may exert long-term effects on maternal metabolic status in late pregnancy by influencing endocrine regulation, placental function, and systemic metabolic networks.

3. Nutritional regulation during early gestation: theoretical evolution, mechanisms, and key nutrients

3.1 Research progress and theoretical evolution of nutritional regulation during early gestation

Improving maternal nutritional status during early gestation in sows has long been recognized as a key strategy to promote successful implantation and enhance embryo survival (Wu et al., 2004; Chen et al., 2019). However, the optimal level of nutritional supply during this period remains controversial, and the underlying concepts have evolved over the past decades alongside the genetic improvement of modern sows.

Early studies conducted in the 1990s generally suggested that high feeding levels during early gestation were detrimental to embryo survival. These studies found that increasing feeding levels led to higher embryonic loss (Pharazyn, 1992; Jindal et al., 1996). For instance, when the feeding level of post-mating sows was increased from 1.5 to 2.0 times the maintenance requirement, the embryo survival rate at GD 28 significantly decreased (Jindal et al., 1996). The mechanistic explanation at that time focused on progesterone (P4) metabolism, suggesting that energy intake exceeding maintenance requirements reduced circulating P4 concentrations, thereby impairing embryo survival (Prime and Symonds, 1993; Mattos et al., 2017). Further research demonstrated that exogenous P4 supplementation could partially reverse the decline in embryo survival in gilts on high feeding levels, supporting a P4-mediated mechanism (Jindal et al., 1997). Based on these findings, a long-term production strategy of restricted feeding during early pregnancy was adopted to reduce embryonic mortality and improve litter size.

However, with the continuous genetic selection of sows for increased litter size and lean growth, the applicability of this traditional “early gestation feed restriction” strategy has been increasingly questioned. Modern sows often experience substantial body weight and reserve losses during lactation (Thaker and Bilkei, 2005; Ferreira et al., 2021), and approximately 90-95% of sows return to estrus within one week after weaning (Poleze et al., 2006), leaving insufficient time for full recovery before mating. Therefore, early gestation is now considered a critical period for restoring

maternal body reserves (Dourmad et al., 1996; Solà-Oriol and Gasa, 2017). Studies have shown that body weight loss exceeding 10% during lactation can negatively affect subsequent reproductive performance (Thaker and Bilkei, 2005). Among the nutrients contributing to weight loss, protein deficiency may exert the most profound impact on subsequent performance (Clowes et al., 2003). Under these conditions, strict feed restriction during early gestation may hinder body reserve recovery and adversely affect reproductive outcomes. In recent years, increasing evidence suggests that moderate increases in feed intake during early gestation do not compromise embryo survival. For instance, increasing feed allowance from 1.2 to 2.4 times maintenance requirements did not negatively affect embryo survival at GD 35 (Quesnel et al., 2010). A meta-analysis further noted that, for modern sow lines, even dietary energy intakes as high as 54 MJ ME·d⁻¹ had no observable negative impact on embryo survival (Leal et al., 2019). In contrast, feed intake below maintenance requirements during early gestation significantly decreased embryo survival (Condous et al., 2014). Moreover, increased feeding levels during this period may improve maternal metabolic status and P4 secretion, thereby exerting positive effects on embryo survival (Athorn et al., 2013), with some studies reporting increased litter sizes (Hoving et al., 2011; Su et al., 2025).

Taken together, these findings indicate that maternal nutritional level during early gestation is a critical determinant of embryo survival, but its effects have evolved with changes in sow genetics. The traditional feed restriction strategy developed for earlier genotypes may no longer be optimal for modern high-producing sows. Therefore, there is a need to re-evaluate nutritional supply strategies during early gestation and to further elucidate the mechanisms by which maternal nutrition regulates embryo survival, in order to develop more precise and efficient nutritional management approaches. However, these studies largely focused on overall energy intake, while the specific roles of individual nutrients, particularly protein and amino acids, remain insufficiently understood.

3.2 Main mechanisms by which early gestational nutrition regulates embryo survival

It has long been recognized that the effects of early gestational nutrition on embryo survival are primarily mediated through the regulation of P4 secretion and metabolism (Jindal et al., 1996). Progesterone, a key steroid hormone secreted by the corpus luteum in sows, plays a crucial role in uterine development, embryo implantation, and pregnancy establishment and maintenance. It promotes the synthesis and secretion of uterine glandular epithelium, leading to the production of histotroph enriched with nutrients and growth factors, which provides essential support for pre-implantation embryos (Large and DeMayo, 2012; Bazer and Johnson, 2014; Okrasa et al., 2014). As already mentioned, earlier studies suggested that energy intake exceeding maintenance requirements could reduce circulating P4 concentrations (Musser et al., 2004). The underlying mechanism may be related to increased feed intake leading to elevated hepatic blood flow, which in turn accelerates the catabolism of progesterone (Mattos et al., 2017). However, recent research has provided new insights into this traditional view. Evidence indicates that although high feed intake may reduce systemic P4 levels, it can simultaneously

enhance the local transfer of progesterone from the ovary to the uterus, thereby increasing uterine P4 concentrations and exerting beneficial effects on embryo survival (Langendijk, 2021). This local counter-current mechanism means that the net effect of high feeding on uterine P4 concentration may differ substantially from its effect on peripheral P4 concentrations. Supporting the functional significance of this local pathway, local uterine P4 originates from two primary sources: systemic P4 delivered via the general circulation and metabolized by the liver, and P4 directly transported to the uterus through a local counter-current transfer mechanism between the ovarian vein and the uterine artery, a pathway that bypasses hepatic first-pass metabolism (Langendijk and Peltoniemi, 2013). Research has shown that P4 concentrations in the uterine artery are significantly higher than those in the peripheral circulation, and this local supply is of great significance for uterine function (Stefańczyk-Krzyszowska et al., 1998). Furthermore, unilateral ovariectomy significantly reduces the embryo survival rate in the corresponding uterine horn, further demonstrating the importance of the local ovarian P4 supply (Athorn et al., 2011). Therefore, the regulation of progesterone by nutrition exhibits a “dual effect,” with the overall outcome depending on the dynamic balance between systemic metabolism and local uterine supply.

In addition to direct regulation of P4, nutrition can influence pregnancy through metabolic hormones. Studies have shown that increased feeding levels during early gestation elevate circulating insulin and insulin-like growth factor 1 (IGF-1) concentrations (Novak et al., 2002; Ferguson et al., 2003). IGF-1 has been demonstrated to promote corpus luteum development and enhance progesterone secretion both in vivo and in vitro (Miller et al., 2003; Ptak et al., 2003; Ptak et al., 2004). Moreover, IGF-1 may indirectly improve embryo survival by enhancing embryonic developmental competence (Block et al., 2003; Langendijk et al., 2008). These findings suggest that nutritional status modulates reproductive outcomes by shaping the endocrine environment through metabolic signaling pathways.

Furthermore, nutrition can regulate embryo implantation and development by modulating endometrial gene expression and epigenetic modifications. Studies have shown that feed restriction during early gestation significantly alters the endometrial transcriptome and DNA methylation patterns, particularly in pathways related to nutrient metabolism and embryo attachment (Zglejc et al., 2018; Zglejc-Waszak et al., 2019). Specific nutrients can directly regulate implantation-related signaling pathways. For instance, arginine has been shown to promote embryo implantation by activating the PI3K/AKT/mTOR/NO signaling pathway (Zeng et al., 2013), while also regulating placental gene expression associated with nutrient metabolism, polyamine synthesis, angiogenesis, and antioxidant responses (Li et al., 2022). Its precursor, N-carbamylglutamate (NCG), can similarly improve pregnancy outcomes by modulating antioxidant and immune-related protein expression (Zhu et al., 2015; Cai et al., 2018).

3.3 Protein nutrition and reproductive performance in sows: traditional perspectives and contemporary challenges

Based on the aforementioned research progress, it is evident that the ongoing genetic advancements in modern sows have significantly altered their nutritional

requirements during early gestation. In addition, currently, most studies have primarily focused on the regulation of overall feeding levels, whereas relatively limited attention has been paid to the specific roles of individual nutrients in embryonic development and implantation. Because increasing feed intake simultaneously elevates the supply of carbohydrates, proteins, lipids, and micronutrients, it remains difficult to disentangle the dominant nutritional factors affecting embryo survival. Among all dietary components, protein serves as a fundamental substrate for tissue synthesis and metabolic regulation. It not only provides the basal substrates for maternal maintenance and growth but also plays a vital role in embryo survival, growth, and placental development (Herring et al., 2018). Therefore, protein nutrition is considered one of the key determinants of pregnancy outcomes. Notably, metabolomics studies in pregnant sows have shown that, compared with high-prolific sows, low-prolific sows exhibit significant disturbances in amino acid metabolism in serum, urine, and follicular fluid, suggesting a close association between amino acid metabolic status and reproductive performance (Chen et al., 2019).

Early research suggested that a 10% dietary protein level fails to provide sufficient amino acids for embryo survival, whereas levels between 14% and 16% provide an excess of amino acids, which may generate high ammonia concentrations and adversely affect embryos (Wu et al., 2017). However, other studies have indicated that adjusting protein levels within a certain range has a limited impact on litter size. For instance, feeding protein levels from 10.5% to 13.5% during mid-to-late gestation showed no significant effect on litter size across groups (Fang et al., 2019). Similarly, studies by Kim et al. demonstrated that feeding 11% to 16% protein throughout gestation in multiparous sows did not significantly influence litter size (Kim et al., 2023). Research on primiparous sows also demonstrated that increasing protein levels (11% to 17%) had a limited effect on litter size, yet it significantly improved maternal body reserves as well as piglet weaning weight and weight gain (Jang et al., 2014). Furthermore, in high-parity sows, moderately increasing amino acid intake has been shown to enhance farrowing performance by improving redox status (Hu et al., 2025). Despite these findings, the optimal level of protein intake during early gestation remains controversial. On the one hand, insufficient protein intake may restrict amino acid availability for placental transport, impair placental function, and lead to intrauterine growth restriction (IUGR). On the other hand, excessive protein intake may increase nitrogen metabolic burden and ammonia toxicity, thereby negatively affecting embryonic development (Herring et al., 2018). Importantly, most existing studies have focused on protein supply during mid-to-late gestation or across the entire gestation period, whereas investigations specifically targeting the early gestational window remain scarce. A recent study from our laboratory demonstrated that moderately increasing dietary protein intake during early gestation significantly improved embryonic survival rate at GD 30 in gilts, suggesting a stage-specific role of protein nutrition during this critical period (Zhao et al., 2022). Whether this effect extends to multiparous sows and translates into improved farrowing outcomes under commercial production conditions needs further investigation. These inconsistent findings highlight that the effects of dietary

protein on reproductive performance are context-dependent, influenced by physiological stage, parity, and metabolic status.

3.4 Amino acids as substrates for fetal and placental synthesis and as metabolic signaling molecules in the mother

Building upon the role of protein nutrition, amino acids, as its fundamental components, exert multiple biological functions during pregnancy (Wu et al., 2017). In addition to serving as structural substrates for fetal and placental growth, amino acids also function as signaling molecules that regulate the mTOR pathway, cell proliferation, angiogenesis, and redox balance.

Pre-implantation embryos exhibit a high dependence on amino acids, and their development is accompanied by the dynamic expression of multiple amino acid transporters to meet stage-specific metabolic demands (Van Winkle, 2001). It has been reported that essential amino acids primarily promote the development of the inner cell mass, whereas non-essential amino acids facilitate blastocyst expansion and hatching (Lane et al., 2001). In in vitro culture systems, non-essential amino acids generally support embryonic development up to the 8-cell stage, after which the requirement for essential amino acids progressively increases (Lane et al., 2001). Moreover, supplementation with specific amino acids has been shown to improve the in vitro development of pre-implantation embryos in mammals (Ho et al., 1995). Recent metabolomic analyses of uterine luminal fluid during the peri-implantation period in sows have revealed that amino acids represent the most abundant class of metabolites, with amino acid-related pathways being significantly enriched (Liu et al., 2025). Compared with estrous sows, the total concentrations of several amino acids, including arginine, proline, glutamine, glutamate, histidine, lysine, phenylalanine, and cysteine are significantly increased in uterine luminal fluid from day 10 to day 16 of gestation (Liu et al., 2025). Notably, a marked elevation of specific amino acids, particularly arginine, glutamine, proline, lysine, and phenylalanine, has been observed at GD 16 (the window of implantation), which is closely associated with the activation of the mTOR signaling pathway and proliferation of trophoblast cells (Liu et al., 2025). Collectively, these findings indicate that amino acids are not only nutritional substrates but also key regulators of critical events during early embryonic development and implantation.

Certain amino acids modulate cellular signaling pathways, metabolic processes, and physiological functions, thereby playing essential roles in maintaining pregnancy and promoting embryonic development. Among them, arginine is one of the most extensively studied functional amino acids. In sows, supplementation with arginine during either early or mid-to-late gestation has been associated with improved embryo survival and increased litter size (Gao et al., 2012; Wu et al., 2013; Li et al., 2014; Guo et al., 2017). The underlying mechanisms may include: (1) promoting the synthesis of nitric oxide (NO) and polyamines, thereby enhancing placental angiogenesis and blood flow to improve maternal-fetal nutrient exchange; (2) improving corpus luteum development and function, leading to enhanced P4 secretion to support pregnancy; (3) facilitating cell proliferation, migration, and embryo attachment; and (4) improving antioxidant capacity through regulation of redox-related processes (Wu et al., 2013; Costa et al., 2019). In addition, arginine

and its endogenous synthesis activator, N-carbamylglutamate (NCG), can activate PI3K/Akt/mTOR signaling pathways, thereby promoting embryo implantation and placental development (Zeng et al., 2008; Zeng et al., 2012; Zeng et al., 2013). Other amino acids also play important roles during pregnancy. For example, leucine and glutamine can promote trophoblast cell proliferation via the mTOR pathway (Kim et al., 2013). Glycine, as an important osmolyte, is involved in oocyte maturation, fertilization, and early embryonic development (Xia et al., 1995), and participates in one-carbon metabolism to support DNA synthesis (Bässler, 1997). Proline and its metabolite polyamines are critical regulators of cell proliferation and differentiation (Wu et al., 2017; Li and Wu, 2018). Taken together, these findings suggest that protein nutrition during early gestation may influence embryo survival and subsequent reproductive performance through amino acid-mediated nutritional and signaling pathways.

4. The role of oxidative stress during pregnancy

The preceding discussion established that both insufficient and excessive protein intake can impair pregnancy outcomes, but through what common mechanistic pathway? Emerging evidence points to oxidative stress as a converging mediator linking nutritional imbalance to placental dysfunction and impaired fetal development, a perspective that is developed in the following section.

4.1 Oxidative stress: definition, sources, and assessment

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant defense mechanisms to eliminate them (Freinbichler et al., 2011). ROS are highly reactive oxygen-containing molecules, mainly including superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2) (Droge, 2002). Under physiological conditions, ROS act as signaling molecules involved in regulating cell proliferation, differentiation, and immune responses (Covarrubias et al., 2008). However, excessive accumulation of ROS can cause oxidative damage to DNA, RNA, lipids, and proteins, leading to irreversible cellular injury and dysfunction of tissues or organs (Demirci-Çekiç et al., 2022). In practical research, oxidative stress is typically evaluated through various biomarkers, including malondialdehyde (MDA), a product of lipid peroxidation; 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of DNA damage; and the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (S et al., 2024). Importantly, oxidative stress is highly sensitive to maternal nutritional status and metabolic regulation, making it a key mechanistic link between nutrition and reproductive outcomes.

4.2 Oxidative stress during pregnancy

Pregnancy is a highly dynamic process of metabolic adaptation, characterized primarily by significant maternal adaptive changes to meet the nutritional demands of fetal growth and development (Napso et al., 2018). This process exhibits distinct phase-specific characteristics over time. Early pregnancy is mainly characterized by

an anabolic state to accumulate maternal reserves, accompanied by increased insulin sensitivity and enhanced energy storage in maternal tissues. In contrast, during late pregnancy, as fetal growth and mammary gland development accelerate sharply, the maternal metabolism shifts to a catabolic state, manifested by increased insulin resistance and enhanced mobilization of energy reserves (Sferruzzi-Perri et al., 2020). Throughout this process, oxidative stress shows a progressive increase. In early pregnancy, the placenta exists in a relatively low-oxygen environment, and ROS levels remain low. As placental vascularization develops and fetal metabolic demand increases, mitochondrial oxidative phosphorylation is enhanced, leading to a gradual rise in ROS production. Studies have shown that the levels of ROS, 8-OHdG, and lipid peroxidation markers such as thiobarbituric acid reactive substances (TBARS) in the serum of late-gestation sows are significantly higher than those in early gestation (Tan et al., 2015), indicating that sows experience an increase in systemic oxidative stress during late pregnancy. Meanwhile, due to increased metabolic burden, the activity of antioxidant enzymes and total antioxidant capacity tend to decline during late gestation (Berchieri-Ronchi et al., 2011; Guo et al., 2022), further exacerbating redox imbalance. This oxidative stress state has been closely associated with a range of adverse reproductive outcomes, including intrauterine growth restriction (IUGR), prolonged parturition, reduced total and live litter size, and decreased litter weight gain (Oliviero et al., 2010; Ruediger and Schulze, 2012; Zhang et al., 2020). In addition, increased feed intake and excessive body condition during late gestation may further aggravate oxidative stress, insulin resistance, and systemic inflammation. These late gestational metabolic disturbances can also negatively affect feed intake and milk production during lactation (Black et al., 1993; Silanikove et al., 2009), thereby exerting carry-over effects across physiological stages. For modern high-producing sows, characterized by larger litter size, faster fetal growth, and greater metabolic load, the risk of redox imbalance is further increased. Therefore, oxidative stress has emerged as a key physiological constraint limiting the reproductive potential of high-producing sows.

4.3 Effects of oxidative stress on placental function

4.3.1 Structural and functional basis of the placenta

The placenta is the first organ formed during mammalian pregnancy. It is composed of maternal endometrium and fetal chorion and serves as a vital life-support system for fetal growth and development. Its primary functions include nutrient transport, gas exchange, removal of metabolic waste, and acting as a maternal-fetal barrier to protect the embryo from harmful external substances (Furukawa et al., 2014). In addition, the placenta functions as a dynamic endocrine organ that secretes hormones, cytokines, and growth factors, which modulate maternal physiology and regulate fetal development throughout gestation (Enders and Blankenship, 1999). Placental efficiency, commonly defined as the ratio of fetal weight to placental weight, is widely used as an integrative indicator of placental function. It reflects the overall capacity of the placenta in nutrient transport, metabolic regulation, and blood supply, and is closely associated with birth weight and within-litter uniformity (Hayward et al., 2016). Impairment of placental function

can lead to adverse pregnancy outcomes, including intrauterine growth restriction (IUGR), stillbirth, low birth weight, and preterm delivery (Brosens et al., 2011).

The porcine placenta is classified as an epitheliochorial type with a diffuse distribution pattern. The maternal and fetal circulations are separated by six distinct tissue layers: maternal vascular endothelium, maternal connective tissue, uterine luminal epithelium, fetal trophoblast epithelium, fetal placental mesenchyme, and fetal vascular endothelium (Enders and Blankenship, 1999) (Figure 1-2). This non-invasive characteristic limits the passive diffusion of macromolecules, making the porcine placenta highly dependent on efficient active transport systems and trophoblast-mediated endocytosis for nutrient exchange.

Given the technical limitations of sampling and morphometric analysis in the diffuse porcine placenta, mechanistic studies of placental biology have frequently employed the mouse placenta as a complementary model system. As a mechanistic model, the mouse placenta is highly invasive and classified as a hemochorial type. In this type, maternal tissues are eroded, and fetal chorionic villi are directly bathed in maternal blood, allowing substance exchange across only three fetal-derived layers. At the anatomical level, the mouse placenta consists of the maternal decidua and two fetal-derived regions: the junctional zone and the labyrinth zone (Panja and Paria, 2021). Among these, the labyrinth zone serves as the primary site for maternal-fetal exchange. At the microscopic interface, the mouse placenta exhibits a specialized hemotrichorial barrier structure. Maternal blood is separated from fetal capillaries solely by fetal-derived tissues, including, from maternal to fetal side: a layer of sinusoidal trophoblast giant cells (S-TGC) directly exposed to maternal blood, two layers of multinucleated syncytiotrophoblasts (Syncytiotrophoblast I and II), and the innermost fetal endothelial cells (Panja and Paria, 2021) (Figure 1-3).

Although porcine and mouse placentas differ substantially in their anatomical structure and maternal-fetal interface, they share several core biological functions that are essential for fetal development (Enders and Blankenship, 1999). These structural differences influence the mode and efficiency of maternal-fetal exchange and should therefore be carefully considered when extrapolating findings across species (Furukawa et al., 2014). Nevertheless, both placentas are responsible for supporting fetal growth, mediating the transfer of glucose, amino acids, lipids, and other nutrients, regulating maternal-fetal metabolic exchange, and maintaining placental redox balance and mitochondrial function (Carter, 2012; Lager and Powell, 2012; Fisher et al., 2020). Several key molecular systems involved in these processes are broadly conserved or functionally comparable across mammalian placentas, including solute carrier transporters for amino acids and glucose, components of mitochondrial energy metabolism, oxidative stress-related pathways such as the Nfe2l2 antioxidant system, and regulators of mitochondrial dynamics (Carter, 2012). Therefore, although the mouse placenta cannot fully replicate the anatomical characteristics of the porcine placenta, it provides a useful mechanistic model for investigating how maternal nutritional imbalance affects placental function and fetal development. In the context of this thesis, the mouse model was not introduced to directly mimic pregnancy in commercial sows, but rather to provide a highly controlled experimental system in which the mechanistic consequences of dietary protein imbalance could be examined more precisely.

Accordingly, findings obtained from mice should be interpreted as mechanistic evidence supporting potential biological pathways, rather than as direct predictions of placental responses in sows.

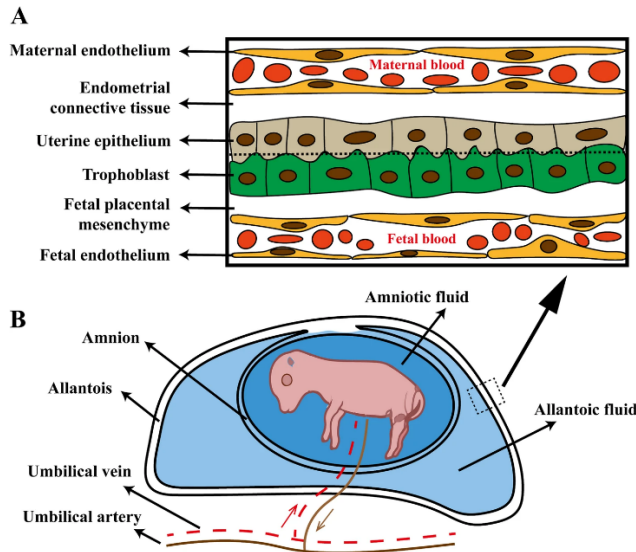


Figure 1-2: Schematic illustration of the porcine placental barrier and conceptus. From Chengquan Tan et al., 2022.

(A) The porcine placental barrier consists of six tissue layers separating maternal and fetal blood: maternal vascular endothelium, maternal connective tissue, uterine luminal epithelium, fetal trophoblast epithelium, fetal placental mesenchyme, and fetal vascular endothelium. (B) The fetus is enclosed by the amnion and surrounded by amniotic and allantoic fluids. The allantois contributes to placental vascular development, while the umbilical vein and artery connect the fetus with the placenta for nutrient, gas, and waste exchange.

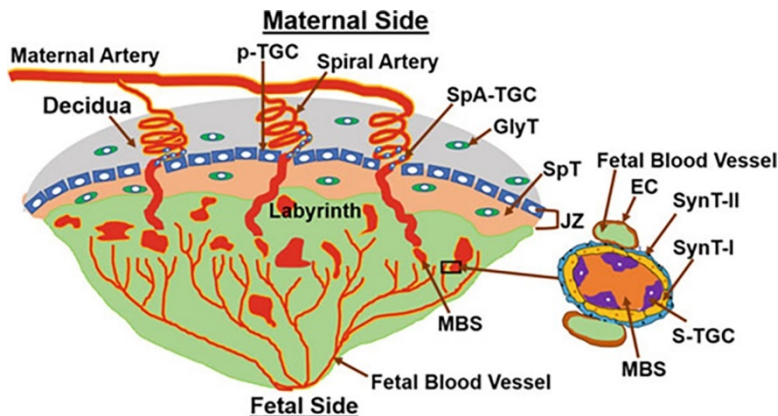


Figure 1-3: Schematic representation of the mature mouse placenta. From Sourav Panja and Bibhash C. Paria, 2021.

The mature mouse placenta consists of the maternal decidua and fetal-derived regions, including the junctional zone (JZ) and labyrinth. Maternal arteries and spiral arteries supply maternal blood to the maternal blood sinusoids (MBS), while fetal blood vessels lined by endothelial cells (EC) are distributed on the fetal side. The figure also shows major trophoblast populations, including parietal trophoblast giant cells (p-TGC), spiral artery-associated TGC (SpA-TGC), sinusoidal TGC (S-TGC), spongiotrophoblasts (SpT), glycogen trophoblasts (GlyT), and syncytiotrophoblast layers I and II (SynT-I and SynT-II). In the right inset, the labyrinth exchange barrier is shown in more detail. Maternal blood in the maternal blood sinusoids is separated from fetal blood by a hemotrichorial barrier composed of three trophoblast layers, including S-TGC, SynT-I, and SynT-II, followed by fetal endothelial cells on the fetal side.

4.3.2 Regulation of placental nutrient transport systems

Fetal growth relies heavily on a continuous supply of nutrients, a process largely dependent on the transport capacity of the placenta. In litter-bearing species such as pigs, limited uterine space leads to substantial competition for nutrients among fetuses, making placental efficiency a key determinant of embryo survival and fetal development (Wilson et al., 1999; Vonnahme et al., 2002). Placental nutrient transport is regulated by multiple factors, including nutrient concentration gradients between maternal and fetal circulation, uteroplacental blood flow, effective exchange surface area, and intrinsic placental metabolic activity. In addition, it critically depends on the expression and functional activity of specific transport proteins within the placental barrier. At the molecular level, nutrient transport across the placenta is primarily mediated by members of the solute carrier (SLC) transporter family (Broer, 2008).

Among these, glucose and amino acids are essential substrates for fetal development, and their placental transport is of particular importance (Lager and Powell, 2012). Glucose serves as the primary energy source for both the fetus and placenta. Since the fetus has a limited capacity for endogenous glucose production, its supply depends almost entirely on placental transfer from the maternal circulation. Placental glucose transport occurs mainly via facilitated diffusion mediated by glucose transporters (GLUTs), among which GLUT1 (Slc2a1) is the predominant isoform expressed during pregnancy (Das et al., 1998). In addition, GLUT3 (Slc2a3), characterized by high affinity for glucose, also contributes to this process. In mice, disruption of placental GLUT3 expression has been associated with early pregnancy loss and fetal growth restriction (Ganguly et al., 2007), indicating that glucose transport capacity directly determines fetal energy supply and growth potential.

Unlike glucose, which is primarily transported across the placenta by facilitated diffusion, placental amino acid transfer is mediated by multiple transporter systems involving active transport, amino acid exchange, and facilitated transport mechanisms. These coordinated transport mechanisms enable the placenta to transfer amino acids against concentration gradients and contribute to fetal amino acid concentrations that may exceed maternal levels (Cetin et al., 2005). Among the best-characterized transport systems are System A and System L. System A transporters (SNAT1, SNAT2, and SNAT4) primarily mediate the uptake of small

neutral non-essential amino acids such as alanine, glycine, and serine (Jansson, 2001; Zhang et al., 2015), providing substrates for subsequent exchange of essential amino acids. In human and rat placental microvillous membranes, SNAT1 is considered the major contributor to system A-mediated amino acid uptake (Takahashi et al., 2017). The remaining uptake is mainly mediated by SNAT4 in humans and SNAT2 in rats (Takahashi et al., 2017). System L transporters are mainly responsible for the exchange of essential neutral amino acids, facilitating transport against concentration gradients via the exchange of intracellular non-essential amino acids with extracellular essential amino acids (Verrey, 2003). Other transport systems include cationic amino acid transporters (e.g., CAT1), which mediate the transport of amino acids such as arginine, and anionic transport systems involved in the transport of acidic amino acids such as glutamate. Importantly, the placenta itself exhibits active amino acid metabolism. Amino acids can be interconverted within placental tissues and participate in protein synthesis, energy production, and various biosynthetic pathways, thereby further influencing nutrient allocation between the mother and fetus (Schneider et al., 1979).

Studies in humans indicate that in pathological pregnancies, such as preeclampsia, intrauterine growth restriction (IUGR), maternal obesity, and gestational diabetes mellitus, the expression and activity of placental macronutrient transporters and associated metabolic enzymes are altered (Jansson et al., 2002; Jansson et al., 2013). Therefore, alterations in placental transport function constitute an important basis for various adverse pregnancy outcomes. Notably, oxidative stress has been shown to directly inhibit transplacental transport of glucose and amino acids by downregulating transporter expression or altering their activity (Khullar et al., 2004; Lappas et al., 2012).

4.3.3 Placental mitochondrial dysfunction and oxidative stress

Evidence from human pregnancies and experimental animal models suggests that placental oxidative stress acts as a critical link between maternal metabolic disturbances and adverse pregnancy outcomes, including preeclampsia, IUGR, gestational diabetes mellitus, and preterm birth (Wisdom et al., 1991; Grzeszczak et al., 2023). Oxidative stress (OS) emerges at the maternal-fetal interface from early pregnancy and progressively increases as gestation advances. With placental development, vascular expansion, and the rising metabolic demands of the fetus, placental mitochondrial content and the activity of the mitochondrial electron transport chain (ETC) are continuously enhanced, leading to increased production of reactive oxygen species (ROS) (Pereira et al., 2015). Under physiological conditions, moderate levels of ROS are essential for normal placental development and function, including trophoblast migration and invasion, placental remodeling, and angiogenesis (Schoots et al., 2018). However, when ROS production exceeds the capacity of antioxidant defense systems, pathological oxidative stress occurs, resulting in damage to placental structure and function. This imbalance is closely associated with adverse pregnancy outcomes, such as miscarriage, fetal growth restriction (FGR), preterm birth (PTB), and fetal death (Grzeszczak et al., 2023). To maintain redox homeostasis, the placenta has developed a comprehensive antioxidant defense system. This includes enzymatic antioxidants, such as SOD, CAT, GPx, glutathione reductase (GR), and glutathione S-transferase, as well as

non-enzymatic antioxidants, including glutathione (GSH), selenium, vitamins C and E, nicotinamide adenine dinucleotide (NADH), and NADPH (Silvestro et al., 2020). These antioxidant systems are dynamically upregulated during pregnancy to adapt to increasing oxidative pressure. At the molecular level, the Keap1-Nfe2l2 signaling pathway is a central regulator of placental redox balance. Under oxidative stress conditions, Nfe2l2 is activated and translocates into the nucleus, where it induces the expression of multiple antioxidant and detoxification-related genes, thereby enhancing cellular antioxidant capacity (Chigusa et al., 2012). Reduced Nfe2l2 activation has also been observed in human placentas from pregnancies complicated by preeclampsia (Chigusa et al., 2012). When this pathway is impaired or imbalanced, oxidative stress is continuously amplified, exerting long-term detrimental effects on placental function.

The placenta is a highly metabolically active organ enriched with mitochondria. Mitochondria play a dual role in oxidative stress, serving both as the primary source of ROS and as a major target of oxidative damage. As the main site of ATP production, mitochondria inevitably generate ROS as byproducts during oxidative phosphorylation via the ETC (Olszewska and Szewczyk, 2013). Complexes I and III are recognized as the principal sites of ROS generation within mitochondria (Murphy, 2009). Excessive ROS production can directly impair mitochondrial structure and function. Placental mitochondrial dysfunction is considered a key mechanistic basis for adverse pregnancy outcomes (Vornic et al., 2024). A systematic review of human studies identified altered mitochondrial DNA content, impaired oxidative phosphorylation, changes in electron transport chain activity, reduced ATP production, and disturbed mitochondrial quality control in placentas associated with adverse pregnancy outcomes (Voros et al., 2025). One of the most frequently reported alterations is the disruption of mtDNA integrity and copy number. A decrease in mtDNA copy number may reflect reduced mitochondrial abundance or impaired mitochondrial biogenesis, whereas an increase may represent a compensatory biogenesis or the accumulation of dysfunctional mitochondria (Filograna et al., 2021). Given that placental growth and function rely heavily on sufficient mitochondrial abundance to meet high metabolic demands, a reduction in mtDNA directly compromises the placenta's capacity for energy production. Furthermore, a significant reduction in ATP production indicates defects in the oxidative phosphorylation of the ETC (Khan et al., 2015). Dysfunction of key ETC complexes impairs electron transport, leading to electron leakage and excessive ROS generation. Moreover, dysregulation of mitochondrial dynamics is another important feature of placental dysfunction. Mitochondrial fusion is regulated by proteins including MFN1, MFN2, and OPA1, whereas mitochondrial fission involves DRP1 and FIS1 (Nan et al., 2017). Alterations in mitochondrial fusion and fission have also been reported in placental disorders such as preeclampsia and intrauterine growth restriction, although the direction of change may vary according to the condition and experimental model (Holland et al., 2017). Dysregulation of mitochondrial dynamics may result in abnormal mitochondrial morphology, decreased membrane potential, and impaired energy metabolism. In summary, oxidative stress disrupts mitochondrial structure and function, interferes with energy

metabolism and cellular homeostasis, and represents a central molecular mechanism underlying placental dysfunction and impaired fetal development.

4.3.4 Placental plasticity and the threshold of metabolic compensation

Although oxidative stress has the potential to impair placental function, the placenta is not a static organ but rather a dynamic system with remarkable structural and functional plasticity. Placental plasticity refers to the ability of the placenta to adapt to changes in maternal nutritional status, metabolic environment, and stress levels through structural remodeling and functional regulation, thereby maintaining fetal growth and development (Sferruzzi - Perri et al., 2023).

Under conditions of nutritional or metabolic stress, the placenta can undergo adaptive adjustments through multiple mechanisms. At the structural level, the placenta can increase the degree of folding and expand the exchange surface area to enhance the efficiency of maternal-fetal nutrient exchange. At the functional level, the placenta can upregulate the expression of specific nutrient transporters, such as amino acid and glucose transporters, thereby improving the supply of key nutrients. In addition, the placenta can modulate its own metabolic pathways, including amino acid and lipid metabolism, to reallocate nutrient resources in favor of fetal development. At the mitochondrial level, the placenta can also partially compensate for oxidative stress by regulating mitochondrial biogenesis and antioxidant system activity. For example, under increased oxidative pressure, an elevation in mitochondrial number or mitochondrial DNA (mtDNA) copy number may occur as an adaptive response to maintain energy supply. However, when the intensity of oxidative stress exceeds the regulatory capacity of the placenta, these compensatory mechanisms fail, leading to placental decompensation, which ultimately manifests as fetal growth restriction or pregnancy failure. Notably, placental plasticity exhibits a clear “threshold effect” and “stage-specificity.” Under mild or transient stress conditions, the placenta can maintain functional stability through adaptive remodeling. In contrast, under persistent or excessive stress, the regulatory capacity of the placenta becomes limited, and abnormal functional reprogramming may occur, potentially leading to long-term adverse effects on fetal development.

Therefore, placental plasticity represents not only an important adaptive mechanism by which the mother responds to environmental changes, but also a key regulatory node linking maternal nutritional status, oxidative stress levels, and fetal developmental outcomes. This highlights the importance of maintaining appropriate redox balance during pregnancy to preserve placental function and optimize reproductive outcomes. This highlights the importance of maintaining appropriate redox balance during pregnancy, particularly through nutritional regulation, to preserve placental function and optimize reproductive outcomes.

5. Nutritional modulation of maternal metabolism and oxidative stress: emerging strategies to improve reproductive performance

5.1 Functional nutritional interventions targeting oxidative stress and reproductive performance in sows

Beyond macronutrients such as protein, increasing attention has recently focused on the role of functional nutritional additives in improving reproductive performance in sows. Unlike conventional nutrients that primarily provide energy or structural substrates, these interventions exert their effects mainly by modulating maternal redox status, inflammatory responses, and metabolic homeostasis.

Regarding antioxidant nutrition, current research on functional nutritional additives primarily focus on vitamins, trace elements, and plant-derived bioactive compounds (Li et al., 2022). Selenium, as an essential component of glutathione peroxidase, plays a critical role in maintaining the maternal antioxidant defense system. Supplementation with organic selenium has been shown to significantly enhance antioxidant capacity in sows and improve piglet survival rate and litter size at weaning (Chen et al., 2016). Vitamin E, a lipid-soluble antioxidant, contributes to the stabilization of cellular membranes by inhibiting lipid peroxidation. Its supplementation has been demonstrated to increase antioxidant enzyme activities and improve reproductive performance in sows (Mahan, 1994). In addition, plant-derived functional compounds have emerged as a major research focus. Plant extracts are rich in bioactive substances such as polyphenols, flavonoids, alcohols, and terpenoids, which exhibit potent antioxidant and anti-inflammatory properties (Li et al., 2022). These substances improve the redox balance in sows by scavenging reactive oxygen species, enhancing antioxidant enzyme activity, and modulating immune responses (Chen et al., 2023).

Despite the diversity of these nutritional interventions, their underlying mechanisms appear to converge. Specifically, they consistently improve reproductive performance by regulating maternal oxidative stress and metabolic status. Therefore, maintaining redox balance and metabolic homeostasis in the maternal organism may represent a central mechanism linking nutritional regulation to reproductive outcomes. These findings further support the concept that targeting maternal oxidative stress and metabolic status represents a central strategy for improving reproductive outcomes.

5.2 The role of gut microbiota and maternal metabolism in pregnancy regulation

In recent years, the gut microbiota has been increasingly recognized as a key link between nutrition and host metabolism, playing an important regulatory role during pregnancy (Sanz et al., 2025). Some studies in humans have shown that the composition of the gut microbiota undergoes significant alterations throughout gestation and is closely associated with maternal metabolic status (Koren et al., 2012). These changes may influence maternal energy metabolism, inflammatory responses, and insulin sensitivity. Gestation associated changes in gut microbiota

composition have also been observed in pigs (Ji et al., 2019; Liu et al., 2019). During gestation, the maternal gut microbiota exhibits pronounced temporal dynamics. In particular, during late gestation and the peripartum period, the microbial community undergoes substantial dysbiosis and remodeling (Koren et al., 2012; Huang et al., 2019). In humans, this included an increase in the relative abundance of *Proteobacteria* and *Actinobacteria* from early to late pregnancy (Koren et al., 2012). These microbial shifts occur in parallel with changes in the maternal inflammatory status (Shao et al., 2019). Moreover, pregnancy-associated alterations in the gut microbiota have been associated with metabolic disorders such as gestational diabetes mellitus (Crusell et al., 2018).

Pregnancy-associated changes in maternal physiology provide a biological explanation for these microbial shifts. Changes in hormonal signalling, immune regulation and maternal metabolism may alter the intestinal environment and contribute to gut microbial remodeling (Amato et al., 2024). Experimental studies using progesterone supplementation in mice and in vitro cultures have shown that progesterone can promote *Bifidobacterium* growth (Nuriel-Ohayon et al., 2019). These findings support a role for pregnancy-associated hormonal changes in shaping the gut microbiota. Dietary factors can also affect the maternal gut microbiota, as demonstrated by dietary fibre interventions in pregnant sows (Liu et al., 2021). Therefore, microbial changes across gestation probably reflect the combined effects of pregnancy-associated physiological adaptations and external factors, although their relative contributions remain incompletely understood.

In addition to compositional changes, the gut microbiota has been implicated in the regulation of host metabolic homeostasis and redox balance. For example, the genus *Bacteroides* has been positively associated with antioxidant capacity and reproductive performance in sows, whereas *Phascolarctobacterium* and *Streptococcus* show negative correlations (Wang et al., 2018; Wang et al., 2019). Meanwhile, metabolomics studies have revealed significant alterations in the maternal serum metabolome during pregnancy, particularly in amino acid and lipid metabolic pathways (Mitro et al., 2021). Accumulating evidence suggests that pregnancy-associated changes in the gut microbiota are closely linked to maternal metabolic adaptations, and these interactions may influence maternal metabolism through host-microbiota crosstalk (Koren et al., 2012; Wang et al., 2018; Liu et al., 2019). Together, these findings suggest that the gut microbiota acts as a potential mediator linking nutrition, metabolism, and oxidative stress during pregnancy.

5.3 Bile acids and metabolic regulation

In recent years, the physiological roles of bile acids have expanded from their traditional function as lipid digestion molecules to important regulators of metabolism. Bile acids are amphipathic sterol derivatives synthesised from cholesterol in the liver and are broadly classified as primary or secondary bile acids depending on whether they undergo microbial biotransformation in the intestine (Ma and Patti, 2014; Hylemon et al., 2017). Primary bile acids are synthesized from cholesterol in the liver, secreted into the intestine, and subsequently transformed into secondary bile acids through the action of the gut microbiota (Jia et al., 2018; Winston and Theriot, 2020). In addition to facilitating the absorption of lipids and

fat-soluble vitamins, bile acids act as signaling molecules that regulate host metabolism and immune function by activating receptors such as the farnesoid X receptor (FXR) and the G protein-coupled receptor TGR5 (Fiorucci and Distrutti, 2015). Accumulating evidence has demonstrated that bile acids are involved in the regulation of lipid metabolism, glucose homeostasis, and energy balance (Ma et al., 2006; Fiorucci et al., 2010; de Aguiar Vallim et al., 2013). Furthermore, bile acids and their derivatives can alleviate oxidative damage by reducing reactive oxygen species (ROS) production, inhibiting lipid peroxidation, and modulating the expression of antioxidant enzymes (Punzo et al., 2024).

5.4 Potential links between CDCA and amino acid metabolism

Chenodeoxycholic acid (CDCA) is one of the major primary bile acids (Joyce and Gahan, 2016). CDCA is synthesized from cholesterol in the liver through the classical and alternative bile acid synthesis pathways and can be conjugated with glycine or taurine before secretion into bile (Chiang and Ferrell, 2018). Bile containing these conjugates may be stored in the gallbladder before release into the small intestine, where bile acids contribute to lipid digestion and absorption (Winston and Theriot, 2020). In the intestine, microbial enzymes can remove the conjugated amino acids, and some of the resulting free CDCA can be converted into lithocholic acid through 7α -dehydroxylation (Fleishman and Kumar, 2024). Most bile acids are reabsorbed from the intestine and returned to the liver through the portal circulation. Active reabsorption of conjugated bile acids occurs mainly in the terminal ileum (Durník et al., 2022). Hepatocytes take up the returning bile acids and secrete them into bile again. This enterohepatic circulation enables repeated use of the bile acid pool. New synthesis in the liver replaces the small fraction lost in the faeces (Chiang and Ferrell, 2018). The synthesis of CDCA and its participation in bile acid metabolism and enterohepatic circulation are illustrated in Figure 1-4. Previous studies have demonstrated that CDCA can improve metabolic disorders by regulating lipid metabolism and inflammatory responses (Zheng et al., 2024). CDCA has also been shown to upregulate the expression of anti-inflammatory genes while suppressing pro-inflammatory cytokines, thereby enhancing insulin sensitivity (Shihabudeen et al., 2015). In addition, both CDCA and ursodeoxycholic acid (UDCA) can attenuate lipopolysaccharide (LPS)-induced inflammatory responses by reducing pro-inflammatory cytokine levels and alleviating oxidative stress (Milivojac et al., 2024). Further studies have indicated that CDCA can mitigate high-fat diet-induced oxidative stress by improving lipid metabolic disturbances and enhancing antioxidant capacity (Zheng et al., 2024). Moreover, in both aquatic animal models and porcine intestinal epithelial cell systems, CDCA has been reported to enhance antioxidant capacity and improve intestinal health (Xie et al., 2021; Xu et al., 2022). Collectively, these findings suggest that CDCA plays a critical role not only in metabolic regulation but also in improving physiological status through modulation of oxidative stress and inflammatory responses, highlighting its potential as a functional nutritional regulator.

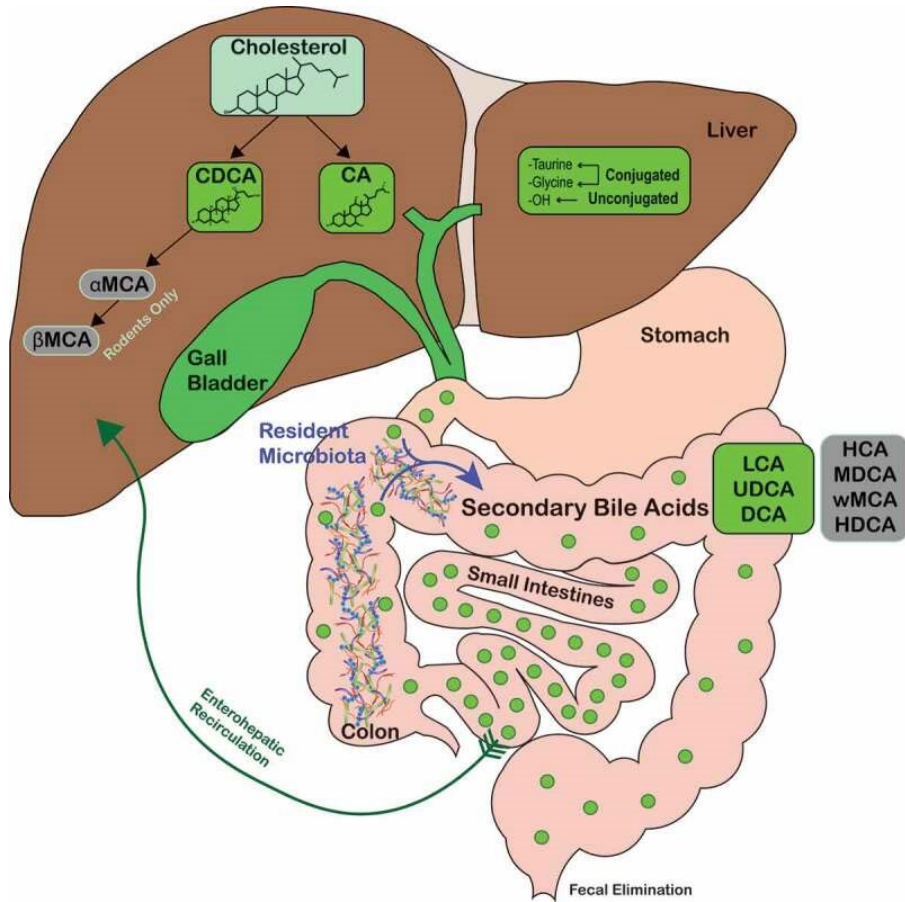


Figure 1-4: Hepatic production, intestinal microbial transformation and enterohepatic recycling of bile acids. From Winston and Theriot, 2020.

CDCA is formed from cholesterol in the liver and can undergo conjugation with glycine or taurine before biliary secretion. Bile acids enter the intestine, where microbial activity modifies part of the pool. Intestinal absorption and return through the portal circulation allow hepatic uptake and repeated secretion into bile, while unabsorbed bile acids are eliminated in the faeces. The figure presents a general overview rather than a sow-specific pathway. Grey-shaded metabolites depict rodent-associated pathways as presented in the original figure. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; αMCA, α-muricholic acid; βMCA, β-muricholic acid; ωMCA, ω-muricholic acid.

Our previous results demonstrated that dietary supplementation with CDCA during early pregnancy improved embryo implantation, reduced maternal inflammation, enhanced antioxidant capacity, and modulated gut microbiota and host metabolism in sows (Chen et al., 2023). In that study, CDCA supplementation also increased the abundance of multiple amino acids in serum, and pathways related

to amino acid metabolism, protein digestion, and absorption were highly enriched (Chen et al., 2023). These findings suggest that amino acid-related metabolic regulation may contribute to the beneficial effects of CDCA during early pregnancy. However, these previous findings mainly focused on the early gestational responses to CDCA supplementation, particularly around the implantation period. Whether these early metabolic changes persist beyond implantation and influence maternal physiological status during late gestation remains unclear. In particular, it is still unknown whether early gestational CDCA supplementation can improve farrowing outcomes by inducing sustained changes in maternal oxidative stress, inflammatory status, insulin-related metabolism, serum metabolomic profiles, and gut microbiota-associated metabolic functions.

As discussed above, reproductive outcomes may not be determined solely by nutrient supply, but also by the capacity of the mother to maintain metabolic and redox homeostasis throughout pregnancy. Therefore, in this thesis, CDCA was further investigated not simply as a bile acid supplement, but as a metabolism-targeted nutritional intervention. By examining maternal metabolic status during late gestation, host-microbiota interactions, and farrowing performance, this study aimed to determine whether CDCA supplementation during early gestation could exert sustained metabolic effects and improve reproductive performance in sows.

Collectively, previous studies suggest that CDCA has the potential to regulate maternal metabolism, oxidative stress, inflammation, gut microbiota, and amino acid-related metabolic pathways. These properties make CDCA a promising candidate for metabolism-targeted nutritional intervention during pregnancy. However, whether CDCA supplementation during early gestation can induce sustained metabolic effects beyond the implantation period and ultimately improve farrowing outcomes in sows remains to be clarified.

6. Research gaps

Despite the increasing recognition of early gestation as a critical window for nutritional regulation in modern high-producing sows, several important questions remain unresolved. First, although increased feed allowance during early gestation has been re-evaluated in modern sow production, most previous studies have focused primarily on overall energy intake, while the specific contribution of dietary protein has received relatively limited attention. Protein and amino acids are essential for embryo development, the composition of uterine histotroph, maternal tissue recovery, and placental growth. However, both insufficient and excessive protein supply may impair pregnancy outcomes. Therefore, whether moderate adjustment of dietary protein during early gestation is sufficient to improve reproductive performance in multiparous sows under commercial production conditions requires further investigation.

Second, the biological mechanisms linking maternal dietary protein intake to reproductive outcomes remain incompletely understood. Existing evidence indicates that amino acid metabolism, oxidative stress, and placental nutrient transport are involved in the regulation of pregnancy. However, it remains unclear whether changes in maternal dietary protein supply affect pregnancy outcomes primarily

through altered nutrient availability, or through broader maternal metabolic responses, including redox imbalance, amino acid metabolic remodeling, and placental functional adaptation. In particular, how maternal dietary protein intake is translated into changes in placental function and fetal development remains to be further elucidated.

Third, if maternal metabolic homeostasis are important mediators of nutritional effects during pregnancy, strategies targeting metabolic regulation rather than nutrient supply alone may represent a promising approach to improve reproductive performance. Bile acids, especially chenodeoxycholic acid (CDCA), have been identified as important signaling molecules involved in glucose and lipid metabolism, inflammation, oxidative stress, gut microbiota regulation, and amino acid-related metabolic pathways. However, whether CDCA supplementation during early gestation can induce sustained improvements in maternal metabolic status and reproductive outcomes in sows, and how these effects are associated with host-microbiota metabolic interactions, remain to be clarified.

Chapter 2

Aims and outlines of the thesis

1. Aims of the thesis

Our previous studies demonstrated that moderately increasing dietary protein levels during early gestation improved nutrient transport and enhanced embryo survival in gilts. However, whether this strategy can be extended to multiparous sows remains unclear. Under practical production conditions, dietary crude protein (CP) levels typically range from 12%-15%, yet it is still unknown whether variations within this range can substantially influence reproductive outcomes or maternal metabolic homeostasis. In addition, the underlying mechanisms by which maternal dietary protein intake affects reproductive performance remain poorly understood, particularly in relation to metabolic responses and placental function.

Based on these considerations, this thesis aimed to investigate whether dietary protein levels during early gestation influence reproductive performance and pregnancy-related physiological responses in multiparous sows. To further explore the biological consequences of protein imbalance, a mouse model was introduced as a controlled mechanistic system, with particular focus on pregnancy outcomes, placental function, and related molecular mechanisms. Finally, early gestational supplementation with chenodeoxycholic acid (CDCA) was evaluated as a potential nutritional strategy to modulate maternal metabolic status and improve reproductive outcomes in sows.

Therefore, the specific objectives of this thesis were:

(1) to evaluate whether dietary protein levels within the practical commercial feeding range during early gestation affect reproductive performance in multiparous sows, and to determine their effects on reproductive hormones, maternal metabolism, systemic redox status, amino acid profiles, and placental nutrient transport;

(2) to investigate, using a mouse model under controlled nutritional conditions, the metabolic and mechanistic basis by which protein imbalance affects pregnancy outcomes, with particular focus on placental oxidative stress, mitochondrial function, and nutrient transport systems;

(3) to determine whether CDCA supplementation during early gestation can exert sustained metabolic regulatory effects by modulating maternal metabolic status, serum metabolomic profiles, and gut microbiota, and to evaluate its potential to improve reproductive performance in sows.

To achieve these goals, three animal experiments were conducted. The overall experimental design and research framework are shown in Figure 2-1.

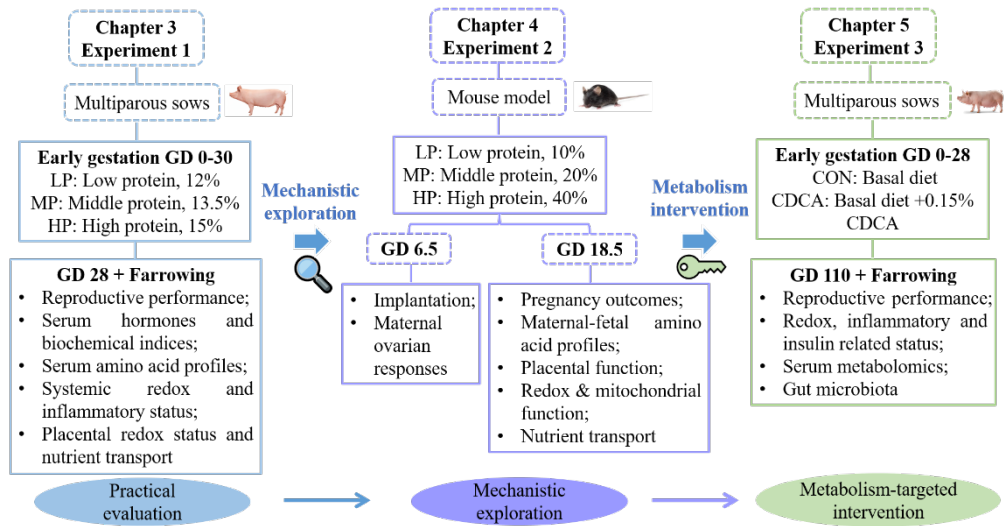


Figure 2-1: The technical route of the thesis.

2. Outline of the thesis

This thesis is composed of six main chapters, structured as follows:

Chapter 1 provides a comprehensive literature review focusing on the physiological importance of early gestation, the role of nutritional regulation in reproductive performance, the functions of protein and amino acids during pregnancy, the role of placental nutrient transport and redox homeostasis, and the potential of bile acid-related metabolic regulation as a nutritional strategy.

Chapter 2 presents the aims and overall framework of the thesis.

Chapter 3 (Experiment 1) aims to evaluate whether dietary protein levels within the range commonly used in commercial production during early gestation influence reproductive performance in multiparous sows. We hypothesized that increasing maternal dietary protein supply during early gestation can improve reproductive performance and might be accompanied by changes in maternal amino acid availability, metabolic and redox status, and placental nutrient transport. To test this hypothesis, multiparous sows were fed isocaloric diets containing 12%, 13.5%, or 15% crude protein from GD 0 to GD 30. Maternal metabolic, hormonal, inflammatory, oxidative stress, and amino acid-related responses were evaluated, together with placental functional indicators and reproductive performance.

Chapter 4 (Experiment 2) aims to explore whether a more pronounced maternal dietary protein imbalance affects implantation, fetal growth, placental function, and the potential mechanism. We hypothesized that an imbalanced maternal dietary protein and amino acid supply would affect reproductive outcomes, potentially through disturbances in maternal-fetal nutrient distribution, placental redox homeostasis, mitochondrial function, and nutrient transport. To test this hypothesis under more controlled nutritional conditions, pregnant mice were fed isocaloric

purified diets containing 10%, 20%, or 40% protein throughout gestation. Implantation-related responses were examined on GD 6.5, while maternal-fetal nutrient distribution, fetal development, placental morphology, oxidative status, mitochondrial function, and nutrient transport were evaluated on GD 18.5. The mouse model was used to investigate mechanisms that could not be examined comprehensively in the sow experiment rather than to directly reproduce commercial sow nutrition.

Chapter 5 (Experiment 3) extends the focus of Experiments 1 and 2 from nutritional imbalance to a metabolism-targeted nutritional intervention. The aim was to evaluate whether a metabolism-targeted nutritional intervention during early gestation can affect maternal physiological status and reproductive performance later in pregnancy. Based on our previous findings concerning the metabolic, antioxidant, anti-inflammatory, and gut microbiota-related effects of chenodeoxycholic acid (CDCA), we hypothesized that CDCA supplementation during early gestation would produce sustained improvements in maternal metabolic and redox homeostasis and potentially improve reproductive performance. To test this hypothesis, multiparous sows received either a control diet or a diet supplemented with 0.15% CDCA from GD 0 to GD 28, followed by a common diet for the remainder of gestation. Maternal inflammatory, oxidative stress, insulin-related metabolic, serum metabolomic, and gut microbiota were evaluated during late gestation, and reproductive performance was recorded at farrowing.

Chapter 6 integrates the findings of the three experimental chapters and discusses how maternal dietary protein supply, maternal metabolic homeostasis, placental adaptation, and metabolism-targeted nutritional interventions may collectively influence fetal development and reproductive performance. It also addresses the limitations of the studies and proposes directions for future research and practical nutritional strategies for gestating sows.

Chapter 3

Effects of dietary protein levels during early gestation on reproductive performance, oxidative stress, and placental nutrient transport in sows

This chapter is based on the following publication:

Zhao Ying, He Liyuan, Ming Dongxu, Gao Ge, Schroyen Martine, Pi Yu, Sun Wenjuan, Li Yanpin, Li Xilong

Effects of dietary protein levels during early gestation on reproductive performance, oxidative stress, and placental nutrient transport in sows

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Chapter 3. Effects of dietary protein levels during early gestation on reproductive performance, oxidative stress, and placental nutrient transport in sows

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Keywords: maternal dietary protein, early gestation, reproductive performance, placental function, sow

1. Abstract

Early gestation (gestation day 0 to gestation day 30) is a critical window for embryo implantation and placental development. This study evaluated the effects of maternal dietary protein during early gestation on reproductive performance, maternal metabolism, and placental function. Forty-five sows were randomly allocated into three iso-caloric diets containing 12% (low protein, LP), 13.5% (medium protein, MP), or 15% (high protein, HP) crude protein during early gestation. Crystalline amino acids were supplemented to maintain constant ratios of standardized ileal digestible (SID) essential amino acids to lysine. The Lys:NE ratios were 2.66, 2.66, and 2.98 g SID Lys/Mcal NE, respectively. Litter size and live birth rate were not significantly affected ($P > 0.05$). Sows fed the HP diet exhibited increased serum alanine transaminase (ALT) ($P < 0.05$), elevated malondialdehyde (MDA) concentration ($0.05 \leq P < 0.10$), and enhanced catalase (CAT) activity ($P < 0.05$) at GD 28. The LP group showed higher serum LDL ($P < 0.05$), HDL, and total cholesterol ($0.05 \leq P < 0.10$). No differences were observed in serum glucose, insulin, progesterone (P4), estradiol (E2) or inflammatory factor levels among groups ($P > 0.05$). Serum amino acid profiling revealed higher circulating levels of valine, isoleucine, leucine, tryptophan, and phenylalanine in the HP group ($P < 0.05$). Placental oxidative stress and nutrient transport were not affected ($P > 0.05$). Overall, dietary protein within 12%-15% during early gestation did not affect reproductive outcomes or placental function, but influenced maternal oxidative status, amino acid profiles, and lipid metabolism on GD 28. These findings reveal a dissociation between maternal metabolic responses and reproductive outcomes, suggesting that metabolic perturbations induced by protein intake may not translate into phenotypic changes within the commercial range, but could become critical when metabolic thresholds are exceeded.

2. Introduction

Early gestation represents a critical period for embryo implantation and rapid placental development, and plays a decisive role in determining pregnancy success. In pigs, embryonic loss is particularly severe, with 20% - 45% of embryos lost and nearly 75% of these losses occurring during early gestation (day 0 to day 30) (Pope, 1988; Ford et al., 2002; Geisert and Schmitt, 2002). During this period, porcine embryos are highly sensitive to maternal nutritional status, and inappropriate nutrient supply may compromise embryo survival. Previous studies have shown that increasing feed intake to two times the maintenance requirement can reduce embryo survival on day 28 of gestation, likely due to disruptions in progesterone (P4) secretion (Jindal et al., 1996). However, modern hyperprolific sows, characterized by higher litter sizes and lower body fat reserves, demand more nutrient-dense diets (Boyd et al., 2000). Early gestation also serves as a crucial window for recovering the body weight and backfat lost during lactation for multiparous sows (Thaker and Bilkei, 2005). Moderate increases in nutrient intake during early gestation have been reported to improve reproductive outcomes (Quesnel et al., 2010; Hoving et al.,

2011), challenging the traditional opinion that feed restriction in pregnant sows improves embryo survival (Jindal et al., 1996).

Dietary protein is a key component of maternal nutrition that provides amino acids for fetal survival, growth, and development (Herring et al., 2018). Our earlier study demonstrated that increasing dietary protein intake by 20% or 40% compared with a basal intake of 280 g/day during early gestation improved embryonic survival rate on gestation day (GD) 30 in sows, which may relate to amino acid transport from mother to fetus (Zhao et al., 2022). However, the effect of maternal dietary protein level during early gestation on the reproductive performance remains to be clarified. Pregnancy is often accompanied by heightened oxidative stress, particularly under conditions of nutritional imbalance, which may impair placental function and fetal development (Mitchell et al., 2009; Kim et al., 2013; Zhao et al., 2013; Morales et al., 2022; Yang et al., 2023). Protein nutrition also affects fetal growth by regulating the placental transport of critical amino acids, thereby influencing fetal nutrient availability (Wu et al., 1998). It has been found that amino acid mixture supplementation or high amino acid intake in late pregnancy can alleviate oxidative stress and improve reproductive performance (Yuan et al., 2022; Hu et al., 2025). Crucially, the placenta is a highly plastic organ capable of functional and structural adaptations in response to the maternal nutritional environment. This plasticity enables the placenta to adapt to maternal nutritional changes by adjusting nutrient transport efficiency, thereby maintaining fetal development even when maternal nutrition varies (Díaz et al., 2014; Sferruzzi - Perri et al., 2023). Nevertheless, the effects of maternal dietary protein intake during early gestation on placental function remain unclear.

In commercial sow production, the crude protein content of gestation diets typically ranges from 12%-15% (Shields Jr et al., 1985; Schoknecht et al., 1994; Wu et al., 1998; Theil et al., 2002; Sarr et al., 2010; Metges et al., 2014; Sui et al., 2014). To better reflect practical production conditions, this study aimed to define whether the commercial maternal dietary protein between 12%-15% during early gestation enhances reproductive performance, while also assessing its effect on maternal health, nutrient transporters and oxidative stress in the placenta in multiparous sows. We hypothesise that maternal dietary protein intake can affect fetal survival through adjusting maternal metabolism and placental function. These findings will help clarify the regulatory roles of protein nutrition during early gestation and provide a theoretical basis for optimizing protein feeding strategies in gestating sows.

3. Materials and methods

3.1 Ethical approval

This study was conducted at Xiaqiu Sow Farm, Huanshan Group Co., Ltd., Qingdao, China. All animal procedures complied with Animal Care and Use Committee of the Feed Research Institute, Chinese Academy of Agricultural Sciences (Protocol No. IFR-CAAS-20230902).

3.2 *Animals and experimental design*

A total of 45 Landrace × Large White post-weaning sows (parity 2 to 5) with mean backfat thickness (14.65 ± 0.2 mm) were selected. After artificial insemination, sows were randomly assigned to three groups based on backfat thickness, parity, and previous litter size. Each treatment included 15 replicate pens, and the sows were housed in individual pens. The day of the first insemination was designated as gestational day 0 (GD 0). During early gestation (GD 0-GD 30), the sows were fed experimental diets containing different levels of crude protein: low protein (LP, 12% crude protein), medium protein (MP, 13.5% crude protein), and high protein (HP, 15% crude protein). All diets were formulated to meet the nutritional requirements established by the NRC (NRC, 2012). Based on the NRC (2012) model estimated for multiparous sows (Parity 3) in early gestation (<90 days), the theoretical dietary requirements are 0.37% for SID Lysine, and approximately 166 g/d for CP. The CP and SID Lys provided in all groups exceeded physiological needs. The diets were formulated to be iso-caloric, with a Net Energy (NE) content of approximately 2180 kcal/kg. To ensure nutritional balance, crystalline amino acids were supplemented to maintain constant ratios of standardized ileal digestible (SID) essential amino acids to lysine across treatments. The ingredient composition and nutrient levels for each group are shown in Table 3-1. Specifically, the calculated Lys:CP ratios (SID Lys % / CP %) were 4.83%, 4.26%, and 4.33% for the LP, MP, and HP groups, respectively. The Lys:NE ratios were 2.66, 2.66, and 2.98 g SID Lys/Mcal NE, respectively. The crude protein content of diets was analyzed according to AOAC methods (AOAC, 2006). All sows were fed 2.5 kg/day during early gestation, which were provided twice daily at 8:00 and 14:00 (half of the meal each time). All sows were fed the same gestation diets (MP) after the early gestation period. The daily feed allowance was adjusted according to body condition, ranging from 2.4-2.8 kg/d during mid-gestation (GD 31 to 90) and 3.0-3.4 kg/d during late gestation (GD 91 to farrowing). The sows had ad libitum access to water throughout the experiment. On GD 107, sows were moved to farrowing rooms and kept in individual farrowing pens (2.2×1.5 m). All sows were housed in controlled environment conditions, with the temperature of sow house maintained at 21-25 °C.

Table 3-1: Diet composition and nutritional components (as-feed basis, %).

Items	Group ⁴		
Feedstuff	LP	MP	HP
Germinated wheat	11.28	20.00	25.00
Corn	38.23	15.49	12.30
Barley	0.00	15.00	15.00
Soybean skin	14.76	10.22	10.11
Wheat bran	15.00	10.00	10.00
Wheatmeal	3.00	10.00	10.00
Soybean meal (46)	4.45	7.23	11.03
Rice bran meal	5.00	5.00	0.00
Mei chao neng premix feed ¹	1.50	1.50	1.50
Monocalcium phosphate (22/17.5)	1.39	1.38	1.39
Soybean oil	2.26	1.30	0.83

1% 5056 MV-YZ premix feed ²	1.00	1.00	1.00
Limestone	0.73	0.75	0.72
NaHCO ₃	0.45	0.45	0.45
NaCl	0.30	0.30	0.30
L- Lysine hydrochloride, 98%	0.28	0.17	0.16
Choline chloride, 60%	0.15	0.15	0.15
L-Threonine, 98%	0.13	0.07	0.07
DL-Methionine	0.05	0.00	0.01
L-Valine, 98%	0.03	0.00	0.00
L-Tryptophan	0.01	0.00	0.00
Total	100.00	100.00	100.00
Calculated nutrient content, %			
³ DM, %	88.17	88.13	88.03
³ CP, %	12.00	13.62	15.00
³ DE	2991.81	3023.81	3059.65
³ NE	2180	2180	2180
³ NDF	22.29	20.03	19.23
³ ADF	10.28	8.73	8.37
Ca, %	0.80	0.80	0.80
Available P, %	0.38	0.38	0.38
³ SID Lys, %	0.58	0.58	0.65
SID Met, %	0.20	0.17	0.20
SID Thr, %	0.42	0.42	0.47
SID Trp, %	0.12	0.12	0.16
SID Val, %	0.44	0.50	0.56
Lys:CP ratio, %	4.83	4.26	4.33
Lys:NE ratio, g/Mcal	2.66	2.66	2.98
Analyzed nutrient level, %			
DM	88.78	88.31	89.64
CP	13.03	14.13	15.65

¹ Mei chao neng premix feed: Emulsified oils (including coconut oil, linseed oil, palm oil, soybean oil, and modified phospholipid oil), short-chain fatty acids (SCFA), extruded corn, nutritional additives, vitamin E, etc. The product contains crude fat $\geq 50\%$, moisture $\leq 6\%$, crude protein $\geq 4\%$, and vitamin E ≥ 120 mg/kg.

² 5056 MV-YZ premix feed supplied per kg of diet: Zinc, 125 ppm; iron, 100 ppm; manganese, 50 ppm; copper, 15 ppm; iodine, 0.35 ppm; selenium, 0.3 ppm; vitamin A, 9920 IU/kg; vitamin D, 1985 IU/kg; vitamin E, 66 IU/kg; vitamin K, 4.4 mg/kg; choline, 660 mg/kg; niacin, 44 mg/kg; riboflavin, 10 mg/kg; pantothenic acid, 33 mg/kg; vitamin B12, 37 μ g/kg; folic acid, 1325 μ g/kg; biotin, 220 μ g/kg; thiamine, 2.2 mg/kg; pyridoxine, 3.3 mg/kg.

³ DM, Dry matter; CP, Crude protein; DE, Digestible energy; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; SID, Standardised ileal digestibility.

⁴ LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%).

3.3 Data collection

On GD 14 and GD 28, the backfat thickness of all sows was measured at the last rib, 65 mm away from the midline. On GD 28, 10 mL blood samples were collected from 12 randomly selected sows via the precaval vein at 2 h after the morning feeding and centrifuged at 3000 rpm for 15 min to obtain serum. The serum was stored at -80 °C until further analysis. At farrowing, placental samples were collected from 11 randomly selected sows and preserved at -80 °C. All placental samples were collected from the central region of the placenta on the fetal side, avoiding the placental margins, large vessels, and any visibly damaged areas. Reproductive performance, including the total piglets born, live piglets, stillborn, and mummified fetuses, was recorded. The percentage of piglets born alive was calculated using the following equation: percentage of piglets born alive (%) = (Number of live piglets / Number of total piglets born) × 100. Sows that returned to estrus, failed to conceive, or aborted were excluded from the trial. At the time of delivery, the number of pregnant sows remaining in each group was 14, 13 and 12 for LP, MP and HP respectively.

3.4 Serum hormone and biochemical index analysis

P4 in the serum was measured by competitive radioimmunoassay (RIA) at the Beijing North Biotechnology Company and estradiol (E2) was measured using an ELISA kit (Wuhan Huamei Biotech Co., LTD, China), following the manufacturers' protocols. The serum biochemical indices, including alanine transaminase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol (TC), total protein (TP), and glucose (GLU) were analyzed using an automated biochemical analyzer (XL-200, ERBA Mannheim GmbH, Germany) through corresponding commercial kits (Maccura Biotechnology Co., Ltd., China).

3.5 Serum oxidative stress and inflammatory markers

Oxidative stress markers in serum, including malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), were measured using specific commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Serum inflammatory cytokines, including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α), were quantified using ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, China).

3.6 Amino acids concentration in serum

Amino acid concentrations in maternal serum were analyzed using high-performance liquid chromatography (HPLC) (Dai et al., 2014). Briefly, samples were deproteinized with an equal volume of 1.5 M HClO₄, and treated with half volume of 2 M K₂CO₃. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was collected. And 100 μ L of the supernatant was mixed with 400 μ L of 0.1 M HCl and 10 μ L of internal standard solution, filtered, and analyzed by HPLC for quantification of 16 amino acids (Wu et al., 1995).

3.7 Placental oxidative stress and energy status

The concentration of MDA and the activities of SOD, GPx, and CAT in placenta were measured using the same assay kits described above. The concentration of ATP in the placenta was measured using the Enhanced ATP Assay Kit (Shanghai Beyotime Biotechnology Co., Ltd, China). And the protein concentration of placental tissue was determined using the BCA protein assay kit (Beijing Huaxing Bochuang Gene Technology Co., Ltd, China), following the manufacturer's protocols.

3.8 RT-qPCR

Total RNA was extracted from placental tissue using a Tissue/Cell RNA Rapid Extraction Kit (Aidlab Biotechnologies Co., Ltd, China). RNA concentration and purity were measured using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). A total of 1000 ng RNA was reverse-transcribed into cDNA using the PrimeScript® RT Reagent Kit with gDNA Eraser (Takara Biomedical Technology Co., Ltd., China). The synthesized cDNA was diluted fivefold. Quantitative PCR was performed using TB Green Premix Ex Taq™ (Takara Biomedical Technology Co., Ltd., China) on a CFX96 Real-Time PCR System (Bio-Rad, USA). The RT-qPCR was performed in a 20 µL reaction system. Product specificity was confirmed by single sharp peak in the melting curve analysis and a single band of expected size in agarose gel electrophoresis. The relative expression levels of target genes were normalized to GAPDH, and calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table 3-2.

Table 3-2: Primer sequences for quantitative real-time PCR in this study.

Gene ¹	Accession number	Primer sequence (5'-3')	Size (bp)
<i>GAPDH</i>	NM_001206359.1	F: CAATGACCCCTTCATTGACC R: CTCCTGGAAGATGGTGATGG	139
<i>CAT</i>	NM_214301.2	F: CCTGCAACGTTCTGTAAGGC R: GCTTCATCTGGTCACTGGCT	72
<i>NQO1</i>	NM_001159613.1	F: GGACATCACAGGTAAACTGA R: TATAAGCCAGAGCAGTCTCG	68
<i>SOD1</i>	NM_001190422.1	F: GAAGACAGTGTTAGTAACGG R: CAGCCTTGTGTATTATCTCC	93
<i>GPXI</i>	NM_214201.1	F: TCTCCAGTGTGTCGCAATGA R: TCGATGGTCAGAAAGCGACG	104
<i>BOAT1</i>	XM_003359855.5	F: CACAACAACCTGCGAGAAGGA R: CCGTTGATAAGCGTCAGGAT	155
<i>SNAT1</i>	XM_084957220.1	F: AAGAACCTGGGCTATCTCGG R: TGTTGCGTTAGGACTCGTTG	138
<i>SNAT2</i>	NM_001317081.1	F: CCGCAGCCGTAGAAGAATGATG R: AGCAATTCCGTCTCAACGTGGTC	123
<i>4F2HC</i>	XM_085010884.1	F: CTCGAACCCACCAAGGAC R: GAGGTGAGACGGCACAGAG	174
<i>GLUT1</i>	XM_021096908.2	F: CGTCGCTGGCTTCTCCAACCTG R: CAGGAGCACCGTGAAGATGATG	109

¹*GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *CAT*, Catalase; *NQO1*, NAD(P)H: quinone oxidoreductase 1; *SOD1*, Superoxide dismutase 1; *GPXI*, Glutathione peroxidase 1; *BOAT1*, Broad-neutral amino acid transporter 1; *SNAT1*, Sodium-coupled neutral amino acid transporter 1; *SNAT2*, Sodium-coupled neutral amino acid transporter 2; *4F2HC*, 4F2 cell-surface antigen heavy chain; *GLUT1*, Glucose transporter 1.

3.9 Statistics analysis

The individual sow was considered as an experimental unit. All data were tested for outliers and normality prior to analysis. Data are presented as means $\pm$ SEM. Statistical analyses were performed using IBM SPSS Statistics 20 and visualized with GraphPad Prism 10.1.2. One-way analysis of variance (ANOVA) was used to analyze group differences, followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $P < 0.05$, and $0.05 \leq P < 0.10$ was considered a trend.

4. Results

4.1 Body condition and reproductive performance

As shown in **Table 3-3**, dietary protein levels had no significant effect on the backfat thickness during early gestation, indicating that dietary protein levels ranging from 12%-15% exerted similar effects on body recovery. The total number of piglets born, the number of live piglets, stillbirths, mummified fetuses, and live birth showed no significant differences among the three groups ($P > 0.05$).

Table 3-3: Effect of different protein levels during early gestation on backfat thickness and reproductive performance in multiparous sows.

Items	Treatment groups ¹			SEM	P-value
	LP	MP	HP		
Backfat thickness, mm					
First insemination	14.66	14.71	14.57	0.60	0.973
GD 14	15.06	14.71	14.64	0.70	0.807
GD 28	15.22	14.64	14.85	0.74	0.718
Litter size					
Total born	14.79	13.85	13.83	0.72	0.555
Born live	12.64	12.15	12.75	0.74	0.842
Stillborn	1.14	1.00	0.75	0.36	0.767
Mummy	1.00	0.69	0.33	0.37	0.510
Percentage of piglets born alive, %	86.43	87.99	92.25	5.23	0.550

¹ LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). n = 14 in LP, n = 13 in MP, and n = 12 in HP.

4.2 Serum hormone levels

From **Table 3-4**, serum hormone analysis on GD 28 revealed no significant differences in P4, E2, or insulin concentrations among the LP, MP, and HP groups ($P > 0.05$).

Table 3-4: Effect of different protein levels during early gestation on serum hormones in multiparous sows on GD 28.

Items ²	Treatment groups ¹			SEM	P-value
	LP	MP	HP		
P4, ng/mL	1.49	1.36	1.33	0.24	0.778
E2, pg/mL	122.19	118.35	112.41	26.76	0.968
INS, μ IU/mL	28.93	33.26	36.57	4.19	0.487

¹ LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). n = 12.

² P4, Progesterone; E2, Estradiol; INS, Insulin.

4.3 Serum biochemical parameters

Table 3-5 shows the results of serum biochemical analysis. The ALT activity was significantly higher in the HP group compared to the MP group ($P < 0.05$), and showed a tendency to be higher than the LP group ($0.05 \leq P < 0.10$). In contrast, the LP group exhibited significantly elevated LDL levels relative to the HP group ($P < 0.05$), and tended to have higher HDL and total cholesterol levels ($0.05 \leq P < 0.10$). No significant differences were observed in AST, glucose, or total protein among groups ($P > 0.05$).

Table 3-5: Effect of different protein levels during early gestation on serum biochemistry in multiparous sows on GD 28.

Items ²	Treatment groups ¹			SEM	P-value
	LP	MP	HP		
ALT, IU/L	43.78 ^{ab, y}	40.34 ^b	50.14 ^{a, x}	1.94	0.005
AST, IU/L	25.03	23.38	22.01	1.45	0.390
GLU, mmol/L	3.28	3.12	3.17	0.15	0.730
HDL, mmol/L	0.80 ^x	0.72	0.70 ^y	0.03	0.051
LDL, mmol/L	1.15 ^a	1.02 ^{ab}	0.98 ^b	0.04	0.040
TC, mmol/L	1.92 ^x	1.80	1.72 ^y	0.06	0.098
TP, g/L	64.01	64.39	66.10	1.25	0.473

¹ LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). n = 12.

² ALT, Alanine transaminase; AST, Aspartate Aminotransferase; GLU, Glucose; HDL, High-density lipoprotein cholesterol; LDL, Low-density lipoprotein cholesterol; TC, Total cholesterol; TP, Total protein.

^{a, b} Different superscripts within the same row indicate significant differences between means ($P < 0.05$); ^{x, y} indicates a trend toward significance ($0.05 < P < 0.1$). Values sharing the same letter are not significantly different.

4.4 Oxidative stress in serum

Markers of antioxidant status in serum on GD 28 are presented in **Table 3-6**. CAT activity in the HP group was significantly higher than in both LP and MP groups ($P < 0.05$). Additionally, MDA levels showed a tendency to increase in the HP group compared to MP ($0.05 \leq P < 0.10$), and SOD activity tended to be higher than that in LP ($0.05 \leq P < 0.10$). Furthermore, SOD levels in the MP group were significantly higher than in the LP group ($P < 0.05$).

Table 3-6: Effect of different protein levels during early gestation on serum oxidative stress in multiparous sows on GD 28.

Items ²	Treatment groups ¹			SEM	P - value
	LP	MP	HP		
MDA, nmol/mL	3.13	3.02 ^y	3.74 ^x	0.20	0.053
CAT, U/mL	3.00 ^b	2.94 ^b	3.87 ^a	0.23	0.045
SOD, U/mL	1053 ^{b,y}	1189 ^a	1176 ^{ab,x}	38.80	0.034
GPx, U/mL	1810	1765	1884	56.18	0.383

¹ LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). n = 12.

² MDA, Malondialdehyde; CAT, Catalase; SOD, Superoxide dismutase; GPx, Glutathione peroxidase.

^{a,b} Different superscripts within the same row indicate significant differences between means ($P < 0.05$); x,y indicates a trend toward significance ($0.05 < P < 0.1$). Values sharing the same letter are not significantly different.

4.5 Inflammatory cytokines in serum

To assess systemic inflammatory responses, serum levels of IL-1 β , IL-6, and TNF- α were measured (**Figure 3-1**). No significant differences were detected among the three dietary groups ($P > 0.05$).

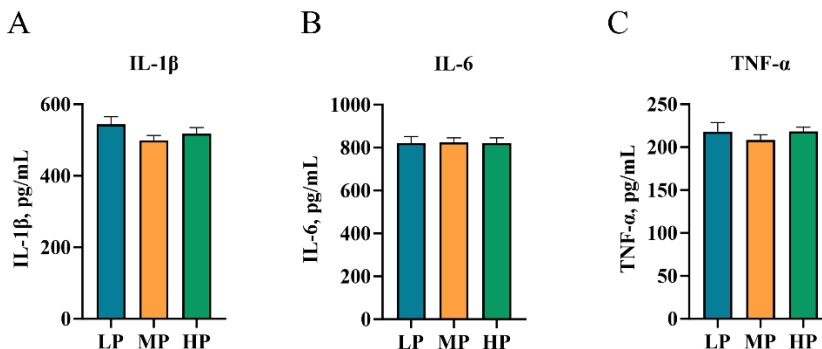


Figure 3-1: Effect of different protein levels during early gestation on serum inflammatory response in multiparous sows.

(A-C) showed the content of IL-1 β , IL-6, TNF- α in serum respectively. n = 12. LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). Data are presented relative to the MP group. Data are means $\pm$ SEM. IL-1 β , Interleukin-1 β ; IL-6, Interleukin-6; TNF- α , Tumor necrosis factor-alpha.

4.6 Amino acid concentrations in serum

Amino acid profiling of serum at GD 28 revealed several differences among dietary treatments (**Table 3-7**). The HP group showed significantly higher

concentrations of valine (Val), isoleucine (Ile), serine (Ser), and tyrosine (Tyr), compared to the MP group ($P < 0.05$), while leucine (Leu) exhibited increasing trends ($0.05 \leq P < 0.10$). Compared with the LP group, the HP group exhibited significantly elevated levels of tryptophan (Trp), isoleucine (Ile), and phenylalanine (Phe) ($P < 0.05$). Interestingly, the LP group had the highest glycine (Gly) levels, which were significantly greater than those observed in the MP group ($P < 0.05$).

Table 3-7: Effect of different protein levels during early gestation on serum amino acid profiles in multiparous sows on GD 28.

Items	Treatment groups ¹ , $\mu\text{mol/L}$			SEM	P-value
	LP	MP	HP		
Lysine	327.05	273.58	308.21	18.90	0.138
Methionine	78.77	72.17	72.45	3.92	0.426
Threonine	344.55	302.67	330.58	17.79	0.238
Tryptophan	144.68 ^b	161.87 ^{ab}	170.17 ^a	5.52	0.002
Valine	419.51 ^{ab}	395.13 ^b	469.20 ^a	19.92	0.042
Leucine	238.57	225.61 ^y	267.51 ^x	12.49	0.073
Isoleucine	147.41 ^b	159.07 ^b	191.17 ^a	8.40	0.003
Phenylalanine	96.69 ^b	106.23 ^{ab}	115.96 ^a	4.92	0.030
Histidine	96.49	80.88 ^x	103.68 ^y	7.41	0.102
Arginine	339.15	301.52	359.82	20.78	0.156
Alanine	538.59	501.08	466.21	28.38	0.222
Aspartate	21.10	22.81	20.52	1.94	0.702
Glutamate	271.25	277.45	245.66	22.29	0.591
Glycine	1049.14 ^a	808.52 ^b	888.40 ^{ab}	53.47	0.008
Serine	147.99 ^{ab}	119.35 ^b	151.64 ^a	9.30	0.036
Tyrosine	104.79 ^{ab}	92.78 ^b	117.43 ^a	5.10	0.007

¹ LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). n = 12.

^{a,b} Different superscripts within the same row indicate significant differences between means ($P < 0.05$); ^{x,y} indicates a trend toward significance ($0.05 < P < 0.10$). Values sharing the same letter are not significantly different.

4.7 Placenta oxidative stress

Figure 3-2 presents the MDA level, activities of oxidative stress-related enzymes, and ATP content in placental tissue. **Figure 3-2F-I** shows the relative mRNA expression of antioxidant enzyme-related genes in the placenta. No significant differences were observed in MDA level, CAT, SOD, and GPx activity, or in the mRNA expression of antioxidant enzymes among groups ($P > 0.05$). Similarly, placental ATP content did not differ significantly among treatments ($P > 0.05$).

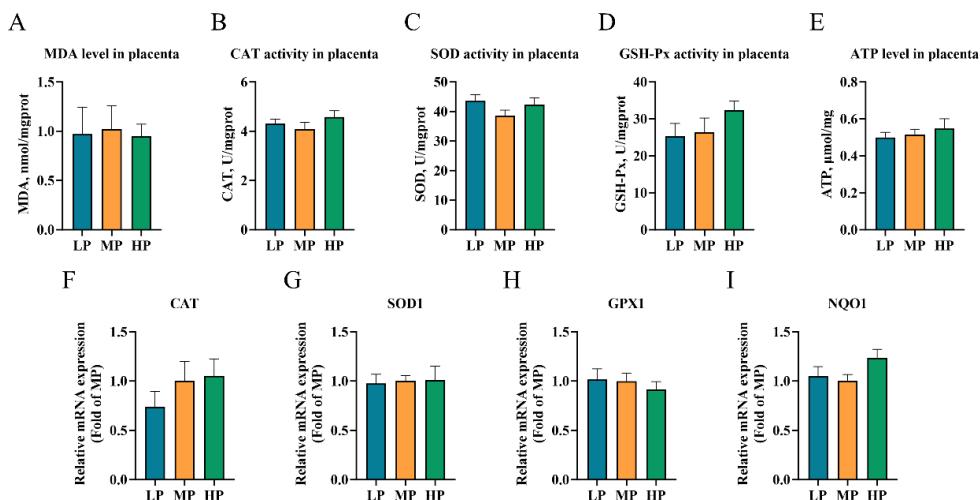


Figure 3-2: Effect of different protein levels during early gestation on placental oxidative stress in multiparous sows.

(A) was the MDA level in placenta. (B-D) showed the antioxidant enzyme activity in placenta, including CAT, SOD, GPx, respectively. (E) showed the ATP levels in placenta. (F-I) was the mRNA expression of CAT, NQO1, SOD1, and GPX1. $n = 11$. LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). Data are presented relative to the MP group. Data are means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. MDA, Malondialdehyde; CAT, Catalase; SOD, Superoxide dismutase; GPx, Glutathione peroxidase; NQO1, NAD(P)H: quinone oxidoreductase 1.

4.8 Nutrient transportation

Finally, we evaluated the mRNA expression of several placental transporters for amino acids and glucose (**Figure 3-3**). We observed no significant differences in the expression levels of *B0AT1*, *SNAT1*, *SNAT2*, *4F2HC*, and *GLUT1* among the three groups ($P > 0.05$).

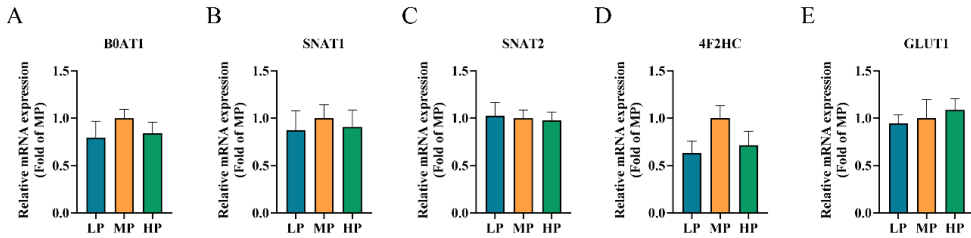


Figure 3-3: Effect of different protein levels during early gestation on placental nutrient transport in multiparous sows.

(A-E) was the mRNA expression of B0AT1, SNAT1, SNAT2, 4F2HC, GLUT1 in placenta. n = 11. LP = low protein diet (12%); MP = medium protein diet (13.5%); HP = high protein diet (15%). Data are presented relative to the MP group. Data are means $\pm$ SEM. *B0AT1*, Broad-neutral amino acid transporter 1; *SNAT1*, Sodium-coupled neutral amino acid transporter 1; *SNAT2*, Sodium-coupled neutral amino acid transporter 2; *4F2HC*, 4F2 cell-surface antigen heavy chain; *GLUT1*, Glucose transporter 1.

5. Discussion

In this study, we evaluated the effects of maternal dietary protein levels during early gestation on reproductive performance, maternal metabolism, oxidative stress status, amino acid availability, and placental nutrient transport in sows. Our results showed that dietary protein levels from 12%-15% did not significantly influence litter size and live birth rate, while several important physiological and metabolic changes involving hepatic function and redox balance during early gestation were observed.

In our present study, maternal dietary protein levels between 12%-15% during early gestation did not significantly influence litter size or live births. This is consistent with earlier reports showing that feeding 11% to 17% protein throughout gestation does not affect litter performance (Jang et al., 2014; Kim et al., 2023). Another study also indicated that extremely low or high protein levels did not affect litter size (Rehfeldt et al., 2011). Our previous study demonstrated that maternal higher protein intake (340 g/d and 400 g/d) significantly improved the embryonic survival rate at GD 30 compared to sows fed with low protein (280 g/d) (Zhao et al., 2022). However, in the present study, no significant difference was observed in the live birth rate, although the value in the HP group (375 g/d) was numerically higher compared with the MP (340 g/d) and LP (300 g/d) groups. This indicates that the beneficial effect of higher protein intake is more pronounced during implantation period but may not persist throughout gestation. One of the possible reasons is the difference in dietary protein levels between the two studies, and another is that previous studies used gilts while we used multiparous sows. The estimated requirement for the multiparous sows in the present study was approximately 166 g/d CP, significantly lower than requirement of gilts which was about 204 g/d (NRC, 2012). Therefore, the dietary protein levels provided here (300 g/d) exceeded the

requirement of sows, explaining why the live birth rate was not improved. Nutritional modulation during early gestation is known to affect embryo implantation and survival through changes in ovarian E2 and P4 (Hazeleger et al., 2005; Lucy, 2008). We did not observe a significant difference of serum hormone concentrations (P4 and E2) among the three groups although our previous study showed that the E2 level in sows fed 400 g/d protein was significantly higher than 280 g/d (Zhao et al., 2022). This may be because of the different level of protein we used. Given that cholesterol serves as a precursor for steroid hormone synthesis (Miller and Bose, 2011), particularly P4 and E2, the lipid profile may influence endocrine support of early pregnancy. The increase in serum total cholesterol, HDL, and LDL levels in LP may reflect a compensatory adjustment in lipid metabolism, providing sufficient substrates for steroid hormone biosynthesis, highlighting that maternal lipid metabolism during early gestation is highly sensitive to protein availability to support steroidogenesis.

In this study, maternal serum indicators revealed distinct metabolic responses to altered protein intake during early gestation, especially the effect of high protein intake on liver health and serum oxidative balance. Liver is a central organ regulating glucose homeostasis and insulin resistance, and ALT and AST are classical biochemical indicators for assessing hepatic function (Ballestri et al., 2016). The elevation of serum ALT activity without a corresponding increase in AST suggests that HP intake may enhance transamination and amino acid catabolism, indicating a metabolic burden on the liver rather than severe liver injury. The increased MDA concentrations indicated an elevated oxidative challenge on GD 28. At the same time, antioxidant enzymes including CAT and SOD were also upregulated, reflecting the activation of antioxidant defenses system. The maintenance of glucose, insulin, or inflammatory factors indicated that maternal dietary protein level did not interrupt energy homeostasis or systemic inflammatory response. These results suggest that high protein intake imposes a mild metabolic load on the liver, triggering oxidative stress and compensatory activation of antioxidant defenses, yet systemic glucose homeostasis and energy metabolism remain intact, indicative of an adaptive rather than pathological response.

Maternal circulating amino acid concentrations at GD 28 further support the metabolic impact of dietary protein intake. Specifically, the significantly elevated concentrations of Val and Trp in the HP group corresponded to the increased dietary ratios relative to Lys. In contrast, serum concentrations of amino acids with constant dietary ratios across treatments, such as Thr, did not differ significantly. This confirms that the variations in serum amino acid levels were primarily driven by dietary nutrient supply. Consistent with earlier studies, protein restriction significantly reduces the concentrations of essential amino acids, including branched-chain amino acids and phenylalanine (Bhasin et al., 2009), whereas high protein intake elevates them, as observed in our HP group and aligned with previous reports (Che et al., 2019). Additionally, Gly was the most abundant amino acid detected (Wu et al., 1998). Amino acids serve not only as substrates for fetal growth and placental development but also as key regulators of signaling pathways. Specifically, branched-chain amino acids (BCAAs), which were elevated in the HP

group, are potent activators of the mTOR signaling pathway (Kim, 2009). This pathway plays a critical role in early placental angiogenesis and embryo implantation. Theoretically, the enriched amino acid environment in the HP group provided a stronger signaling potential for fetal development. However, the lack of difference in reproductive performance in the current study suggests that the serum amino acid concentrations in the LP group were already sufficient to fully activate these key pathways. Consequently, the elevated amino acid levels in the HP sows likely represented a nutritional surplus that was catabolized rather than utilized for additional fetal development. Furthermore, since all sows were switched to a common diet during mid and late gestation, any signaling advantages established during the first 30 days may have been diluted over the subsequent months. Thus, while early high-protein intake altered the maternal metabolic milieu, it was insufficient to permanently reprogram reproductive outcomes in multiparous sows.

The placenta exhibits high metabolic activity and flexibility, and its metabolism can be modulated under adverse maternal environments to maintain fetal health (Vaughan and Fowden, 2016). In the present study, no significant differences were observed in placental oxidative stress markers among dietary protein treatments. Previous studies showing that higher amino acid intake during gestation in high-parity sows can elevate serum SOD activities in sows (Hu et al., 2025), while higher dietary energy or amino acid intake in late gestation increased oxidative markers such as MDA (Che et al., 2019). This result may be attributed to the fact that the protein intervention was limited to early gestation, whereas all sows were fed the same diet during mid and late gestation. The subsequent same feeding may have allowed the placenta to restore oxidative balance and maintain normal antioxidant capacity. Furthermore, the unchanged ATP level suggests that mitochondrial energy production capacity was not compromised by 12%-15% protein intake in early gestation. This indicates that dietary protein intake during early gestation did not impact placental oxidative stress at parturition.

Contrary to expectations, no significant differences were observed in the mRNA expression of key placental amino acid and glucose transporters among the three groups although the maternal supply was changed on GD 30. Previous studies have shown that maternal dietary protein restriction can downregulate the expression of placental amino acid transporters, thereby reducing the placental capacity for amino acid transfer (Malandro et al., 1996; Rosario et al., 2011; Pantham et al., 2015). Our previous study found that increased protein level tended to improve the mRNA level of *SNAT1* in the endometrium on GD30 (Zhao et al., 2022). These findings indicate that within the practical protein range of 12%-15% during early gestation, the placenta may maintain a relatively stable capacity for nutrient transport, ensuring an adequate supply of amino acids and glucose to support early conceptus development. From a practical perspective, these results imply that maternal dietary protein level between 12% and 15% during the peri-implantation period does not compromise placental nutrient transfer efficiency in sows. Although no significant differences in placental functional indicators were detected at farrowing, early gestation remains an important period for conceptus development and the establishment of placental structure and function. Nutritional exposure during this period could theoretically influence later placental function through changes in early trophoblast development,

vascularization, nutrient-sensing pathways, or epigenetic regulation of gene expression. For example, maternal nutrition may alter DNA methylation, histone modifications, or other regulatory processes established during early development (Peral-Sanchez et al., 2021).

Importantly, although no significant effects on reproductive performance or placental function were observed, the consistent alterations in maternal metabolic indicators suggest that dietary protein intake within the commercial range is sufficient to induce systemic metabolic responses without exceeding the adaptive capacity of the placenta.

6. Conclusion

Taken together, our findings indicate that maternal dietary protein levels of 12%-15% during early gestation in multiparous sows support normal reproductive outcomes and did not compromise placental function. High protein intake induces a mild oxidative stress accompanied by adaptive antioxidant defense, without disrupting systemic glucose homeostasis or inflammatory response on GD 28. Placental oxidative status, energy production, and nutrient transporter expression remain stable across these protein levels. Collectively, these findings indicate that protein levels commonly used in commercial swine production are unlikely to provide additional reproductive benefits in multiparous sows. Importantly, these findings highlight that maternal metabolic responses are sensitive to dietary protein supply, supporting the need for further mechanistic studies to determine the conditions under which metabolic imbalance may translate into reproductive outcomes.

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8. Transition to Chapter 4

Chapter 3 showed that varying dietary protein levels from 12% to 15% during early gestation induced maternal metabolic responses but did not markedly affect reproductive performance or placental function at farrowing. This dissociation between metabolic perturbation and phenotypic outcomes raises a critical question of whether more pronounced nutritional challenges, particularly under conditions of metabolic overload, could exceed the adaptive capacity of the placenta and affect reproductive outcomes. Chapter 4 addresses this question using a controlled mouse model to further investigate the underlying mechanisms.

Chapter 4

Impact of extreme maternal dietary protein stress on fetal development and mechanisms of placental mitochondrial dysfunction in mice

This chapter is based on the following publication:

Zhao Ying, Long Cai, Liyuan He, Ge Gao, Martine Schroyen, Yu Pi, Wenjuan Sun, Dongxu Ming, Yanpin Li, and Xilong Li

Maternal high-protein diet impairs fetal growth through placental dysfunction in mice

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Chapter 4. Impact of extreme maternal dietary protein stress on fetal development and mechanisms of placental mitochondrial dysfunction in mice

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Keywords: high-protein diet, placental function, amino acid transport, oxidative stress, mitochondrial function, fetal development

1. Abstract

Maternal dietary protein intake plays a vital role in pregnancy outcomes. Building upon the mild metabolic perturbations observed in multiparous sows fed 15% CP, the present study aimed to determine whether more extreme protein intake could exceed the threshold of maternal and placental adaptation and impair reproductive performance. We investigated the effect of different levels of maternal dietary protein during pregnancy on embryo implantation, placental development, and fetal growth in mice. Pregnant C57BL/6 mice were fed isocaloric purified diets containing 10% (low protein, LP), 20% (medium protein, MP), or 40% (high protein, HP) protein. Implantation was assessed on day 6.5, and gestational outcomes were analyzed on day 18.5. Maternal dietary protein did not affect implantation number or litter size. However, HP intake reduced fetal weight and placental efficiency. Morphologically, HP intake decreased the placental labyrinth zone/total area ratio compared to LP. It was further revealed that maternal HP intake reduced fetal serum glucose, increased fetal serum urea nitrogen, and activated hepatic gluconeogenic and amino acid catabolic pathways compared to MP. Mechanistically, HP intake induced placental oxidative stress, evidenced by enhanced malondialdehyde content, and decreased the activity of placental catalase, superoxide dismutase, and glutathione peroxidase. The expression of genes involved in antioxidant defense and amino acid transport was also suppressed in placenta. Moreover, maternal HP intake reduced ATP production, lowered mitochondrial DNA copy number, and downregulated gene expression of electron transport chain components. Excessive maternal dietary protein intake under isoenergetic conditions disrupts placental redox homeostasis and mitochondrial function, and impairs nutrient transfer. These alterations compromise placental efficiency and fetal growth, highlighting the potential risks associated with high-protein dietary patterns during pregnancy. These findings demonstrate that when maternal metabolic perturbations exceed the adaptive capacity of the placenta, they translate into functional impairment and fetal growth restriction.

2. Introduction

Protein intake during gestation is especially crucial for maternal physiology, fetal development and long-term offspring health (Elango and Ball, 2016). In chapter 3, we found that variations in dietary protein levels within the practical commercial range (12-15%) altered maternal metabolic status, including oxidative balance and amino acid profiles, without affecting reproductive performance. However, it remains unclear whether more extreme protein conditions could affect the pregnancy outcomes. The adverse impacts of protein restriction have been well studied (Langley-Evans et al., 1996; Petry et al., 2001; Fernandez-Twinn et al., 2006; Guzman et al., 2006; Zohdi et al., 2014), while the consequences of high protein (HP) intake on placental function and fetal development remain less elucidated. Current evidence regarding the impact of HP intake on fetal development is contradictory, with some studies reporting beneficial outcomes such as reduced incidence of low birth weight (LBW), small for gestational age (SGA), and intrauterine growth

restriction (IUGR) (Mikolainis, 1989; Yang et al., 2022; Zhang et al., 2025), while others have observed adverse effects including decreased ponderal index and lower birth weight (Andreasyan et al., 2007; Rehfeldt et al., 2012). To date, few studies have concurrently evaluated the impact of maternal low- and high-protein intake during pregnancy on fetal development and maternal physiological outcomes. Addressing this question is critical for understanding the mechanistic link between maternal metabolic responses and reproductive outcomes.

Embryo implantation and placental development are critical events that determine pregnancy success, and their outcomes are highly sensitive to maternal nutritional status (Cross and Mickelson, 2006). The placenta adapts dynamically to maternal nutritional status to optimize nutrient transport and fetal growth (Cetin and Alvino, 2009; Fowden et al., 2009), yet such adaptations may not always be beneficial (Gluckman et al., 2008). These adaptations rely on finely tuned amino acid (AA) transport systems and optimal mitochondrial function to meet the high energy demands of fetal growth. Any disruption in these systems, whether due to nutrient scarcity or overload, can compromise fetal development. Mitochondria are the primary generators of ATP and a major source of reactive oxygen species (ROS). An imbalance between ROS production and antioxidant defense is closely associated with placental dysfunction and adverse pregnancy outcomes such as gestational morbidities, poor fetal growth, or premature birth (Gupta et al., 2005; Luo et al., 2006; Holland et al., 2017). Pregnancy pathologies are related to abnormal mitochondrial structure and function (Hung et al., 2002). Crucially, mitochondrial function is sensitive to metabolic status. While protein restricted diets are known to induce oxidative stress (Lo et al., 2023), whether and how maternal HP intake disrupts placental redox and mitochondrial homeostasis remains unclear.

Notably, in isoenergetic dietary models, an increase in protein intake causes a proportional reduction in carbohydrates. Therefore, an HP diet creates a low-carbohydrate environment, whereas a low-protein (LP) diet results in high-carbohydrate intake. Consequently, this study aimed to evaluate the effects of maternal dietary protein levels and the associated change in carbohydrates on early embryo implantation and fetal growth in mice, with an emphasis on placental AA transporters, redox balance, and mitochondrial function.

3. Materials and methods

3.1 *Animal treatments and sample collection*

All animal procedures were approved by the Ethics Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences (Protocol No. IFR-CAAS-20240820). Eight-week-old female C57BL/6 mice were purchased from SPF (Beijing, China) Biotechnology Co., Ltd. and housed under controlled conditions with free access to food and water at the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences. After a one-week acclimation period, females were mated with male C57BL/6 mice overnight at a 2:1 ratio. Mated females (vaginal plug = gestational day 0.5, GD 0.5) were randomly assigned to one of three diets containing 10% protein (LP), 20%

protein (MP), 40% protein (HP) while providing the same calculated metabolizable energy. Carbohydrates were adjusted inversely to the protein content. The composition of these diets is shown in **Table 4-1**. The MP group (standard protein and carbohydrate level) served as the control.

Two experiments were performed. In experiment 1, a total of 45 vaginal plug-positive females were randomly assigned to one of three groups (n = 15 per group). On GD 6.5, blood was collected via orbital bleeding. The number of embryo implantation sites in both uterine horns was recorded. The number of confirmed pregnancies per group was: LP = 12, MP = 13, HP = 11. In experiment 2, another 45 vaginal plug-positive mice were selected and they were sacrificed on GD 18.5. Feed intake was recorded daily throughout gestation. On GD 18.5, blood was collected. After uterus was exposed, the number of total and live fetuses, individual fetal and placental weights were recorded. Fetal blood was collected via decapitation of the fetuses immediately after removal from the uterus. Amniotic fluid, uterine tissue, placentas, maternal serum, and fetal serum were collected and stored at -80°C . Placentas were fixed in 4% paraformaldehyde. The number of mice confirmed pregnant per group was: LP = 11, MP = 10, HP = 12. The indices were calculated as follows: Weight gain (g) = final weight-initial weight. Placental efficiency = fetal weight / placental weight (Hayward et al., 2016; Mellott et al., 2025).

Table 4-1: Diet composition (as-feed basis, %).

Composition	Low protein	Medium protein	High protein
Casein	10.00	20.00	40.00
Corn starch	49.75	39.75	19.75
Dextrin	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00
Soybean oil	7.00	7.00	7.00
Cellulose	5.00	5.00	5.00
Mineral mix	3.50	3.50	3.50
Vitamin mix	1.00	1.00	1.00
Cystine	0.30	0.30	0.30
Choline	0.25	0.25	0.25
Total	100.00	100.00	100.00
Calculated values			
Protein, g/100 g	10.00	20.00	40.00
Fat, g/100 g	7.00	7.00	7.00
Carbohydrate, g/100 g	73.95	63.95	43.95
Protein, % of total energy	10.00	20.00	40.00
Fat, % of total energy	15.80	15.80	15.80
Carbohydrate, % of total energy	74.20	64.20	44.20
Metabolizable energy, kcal/g	3.98	3.98	3.98

Calculated using energy factors of 4 kcal/g for protein, 9 kcal/g for fat, and 4 kcal/g for carbohydrate, following the calculation used for the reference AIN-93G diet.

Vitamin mix supplied per kg of diet: Vitamin A: 8 mg, Vitamin D3: 2.5 mg, Vitamin E: 150 mg, Vitamin K: 0.75 mg, Biotin: 0.2 mg, Vitamin B12: 25 mg, Folic acid: 2 mg, Niacin: 30 mg, Calcium pantothenate: 16 mg, Vitamin B6: 7 mg, Vitamin B2 (Riboflavin): 6 mg, Vitamin B1 (Thiamine): 6 mg, Sucrose: 9.747 g.

Mineral mix supplied per kg of diet: Calcium carbonate: 12.495 g, Potassium phosphate: 6.86 g, Potassium citrate: 2.477 g, Sodium chloride: 2.59 g, Potassium sulfate: 1.631 g, Magnesium oxide: 840 mg, Ferric citrate: 212.1 mg, Zinc carbonate: 57.75 mg, Sodium metasilicate: 50.75 mg, Manganese carbonate: 22.05 mg, Copper carbonate: 10.5 mg, Potassium chromium sulfate: 9.625 mg, Boric acid: 2.853 mg, Potassium iodate: 0.35 mg, Sodium fluoride: 2.223 mg, Nickel carbonate: 1.113 mg, Lithium chloride: 0.609 mg, Sodium tungstate: 0.359 mg, Ammonium molybdate: 0.278 mg, Ammonium vanadate: 0.231 mg, Sucrose: 7.736 g.

3.2 Concentrations of glucose and blood urea nitrogen (BUN) in serum and amniotic fluid

The concentrations of glucose and BUN in maternal serum, fetal serum, and amniotic fluid were determined using a commercial kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

3.3 Concentrations of amino acids in serum and amniotic fluid

Amino acid concentrations were analyzed using high-performance liquid chromatography (HPLC) as previously described (Dai et al., 2014). Briefly, samples of maternal serum, amniotic fluid, and fetal serum, collected on GD 18.5, were deproteinized using 1.5M HClO₄, neutralized with 2M K₂CO₃, and centrifuged. Supernatants were mixed with 0.1M HCl and internal standard, then filtered and analyzed for 16 types of amino acids. For fetal blood and amniotic fluid, the available volume was sometimes insufficient, and six to seven samples per group were analyzed.

3.4 RNA extraction and RT-qPCR

Total RNA was extracted from homogenized tissues following a previous protocol (Wu et al., 2024). RNA quality was assessed with the NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, USA), and 1000 ng of RNA was reverse-transcribed using the PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, China). The RT-qPCR was conducted using TB Green Premix Ex Taq™ Kit (Takara, China) on a CFX96 Real-Time PCR System (Bio-Rad, USA), normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and analyzed by the 2^{-ΔΔCt} method. Notably, GAPDH and β-actin were initially examined as candidate reference genes. GAPDH showed lower variation in Ct values than β-actin and similar Ct values across the dietary treatment groups and was therefore selected as the reference gene for normalization. Primer sequences are listed in **Table 4-2**.

Table 4-2: Primer sequences used for real-time quantitative PCR.

Gene	Accession number	Primer sequence (5'-3')	Size (bp)
<i>Cyp11a</i>	NM_001346787.1	F: TGCTCAACCTGCCTCCAGACTT R: ACTGGCTGAAGTCTCGCTTCTG	150
<i>Cyp19a</i>	NM_001348171.1	F: CGAAGCAGCAATCCTGAAGGAG R: CCAAGTCCACAACAGGCTGGTA	134
<i>Star</i>	NM_011485.5	F: AACGGGGACGAAGTGCTAAG R: CCGTGTCTTTTCCAATCCTCTG	184
<i>3β-hsd</i>	NM_001304800.1	F: AGAACTGCAGGAGGTCAGAGCT R: GGCATCCAGAATGTCTCCTTCC	118
<i>Esr</i>	NM_001302531.2	F: AATGCAAGAACGTTGTGCCC R: TCTGCTTCCGGGGGTATGTA	181
<i>Pgr</i>	NM_008829.2	F: TATGGCGTGCTTACCTGTGG R: TGCCAGCCTGACAACACTT	160
<i>Lat2</i>	NM_016972.2	F: GCATACGTCACTGCAATGTCCC R: GGAGCCATTGACTCCACCAAAC	144
<i>Lat3</i>	NM_001081349.2	F: TTCCTGTGGAGCCTTGTCACCA R: CTCCACCTTCTGTCTCTGCTCA	138
<i>Lat4</i>	NM_001199283.2	F: CAGCATCCTTGAGTTCCTGGTC R: TGATGTAGCCGATGACAGGAGC	116
<i>4F2hc</i>	NM_008577.5	F: GAGCGTACTGAATCCCTAGTCAC R: GCTGGTAGAGTCGGAGAAGATG	127
<i>Cat1</i>	NM_001429138.1	F: CTCCTGGCTTACTCTTTGGTGG R: GATCTAGCTCCTCGGTGGTTCT	106
<i>Snat2</i>	NM_001355633.1	F: GCGTTGGCATTCAATAGCACCG R: TCGTAGATGGGAAGAACAGCGG	134
<i>y+Lat1</i>	NM_001253679.1	F: AAGGTGTTGGCGCTGATTGCAG R: AGAGTGCCAGAGCAATGTCACC	127

<i>Nfe2l2</i>	NM_010902.5	F: CAGCATAGAGCAGGACATGGAG R: GAACAGCGGTAGTATCAGCCAG	107
<i>Gpx4</i>	NM_001037741.4	F: GCTGGGAAATGCCATCAAATGGA R: ACGGCAGGTCCTTCTCTATCAC	116
<i>Nqo1</i>	NM_008706.5	F: GCCGAACACAAGAAGCTGGAAG R: GGCAAATCCTGCTACGAGCACT	120
<i>Sod1</i>	NM_011434.2	F: GGTGAACCAGTTGTGTTGTCAAG R: ATGAGGTCCTGCACTGGTACAG	114
<i>Cat</i>	NM_009804.2	F: CGGCACATGAATGGCTATGGATC R: AAGCCTTCCTGCCTCTCCAACA	132
<i>Mfn2</i>	NM_001355590.1	F: AAGCAGATTACGGAGGAAGTGGAA R: GAGCAGCGGTCAGACAGGTT	191
<i>Nrf1</i>	NM_001164227.2	F: CTGTGGCTGATGGAGAGGTGGAA R: GATGCTTGCGTCGTCTGGATGG	79
<i>Fis1</i>	NM_001163243.1	F: TTGAATATGCCTGGTGCCTGGTT R: GCTGTTCCTCTTTGCTCCCTTTG	105
<i>Ppargc1a</i>	NM_008904.3	F: TCGCTGCTCTTGAGAATGGATATACTT R: AATCGTCTGAGTTGGTATCTAGGTCTG	126
<i>Tfam</i>	NM_009360.4	F: GAGGCAAAGGATGATTCCGGCTC R: CGAATCCTATCATCTTTAGCAAGC	116
<i>Ndufs8</i>	NM_001271444.1	F: GTTCATAGGGTCAGAGGTCAAG R: TCCATTAAGATGTCTGTGCG	112
<i>Sdhb</i>	NM_001355515.1	F: TGCGGACCTATGGTGTGGATG R: CCAGAGTATTGCCTCCGTTGATG	133
<i>Uqcrc1</i>	NM_025407.2	F: AGTGTGGATTGACGCTGGCAGT R: CCTCCTTCTCTAAGGCATTGCC	122
<i>Cox4i1</i>	NM_009941.3	F: TCATTGGCTTCACTGCGCTCGT R: TCCAGCATTGCTTGGTCTGCA	112

<i>Atp5pb</i>	NM_007505.2	F: CATTGGTGATGGTATTGCGC R: TCCCAAACACGACAACTCC	134
<i>Glut1</i>	NM_001424864.1	F: GCTTCTCCAACCTGGACCTCAAAC R: ACGAGGAGCACCGTGAAGATGA	103
<i>Pck1</i>	NM_011044.3	F: GGCGATGACATTGCCTGGATGA R: TGTCTTCACTGAGGTGCCAGGA	105
<i>G6pc</i>	NM_008061.4	F: AGGTCGTGGCTGGAGTCTTGTC R: GTAGCAGGTAGAATCCAAGCGC	141
<i>Pygl</i>	NM_133198.2	F: GGCAGAAGTGGTGAACAATGACC R: TCCGATAGGTCTGTGGCTGGAA	114
<i>Lat1</i>	NM_011404.3	F: CTA CTCTTTGGTGTCTGGTGGAA R: GAGGTACCACCTGCATCAACTTC	101
<i>Bcat2</i>	NM_009737.4	F: CAAAGGTGGAGACCAGCAGGTA R: TGGCGGATACACTCCAACAGCT	126
<i>Gpt1</i>	NM_182805.3	F: CCACTCAGTCTCTAAGGGCTAC R: ACACAACCGCACGCTCATCAGT	121
<i>Got1</i>	NM_010324.2	F: TGCTACTGGGATGCGGAGAAGA R: TGCATGACAGCAGCGATCTGCT	164
<i>Gapdh</i>	NM_001289726.2	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA	123
<i>mt-Co2</i>	NC_005089.1	F: ATAACCGAGTCGTTCTGCCAAT R: TTTCAGAGCATTGGCCATAGAA	180
<i>Rps18</i>	NC_000083.7	F: TGTGTTAGGGGACTGGTGGACA R: CATCACCCACTTACCCCCAAAA	195

3.5 Protein extraction and Western blot

The placental protein was extracted and quantified by BCA assay (Beijing Huaxing Bochuang Gene Technology Co., Ltd.). Equal amounts (40 µg) were loaded onto 12% SDS-PAGE gels (Bio-Rad, USA). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, blocked and incubated with primary antibodies. After washing, membranes were incubated with secondary antibodies

and visualized using an Odyssey CLx infrared imaging system (LI-COR Biosciences, USA). Band intensities were quantified with ImageJ and normalized to actin beta (ACTB). Antibodies are listed in **Table 4-3**.

Table 4-3: Antibody information.

Target	Supplier	Catalog	Appli -cation	Type	Source	Diluti- on	Size (kDa)
ACTB	CST	4970S	WB	Monoclonal	Rabbit	1:1000	45
NFE2L2	Proteintech	16396	WB	Polyclonal	Rabbit	1:2000	68
KEAP1	Abcam	Ab196346	WB	Polyclonal	Rabbit	1:1000	70
BAX	Affinity	AF0120	WB	Polyclonal	Rabbit	1:1000	21
BCL2	Affinity	AF6138	WB	Polyclonal	Rabbit	1:1000	26
MFN2	Affinity	DF8106	WB	Polyclonal	Rabbit	1:1000	86
DNM1L	Affinity	DF7037	WB	Polyclonal	Rabbit	1:1000	79
CD31	Servicebio	GB113151	IHC	Polyclonal	Rabbit	1:100	-

3.6 Histological analysis

Fixed placentas were embedded and sectioned into 4 μm -thick slices. Hematoxylin and eosin (HE) staining, periodic acid-Schiff (PAS) staining, and immunohistochemistry (IHC) for CD31 were performed on 6 placentas per group randomly by Wuhan Servicebio Technology CO., LTD. Antibody information is provided in **Table 4-3**. Placental tissue sections were scanned using a PANNORAMIC MIDI digital slide scanner (3DHISTECH Ltd., Budapest, Hungary). Image analysis of HE sections was conducted using CaseViewer 2.3 software. The areas of the whole placenta, decidua (DEC), labyrinth zone (LZ), and junctional zone (JZ) were quantified as previously described (He et al., 2016). The proportion of PAS-positive glycogen area in JZ and the CD31-positive area in LZ were quantified using ImageJ software.

3.7 Oxidative stress and ATP content

The activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and malondialdehyde (MDA) level in the placenta were measured using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The

concentration of ATP in the placenta was measured using the Enhanced ATP Assay Kit (Shanghai Beyotime Biotechnology Co., Ltd.).

3.8 *mtDNA copy number*

Mitochondrial DNA (mtDNA) copy number was quantified by qPCR, as previously described (Eaton et al., 2007). Total DNA was extracted from placental tissues using a commercial genomic DNA extraction kit (TIANamp genomic DNA Kit, China), and 10 ng of DNA was used in the qPCR reaction. The mtDNA copy number was calculated by normalizing the expression level of mitochondrially encoded cytochrome c oxidase II (mt-Co2) to nuclear ribosomal protein S18 (Rps18). Primer sequences for mice mt-Co2 and Rps18 were obtained from a previous study and provided in **Table 4-2** (Watson and Cross, 2005; Zhao et al., 2020).

3.9 *Statistical analysis*

Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using IBM SPSS Statistics 20 and visualized with GraphPad Prism 10.1.2. One-way analysis of variance (ANOVA) was used to determine differences between groups. Multiple comparisons of significant differences were performed using the Tukey test. For unequal variances (Levene's test, $P < 0.05$), Welch's ANOVA with Games-Howell correction was used. Different superscript letters indicate significant differences between groups. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and $0.05 \leq P < 0.10$ indicates a trend toward significant difference.

4. Results

4.1 *Effects of different maternal dietary protein levels on feed intake, weight gain, and reproductive performance.*

Detailed maternal average daily feed intake (ADFI) data throughout gestation are presented in **Table 4-4**. There were no significant differences in ADFI among groups during GD 0.5 - 6.5 or GD 6.5 - 13.5, while it was significantly decreased with the protein level increased during GD 13.5 - 18.5 ($P < 0.001$). However, total gestational ADFI did not differ between HP and MP. Despite the reduced feed intake in late gestation, maternal weight gain and uterus weight remained unaffected among groups (**Table 4-5**). Regarding the reproductive performance (**Table 4-5**), no significant differences were observed in implantation numbers in mice at GD 6.5, or total litter size, live litter size, total litter weight and average placenta weight at GD 18.5 ($P > 0.05$). However, average fetal weight was significantly lower in HP relative to MP, accompanied by reduced placental efficiency ($P < 0.05$).

Table 4-4: Maternal average daily feed intake during overall gestation.

Items	Treatment groups ¹			SEM	P-value
	LP	MP	HP		
ADFI ² (g, GD 0.5-6.5)	2.69	2.65	2.78	0.07	0.479
ADFI (g, GD 6.5-13.5)	3.10	3.10	3.19	0.11	0.830
ADFI (g, GD 13.5-18.5)	4.76 ^a	4.16 ^b	3.51 ^c	0.10	< 0.001
ADFI (g, GD 0.5-18.5)	3.42 ^a	3.28 ^{ab}	3.14 ^b	0.06	0.035

¹ LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%).

² ADFI: Average daily feed intake.

Table 4-5: Effect of different protein levels on implantation and reproductive performance in pregnant mice.

Items	Treatment groups ¹			SEM	<i>P</i> -value
	LP	MP	HP		
GD 6.5²					
Number of embryo implantation sites	9.25	8.62	8.73	0.25	0.491
Left number of implanted embryos	4.83	5.00	4.55	0.49	0.658
Right number of implanted embryos	4.42	3.62	4.18	0.41	0.405
GD 18.5³					
Total litter size	8.64	8.90	9.25	0.37	0.481
Live litter size	8.27	8.40	8.92	0.39	0.453
Weight gain during pregnancy (g)	13.54	14.75	13.90	0.47	0.219
Uterus weight (g)	11.52	11.83	11.76	0.45	0.874
Total litter weight (g)	9.03	9.43	9.33	0.37	0.752
Average fetal weight (g)	1.09 ^{ab}	1.13 ^a	1.05 ^b	0.02	0.013
Average placenta weight (g)	0.10	0.10	0.10	0.00	0.377
Placental efficiency	11.50 ^{ab}	12.06 ^a	10.55 ^b	0.30	0.005

¹ LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%).

² At GD 6.5, the replicates were 12, 13, and 11 in LP, MP, and HP groups, respectively.

³ At GD 18.5, the replicates were 11, 10, and 12 in LP, MP, and HP groups, respectively.

^{a,b} indicates significant differences between means ($P < 0.05$). Within the same row, values sharing the same letter are not significantly different.

4.2 Effects of different maternal dietary protein levels on maternal hormones, serum biochemical index and gene expression at GD 6.5.

Since steroid hormones are critical for implantation, we examined maternal serum hormone levels at GD 6.5. As shown in Figure 4-1A, the estradiol level was significantly elevated in the LP group compared to the MP group ($P < 0.05$). Conversely, no significant differences were observed in serum progesterone (Figure 4-1 B) and insulin (Figure 4-1 C) concentrations among the groups ($P > 0.05$). Moreover, the levels of high-density lipoprotein (HDL) and total cholesterol (TC) in the LP group were significantly higher than those in both the MP and HP groups ($P < 0.05$) (Figure 4-1D and F). The concentration of low density lipoprotein (LDL) in LP was also significantly higher than that in the HP group ($P < 0.05$) (Figure 4-1 E). Ovarian expression of steroidogenesis-related genes (Figure 4-1G) revealed that Cytochrome P450 family 11 subfamily A member 1 (*Cyp11a*) was significantly downregulated in LP compared to MP. For Cytochrome P450 family 19 subfamily A member 1 (*Cyp19a*), the LP group showed an upward trend compared to the MP group ($0.05 \leq P < 0.1$), and was significantly higher than the HP group ($P < 0.05$). Additionally, the expression of *Cyp19a* in HP exhibited a decreasing trend compared to MP group ($0.05 \leq P < 0.1$). The expression of steroidogenic acute regulatory protein (*Star*) was significantly downregulated in the HP group compared to both the LP ($P < 0.001$) and MP ($P < 0.05$) groups, while exhibiting an upward trend in the LP group relative to the MP group ($P = 0.066$). Interestingly, the expression of 3β -hydroxysteroid dehydrogenase (*3\beta*-*hsd*) remained unchanged across all dietary groups ($P > 0.05$), which is consistent with the stable serum progesterone levels observed. Furthermore, we detected the relative mRNA expression of estrogen receptor (*Esr*) and progesterone receptor (*Pgr*) in the implantation site (Figure 4-1 H). Compared to the MP group, the expression of *Esr* was significantly decreased in both the LP ($P < 0.05$) and HP ($0.05 \leq P < 0.1$) groups. Notably, the expression of *Pgr* was significantly lower in the HP group compared to both the LP and MP groups ($P < 0.05$). These results indicated that varying maternal dietary protein levels can significantly modulate lipid metabolism and hormone signaling pathways, even without altering the absolute number of embryo implantations.

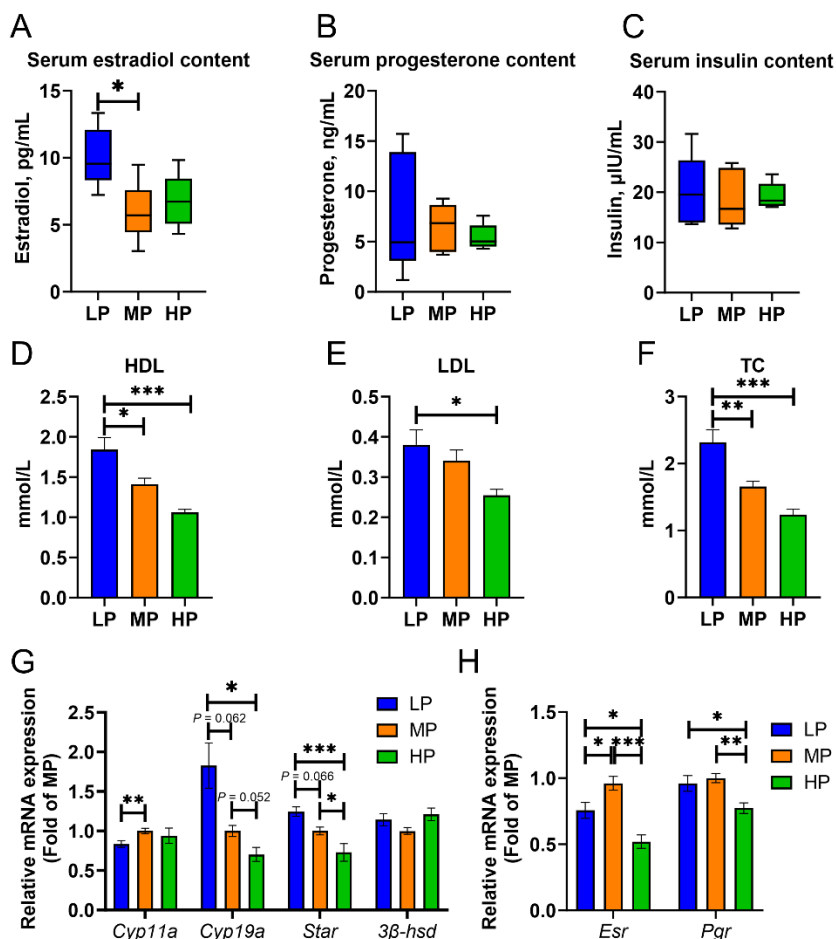


Figure 4-1: Serum hormone synthesis related index in mice at GD 6.5.

(A-C) The estradiol, progesterone, and insulin levels in serum at GD 6.5 (n = 6), respectively. (D-F) Serum concentrations of high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (TC) at GD 6.5 (n = 6). (G) The relative mRNA expression in ovary in mice at GD 6.5 (n = 10). (H) The relative mRNA expression in implantation site in mice at GD 6.5 (n = 10). LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%). Data are means $\pm$ SEM. The gene expression data are presented relative to the MP group. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

4.3 Effects of different maternal dietary protein levels on glucose and BUN concentrations in maternal serum, amniotic fluid and fetal serum at GD 18.5.

As shown in **Table 4-6**, compared to MP, glucose concentrations in maternal serum and amniotic fluid were significantly elevated in LP ($P < 0.01$), whereas no

significant difference was observed between MP and HP. Notably, fetal serum glucose was significantly reduced in HP ($P < 0.05$). The BUN concentration in maternal serum, amniotic fluid, and fetal serum exhibited a dose-dependent increase with dietary protein levels ($P < 0.05$), confirming the altered nitrogen metabolism.

Table 4-6: Concentrations of glucose (mmol/L) and BUN (mmol/L) in maternal serum, amniotic fluid and fetal serum of mice fed different protein levels on GD 18.5.

Items	Treatment groups ¹			SEM	<i>P</i> -value
	LP	MP	HP		
Maternal serum					
Glucose (mmol/L)	6.33 ^a	4.72 ^b	4.19 ^b	0.36	0.001
BUN (mmol/L)	2.65 ^c	4.19 ^b	5.16 ^a	0.21	0.000
NEFA (μM)	30.36 ^b	32.99 ^{ab}	37.56 ^a	1.08	0.004
Amniotic fluid					
Glucose (mmol/L)	2.03 ^a	1.48 ^b	1.45 ^b	0.13	0.007
BUN (mmol/L)	3.15 ^c	5.60 ^b	7.16 ^a	0.29	0.000
Fetal serum					
Glucose (mmol/L)	3.83 ^{ab}	3.86 ^a	2.65 ^b	0.39	0.031
BUN (mmol/L)	4.47 ^c	7.91 ^b	9.81 ^a	0.32	0.000

¹ LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%).

² Replicates were n = 10 for maternal serum, n = 6 for amniotic fluid, and n = 7 for fetal serum.

^{a,b} indicates significant differences between means ($P < 0.05$); ^{x,y} indicates a trend toward significance ($P < 0.10$). Within the same row, values sharing the same letter are not significantly different.

BUN, Blood urea nitrogen; NEFA, non-esterified fatty acids.

4.4 Effects of different maternal dietary protein levels on amino acid profiles in maternal serum, amniotic fluid and fetal serum at GD 18.5.

Amino acid profiles are detailed in Table 4-7. In maternal serum, the level of valine (Val) was significantly higher in HP than MP ($P < 0.05$). No other amino acids were significantly changed in HP or LP compared to MP. However, the levels of branched chain amino acid (BCAA, including valine, leucine, and isoleucine), and histidine

(His), threonine (Thr), tyrosine (Tyr), and methionine (Met) were elevated in HP relative to LP ($P < 0.05$). In amniotic fluid, we found that there was no significant difference between LP and MP or between HP and MP, except lysine (Lys) was significantly higher in LP than in MP, and arginine (Arg) was significantly lower in

HP than in LP ($P < 0.05$). However, the HP showed higher level of Val and Ile but significantly lower level of non-essential AA (NEAA), including Asp, glutamate (Glu), serine (Ser), glycine (Gly), Arg, and alanine (Ala) compared to LP ($P < 0.05$). Specifically, Ala tended to be higher in LP than in MP ($0.05 \leq P < 0.10$), and was significantly higher than in HP ($P < 0.05$). In fetal serum, many AA levels showed no significant difference among the groups. The LP exhibited elevated Ala and Lys but decreased Trp compared to MP ($P < 0.05$). Met was substantially higher in HP compared to LP ($P < 0.05$), and showed a trend to be higher than MP ($0.05 \leq P < 0.10$).

Table 4-7: Concentrations of amino acids ($\mu\text{mol/L}$) in maternal serum, amniotic fluid and fetal serum of mice fed different protein levels on GD 18.5.

Items	LP	MP	HP	SEM	P-value
Serum					
Asp	13.20 ^y	13.91	20.13 ^x	2.067	0.052
Glu	50.84	55.39	64.65	8.065	0.534
Ser	103.23	103.25	113.27	10.322	0.747
His	30.90 ^b	40.55 ^{ab}	49.21 ^a	4.337	0.024
Gly	64.95	71.62	71.38	5.160	0.577
Thr	127.87 ^b	186.70 ^{ab}	244.22 ^a	17.818	0.001
Arg	7.10	4.93	4.44	1.538	0.467
Ala	353.66	294.14	353.93	39.817	0.517
Tyr	34.86 ^b	45.49 ^{ab}	55.46 ^a	5.196	0.032
Val	83.20 ^b	107.50 ^b	159.34 ^a	13.273	0.003
Met	37.01 ^b	44.36 ^{ab}	58.69 ^a	4.174	0.041
Trp	42.97 ^y	54.94 ^x	56.12 ^x	3.713	0.041
Ile	33.75 ^b	43.68 ^{ab}	55.46 ^a	4.673	0.014
Phe	31.99 ^y	41.91 ^x	54.57 ^x	4.749	0.023
Lys	360.80	392.33	355.17	25.280	0.552
Leu	56.33 ^b	74.64 ^{ab}	92.36 ^a	8.421	0.024
Amniotic fluid					
Asp	20.08 ^a	17.34 ^{ab}	14.98 ^b	1.118	0.021
Glu	114.10 ^a	100.54 ^{ab}	72.28 ^b	10.942	0.049
Ser	171.64 ^a	156.62 ^{ab}	139.61 ^b	8.244	0.053
His	84.20	97.55	81.95	4.892	0.104
Gly	169.17 ^a	157.65 ^{ab}	138.68 ^b	7.683	0.044
Thr	246.91	294.53	266.95	15.729	0.166
Arg	87.54 ^a	84.68 ^a	60.45 ^b	5.992	0.016
Ala	564.21 ^{a,x}	485.37 ^{ab,y}	419.90 ^b	23.387	0.003
Tyr	186.16	202.83	222.45	14.840	0.257
Val	250.17 ^b	275.32 ^{ab}	295.78 ^a	10.579	0.059
Met	128.50	125.67	118.50	5.898	0.483
Trp	53.51	51.22	53.23	4.226	0.930
Ile	87.66 ^b	94.69 ^{ab}	109.06 ^a	4.688	0.034

Phe	116.08	114.87	123.17	6.318	0.626
Lys	799.53 ^a	688.24 ^b	633.69 ^b	28.699	0.003
Leu	220.32	242.12	260.39	12.768	0.138
Fetal serum					
Asp	46.79	36.82	41.76	3.169	0.121
Glu	234.44	216.20	212.44	13.158	0.498
Ser	115.58	92.45	93.93	7.574	0.088
His	85.49	96.44	99.49	5.094	0.166
Gly	159.75	132.86	137.96	14.983	0.447
Thr	157.10	160.11	198.01	17.052	0.251
Arg	35.36	37.80	23.60	9.280	0.573
Ala	522.87 ^a	370.27 ^b	395.86 ^b	23.014	0.001
Tyr	102.41	118.39	131.42	8.743	0.143
Val	177.13	179.71	187.17	7.936	0.733
Met	75.72 ^{b,xy}	77.29 ^{ab,y}	89.74 ^{a,x}	3.332	0.030
Trp	36.59 ^b	48.64 ^a	36.94 ^b	2.620	0.006
Ile	57.65	57.97	65.35	2.860	0.225
Phe	69.78	76.34	78.99	4.956	0.504
Lys	669.33 ^a	582.08 ^b	560.75 ^b	21.732	0.014
Leu	109.33	102.78	113.66	6.685	0.567

¹ LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%).

² Replicates were n = 10 for maternal serum, n = 6 for amniotic fluid, and n = 7 for fetal serum.

^{a,b} indicates significant differences between means ($P < 0.05$); ^{xy} indicates a trend toward significance ($P < 0.10$). Within the same row, values sharing the same letter are not significantly different.

Asp, Aspartate; Glu, Glutamate; Ser, Serine; His, Histidine; Gly, Glycine; Thr, Threonine; Arg, Arginine; Ala, Alanine; Tyr, Tyrosine; Val, Valine; Met, Methionine; Trp, Tryptophan; Ile, Isoleucine; Phe, Phenylalanine; Lys, Lysine; Leu, Leucine.

4.5 Effects of different maternal dietary protein levels on placenta morphology and nutrient transporters in placentas of mice at GD 18.5.

Since the placenta acts as the primary interface for nutrient transfer, we investigated whether the placenta morphology and nutrient transporter function was compromised in the HP group. **Figure 4-2** shows the HE staining results, which illustrate the placental morphology, which consists of the maternal DEC, JZ, and LZ. According to **Figure 4-2D**, no significant differences were observed in the decidual/total area ratio among groups. There was no significant difference in the JZ/total area ratio or LZ/total area ratio between MP and HP or MP and LP. However, the JZ/total area ratio was significantly increased and the LZ/total area ratio was decreased in HP compared to LP ($P < 0.05$). Glycogen storage (PAS staining) and vascular coverage (CD31) did not differ significantly among groups (**Figure 4-2B, C, E, F**). We further analyzed placental nutrient transporter genes

(Figure 4-2G). Compared to MP, L-type amino acid transporter 3 (*Lat3*), L-type amino acid transporter 4 (*Lat4*), cationic amino acid transporter 1 (*Cat1*), and glucose transporter 1 (*Glut1*) were significantly downregulated in HP ($P < 0.05$), and L-type amino acid transporter 2 (*Lat2*) and hexokinase 1 (*Hk1*) showed a downward trend ($0.05 \leq P < 0.10$). Conversely, CD98 heavy chain (*4F2hc*) and y+L amino acid transporter 1 (*y+Lat1*) were upregulated in LP.

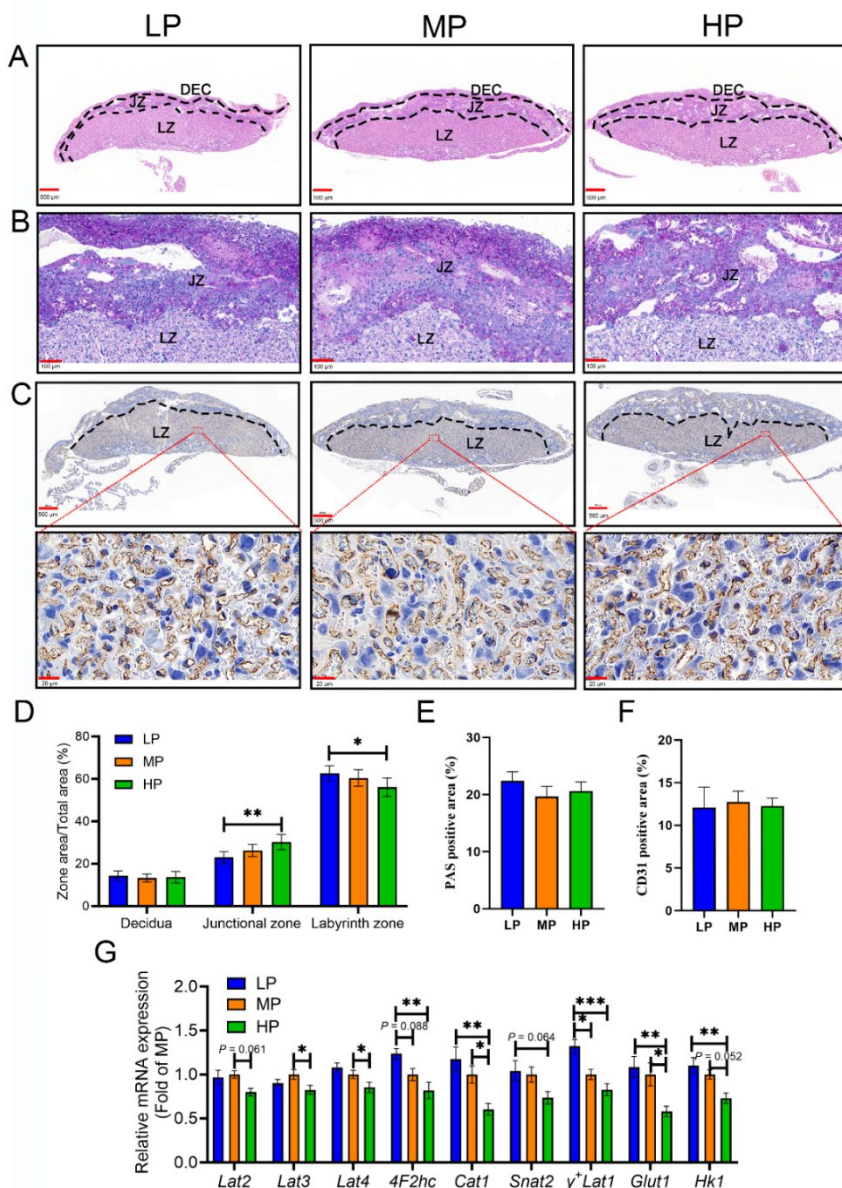


Figure 4-2: Effects of different protein levels on the placenta structure, glycogen storage, vascularization, and nutrient transport of mice on GD 18.5.

(A) Representative images of HE stained placental cross-sections of LP, MP, and HP groups, scale bar = 500 μm . (B) Representative images of PAS staining showing glycogen, scale bar = 100 μm . (C) Representative images of IHC staining for CD31 in the labyrinth zone, scale bar = 500 μm and 20 μm . (D) Quantitative analysis of the proportional areas of decidua, junctional zone, and labyrinth zone to total placental sectional area ($n = 6$). (E) Quantitative analysis of PAS-positive area percentage ($n = 6$). (F) Quantification of CD31-positive area percentage ($n = 6$). (G) The mRNA expression of amino acid transporter, glucose transporter/metabolism related genes in placenta ($n = 10$). LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%). Data are means $\pm$ SEM, * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

4.6 Effects of different maternal dietary protein levels on placental oxidative stress status of mice at GD 18.5.

To explore the mechanisms underlying the observed morphological and transport damage, we assessed the placental oxidative stress status. From **Figure 4-3 A-D**, in the HP group, the lipid peroxidation marker MDA was significantly elevated, while the key antioxidant enzyme activities, including SOD, CAT, and GPx, were significantly decreased compared to MP ($P < 0.05$). Consistently, mRNA levels of antioxidant genes such as glutathione peroxidase 4 (*Gpx4*), NAD(P)H dehydrogenase quinone 1 (*Nqo1*), superoxide dismutase 1 (*Sod1*) and catalase (*Cat*) were significantly decreased in HP (**Figure 4-3 E**). In contrast, MDA levels in LP were significantly lower than in MP ($P < 0.05$), with a decreasing trend of SOD observed in the LP group ($0.05 \leq P < 0.10$). Regarding the NFE2L2 pathway (**Figure 4-3 F-I**), the protein expression of nuclear factor erythroid derived 2 like 2 (NFE2L2), the master transcriptional regulator of antioxidant defense, was significantly upregulated in LP compared to MP ($P < 0.05$), while kelch-like ECH-associated protein 1 (KEAP1), a negative regulator of NFE2L2, was significantly decreased ($P < 0.05$). Additionally, the pro-apoptotic protein, BCL2-associated X protein (BAX) was also significantly decreased in LP ($P < 0.05$).

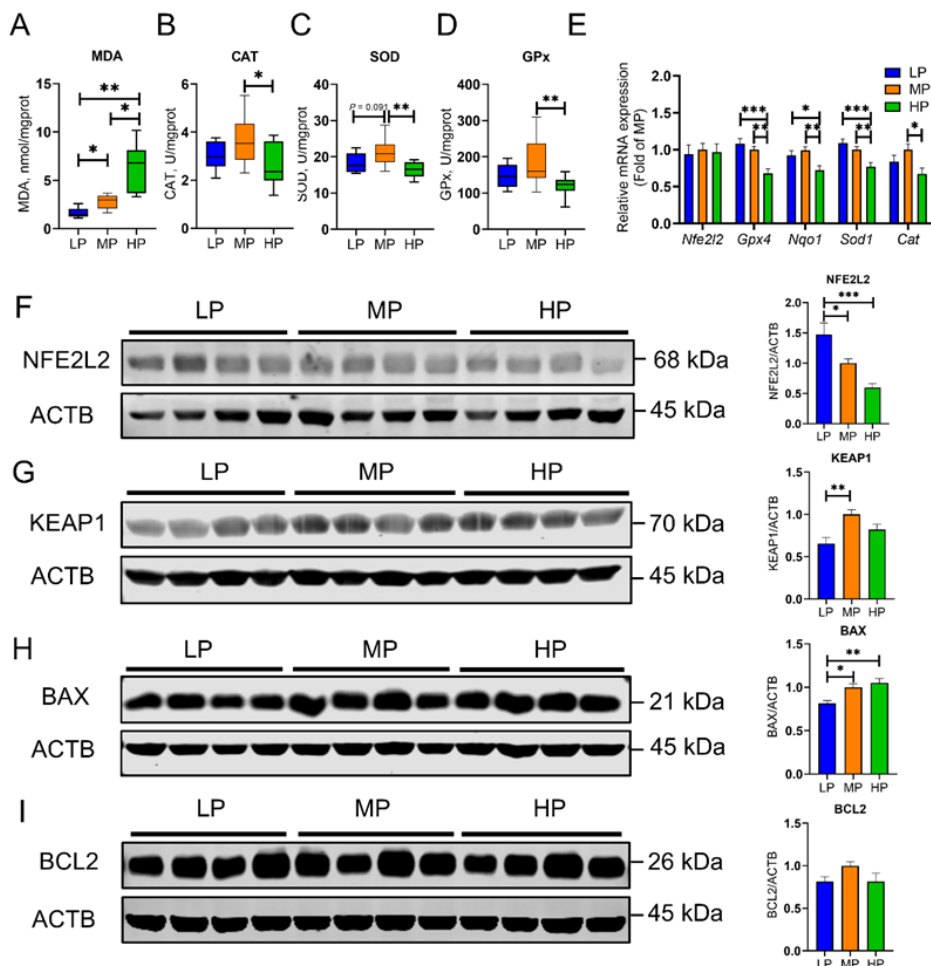


Figure 4-3: Placental oxidative stress status in mice fed with different protein levels at GD 18.5.

(A-D) The MDA level and antioxidant enzyme levels in placenta of mice at GD 18.5 (n = 10). (E) The relative mRNA expression of oxidative stress related genes in placenta (n = 10).

(F-I) The expression of oxidative stress and apoptosis related proteins (n = 8). LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%). Data are presented relative to the MP group. Data are means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

4.7 Effects of different maternal dietary protein levels on mitochondrial function in placenta of mice at GD 18.5.

Given the elevated oxidative stress in HP, we further examined mitochondrial function (Figure 4-4). According to the protein results of mitofusin 2 (MFN2) which mediates mitochondrial fusion, and dynamin 1-like (DNM1L) which drives mitochondrial fission (Figure 4-4 A-B), MFN2 levels were significantly increased while DNM1L levels were significantly decreased in the LP group ($P < 0.05$), suggesting the enhanced mitochondrial fusion in LP. From Figure 4-4 C, ATP level had no significant difference in HP compared to MP, while it was significantly lower than in LP ($P < 0.05$). Furthermore, the relative mtDNA copy number of *mt-Co2* (Figure 4-4 D) was significantly reduced in HP compared to both MP and LP ($P < 0.05$). Gene expression analysis (Figure 4-4 E-F) showed that mitochondrial biogenesis and dynamic genes such as *Mfn2*, mitochondrial fission 1 (*Fis1*), peroxisome proliferative activated receptor gamma coactivator 1 alpha (*Ppargc1a*), and transcription factor A mitochondrial (*Tfam*), as well as mitochondrial electron transport genes such as NADH: ubiquinone oxidoreductase core subunit S8 (*Ndufs8*), succinate dehydrogenase complex subunit B (*Sdhb*), and cytochrome c oxidase subunit 4I1 (*Cox4i1*) were significantly downregulated in HP compared to MP ($P < 0.05$).

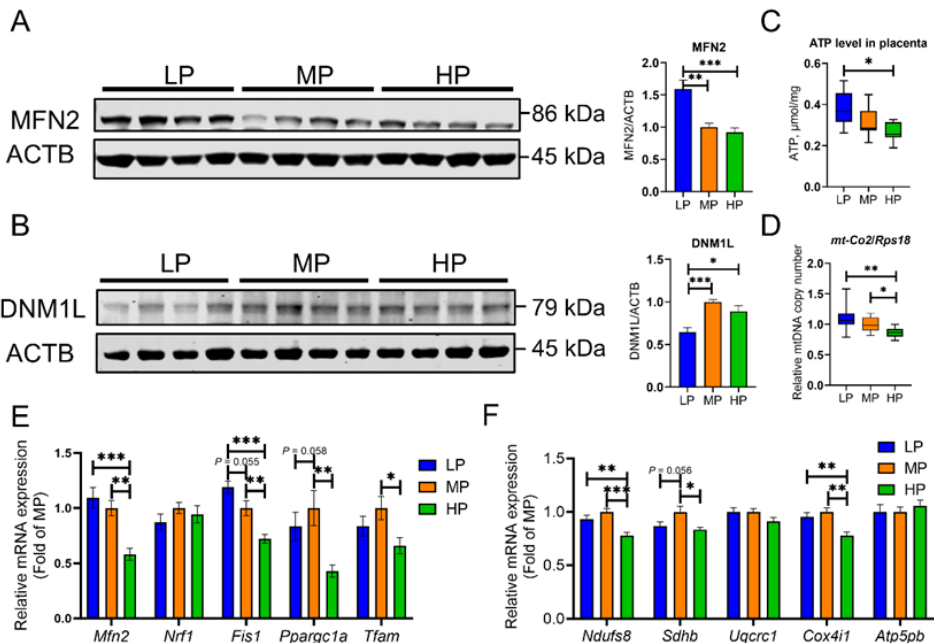


Figure 4-4: Mitochondrial function of placenta in mice fed with different protein levels at GD 18.5.

(A-B) The expression of mitochondrial fusion and fission proteins (n = 8). (C) The ATP

content in placenta (n = 8). (D) The mtDNA copy number in placenta (n = 10). (E-F) The mRNA expression of mitochondrial function related genes in placenta (n= 10). LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%). Data are presented relative to the MP group. Data are means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

4.8 Effects of different maternal dietary protein levels on fetal glucose and amino acid metabolism at GD 18.5.

Finally, we evaluated fetal metabolic adaptations in the liver (Figure 4-5). In the HP group, genes related to glucose metabolism, including glucose transporter 1 (*Glut1*), phosphoenolpyruvate carboxykinase 1 (*Pck1*), and glycogen phosphorylase L (*Pygl*) were significantly upregulated compared to MP ($P < 0.05$), with glucose-6-phosphatase catalytic subunit (*G6pc*) showing an increasing trend ($0.05 \leq P < 0.10$). Figure 4-5B showed that amino acid transporters *Lat1* and *Lat2*, and catabolic enzymes, such as branched-chain amino acid transaminase 2 (*Bcat2*), glutamic-pyruvic transaminase 1 (*Gpt1*), and glutamic-oxaloacetic transaminase 1 (*Got1*), were significantly increased in HP ($P < 0.05$).

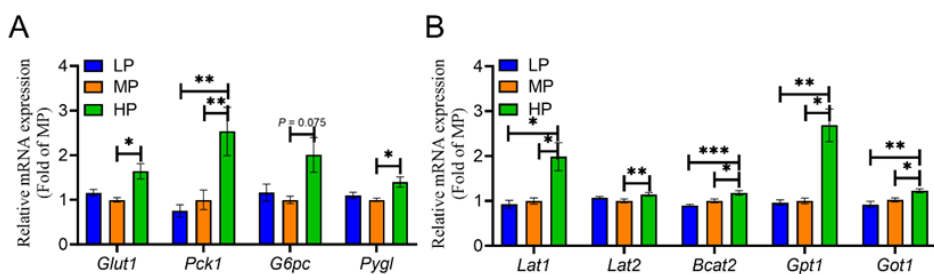


Figure 4-5: Fetal hepatic amino acid and glucose metabolism related genes expression at GD 18.5.

(A) Relative mRNA expression of genes related to glucose uptake and gluconeogenic genes in fetal liver (n = 10). (B) Relative mRNA expression of genes related to amino acid transport and catabolism in fetal liver (n = 10). LP, low protein diet (10%); MP, medium protein diet (20%); HP, high protein diet (40%). Data are presented relative to the MP group. Data are means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

5. Discussion

This study provides a comprehensive investigation into the effects of varying maternal dietary protein levels on pregnancy outcomes. Neither low nor high protein diets significantly altered the number of implanted embryos at GD 6.5 or litter size at GD 18.5. These findings are consistent with previous reviews indicating that maternal low- or high-protein diets during pregnancy have minimal impact on litter size in rats (Ajuogu et al., 2020). However, maternal dietary protein imbalance still

induced clear endocrine and metabolic alterations during early gestation. These findings suggest that early pregnancy possesses a substantial compensatory capacity that maintains implantation success despite nutritional disturbance. However, as gestation progressed and fetal nutrient demand increased, excessive protein intake progressively impaired placental function and fetal growth.

Indeed, changes in circulating lipid profiles, ovarian steroidogenic gene expression, and implantation-related receptor signaling indicate that maternal physiological adaptation had already been initiated at GD 6.5. Steroid hormones synthesized by the corpus luteum are essential regulators of uterine receptivity and embryo implantation. Cholesterol, the precursor for all steroid hormones, is transported from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (*Star*), where it is converted into pregnenolone by cholesterol side-chain cleavage enzyme (*Cyp11a1*) (Miller and Bose, 2011; Miller, 2013). Pregnenolone is further converted into progesterone by 3 β -hydroxysteroid dehydrogenase (*3 β -hsd*), and progesterone is subsequently metabolized into androgens and estrogens via *Cyp11a1* and *Cyp19a1*, respectively (Ye et al., 2021). In the LP group, significantly elevated serum HDL and total cholesterol, along with an upregulation of ovarian *Star* and *Cyp19a1*, suggested a compensatory increase in estrogen synthesis. However, excessive estradiol has been reported to exert toxic effects on embryos, inhibiting blastocyst implantation both in vivo and in vitro, as well as hindering early post-implantation development (Valbuena et al., 2001; Chang et al., 2022). Furthermore, the downregulation of *Cyp11a1* and estrogen receptor (*Esr*) at implantation sites may reflect impaired initiation of steroid biosynthesis and reduced endometrial responsiveness. Conversely, the HP group exhibited a downward trend in circulating cholesterol and HDL, along with a decreased expression of *Star* and *Cyp19a1*, indicating suppressed ovarian steroidogenesis. Together, these results suggest that inappropriate maternal dietary protein intake may disrupt the uterine microenvironment by altering systemic hormone levels and local implantation-related molecular signals, although these early alterations were insufficient to directly impair implantation establishment.

As gestation progressed, the detrimental impact of HP intake became evident through reduced average fetal weight and decreased placental efficiency. Placental efficiency, a key indicator of placental function (Hayward et al., 2016; Mellott et al., 2025), was notably reduced in HP, implying that each unit of placental tissue supports less fetal growth. However, when interpreting the adverse late-gestation outcomes observed in the HP group, the reduced ADFI should also be considered. The lower feed intake in HP during late gestation may partly reflect the satiating effect of dietary protein (Mithieux et al., 2005). Because feed intake did not differ during early or mid-gestation, a consistent difference in diet palatability seems less likely to be the main explanation. Although the HP group exhibited lower ADFI during late gestation, feed intake over the whole gestation period and maternal weight gain did not differ significantly between the HP and MP groups. Nevertheless, because late gestation is a period of rapid fetal growth and increasing placental metabolic demand, reduced feed intake during this period may have contributed to the lower fetal weight and placental efficiency. Therefore, these

outcomes may reflect both the effects of the HP dietary treatment and the associated reduction in late-gestation feed intake. The present design cannot determine their relative contributions.

A critical finding of this study is the fetal metabolic adaptation induced by the HP diet. Although the diets were formulated to differ primarily in protein, the HP diet is relatively low in carbohydrates. Notably, the fetal serum glucose level was significantly decreased. Since glucose is the main energy source for fetus and is derived mainly from maternal circulation (Brett et al., 2014), the HP fetuses undergo extensive metabolic changes in response to this environment. The significant upregulation of hepatic glucose transporter *Glut1* and key gluconeogenic enzymes, including *Pck1* and *G6pc* in the HP fetus, indicates an active shift to endogenous glucose production. Crucially, this process is fueled by amino acid catabolism, evidenced by the upregulation of hepatic catabolic enzymes *Gpt1*, *Got1*, *Bcat2* and the markedly elevated BUN levels in fetal serum and amniotic fluid. Collectively, these findings demonstrate that the high fetal amino acids are diverted towards oxidative catabolism to compensate for the carbohydrate deficit, directly contributing to the growth restriction.

To further elucidate the dynamics of nutrient supply, we analyzed AA profiles. As expected, maternal serum in the HP group showed elevated levels of most essential AAs and some non-essential AAs (NEAAs), reflecting the direct dietary input. This is consistent with established evidence that maternal circulating AA concentrations are highly sensitive to dietary protein intake (Bhasin et al., 2009). However, the AA profile in fetal serum did not linearly correlate with the maternal profile. In our study, although we observed a coordinated downregulation of key amino acid transporters, including *Snat2* (e.g., for alanine), *Cat1* (e.g., for lysine), and *Lat2/Lat3/Lat4* (e.g., for BCAAs) in the HP placenta, fetal AA levels did not decrease. Placental amino acid transport is a complex process, and the net transfer rate is determined by multiple factors, including transporter activity, placental surface area, blood flow, maternal amino acid concentrations, and placental and fetal metabolism (Cleal et al., 2018). We attribute this maintenance to increased maternal substrate availability. Furthermore, the altered maternal nutritional environment may impair the efficient utilization of amino acids for fetal protein accretion, contributing to their accumulation in the fetal circulation. Amniotic fluid composition reflects both fetal metabolic status and placental transport activity and it represents the net result of bidirectional exchange between the mother and fetus (Schmidt, 1992; Underwood et al., 2005). We propose that HP fetuses use non-essential amino acids, such as Ala, Gly, and Glu, to fuel hepatic gluconeogenesis, leading to their reduced concentrations in the amniotic fluid. Collectively, the adverse effect of HP intake on fetal growth is driven not only by placental transport regulation, but also by the fetal utilization efficiency driven by the low carbohydrate environment.

The placenta serves as the interface for nutrients and oxygen exchange. To determine whether the HP-induced growth restriction stemmed from structural defects or functional impairment, we performed a detailed histological analysis. The most significant morphology alteration was the altered zone distribution. Specifically, since the LZ is the primary site for maternal-fetal exchange (Watson

and Cross, 2005), this structural reduction implies a diminished functional surface area for nutrient transfer, directly correlating with the reduced placental efficiency. JZ is the major endocrine layer of the placenta, plays a crucial role in maternal adaptation by regulating blood flow (Cross, 2005), and in late gestation, it also contributes to glycogen storage for the fetus (Coan et al., 2006). Given the low-carbohydrate content in the HP diet and the observed fetal hypoglycemia, we employed PAS staining to investigate whether placental glycogen reserves were depleted by the fetuses. Interestingly, despite the structural expansion of the JZ, the glycogen density (PAS-positive area) remained comparable to the MP group. These findings suggest that the placenta may attempt to compensate for maternal metabolic stress through structural adaptation. However, such compensatory responses appear insufficient to fully maintain placental efficiency under sustained excessive protein exposure. Crucially, the lack of difference in vascular coverage (CD31) suggests that the placental insufficiency is not due to angiogenic failure, but rather due to compromised transport efficiency.

Oxidative stress has been shown to disrupt placental structure and is associated with adverse pregnancy outcomes (Myatt and Cui, 2004; Joo et al., 2021). In the HP group, the placental structural and functional impairments are accompanied by a marked disruption of placental redox homeostasis, evidenced by elevated lipid peroxidation levels and decreased activities of antioxidant enzymes which form the primary defense mechanism against oxidative stress (Ighodaro and Akinloye, 2018). NFE2L2 is a critical regulator of antioxidant response (Nguyen et al., 2009), and the inhibited NFE2L2 pathway in HP also supported the oxidative damage. Conversely, the LP diet appeared to activate antioxidant defenses, with lower MDA levels, higher NFE2L2 protein expression, lower KEAP1 protein expression, and lower levels of the pro-apoptotic protein BAX. Since KEAP1 acts as a negative regulator that promotes NFE2L2 degradation (Nguyen et al., 2009), the higher expression of NFE2L2 and lower expression of KEAP1 in LP placentas indicate an adaptive activation of NFE2L2 pathway. Collectively, the results suggest that mild protein restriction may elicit adaptive redox regulation. In contrast, the HP diet appears to exceed the defensive capacity, leading to oxidative damage.

The placenta is a highly metabolically active organ rich in mitochondria, making it particularly vulnerable to oxidative damage. Mitochondria serve as the primary source of placental ATP and dynamically respond to environmental and nutritional cues. In the HP group, we observed clear signs of mitochondrial dysfunction, including reduced ATP production, decreased mtDNA copy number, and the downregulation of genes related to the electron transport chain (ETC) and mitochondrial biogenesis. Impaired placental energy metabolism may constrain energy-dependent nutrient transport processes and thereby contribute to reduced nutrient transport capacity. This aligns with previous findings linking placental mtDNA content to fetal weight (Poidatz et al., 2015). Furthermore, the impaired ETC tends to generate excess ROS, which aggravate oxidative damage and further compromise mitochondrial function (Musatov and Robinson, 2012). MFN2 promotes mitochondrial fusion, which is often associated with improved efficiency and stress resistance (Adebayo et al., 2021). The LP diet promoted mitochondrial

fusion (increased MFN2, decreased DNM1L), suggesting the formation of a stable network resistant to damage. One possible explanation for the impaired mitochondrial function in the HP group is altered energy substrate availability resulting from the lower carbohydrate content of the diet. Glucose is the primary fuel for placental growth and ATP generation. Since the HP diet is inherently low in carbohydrates, the relative glucose scarcity creates a metabolic challenge, especially during late gestation when fetal demand peaks and the ADFI decreases. Taken together, these findings indicate that excessive maternal dietary protein intake induces a coordinated disruption of placental redox balance and mitochondrial function, ultimately impairing nutrient transport and fetal growth. Importantly, these findings suggest that maternal metabolic imbalance acts as an upstream driver that disrupts placental redox homeostasis and mitochondrial function, which in turn impairs nutrient transport and ultimately restricts fetal growth. This raises the possibility that targeting maternal metabolic pathways, particularly those involved in redox regulation and nutrient sensing, may represent an effective strategy to improve pregnancy outcomes by modulating maternal metabolic and redox status.

6. Conclusion

In conclusion, maternal nutritional imbalance may not immediately manifest as reproductive failure during early gestation, but can progressively compromise placental and fetal development through cumulative metabolic stress during late pregnancy. Although both protein deficiency and excess can disrupt physiological balance, our findings highlight that the excessive protein intake was associated with changed placental morphology, downregulation of placental AA transporters, heightened oxidative stress, and mitochondrial dysfunction. Furthermore, the fetus exhibited enhanced gluconeogenesis and amino acid catabolism, leading to growth restriction. This indicates that maternal supply can affect placental adaptation and fetal utilization, potentially contributing to reduced fetal weight. Given that current recommendations for protein intake during pregnancy remain inconsistent, these findings have significant implications for establishing appropriate protein recommendations during pregnancy and supporting healthy fetal growth.

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9. Transition to Chapter 5

Together, Chapters 3 and 4 indicate that maternal metabolic homeostasis and placental adaptation may be more sensitive to changes in nutritional supply than litter size or fetal number. Chapter 4 further showed that a sustained high-protein/low-carbohydrate imbalance was associated with placental oxidative stress, mitochondrial dysfunction, altered nutrient transport, and impaired fetal growth. These findings provide a rationale for investigating nutritional interventions targeting maternal metabolic and redox homeostasis rather than simply increasing protein supply. Therefore, based on our previous findings concerning the metabolic effects of CDCA, Chapter 5 evaluates whether beneficial maternal metabolic and redox responses remain detectable during late gestation after CDCA supplementation has been limited to early gestation.

Chapter 5

Persistent benefits of early gestational chenodeoxycholic acid supplementation on late pregnancy in sows via sustained modulation of the gut-metabolism axis

This chapter is based on the following publication:

Zhao Ying, Xiaolong Wang, Qian Ma, En Liu, Dongyan Zhang, Hui Liu, Long Cai, Jing Wang, Tao Feng, Martine Schroyen, and Meixia Chen

Persistent benefits of early gestational Chenodeoxycholic Acid supplementation on late pregnancy in sows via sustained modulation of the Gut-Metabolism axis.

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Chapter 5: Persistent benefits of early gestational chenodeoxycholic acid supplementation on late pregnancy in sows via sustained modulation of the gut-metabolism axis

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Key words: Chenodeoxycholic acid, early gestation, sow model, metabolic health, gut microbiota, metabolomics, reproductive performance

1. Abstract

These observations in Chapter 3 and 4 highlight maternal metabolic dysregulation as a central driver of adverse pregnancy outcomes and underscore the need for effective nutritional strategies to restore metabolic balance during gestation. Chenodeoxycholic acid (CDCA), a primary bile acid, has been identified in our previous studies as a potent metabolic regulator during early gestation, capable of improving embryo implantation through modulation of maternal metabolism and gut microbiota-host interactions. However, whether early gestational CDCA intervention exerts sustained effects on maternal metabolic health in late pregnancy remains unclear. To address this, multiparous sows ($n = 24$) were randomly assigned to a control diet or a diet supplemented with 0.15% CDCA during early gestation (gestational day 0-28), followed by a common diet thereafter. Reproductive performance, oxidative stress, inflammatory status, gut microbiota, and serum metabolome were evaluated in late gestation. Results showed that early CDCA intervention significantly increased the total and live litter sizes ($P < 0.05$). Furthermore, CDCA treatment alleviated maternal oxidative stress and systemic inflammation, and improved insulin-related metabolic status in late gestation ($P < 0.05$). Untargeted metabolomics revealed a distinct metabolic remodeling, characterized by the enrichment of beneficial metabolites, such as L-ornithine, and the depletion of proinflammatory and oxidative markers, including 1-palmitoylphosphatidylcholine, 2-lysophosphatidylcholine, 3-hydroxyanthranilic acid, and N-carboxymethyllysine. Gut microbiota analysis indicated that CDCA induced targeted modulation rather than global restructuring. Specifically, CDCA suppressed potential harmful bacteria, including *NK4A214_group*, *Clostridium_sensu_stricto_1*, and *Turicibacter*, while enriching beneficial genera like *Subdoligranulum*. Multi-omics integration identified *NK4A214_group* was associated with exacerbated inflammatory status and unfavorable metabolic profiles. Collectively, these findings indicate that early-gestation CDCA supplementation is associated with beneficial maternal metabolic and redox responses during late gestation, together with changes in specific gut microbial taxa and serum metabolites and improved reproductive outcomes. This study provides a potential nutritional strategy to mitigate metabolic stress during pregnancy and supports the concept of early gestational metabolic programming.

Key words: Chenodeoxycholic acid, early gestation, sow model, metabolic health, gut microbiota, metabolomics, reproductive performance

2. Introduction

Successful pregnancy relies on precise maternal metabolic adaptations. Chapter 3 and 4 have demonstrated that maternal nutritional imbalance, particularly in dietary protein levels, can disrupt metabolic homeostasis and impair placental function, ultimately restricting fetal growth. These findings highlight that maternal metabolic status, rather than nutrient supply alone, plays a central role in determining pregnancy outcomes. In modern high-producing sows, the increasing reproductive outcome has imposed substantial metabolic demands during gestation, making maternal metabolic homeostasis a critical determinant of reproductive efficiency. Therefore, targeting maternal metabolic regulation may be an effective approach to improve gestational performance.

Our previous research identified chenodeoxycholic acid (CDCA) as a potent metabolic regulator during early gestation (Chen et al., 2023). CDCA supplementation was shown to enhance embryo implantation by optimizing ovarian progesterone synthesis, remodeling of the maternal amino acid metabolism, improving anti-inflammatory and antioxidant capacity and insulin sensitivity, and reshaping gut microbiota-host interactions (Chen et al., 2023; Chen et al., 2024). However, pregnancy is a continuous physiological process. As gestation advances, maternal systems face escalating metabolic demands, often manifesting as insulin resistance and heightened oxidative stress and low-grade inflammatory state (Palm et al., 2013; Sonagra et al., 2014; Wang et al., 2016; Grzeszczak et al., 2023). While these adaptations are physiologically necessary to support fetal development, their dysregulation can predispose mothers to gestational metabolic disorders, such as gestational diabetes and preeclampsia, and may also increase the risk of preterm birth and stillbirth, and adversely influence fetal programming (Chu et al., 2007; Catalano and Shankar, 2017; Sferruzzi-Perri et al., 2020; Piani and Tossetta, 2024). While nutritional intervention during late pregnancy have proven effective (Long et al., 2021; Zhang et al., 2023; Zhou et al., 2024), it remains largely unexplored whether metabolic modulations applied strictly during early pregnancy can confer sustained benefits into late gestation. This raises an important question as to whether early metabolic modulation by CDCA can exert sustained effects throughout gestation and ultimately improve reproductive outcomes.

To address these questions, we hypothesized that early-gestational CDCA supplementation could induce maternal metabolic programming, conferring sustained anti-inflammatory and antioxidant benefits into late gestation via the modulation of the gut microbiota-metabolome axis. To test this hypothesis, the present study evaluated maternal metabolic health and reproductive performance during late gestation following early-gestational CDCA supplementation. By integrating untargeted metabolomics and microbiome analyses, we aimed to: (1) characterize the late-gestational metabolic effect of early CDCA supplementation; (2) elucidate the metabolic and microbial underpinnings of such sustained effects; and (3) characterize the relationships between gut microbiota and host metabolites in late pregnancy, providing predictive insights into microbial functional adaptations. This investigation advances our understanding of bile acids as pivotal regulators of

pregnancy-related metabolic programming and offers a conceptual framework for developing nutritional strategies aimed at “pregnancy health programming”.

3. Materials and methods

3.1 Animal experiment and sample collection

The study was approved by the Animal Welfare and Ethics Committee of the Beijing Academy of Agriculture and Forestry Sciences (Approval Number: IHVM11-2202-2). The experimental design and sample collection procedures have been described previously (Chen et al., 2023). A total of 24 healthy multiparous (Landrace × Large White) sows were selected and randomly allocated into two groups (n=12) based on body weight, backfat thickness, parity, and previous litter size. In this study, the individual sow was considered the experimental unit. From the fourth day post-weaning, estrus detection was performed twice daily using a teaser boar, and artificial insemination was conducted at the optimal breeding times, with a second insemination occurring 24 hours later. The first day of insemination was designated as day 0 of gestation. From weaning to early gestation (days 0-28), the control group (CON) was fed a basal diet, while the treatment group received a basal diet supplemented with 0.15% CDCA (CDCA). CDCA (Cat. No. S25542, ≥ 98% purity) was purchased from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). For the remainder of the gestation period, both groups were provided with the same amount of gestation diet. Sows were managed under a restrictive feeding strategy to maintain appropriate body condition. Feeding quantities were regulated as follows: 2.5 kg/day pre-mating, 2 kg/day during early gestation (days 0-28), 2.5 kg/day during mid-gestation (days 29-90), and 3 kg/day during late gestation (days 91 to parturition). The sows were housed individually with free access to water. All sows were housed in a uniform environment, with individual pen placement balanced between groups to minimize potential confounders. The basal diet met the nutrient requirements recommended by the National Research Council for pregnant sows (NRC, 2012), and dietary composition and nutrient levels are detailed in Table 5-1. Sows were examined for pregnancy via B-ultrasound at d 28 of gestation. Sows that failed to conceive or experienced abortion during the gestational period were excluded from the study, as maintaining pregnancy is a fundamental biological prerequisite for evaluating late-gestational metabolic adaptations and farrowing outcomes. Ultimately, three sows in the CON group and one sow in the CDCA group were excluded, resulting in a final sample size of n = 9 for the CON group and n = 11 for the CDCA group. On day 110 of gestation, following an overnight fast, 10 mL of blood was collected from the anterior vena cava prior to the morning feeding, centrifuged at 3,000 g for 15 minutes, and the serum was stored at -80 °C for further analysis. To minimize analytical errors caused by sample conditions, six serum samples per group were randomly selected for subsequent biochemical and metabolomics analyses. Additionally, fresh fecal samples were collected from the rectum of each sow on day 110 of gestation and stored at -80 °C for microbiota analysis. The numbers of total born, born live, stillborn, deformed, and mummified piglets were recorded at farrowing.

Table 5-1: Nutrient composition of basal diet (as-fed basis).

Items	Estrus Phase	Pregnancy Phase
Corn	62.39	68.90
Soybean meal	18.50	14.50
Wheat bran	4.65	14.00
Fish meal	3.00	-
Extruded soybean	6.70	-
Monocalcium phosphate	0.95	0.40
Limestone	1.15	1.30
Soybean oil	1.00	-
Salt	0.30	0.40
Sodium bicarbonate	0.10	-
Sodium sulfate	0.10	-
Magnesium sulfate	0.20	-
Potassium chloride	0.15	-
L-Lysine HCl	0.35	0.22
L-Threonine	0.05	0.04
Choline chloride (60%)	0.10	-
Phytase	-	0.02
Vitamin-mineral premix	0.31	0.23
Analyzed nutrient composition	-	-
Total energy, MJ/kg	16.31	15.88
Crude protein, %	15.94	13.12

3.2 Determination of serum antioxidant, inflammatory, and insulin sensitivity related parameters

Oxidative stress related indices, including malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), total antioxidant capacity (T-AOC), and catalase (CAT), inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), as well as metabolic parameters including total cholesterol (TC), glucose (GLU) and insulin (INS) in six randomly selected serum samples per group, were measured

using specific commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR), calculated as $\text{insulin (mIU/L)} \times \text{glucose (mmol/L)} / 22.5$.

3.3 Untargeted serum metabolomics analysis

Non-targeted metabolomics analysis of the six randomly selected serum samples per group was performed using an UHPLC-MS/MS system ($n = 6$ per group). Samples were prepared as follows: 100 μL of serum sample was mixed with 400 μL of extraction solution (acetonitrile: methanol = 1:1, v/v). The mixture was subjected to low-temperature ultrasonic extraction for 30 minutes (5°C , 40 kHz), followed by standing at -20°C for 30 minutes, and then centrifuged at 13,000 g for 15 minutes at 4°C . The supernatant was collected, dried under nitrogen gas, and reconstituted in 120 μL of reconstitution solution (acetonitrile: water = 1:1, v/v). The reconstituted solution underwent a 5-minute low-temperature ultrasonic extraction (5°C , 40 kHz), and the supernatant was collected into sample vials for analysis. A 3 μL aliquot of each sample was injected into an HSS T3 column (100 mm $\times$ 2.1 mm i.d., 1.8 μm). Additionally, to ensure analytical reliability, quality control samples (QC) were inserted at regular intervals. The mobile phases consisted of solvent A (water:acetonitrile = 95:5, v/v, containing 0.1% formic acid) and solvent B (acetonitrile:isopropanol:water = 47.5:47.5:5, v/v/v, containing 0.1% formic acid). The flow rate was set at 0.40 mL/min, and the column temperature was maintained at 40°C . The mass spectrometry conditions were as follows: The mass spectra were acquired in both positive and negative ion modes, with a mass scan range of 70-1050 m/z . The sheath gas flow rate was set to 50 Arb, the auxiliary gas flow rate to 13 Arb, and the auxiliary gas heater temperature to 425°C . The ion spray voltage was set at 3500 V for positive ions and -3500 V for negative ions. The ion transfer tube temperature was 325°C , and the collision energy was set to cycle through 20-40-60 V. The raw data were processed using the metabolomics software Progenesis QI (Waters Corporation, Milford, USA) and the final data matrix was obtained for subsequent analyses. Both MS and MS/MS spectral information were annotated using public metabolite databases, including HMDB (<http://www.hmdb.ca/>) and Metlin (<https://metlin.scripps.edu/>) to identify the metabolites. The preprocessed data was then uploaded to the Majorbio Cloud Platform (<https://cloud.majorbio.com>) for comprehensive data analysis. Next, based on the levels of metabolites in different samples, PCA and PLS-DA analyses were conducted to evaluate the similarity of samples within groups and the differences between groups. Differential metabolites were identified as $\text{VIP} > 1$ and $P\text{-value} < 0.05$. Functional KEGG pathways enrichment analysis was performed using the identified differential metabolites, and correlation analyses were carried out between these differential metabolites and relevant factors.

3.4 Gut microbiota DNA extraction, 16S rRNA sequencing, and data processing

Six fecal samples were randomly selected from each group for bacterial genomic DNA extraction, 16S rRNA sequencing, and data processing. Microbial genomic

DNA from the feces was extracted using a commercial DNA kit (M5636, Omega Bio-tek, Norcross, GA, USA), following the manufacturer's protocol. The purity and concentration of the extracted DNA were assessed using a NanoDrop 2000 spectrophotometer, and its quality was verified by agarose gel electrophoresis. The bacterial 16S rDNA V3 and V4 regions were amplified using specific universal primers (338F: 5'-ACTCCTACGGGAGGCAGCAG-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3'), producing a fragment of approximately 500 bp. The PCR products were recovered using 2% agarose gel electrophoresis and purified using the PCR Clean-Up Kit (YuHua, Shanghai, China). The purified products were quantified using a Qubit 4.0 (Thermo Fisher Scientific, USA). Library construction was performed using the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, USA). The constructed library was sequenced on the Illumina MiSeq PE300 platform (Illumina, San Diego, USA). Quality control of the raw paired-end sequencing reads was performed using fastp (version 0.19.6, <https://github.com/OpenGene/fastp>), and the reads were assembled using FLASH (version 1.2.11, <http://www.cbcb.umd.edu/software/flash>). The optimized sequences were clustered into operational taxonomic units (OTUs) using UPARSE (version 7.1) based on 97% similarity. Taxonomic annotation of OTUs was performed using the RDP classifier (version 2.11, <http://rdp.cme.msu.edu/>) based on the Silva 16S rRNA gene database (v138) with a 70% confidence threshold. All data analyses were performed on the Majorbio Cloud platform, including α -diversity and β -diversity indices, community composition and differences at various taxonomic levels, and correlation analyses between microbial genera and environmental factors. Functional prediction was conducted using PICRUST2 (<http://huttenhower.sph.harvard.edu/galaxy>).

3.5 Data analysis and statistics

Data are presented as means $\pm$ SEM. All data were tested for outliers, normality, and homogeneity of variance prior to analysis. To accommodate the slight imbalance in final sample sizes between groups (CON, $n = 9$; CDCA, $n = 11$), data were analyzed using an unpaired two-tailed Student's t-test performed in SPSS 20. Visualizations were conducted using GraphPad Prism 10.1.2. The correlation analyses among the key parameters were conducted using the Spearman's correlation analysis. Procrustes analysis was performed to assess the association between the microbiome and metabolome based on the R package vegan. Statistical significance was determined at $P < 0.05$, while $0.05 < P < 0.10$ was considered indicative of a trend.

4. Results

4.1 Early gestational dietary CDCA intervention improves the reproductive performance of sows.

Table 5-2 shows that the total number of piglets and the number of live piglets were significantly higher in the CDCA group compared to the CON group ($P <$

0.05). No significant differences were observed in the weight of piglets alive and the numbers of stillborn, deformed, or mummified piglets ($P > 0.05$).

Table 5-2: Effects of CDCA supplementation on the reproductive performance of sows.

Items	CON	CDCA	<i>P</i> -value
Total born, n/litter	14.00 ± 0.76	16.64 ± 0.79	0.029
Born live, n/litter	12.78 ± 0.52	15.00 ± 0.80	0.040
Weight of piglets alive, kg	1.52 ± 0.05	1.50 ± 0.06	0.801
Stillborn, n	0.89 ± 0.35	1.18 ± 0.26	0.412
Deformed, n	0.22 ± 0.22	0.27 ± 0.27	1.000
Mummified, n	0.11 ± 0.11	0.18 ± 0.12	0.824

4.2 Early gestational dietary CDCA intervention alleviates oxidative stress, downregulates inflammatory responses, and improves glucose metabolism during late gestation.

On day 110 of gestation, maternal serum levels of MDA were significantly reduced whereas the activities of T-AOC, SOD, CAT, and GPx were significantly increased in the CDCA group compared to CON (Figure 5-1, $P < 0.05$). Meanwhile, the concentrations of inflammatory cytokines, including IL-1 β , IL-6, TNF- α , and IFN- γ , were significantly decreased ($P < 0.05$). In addition, INS levels were significantly reduced, with a decreasing trend observed in HOMA-IR ($0.05 \leq P \leq 0.10$), whereas no significant differences were observed in GLU or TC levels. These data indicated that the sows receiving CDCA during early gestation had lower inflammatory and oxidative stress indicators and greater antioxidant capacity during late gestation.

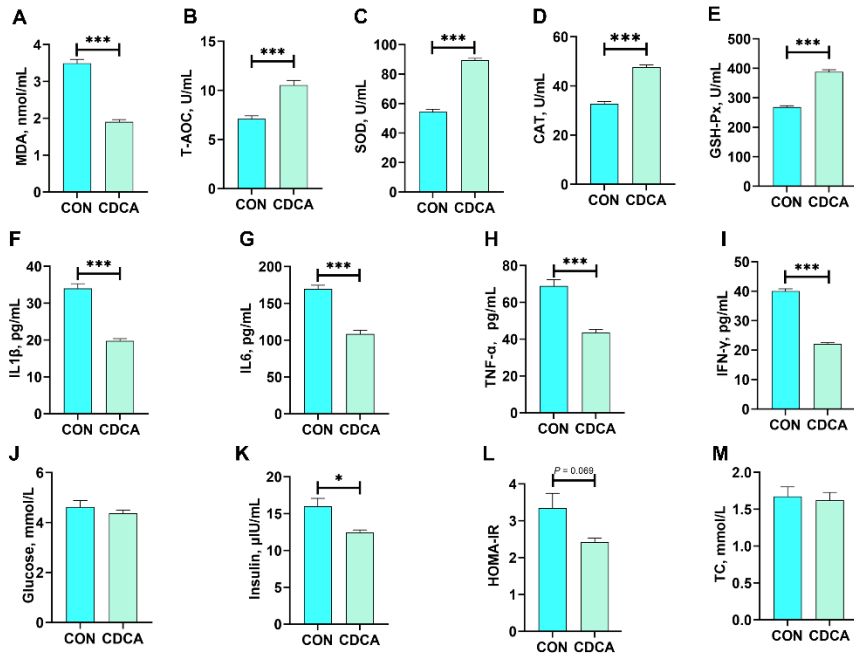


Figure 5-1: Effects of early gestational dietary CDCA intervention on oxidative stress, inflammatory responses, and glucose metabolism in sows on gestational day 110.

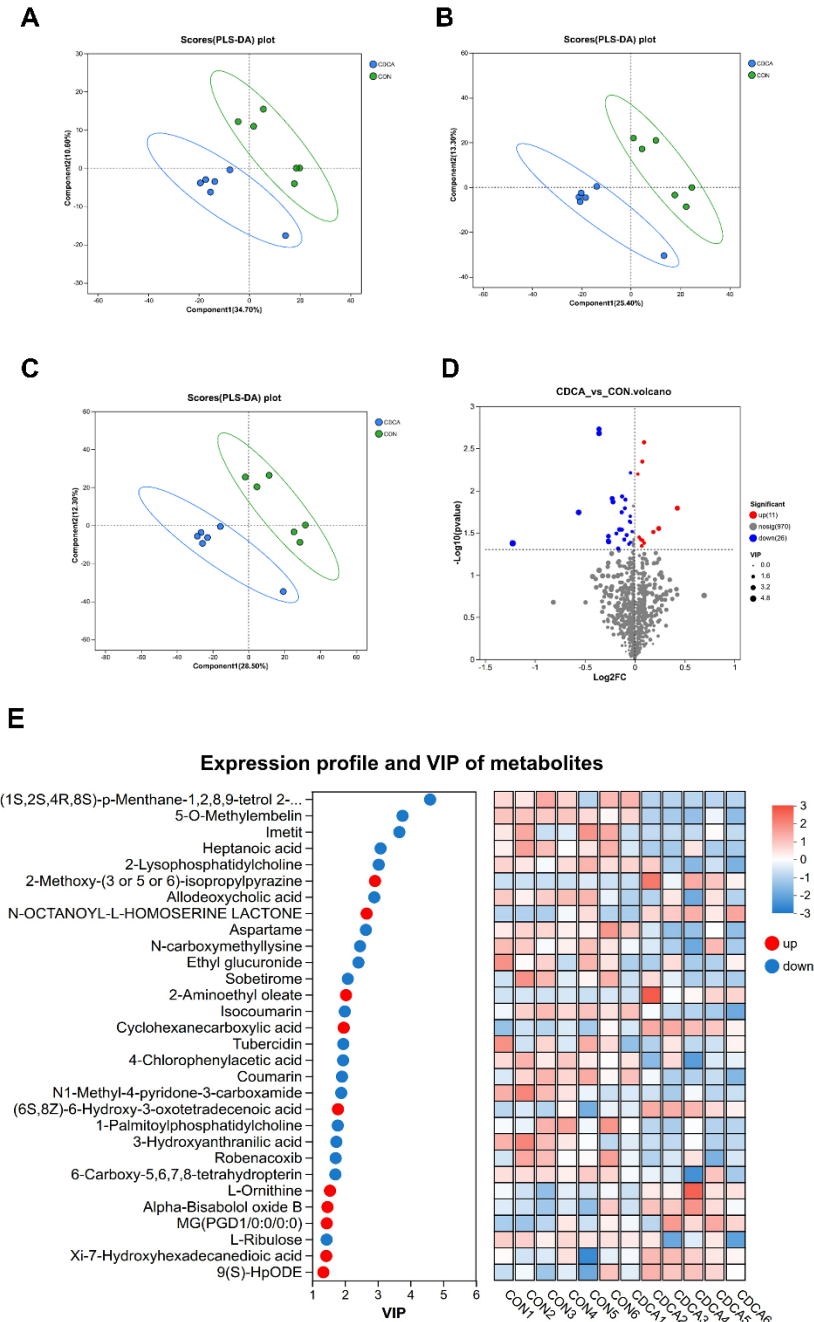
Maternal serum levels of MDA (A), the activities of T-AOC (B), SOD (C), CAT (D), GPx (E) were determined to evaluate oxidative status. Serum concentrations of inflammatory cytokines, including IL-1β (F), IL-6 (G), TNF-α (H), and IFN-γ (I), were measured to assess systemic inflammation. Metabolic parameters, including glucose (J), insulin (K), HOMA-IR (L), and TC (M), were also analyzed. Data are presented as mean ± SEM (n = 6). CON, control group fed a basal diet; CDCA, treatment group fed a basal diet supplemented with 0.15% chenodeoxycholic acid. * indicates $P < 0.05$, ** indicates $0.001 < P < 0.01$, *** indicates $P < 0.001$ between the control (CON) and CDCA groups.

4.3 Early gestational dietary CDCA intervention reshaped maternal metabolic profiles during late gestation.

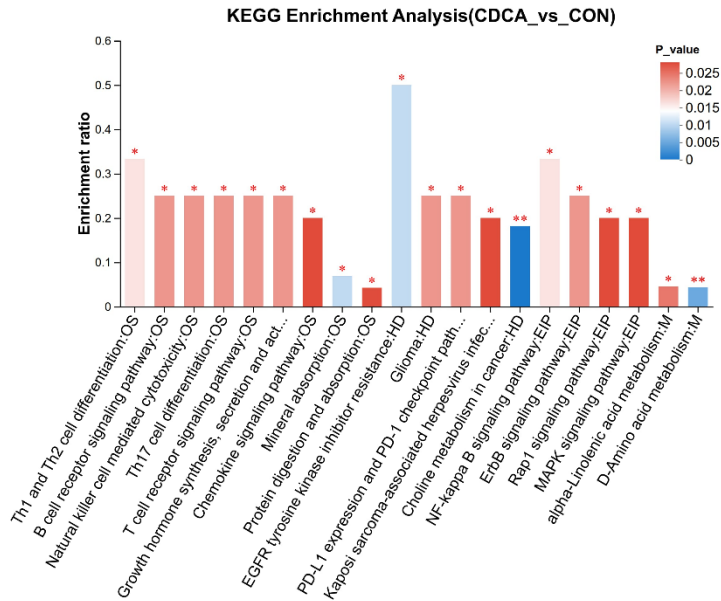
To systematically elucidate how early gestational CDCA intervention modulated maternal metabolic health, metabolic profiles of sows on day 110 of gestation were characterized. A partial least squares discriminant analysis (PLS-DA) revealed distinct separations between the CON and CDCA group (Figure 5-2 A-C), indicating significant metabolic reshaping. Furthermore, 37 significantly different metabolites were identified. Among them, 11 metabolites were significantly upregulated and 26 were significantly downregulated in the CDCA group (Figure 5-2 D). The top 30 differential metabolites based on VIP scores are presented in a heatmap (Figure 5-2E). Notably, the relative levels of metabolites such as 2-lysophosphatidylcholine, N-carboxymethyllysine, N1-methyl-4-pyridone-3-carboxamide, 1-palmitoylphosphatidylcholine, 3-hydroxyanthranilic acid were decreased and levels

of metabolites such as L-ornithine, alpha-bisabolol were increased in the CDCA group.

KEGG pathway enrichment analysis (Figure 5-2F) highlighted pathways related to amino acid and lipid metabolism, such as protein digestion and absorption, D-amino acid metabolism, alpha-linolenic acid metabolism, and choline metabolism pathways, and immune modulation-related pathways, such as the NF-kappa B signaling pathway, Th1/Th2/Th17 cell differentiation pathway, and T cell/B cell receptor signaling pathway were significantly enriched. Furthermore, pathways related to cell growth or repair, such as growth hormone synthesis, MAPK and ErbB signaling were also enriched. Furthermore, KEGG topology analysis (Figure 5-2G) emphasized the centrality of amino acid metabolism (arginine biosynthesis, arginine and proline metabolism, glycine, serine and threonine metabolism, and phenylalanine metabolism) and nicotinate and nicotinamide metabolism. These findings indicate that early CDCA intervention orchestrates systemic metabolic improvements involving nitrogen balance, immune regulation, and cellular processes.



F



G

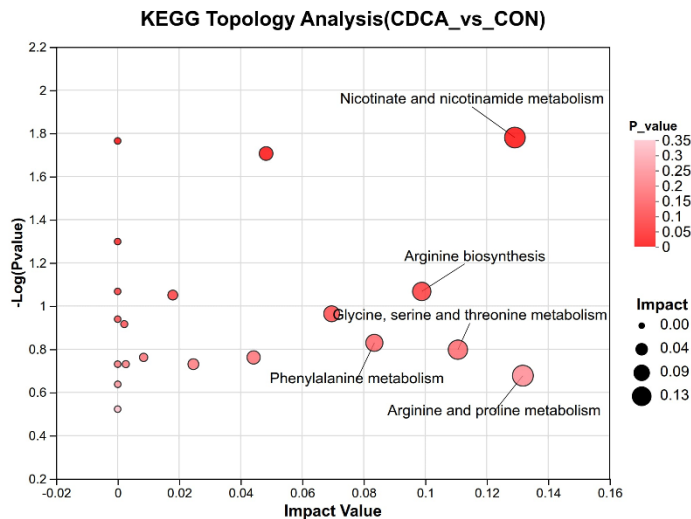


Figure 5-2: Effects of early gestational CDCA supplementation on the serum metabolomic profile of sows at day 110 of gestation.

(A-C) Partial least squares discriminant analysis (PLS-DA) score plots derive from (A) positive ion mode, (B) negative ion mode, and (C) mix mode. (D) Volcano plot of differential metabolites. Red dots represent significantly upregulated metabolites, blue dots

represent significantly downregulated metabolites, and gray dots represent non-significant metabolites. (E) Hierarchical clustering heatmap and Variable Importance in Projection (VIP) scores of the top 30 differential metabolites. In the heatmap, red indicates higher relative abundance, while blue indicates lower abundance. A higher VIP value indicates a greater difference of the metabolite between the two groups. (F) KEGG pathway enrichment analysis. The x-axis represents the pathway name, and the y-axis represents the enrichment ratio, with a higher ratio indicating greater enrichment. The color of the bars indicates the significance of the enrichment. (G) KEGG topology analysis. Each bubble represents a KEGG pathway. The x-axis indicates the relative importance of metabolites within the pathway, and the y-axis represents the enrichment significance. The bubble size corresponds to the importance of the pathway. CON, control group fed a basal diet (n = 6); CDCA, treatment group fed a basal diet supplemented with 0.15% chenodeoxycholic acid (n = 6). * $P < 0.05$, ** $P < 0.01$.

CDCA, chenodeoxycholic acid; CON, the control group; KEGG, Kyoto Encyclopedia of Genes and Genomes; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection.

4.4 Correlation analysis reveals favorable associations between serum metabolites and maternal oxidative stress, immune response, insulin sensitivity and reproductive performance.

To further investigate whether the enhancement of maternal anti-inflammatory and antioxidant capacity is associated with alterations in the maternal metabolome, a correlation analysis was performed. Spearman correlation analysis (Figure 5-3A) revealed that alpha-bisabolol, L-ornithine and other 9 metabolites showed significant positive correlations with serum levels of GPx, CAT, SOD, or T-AOC, or negative correlations with MDA levels, indicating these metabolites may contribute to combating oxidative stress. Moreover, these compounds were negatively correlated with inflammatory markers such as IFN- γ , TNF- α , IL-6, or IL-1 β , suggesting a close association with reduced systemic inflammation. Conversely, 24 metabolites, including N1-methyl-4-pyridone-3-carboxamide, 3-hydroxyanthranilic acid, dodecanedioic acid, 1-palmitoylphosphatidylcholine, and 9(S)-HpOTrE were positively correlated with oxidative stress and inflammation. Notably, 2-methylpropanamine exhibited significant negative correlations with insulin levels and the HOMA-IR index, whereas 1,5-anhydro-D-glucitol positively correlated with insulin levels. Regarding reproductive performance (Figure 5-3B), 10 metabolites were positively related to the number of total piglets and live piglets while 16 metabolites were negatively associated. These results suggest that CDCA-induced metabolic remodeling promotes an antioxidant and anti-inflammatory profile conducive to optimal gestational outcomes.

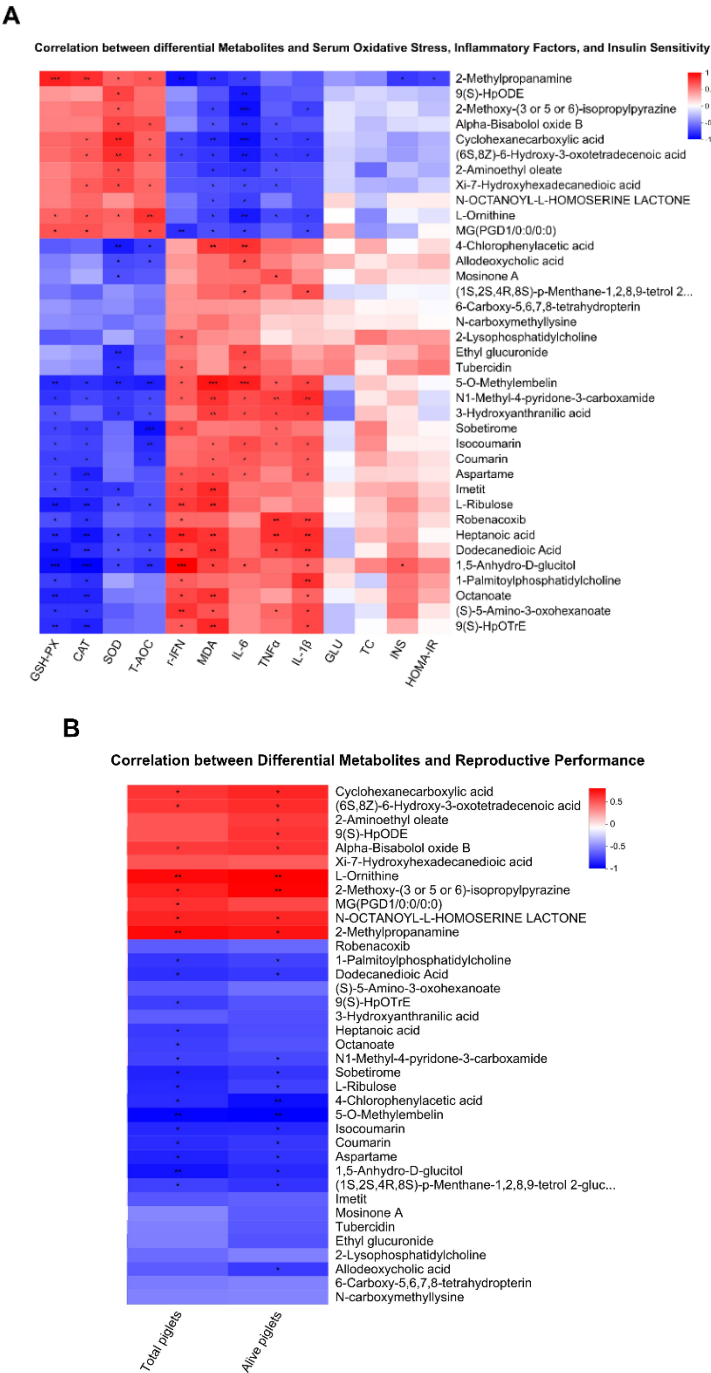


Figure 5-3: Association between serum metabolites and capacity of antioxidant, anti-inflammatory, insulin sensitivity and reproductive performance of sows.

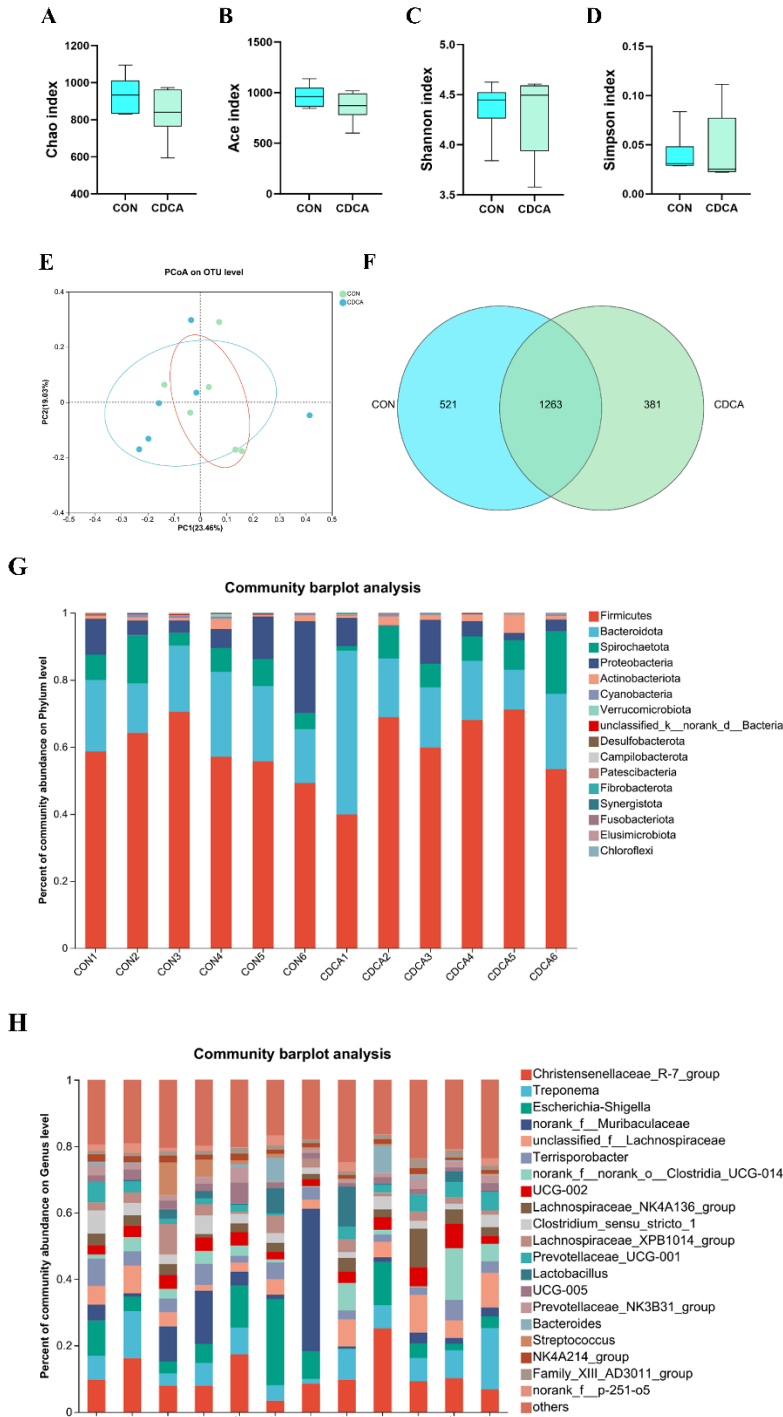
Spearman correlation analysis between the total 37 differentially abundant serum metabolites at day 110 of gestation and serum oxidative stress, inflammatory response, insulin sensitivity (A), and reproductive performance (B). Red represents a positive correlation, while blue represents a negative correlation. CON, control group fed a basal diet (n = 6); CDCA, treatment group fed a basal diet supplemented with 0.15% chenodeoxycholic acid (n = 6). * $P < 0.05$, ** $P < 0.01$.

4.5 Early gestational CDCA supplementation modulates specific microbes in sows during late gestation.

To further analyze whether the positive regulatory effect of early CDCA intervention on late pregnancy was related to its modulation of gut microbiota, the gut microbiota in sows during late pregnancy was analyzed. Alpha diversity indices showed no significant differences between groups (Figure 5-4A-D), and PCoA revealed similar community structures (Figure 5-4E). However, the Venn diagram identified 521 and 381 unique OTUs in the CON and CDCA groups respectively (Figure 5-4F), indicating a certain number of differential microbes existing between the two groups.

Next, we analyzed the composition of the fecal microbiota. At the phylum level, the four dominant phyla, Firmicutes, Bacteroidota, Spirochaetota, and Proteobacteria showed no significant differences (Figure 5-4G), though Fibrobacterota tended to be lower in the CDCA group ($P = 0.052$). The abundances of the top 20 microbial genera were pictured at the genus level, and the top ten genera collectively constituted over 50% of the total microbiota (Figure 5-4H). At the genus level (Figure 5-4I), 23 microbial genera showed a differential trend ($P < 0.1$). Specifically, *NK4A214_group*, *norank_f_norank_o_Bradymonadales*, *Anaerofustis* and *Anaeroplasma* were significantly different between CON and CDCA ($P < 0.05$). The proportions of some genera, such as *Clostridium_sensu_stricto_1*, *NK4A214_group*, *Turicibacter*, *Butyrivibrio*, *Anaeroplasma*, were lower ($P < 0.1$), while the proportion of other genera, such as *Subdoligranulum*, and *Anaerofustis*, were higher in the CDCA group ($P < 0.1$). Collectively, CDCA supplementation did not alter the structure of the gut microbiota but did affect the abundance of some specific microbial genera.

Impact of protein level and early-stage CDCA supplementation during gestation on maternal physiology in sows



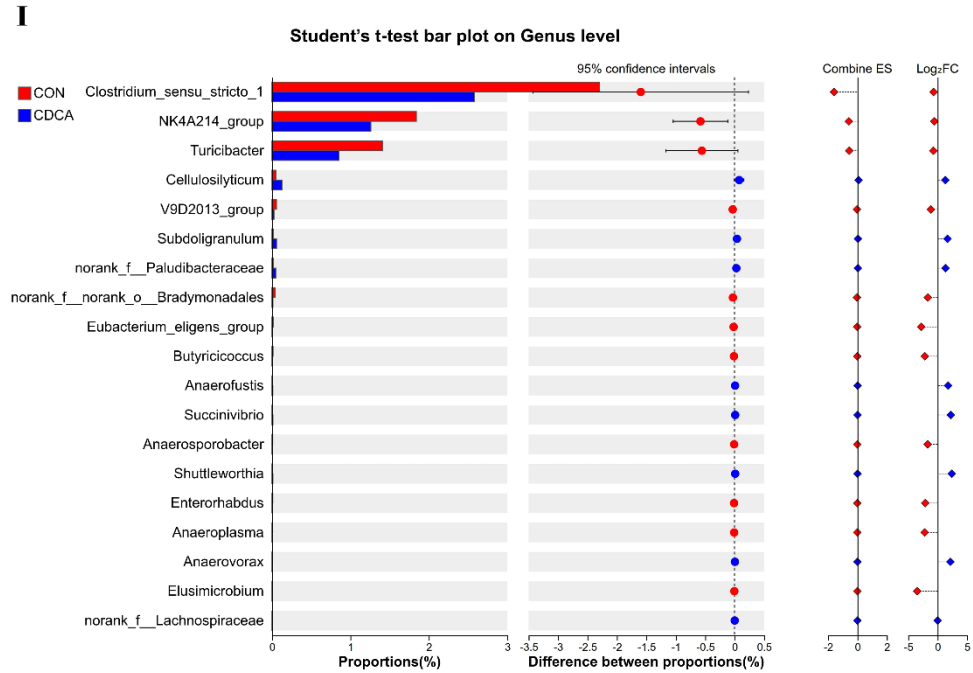


Figure 5-4: Effects of early gestational CDCA supplementation on fecal microbial diversity and composition in sows at day 110 of gestation.

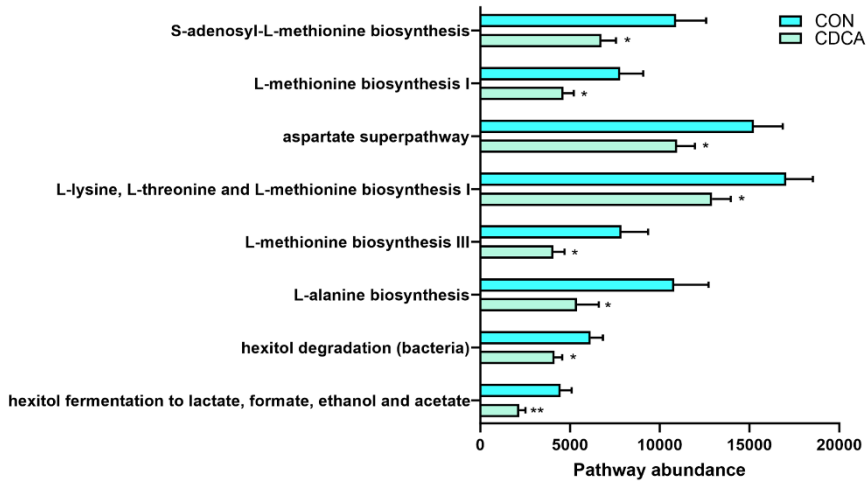
(A-D) Illustrate α -diversity indices of the gut microbiota, including (A) Chao index, (B) ACE index, (C) Shannon index, and (D) Simpson index. (E) Beta-diversity analysis (PCoA) comparing microbial community composition between the groups. (F) Venn diagram depicting shared and unique operational taxonomic units (OTUs) between groups. (G) Relative abundance of major microbial phyla in each fecal sample. (H) Relative proportions of the top 20 most abundant microbial genera. (I) Bar plot showing microbial genera with differential relative abundances between the two groups ($P < 0.1$). CON, control group fed a basal diet ($n = 6$); CDCA, treatment group fed a basal diet supplemented with 0.15% chenodeoxycholic acid ($n = 6$). * $P < 0.05$, ** $P < 0.01$.

4.6 Associations among gut microbiota, systemic metabolites, and maternal metabolic health indicators.

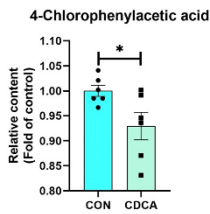
To elucidate the functional changes corresponding to these microbial alterations, we carried out a functional prediction analysis using PICRUSt2. Results showed that maternal CDCA supplementation significantly downregulated microbial amino acid biosynthesis pathways (Figure 5-5A, $P < 0.05$). Consistently, serum metabolomics revealed decreased levels of amino acid derivatives (3-hydroxyanthranilic acid, (S)-5-amino-3-oxohexanoate, 4-chlorophenylacetic acid) and increased L-ornithine in the CDCA group (Figure 5-5B-E). Spearman correlation heatmap revealed specific relationships between the differential microbes and differential metabolites (Figure 5-5F). For example, genera like *Anaerofustis*, *Eubacterium_eligens_group*,

k_norank_d_Bacteria, *NK4A214_group*, and *norank_f_norank_o_Bradymonadales*, showed significant correlations with multiple differential metabolites. Specifically, 4-Chlorophenylacetic acid was negatively correlated with *Anaerofustis* and positively correlated with *Clostridium_sensu_stricto_1*. L-Ornithine was positively correlated with *Anaerofustis*. 3-Hydroxyanthranilic acid was positively correlated with *NK4A214_group*. In addition, (S)-5-Amino-3-oxohexanoate was positively correlated with *Turicibacter*. Procrustes analysis did not identify significant global congruence between the gut microbiota and serum metabolome (Figure 5-5 G, Monte Carlo *P*-value = 0.284, M2 = 0.828). Nevertheless, correlations were observed between selected microbial genera and serum metabolites. These associations do not establish whether the microbial changes contributed to the metabolic responses to CDCA.

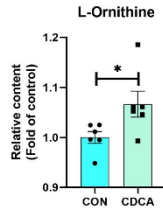
A



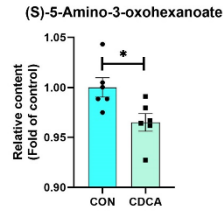
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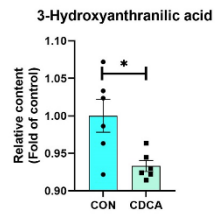
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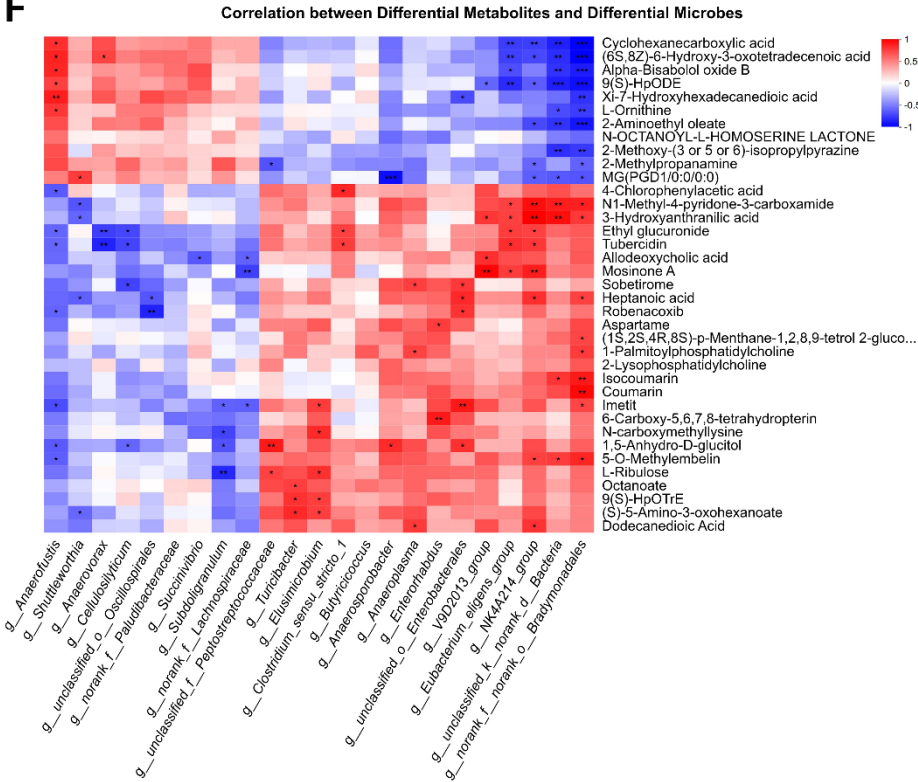
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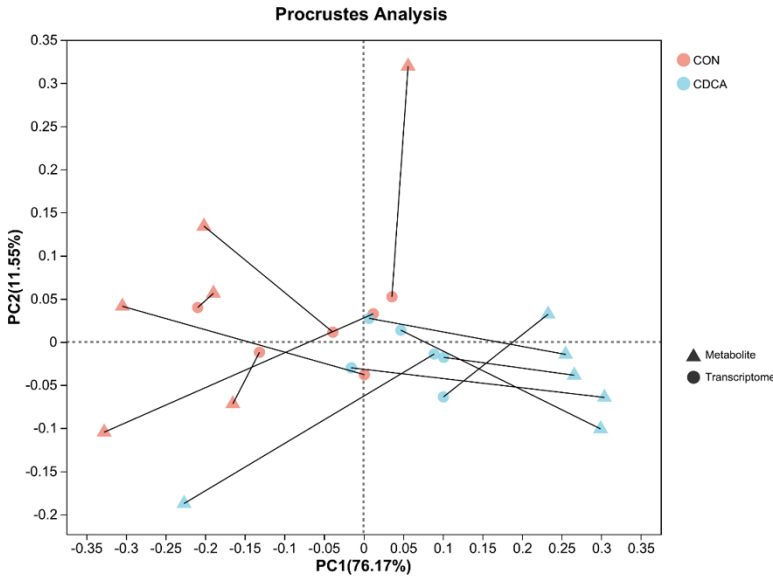


Figure 5-5: Relationship between specific gut microbiota and serum metabolic alterations in sows at day 110 of gestation.

(A) PICRUSt2 functional prediction to infer the functional potential of microbial communities based on the MetaCyc database. (B-E) Relative levels of representative differential metabolites involved in amino acid metabolism, including (B) 4-Chlorophenylacetic acid, (C) L-Ornithine, (D) (S)-5-Amino-3-oxohexanoate, and (E) 3-Hydroxyanthranilic acid. (F) Spearman correlation heatmap between differential serum metabolites and differential microbial genera. Red indicates positive correlation, while blue indicates negative correlation. (G) Procrustes analysis showing the correlation between differential serum metabolites and differential fecal genera. CON, control group fed a basal diet; CDCA, treatment group fed a basal diet supplemented with 0.15% chenodeoxycholic acid. Data are presented as mean $\pm$ SEM (n = 6). * $P < 0.05$, ** $P < 0.01$.

CDCA, chenodeoxycholic acid; CON, the control group; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2; SEM, standard error of the mean.

We further correlated the top 30 most abundant microbial genera with key maternal health indicators (Figure 5-6A). *NK4A214_group*, significantly reduced by CDCA, was positively correlated with serum levels of all measured inflammatory cytokines (TNF- α , IFN- γ , IL-1 β , and IL-6) and MDA, and negatively correlated with antioxidant enzymes SOD and T-AOC. Similarly, the genus *UCG-005* levels were positively correlated with TNF- α and IL-6, and negatively correlated with SOD and T-AOC levels. The genus *Lachnospiraceae_XPB1014_group* was positively correlated with IL-1 β , while *Clostridium_sensu_stricto_1* was positively correlated with IL-6 and negatively correlated with SOD levels. Regarding insulin sensitivity, *Prevotella* and *Escherichia-Shigella* correlated positively with HOMA-IR, whereas *norank_f_norank_o_RF39* showed negative correlation with INS and HOMA-IR. *Rikenellaceae_RC9_gut_group* and *Lachnospiraceae_NK4A136_group* were negatively correlated with GLU. Comprehensive analysis identified *NK4A214_group* as a key CDCA-responsive genus associated with several maternal metabolic, antioxidant capacity, anti-inflammatory activity, insulin sensitivity, and reproductive performance of sows (Figure 5-6 B-J).

Collectively, early-gestation CDCA supplementation was associated with changes in gut microbiota composition and predicted microbial functions alongside improvements in maternal metabolic status during late gestation.

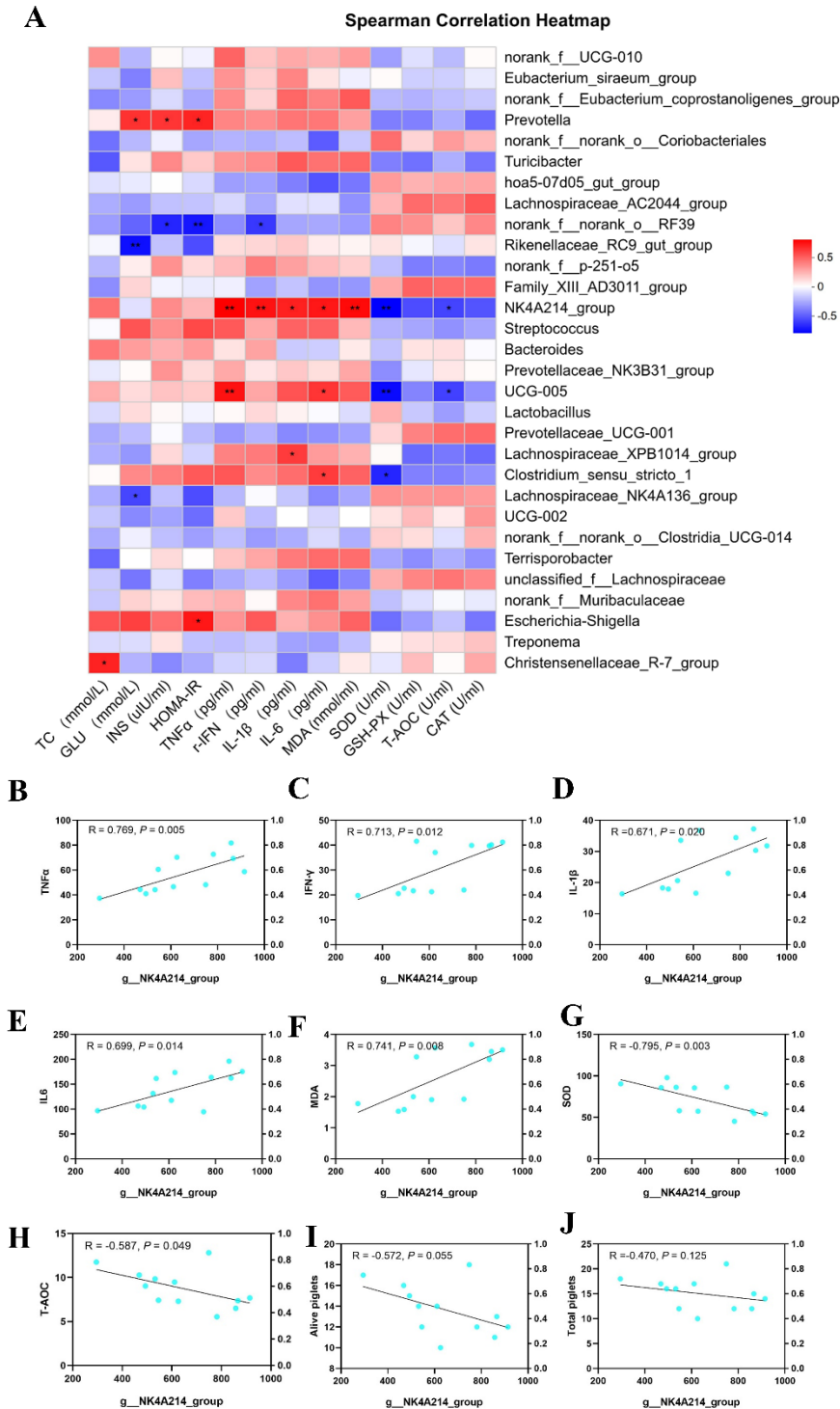


Figure 5-6: Associations between gut microbiota and serum insulin sensitivity, oxidative stress, and inflammatory markers.

(A) Spearman correlation heatmap between the Top 30 most abundant intestinal microbial genera and serum insulin sensitivity, oxidative stress, and inflammatory markers in sows at Day 110 of gestation. Red grids represent positive correlations, while blue grids indicate negative correlations. (B-J) Scatter plots showing the linear correlations between the relative abundance of g_NK4A214_group and serum levels of TNF- α (B), IFN- γ (C), IL-1 β (D), IL-6 (E), MDA (F), SOD (G), T-AOC (H), as well as reproductive performance indicators, including the number of alive piglets (I), and total piglets (J). R values indicate the Spearman correlation coefficient, and *P*-values indicate statistical significance. CON, control group fed a basal diet (n = 6); CDCA, treatment group fed a basal diet supplemented with 0.15% chenodeoxycholic acid (n = 6). * *P* < 0.05, ** *P* < 0.01.

5. Discussion

Our previous observations highlight maternal metabolism as a central determinant of gestational success. Building on this framework, the present study aimed to determine whether targeted metabolic modulation during early gestation could confer sustained benefits. Adverse maternal metabolic conditions during pregnancy are key contributors to declining reproductive capacity in mammals (Parrettini et al., 2020). While late-gestational nutritional interventions have shown promise, the persistent “programming” potential of early interventions remains understudied. Our previous work established CDCA as a metabolic regulator during early gestation (Chen et al., 2023; Chen et al., 2024). In the present study, we demonstrate that beneficial metabolic differences remained detectable during late gestation after early CDCA supplementation. It is important to emphasize that while pregnancy is a dynamic immunological process requiring a baseline level of inflammation for normal progression, modern hyperprolific sows naturally experience metabolic syndrome during late gestation, which often leads to insulin resistance, systemic oxidative stress and a low-grade inflammatory state (Cheng et al., 2018; Gao et al., 2024; Zheng et al., 2024). In the present study, the CDCA-driven reduction in MDA and pro-inflammatory cytokines and increase in antioxidant enzyme activities represents a mitigation of this metabolic burden rather than an inhibition of essential immune processes. The beneficial effects of CDCA-driven mitigation in maternal metabolic burden were accompanied by increased total number of piglets and live births, supporting a beneficial association between early CDCA intervention, maternal physiological status, and reproductive outcomes. These results support the potential value of early gestation as an intervention window for improving maternal health later in pregnancy.

CDCA induces a coordinated metabolic remodeling, encompassing lipid metabolism, amino acid metabolism, and gut microbiota interactions, which collectively contribute to the restoration of maternal metabolic homeostasis. In serum metabolomic profile, the changed metabolites primarily clustered in lipid metabolism, amino acid metabolism, immune regulation and cellular growth. We propose that these metabolic shifts are central to CDCA’s regulatory effects on

maternal health. Regarding the lipid metabolism, we observed a significant downregulation of lysophosphatidylcholines (LPC), including 1-palmitoylphosphatidylcholine and 2-lysophosphatidylcholine. While LPCs serve as important carriers for the placental transfer of polyunsaturated fatty acids (Ferchaud-Roucher et al., 2019), they also function as potent pro-inflammatory mediators linked to oxidative stress (Liu et al., 2020) and insulin resistance (Han et al., 2011). The reduction of LPCs, alongside the downregulation of lipid peroxidation biomarkers dodecanedioic acid (Inouye et al., 2000), and the oxylipin 9(S)-HpOTrE, which correlated with inflammation response (Grantz et al., 2024), indicates an alleviation of systemic metabolic stress and inflammatory status in CDCA-treated sows. Crucially, as the placenta possesses redundant transport mechanisms, this remodeling likely optimized the gestational environment without compromising essential nutrient delivery, which is supported by the significantly improved reproductive performance. Moreover, the upregulation of alpha-bisabolol and its derivatives further strengthened this protective profile through their potent anti-inflammatory and antioxidant properties (Kim et al., 2011; Bastaki et al., 2025).

Secondly, CDCA intervention significantly modulated amino acid metabolism, particularly the arginine biosynthesis and the polyamine pathways, which are critical mechanisms for placental angiogenesis and fetal growth (Wu et al., 2005; Elmetwally et al., 2022). In the present study, arginine biosynthesis emerged as a key pathway, aligning with the specific upregulation of L-ornithine. As a central node linking the urea cycle and polyamine biosynthesis, the elevation of L-ornithine not only indicates improved ammonia detoxification but also provides precursors for polyamines, which are crucial for placental vascularization (Yang et al., 2024). Furthermore, L-ornithine has been shown to exert anti-inflammatory effects (Li et al., 2025) and to correlate negatively with metabolic disorders (Cao et al., 2019). This beneficial shift was paralleled by the depletion of harmful amino acid derivatives associated with systemic inflammation and insulin resistance. Specifically, 3-hydroxyanthranilic acid (3-HAA) and N-carboxymethyllysine were downregulated in CDCA. Both of them typically accumulate under inflammatory states (Moffett and Namboodiri, 2003; Pae et al., 2006) and trigger pro-inflammatory pathways like NF- κ B (Gaens et al., 2010). Furthermore, elevated CML levels have been linked to the development of insulin resistance (Gaens et al., 2014). Consequently, the observed reduction in CML indicates a suppressed inflammation status and mitigated metabolic burden. Additionally, the reduction in N1-methyl-4-pyridone-3-carboxamide (4PY), a marker of vascular inflammation (Ferrell et al., 2024), further supports the protective effect of CDCA on maternal health. Collectively, these amino acid-related metabolic shifts provide a robust molecular basis for the enhanced reproductive performance observed in CDCA-treated sows.

Gut microbiota modulation may be involved in the late-gestation responses observed following early CDCA supplementation. This study found that CDCA addition during early gestation did not significantly affect the global diversity or phylum level composition of the gut microbiota in late gestation, which is consistent with the observations in early gestation (Chen et al., 2023). In addition, at the genus level, compared to our previous finding in early pregnancy, the top 20 abundant

genera showed relatively stable compositions, and the two most abundant genera remained *Christensenellaceae_R-7_group* and *Treponema* (Chen et al., 2023). However, the microbial profile during late gestation was characterized by a dynamic enrichment of potential pathobionts. We observed the proportions of *Escherichia-Shigella*, and *Terrisporobacter* were higher than in early pregnancy. *Escherichia-Shigella* is typically associated with inflammation and insulin insensitivity, and an increase in *Escherichia-Shigella* abundance in late pregnancy sows can lead to elevated endotoxin levels, thereby exacerbating inflammation during late pregnancy in sows (Li et al., 2023). Similarly, *Terrisporobacter* is an anaerobic pathogen whose increase in abundance can lead to oxidative stress (Cheng et al., 2016; Cai et al., 2019). This pro-inflammatory shift in the microbial environment as pregnancy advances highlights the importance of targeted interventions to maintain maternal gut microbiota homeostasis.

Beyond gestational adaptations, CDCA supplementation significantly remodeled the gut microbiota by suppressing potential pathobionts and enriching beneficial bacteria. Regarding the specific impact of CDCA, *NK4A214_group*, *norank_f_norank_o_Bradymonadales*, *Anaeroplasm*, and *Anaerofustis* were the main differential genera between the CDCA and the control group. Among the differential genera, *NK4A214_group* exhibited a strong positive correlation with maternal inflammatory response and a negative correlation with antioxidant enzyme levels. Notably, dietary *Clostridium butyricum* has been shown to improve immune responses and antioxidant capacity while concurrently reducing *NK4A214_group* abundance (Jiao et al., 2024). While some studies suggest that a decrease in this genus may induce metabolic disorders and inflammatory diseases (Burakova et al., 2022), our data indicate that under late-gestation metabolic stress, the enrichment of *NK4A214_group* is closely linked to an exacerbated inflammatory and oxidative stress. This aligns with network ecology analyses identifying this group as a key taxon associated with progression of insulin resistance and type 2 diabetes (Esquivel-Hernandez et al., 2023). Furthermore, CDCA treatment reduced the abundance of *Anaeroplasm*, a genus enriched in high inflammatory models and positively correlated with intestinal inflammation biomarkers (Gates et al., 2023). Other opportunistic pathogens, such as *Clostridium sensu stricto_1* and *Turicibacter*, both strongly associated with gut inflammation, barrier dysfunction, and pro-inflammatory cytokine elevation (Mo et al., 2021; Abdelbary et al., 2022; Luo et al., 2022; Martell et al., 2025), were similarly reduced. Conversely, CDCA promoted the enrichment of *Subdoligranulum*, a key butyrate-producing bacterium associated with improved insulin sensitivity, gut barrier integrity, and healthy metabolic status (Gradisteanu Pircalabioru et al., 2022; Singh et al., 2022). Collectively, these targeted microbial shifts, characterized by the suppression of harmful taxa and the promotion of beneficial bacteria, provide possible biological explanations for the observed improvements in maternal metabolic health.

Beyond compositional changes, the functional prediction of gut microbiota indicated significant downregulation of pathways related to amino acid biosynthesis in the CDCA group. This was consistent with our serum metabolomics data, which showed reduced levels of potentially harmful amino acid-derived metabolites, such

as 3-hydroxyanthranilic acid and 4-chlorophenylacetic acid. This concordance suggests a possible link between predicted microbial functions and the changes in circulating amino acid derivatives following CDCA supplementation. Although Procrustes analysis showed no significant global congruence, Spearman correlation identified specific correlations between differential genera and metabolites. These findings suggest that associations involving individual microbial taxa may be relevant even in the absence of a significant overall relationship between the two datasets. However, they do not demonstrate that the microbial changes induced the serum metabolomic response, and the microbiota and metabolome may represent parallel responses to CDCA supplementation. In particular, *NK4A214* group was reduced by CDCA and correlated positively with several metabolites discussed above as potentially unfavorable and negatively with potentially protective metabolites. This pattern suggests a possible involvement of this genus in the maternal metabolic response to CDCA. Together, these multi-omics findings support the hypothesis that specific microbe-metabolite interactions may contribute to the maternal metabolic changes observed in late gestation following early-gestation CDCA supplementation. Further functional studies are needed to clarify their contribution to maternal metabolic health. Together with our previous findings on protein-induced metabolic imbalance, these results support a conceptual framework in which both nutritional disruption and nutritional intervention converge on maternal metabolic homeostasis as a central determinant of reproductive outcomes.

6. Conclusion

In conclusion, this study demonstrates that early-gestation CDCA supplementation acts as a promising metabolism targeted nutritional intervention to improve reproductive performance. Our findings showed that early-gestation CDCA supplementation was associated with improved reproductive performance and beneficial differences in maternal inflammatory, oxidative, and metabolic status during late gestation. These benefits were accompanied by changes in lipid and amino acid metabolism, and in the relative abundance of specific gut microbial taxa. Because these responses were observed after CDCA supplementation had ended, the findings support the possibility that some benefits extended beyond the supplementation period. However, the continuous maintenance of the early responses throughout gestation and the causal contribution of the gut microbiota-metabolism axis require further investigation. Overall, the findings support maternal metabolism as an important regulatory target during gestation.

7. Acknowledgements

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Chapter 6

**General discussion, general conclusion
and perspective**

1. General discussion

1.1 Introduction

Sow reproductive performance is an important factor affecting the production efficiency and economic benefits of the pig industry. Among the many factors that limit reproductive performance in sows, embryonic loss during early gestation is considered one of the major constraints on litter size in modern highly prolific sows. Increasing evidence has shown that maternal nutrition during gestation plays a key role in regulating reproductive outcomes in sows (Gormley et al., 2024). Early gestation is a nutritionally sensitive period for porcine embryos, and improving the nutritional status of sows during this stage can enhance embryo quality and survival, as well as promote early embryo implantation (Bazer et al., 2004; Chen et al., 2019). Among nutritional factors, protein is particularly important because it not only provides substrates for fetal growth, but also has influence on maternal metabolic homeostasis. This is especially relevant for modern highly prolific sows, where tissue mobilization and body condition loss during lactation may compromise subsequent reproductive performance (Wientjes et al., 2013). It is therefore necessary to re-evaluate whether current commercial feeding programs adequately meet the needs of modern sows for protein and essential amino acids during early gestation.

Previous work from our laboratory showed that increasing dietary protein levels during early gestation improved embryo survival in gilts by modulating nutrient transport (Zhao et al., 2022). However, whether a similar response occurs in multiparous sows remains unclear. Moreover, the biological mechanisms linking maternal dietary protein nutrition to pregnancy outcomes are still not fully understood.

This thesis was initially designed to investigate whether maternal dietary protein level during early gestation affects reproductive performance in multiparous sows and to explore the potential mechanisms. However, the results showed that changes in maternal dietary protein supply are not necessarily reflected in litter size. Instead, maternal metabolic status, amino acid profiles, redox balance, and placental functional indicators appeared to be more sensitive to nutritional disturbance. These findings suggest that maternal metabolic homeostasis and placental functional adaptation may serve as key intermediate links between maternal nutrition and pregnancy outcomes.

To address this question, three related experiments were conducted. First, a sow model was used to evaluate the effects of dietary protein levels within a commercial range during early gestation on reproductive performance, maternal metabolic status, and placental function in multiparous sows (Chapter 3). Then based on the metabolic responses observed in sows, a mouse model was further used to investigate the effects of sustained protein imbalance on pregnancy outcomes, placental function, and fetal development under a more controlled and stronger nutritional challenge (Chapter 4). Finally, within the overall framework of this thesis, CDCA was evaluated as a metabolism-targeted nutritional intervention to examine whether

improving maternal metabolic status during early gestation was associated with maternal health in late gestation and reproductive outcomes (Chapter 5).

Accordingly, this General Discussion addresses three main questions. First, why does changing maternal dietary protein intake within a certain range not necessarily alter implantation number or litter size? Second, how do maternal metabolic status, placental adaptation, and nutrient transport mediate the relationship between maternal nutrition and fetal development? Third, based on the results of the CDCA intervention, what is the potential value of improving pregnancy outcomes through metabolic homeostasis regulation, and what are the implications for precision nutrition strategies? In this context, Chapters 3 and 5 provide an important indication: under a commercial protein supply background, increasing dietary protein alone did not markedly improve litter performance in multiparous sows, whereas early CDCA supplementation was associated with improved maternal metabolic status and increased litter performance.

1.2 Litter size and implantation number show relative robustness to maternal dietary protein variation under the present experimental conditions

The present study showed that, in multiparous sows, increasing dietary CP from 12%-15% during early gestation did not significantly affect total born piglets, live-born piglets, or the live-born rate. Similarly, in the mouse model with a more severe protein challenge, neither LP nor HP treatment altered the number of implantation sites at GD 6.5 or litter size at GD 18.5. These findings suggest that, under the conditions of this study, implantation number and litter size showed a certain degree of robustness to changes in maternal dietary protein intake.

This result should be interpreted in relation to the requirement for protein and amino acids during early gestation. Early gestation is a critical period for embryonic loss, and amino acids are not only structural substrates for embryo and placental development, but also participate in early embryonic development and cell signaling. Previous studies have shown that amino acid requirements increase around the peri-implantation period, and mixtures of amino acids are commonly included in in vitro embryo culture systems, supporting the importance of amino acids for early embryonic development (Van Winkle, 2001). In addition, supplementation with functional amino acids such as arginine during gestation has been reported to improve litter size and embryo survival (Wu et al., 2010; Li et al., 2015). These findings indicate that protein and amino acids do have the potential to regulate pregnancy outcomes.

However, the results of the present study also suggest that once basal protein and amino acid supply has reached an adequate level, further increasing protein intake may not continue to improve implantation number or litter size. This is consistent with several previous studies. In mice, both LP and HP intake during gestation have been reported to have no significant effect on litter size (Ajuogu et al., 2020). In sows, available evidence also suggests that increasing dietary protein or lysine within a certain range has limited effects on litter size (Mahan and Mangan, 1975; Cooper et al., 2001). For example, Cooper et al. found that once lysine intake during

gestation reached approximately 10.6 g/d, further increases in lysine intake did not improve litter size or overall productive performance (Cooper et al., 2001). Similarly, feeding 11% to 16% CP throughout gestation did not affect litter size in multiparous sows (Kim et al., 2023; Johannsen et al., 2024). These findings suggest that the effect of gestational protein nutrition on reproductive performance may reach a plateau once the requirement has been met. Another study reported that increasing SID lysine intake from 11.0 g/d (13.8% CP) to 18.5 g/d (20.2% CP) did not increase total born or live-born piglets in gilts or sows, although it reduced stillbirth rate and thereby improved the proportion of live-born piglets in multiparous sows (Thomas et al., 2021). Overall, previous studies suggest that gestational protein level has a relatively limited effect on litter size, whereas its effect on piglet birth weight may be more evident, especially when protein intake is insufficient (Mahan and Mangan, 1975; Shields et al., 1985).

The lack of significant differences in live-born rate and litter performance in the multiparous sows of the present study may also be related to their nutrient requirements. Compared with gilts, multiparous sows have lower requirements for their own growth and body protein deposition, and may therefore show a weaker reproductive response to additional protein supply. Previous studies have also shown that increasing dietary protein during early gestation mainly promotes body weight recovery and body protein deposition in low-parity sows, while having a smaller effect on growth in high-parity sows (Beaulieu et al., 2009; Johannsen et al., 2024). In addition, during early gestation, the embryo and placenta are still small and their absolute nutrient demands are limited. Therefore, the lowest protein level in the present sow trial, which provided approximately 300 g of protein per day, may already have been sufficient to support early pregnancy maintenance and embryo survival in multiparous sows, without impairing early body condition recovery.

The effect of protein on reproductive performance should also be considered in relation to energy supply. One study reported that increasing feed allowance during early gestation had similar effects on body weight gain and litter size in primiparous and multiparous sows, and that a 30% increase in feed allowance increased litter size (Hoving et al., 2011). In contrast, increasing protein supply alone by 30% (11.78% vs. 14.74%) did not affect reproductive performance (Hoving et al., 2011). This suggests that improvements in reproductive performance during early gestation may not depend solely on dietary protein concentration, but are closely related to total nutrient intake, energy supply, and the balance between energy and protein. It has also been suggested that, when energy supply is insufficient, additional protein intake may be directed more toward catabolic pathways such as deamination and the urea cycle, rather than being fully used for tissue deposition and embryonic development (Pregnancy, 1990).

However, this robustness does not mean protein nutrition is unimportant, it more likely reflects a strong physiological capacity for compensation during gestation, with maternal metabolic status, redox balance, and placental function emerging as more sensitive indicators than litter size itself.

1.3 Maternal dietary protein levels modulate early gestational endocrine environment and implantation-related signaling

Although maternal dietary protein levels did not significantly affect implantation number or litter size in the present study, this does not mean that the endocrine environment and implantation-related regulation during early gestation were completely unaffected. Instead, the results suggest that circulating hormone concentrations and implantation number are relatively robust to changes in protein nutrition, whereas lipid metabolism, steroidogenic potential, and local hormone receptor expression may be more sensitive to changes in protein supply.

In the sow trial, dietary protein levels ranging from 12%-15% did not affect serum P4, E2, or insulin concentrations at GD 28. This suggests that, when basal nutritional requirements are met, protein variation within a commercial range does not markedly disturb the systemic steroid hormone environment or glucose-insulin homeostasis. It should be noted that serum hormones in the sow trial were measured at GD 28, when embryo attachment and implantation in pigs had largely been completed. Therefore, these results more likely reflect the systemic endocrine status after pregnancy establishment, rather than the dynamic hormonal changes occurring during the peri-implantation window. Nevertheless, the stability of circulating hormone concentrations does not mean that endocrine-related metabolic processes were completely unchanged. In both sows and mice, the LP groups showed an increasing trend or elevation in cholesterol-related indicators, such as LDL, HDL, or total cholesterol. Since cholesterol is the precursor for steroid hormone synthesis (Miller and Bose, 2011), these results suggest that, under reduced protein supply, cholesterol metabolism may be adjusted to help maintain the endocrine support required for pregnancy establishment.

The mouse model further showed that, under a stronger protein imbalance, the ovarian expression of *Star*, a key regulator of steroidogenesis, and *Cyp19a1*, an enzyme involved in estrogen synthesis, changed in a protein level-dependent manner. The detailed molecular mechanisms have been discussed in Chapter 4. In parallel with these changes in steroidogenic genes, *Esr* and *Pgr* expression at the implantation site was also affected by maternal dietary protein level. This suggests that protein nutrition may influence not only steroid hormone synthesis, but also the local uterine response to hormone signals. Previous studies also support the idea that nutritional status can regulate ovarian steroidogenesis and uterine receptivity through similar mechanisms. For example, a high-fat diet increased serum E2 and P4 concentrations in mice and upregulated ovarian *Star*, *Cyp11a1*, and *Cyp19a1* expression (Li et al., 2021). Methionine supplementation has also been shown to increase *Star*, *Cyp11a1*, and *Cyp19a1* expression and promote E2 synthesis (Yang et al., 2024). In addition, supplementation with short- and medium-chain fatty acids increased E2 secretion in porcine granulosa cells and improved the expression of genes related to endometrial receptivity (Zeng et al., 2022). Together with the present findings, these studies suggest that nutrition-induced metabolic changes can participate in early pregnancy regulation by modulating steroidogenic potential and local hormonal responsiveness.

1.4 Maternal metabolic homeostasis and amino acid balance as sensitive mediators of nutritional effects during pregnancy

Maternal metabolic homeostasis during pregnancy provides an essential basis for normal pregnancy maintenance and fetal development. During gestation, maternal glucose, lipid, protein, and amino acid metabolism undergo dynamic adaptations to meet the needs of maternal maintenance, embryo and placental development, and fetal growth (Hadden and McLaughlin, 2009; Zhu et al., 2023). These metabolic adaptations are jointly regulated by maternal nutritional status, endocrine changes, and placental-derived signals (Sferruzzi-Perri et al., 2020). Therefore, disruption of maternal metabolic homeostasis during pregnancy may affect placental function, fetal growth quality, and long-term offspring health. Among these metabolic processes, protein and amino acid metabolism play a central role (Hadden and McLaughlin, 2009).

The present study showed that maternal metabolic status was more sensitive to changes in protein nutrition than litter size or implantation number. In the sow trial, dietary protein levels within the commercial range of 12%-15% did not significantly affect reproductive performance, but the HP group showed increased serum concentrations of several free amino acids, together with changes in ALT and redox-related indicators. These findings suggest that maternal amino acid profiles and some metabolic indicators responded to the dietary treatments despite the absence of significant differences in litter performance. However, serum amino acid concentrations alone do not indicate whether the additional dietary nitrogen was used for productive processes or was catabolized and subsequently disposed of as urea. Serum urea or blood urea nitrogen would therefore have provided complementary information on amino acid utilization and nitrogen metabolism. A study reported that a higher-CP diet during late gestation increased maternal serum urea concentrations without improving sow performance, suggesting increased disposal of surplus dietary nitrogen (Kroeske et al., 2021). Because serum urea or BUN was not measured in the present study, it remains unclear whether the absence of an improvement in reproductive performance in the HP group was accompanied by increased catabolism of surplus amino acids.

An important consideration is that the dietary treatments cannot be regarded as differing exclusively in crude protein. During diet formulation, the ratios of SID essential amino acids to SID lysine were considered, and crystalline amino acids were included to adjust the essential amino acid profile and meet the estimated requirements. However, the complete amino acid profile was not standardized across treatments. In particular, non-essential amino acids and their proportions relative to lysine were not specifically controlled when the proportions of protein-containing ingredients were changed. Moreover, the HP diet had a higher SID lysine concentration and a higher SID lysine-to-net-energy ratio. Therefore, the observed maternal metabolic responses may reflect both the change in total dietary protein supply and the accompanying differences in amino acid supply and relative proportions.

In the mouse model, a stronger protein imbalance also altered maternal serum free amino acid profiles, but changes in amniotic fluid and fetal serum amino acids did

not fully follow the changes observed in maternal serum. A previous study also reported that a high-protein/low-carbohydrate diet during pregnancy altered maternal serum amino acid concentrations and placental amino acid extraction, without causing parallel changes in fetal serum amino acid concentrations (Metzler-Zebeli et al., 2012). In addition, BUN concentrations in maternal serum, amniotic fluid, and fetal serum increased in a dose-dependent manner with dietary protein level. Previous studies have also shown that increasing CP during gestation can elevate BUN, suggesting that excessive protein intake may increase amino acid oxidation and nitrogen metabolic burden (Fang et al., 2019). Together, these findings indicate that maternal metabolic homeostasis and amino acid availability may represent more sensitive responses to gestational protein nutrition than reproductive traits themselves.

Protein and amino acid nutritional status is also closely related to maternal glucose homeostasis and insulin sensitivity. Human studies have shown that protein intake and protein source during pregnancy are associated with the risk of GDM, and that branched-chain amino acids and aromatic amino acids are linked to insulin resistance and abnormal glucose metabolism (Wang et al., 2011; Floegel et al., 2013; Pang et al., 2017; Zhou et al., 2018). These findings suggest that the role of protein nutrition during pregnancy is not limited to providing amino acid substrates, but may also involve regulation of insulin secretion, glucose homeostasis, and maternal metabolic adaptation, thereby contributing to pregnancy outcomes (Tremblay et al., 2007). Therefore, the increased amino acids observed in the HP group of the present study should not be interpreted only as increased nutrient supply. They may also reflect changes in maternal amino acid and glucose metabolic homeostasis.

The mouse model further supports the idea that maternal amino acid supply and fetal nutrient acquisition are not simply synchronized. This indicates that maternal-fetal amino acid distribution is not determined only by amino acid concentrations in maternal serum, but is also regulated by placental transport, placental metabolism, and fetal utilization. Previous studies have shown that maternal plasma free amino acid concentrations change across gestation, which may be related to increased placental uptake, changes in maternal insulin and hepatic amino acid use, and enhanced amino acid transfer to the fetus (Metzger et al., 1977; Baird, 1986). This view is also consistent with the maternal nutrient buffering model, which proposes that fetal availability of macronutrients such as glucose, fatty acids, and amino acids is not simply determined by current dietary intake, but is buffered by maternal metabolic regulation, mobilization of nutrient reserves, amino acid partitioning, and placental transport (Thayer et al., 2020).

Therefore, changes in protein intake during pregnancy do not translate in a simple linear manner into changes in implantation number, litter size, or fetal growth. Instead, they may first be reflected in more sensitive processes, including maternal circulating amino acid profiles, nitrogen metabolic burden, glucose-insulin homeostasis, redox status, and placental nutrient allocation.

1.5 Placental functional adaptation links maternal dietary protein nutrition to fetal growth

1.5.1 The placenta as a regulatory center for maternal-fetal nutrient allocation

Both undernutrition and overnutrition can alter maternal metabolism and circulating hormone concentrations, which may in turn regulate placental function (Gaccioli et al., 2013). For example, maternal dietary protein restriction is often associated with reduced serum insulin, IGF-1, and leptin, whereas overnutrition is generally associated with increased circulating insulin, IGF-1, and leptin (Jansson et al., 2006). These metabolic hormones and cytokines not only reflect maternal nutritional status, but can also act as signals to the placenta, regulating placental growth, nutrient-sensing pathways, and transporter activity. Previous studies have shown that IGF-1, insulin, leptin, and certain cytokines can regulate placental amino acid transport activity (Karl et al., 1992; Karl, 1995; Jansson et al., 2003; Jones et al., 2009). Therefore, the impact of maternal nutrition on fetal development depends largely on how the placenta senses, buffers, and translates nutritional and endocrine signals.

In the sow trial, dietary protein levels within the commercial range of 12%-15% during early gestation altered maternal amino acid profiles and some metabolic indicators, but did not significantly affect litter performance, placental oxidative stress, ATP levels, or the expression of nutrient transport-related genes at farrowing. In contrast, in the mouse model, a stronger and sustained high-protein/low-carbohydrate imbalance did not change implantation number or litter size, but reduced fetal weight and placental efficiency, and was accompanied by a lower proportion of the placental labyrinth zone. These findings suggest that the placenta may be a key regulatory site that determines whether changes in maternal nutrition are further translated into altered fetal growth.

This interpretation is consistent with the theory of placental nutrient sensing. The placenta is the central maternal-fetal interface linking maternal nutritional status with fetal development (Carrasco-Wong et al., 2020). It not only carries out basic functions such as nutrient transport, gas exchange, and waste removal, but also plays important endocrine and metabolic regulatory roles (Burton et al., 2010; Burton and Fowden, 2012). Through the secretion of hormones, cytokines, and other signaling molecules, the placenta participates in bidirectional communication among the mother, placenta, and fetus, thereby integrating maternal nutrient supply, maternal metabolic status, and fetal nutrient demand. Previous studies have proposed that the placenta acts as a “nutrient sensor”, sensing maternal nutrients, oxygen availability, metabolic hormones, and fetal demand signals, and regulating placental growth and nutrient transport through nutrient-sensing pathways such as mTOR, AMPK, and AAR (Jansson and Powell, 2006; Díaz et al., 2014). Thus, the placenta is not a passive exchange organ, but a key regulatory hub through which maternal nutrition influences fetal growth.

Within this framework, the sow and mouse results can be understood as two different placental response states under different intensities and durations of nutritional intervention. In the sow trial, dietary protein levels were within the commercial production range and were applied only during early gestation. In

addition, all sows received the same diet during mid- and late gestation, and placental samples were collected at farrowing. Therefore, the lack of marked changes in placental functional indicators at farrowing may suggest that the 12%-15% protein range did not exceed the placental compensatory capacity. It may also indicate that any transient placental response during early gestation had recovered or been masked under the common diet provided later in gestation.

In contrast, the high-protein/low-carbohydrate mouse model provided mechanistic information under a stronger nutritional imbalance. Because mice were fed different protein diets throughout gestation, and because the isocaloric HP diet was accompanied by a reduced carbohydrate proportion, the effects observed in the HP group should be interpreted as the result of sustained high-protein/low-carbohydrate macronutrient imbalance. This treatment not only changed maternal amino acid supply and the structure of available energy substrates, but may also have increased placental redox pressure and energy metabolic burden, eventually shifting the placenta from an adaptive state toward functional impairment.

Therefore, the present study supports a “placental compensatory threshold” model (Gaccioli et al., 2013). Under moderate, short-term, or commercial-range nutritional variation, the placenta may act as a buffer, maintaining relatively stable fetal development through nutrient sensing and functional adaptation. However, when nutritional imbalance is stronger, longer lasting, or accompanied by oxidative stress and mitochondrial dysfunction, placental compensatory capacity may be exceeded. Under these conditions, maternal nutritional disturbance may be translated into placental dysfunction and fetal growth restriction. Thus, the placenta can act both as a buffer against maternal nutritional disturbance and, once compensation fails, as a key site where maternal metabolic alterations are transmitted and amplified into fetal developmental outcomes.

1.5.2 Placental oxidative stress, mitochondrial dysfunction, and altered nutrient transport jointly contribute to placental decompensation

Placental redox homeostasis, mitochondrial energy metabolism, and nutrient transport function are closely linked biological processes. They provide important evidence together for assessing whether the placenta has shifted from an adaptive state toward functional impairment. Previous studies have shown that placental oxidative stress can impair trophoblast function, placental vascular development, and nutrient transport capacity, and is closely associated with adverse pregnancy outcomes such as fetal growth restriction, reduced live-born piglets, and lower litter weight (Cheng et al., 2019; Hussain et al., 2021; Sultana et al., 2023). Mitochondrial dysfunction may further aggravate placental impairment by reducing ATP production and increasing ROS generation (Guo et al., 2013; Khan et al., 2015). Abnormal nutrient transport represents a direct functional manifestation of placental insufficiency impairing fetal growth (Cetin et al., 1992; Jansson et al., 2002; Sibley et al., 2005). In the present study, the sow and mouse models showed different degrees of response in these three aspects, suggesting that whether maternal dietary protein nutrition affects fetal growth may depend on the ability of the placenta to maintain coordination among redox homeostasis, energy metabolism, and nutrient transport.

As noted above, placental redox status, energy metabolism, and nutrient transport remained largely stable at farrowing in the sow trial, whereas these processes were altered in the mouse model under sustained high-protein/low-carbohydrate imbalance. In this model, the placenta showed abnormalities in oxidative stress, mitochondrial function, and nutrient transport capacity. In the HP group, placental MDA was increased, and antioxidant enzyme activities and the NFE2L2/KEAP1-related antioxidant pathway were affected. At the same time, ATP production decreased, mtDNA copy number and ETC-related genes were altered, and the expression of several amino acid and glucose transport-related genes was reduced. These findings are consistent with previous evidence showing reduced placental amino acid transport capacity in IUGR/FGR pregnancies, and with mechanistic studies showing that placental-specific knockdown of key transporters can lead to fetal growth restriction (Cetin et al., 1992; Jansson et al., 2002; Sibley et al., 2005; Vaughan et al., 2021).

It should be noted that, because placental samples in the present study were mainly collected at a single time point, the current data cannot establish a clear causal relationship among oxidative stress, mitochondrial dysfunction, and altered nutrient transport. Therefore, these changes should be interpreted as interrelated functional abnormalities occurring together in a state of placental decompensation. From a biological perspective, placental amino acid transport is an actively regulated process. System A, System L, and cationic amino acid transport systems are involved in the transplacental transport of different types of amino acids, and are regulated by maternal nutritional status, the placental intracellular amino acid pool, nutrient-sensing signals such as mTOR, placental energy status, and fetal demand signals (Cleal and Lewis, 2008; Jansson et al., 2012; Shimada et al., 2024). When placental oxidative stress and mitochondrial energy metabolism are impaired, active nutrient transport may be further limited. Amino acid transport across the placenta, especially active or exchange transport processes that depend on ion gradients and intracellular amino acid pools, requires normal placental energy metabolism. Therefore, under high-protein/low-carbohydrate conditions, even if maternal circulating amino acid concentrations are increased, reduced placental ATP production, mitochondrial dysfunction, and enhanced oxidative stress may limit the ability of the placenta to maintain efficient amino acid transfer to the fetus.

Consistent with this interpretation, fetuses in the HP group also showed metabolic features of limited energy substrate availability and increased amino acid catabolism, including reduced fetal serum glucose, increased BUN, and altered expression of genes related to hepatic gluconeogenesis and amino acid degradation. These findings suggest that fetal growth restriction under high-protein/low-carbohydrate macronutrient imbalance should not be simply interpreted as a result of insufficient protein supply. Rather, it may reflect the combined effects of impaired placental nutrient transport and abnormal fetal metabolic adaptation. Under these conditions, excess amino acids may be used more for catabolism or energy compensation than for fetal tissue deposition (Torres et al., 2023). At the same time, placental oxidative stress and impaired energy metabolism may further limit the effective transfer of nutrients to the fetus, together contributing to fetal growth restriction.

This provides functional evidence supporting the “placental compensatory threshold” model proposed above, although the exact timing and causal pathways still need to be verified in future studies using longitudinal sampling and mechanistic experiments.

1.6 Potential effects of maternal dietary protein nutrition on offspring development and metabolism after birth

Beyond their effects on placental function and fetal development, maternal nutritional conditions during critical periods of gestation may induce lasting changes in offspring growth and metabolism, a process commonly described as developmental or metabolic programming.

Evidence from pigs supports the potential programming effects of maternal dietary protein nutrition. Maternal dietary protein restriction and excess during gestation have been shown to alter gene expression and DNA methylation in the liver and skeletal muscle of offspring, with some effects detected after birth and at the finishing stage (Altmann et al., 2012). Another study also reported that maternal dietary protein during late gestation affected serum urea concentrations and some postnatal performance-related measurements in the offspring (Kroeske et al., 2021). These findings indicate that differences in maternal dietary protein supply during gestation may have consequences for offspring metabolism and development after birth.

In the present sow experiment, the absence of significant differences in litter size or the number of live-born piglets does not exclude possible postnatal programming effects. The dietary treatments altered maternal amino acid availability and several metabolic indicators during early gestation, when conceptus development and placental establishment occur. A testable hypothesis is therefore that the early maternal metabolic environment could influence the subsequent development and metabolic capacity of offspring tissues, including their ability to utilize dietary amino acids after birth. The placental and fetal metabolic responses observed in the mouse experiment provide mechanistic plausibility for this hypothesis, but because the piglets were not followed after birth, the present studies cannot establish persistent programming in pigs. Future studies should follow offspring after birth and evaluate their growth, feed efficiency, amino acid and nitrogen metabolism, and metabolic regulation in the liver and skeletal muscle.

1.7 CDCA as a metabolism-targeted nutritional intervention to improve gestational homeostasis

Although macronutrients such as glucose, fatty acids, and amino acids are essential substrates for fetal growth and metabolic development, the nutrients actually available to the fetus are not determined simply by maternal intake (Thayer et al., 2020). Instead, they are buffered by maternal metabolic regulation, mobilization of tissue reserves, and placental transport function (Thayer et al., 2020). The protein studies described above suggest that, when basal protein and amino acid requirements are already met, further increasing the supply of a single macronutrient may not necessarily improve pregnancy outcomes and may instead increase the

maternal metabolic burden. In this context, maintaining maternal metabolic homeostasis, redox balance, and placental functional adaptation may represent a more important nutritional target.

Based on this concept, CDCA was used in this thesis as a metabolism-targeted nutritional intervention to examine whether regulating maternal metabolic status during early gestation could have lasting effects on maternal health and reproductive outcomes in late gestation. The results showed that CDCA supplementation during early gestation reduced maternal inflammatory factors and oxidative stress, improved antioxidant capacity, and modulated insulin-related metabolic status in late gestation. Moreover, the increases in total born and live-born piglets in the CDCA group were not accompanied by a reduction in individual live-born piglet weight. These findings suggest that the benefits of early metabolism-targeted nutritional intervention may remain detectable during late gestation after the intervention has ended. It is worth noting that the basal dietary protein level in the CON group of the CDCA trial in Chapter 5 was close to that of the MP group in Chapter 3, in which litter performance was not improved. However, without increasing dietary protein supply, CDCA supplementation during early gestation increased both total born and live-born piglets. This result provides important support for the shift from a nutrient supply based approach toward metabolic homeostasis regulation.

The potential mechanisms underlying this “early intervention and late benefit” effect can only be inferred indirectly from the present results and remain speculative. One possibility is that early gestation (GD0-28) represents an active window for remodeling of the maternal gut microbiota and host metabolic adaptation. One possibility is that the microbial and metabolic responses initiated during early gestation contributed to the differences observed during late gestation. However, the temporal continuity of these responses cannot be confirmed because corresponding measurements were not obtained during mid-gestation. Another possibility is that the improvement in maternal metabolism and redox status induced by CDCA may reduce the cumulative metabolic burden during mid-gestation, thereby delaying or alleviating the increase in physiological insulin resistance and oxidative stress during late gestation. Because late gestation is characterized by rapid fetal growth, increased maternal energy demand, and enhanced lipid mobilization, sows are more likely to experience insulin resistance, oxidative stress, and increased inflammatory pressure during this stage (Duhig et al., 2016). Therefore, improving maternal metabolic status during early gestation may have sustained physiological relevance. However, the present study cannot distinguish between these possible mechanisms, and future studies with longitudinal sampling, dynamic monitoring of gut microbiota, and causal mechanistic experiments are still needed.

The effects of CDCA can also be understood from the perspective of bile acid signaling. Bile acids are not only involved in lipid digestion and absorption, but also act as important endogenous signaling molecules. Through receptors such as FXR and TGR5, they participate in the regulation of glucose and lipid metabolism, energy homeostasis, inflammation, and immune responses (Baars et al., 2015; Chiang and Ferrell, 2020). As a primary bile acid, CDCA is one of the important natural ligands of FXR, and may therefore influence maternal metabolism and inflammatory status

through bile acid receptor-related signaling (Parks et al., 1999). In addition, bile acids and gut microbiota interact bidirectionally. Gut microbiota can modify bile acid composition, while bile acids can also influence gut microbial structure, intestinal barrier function, and host metabolism through receptor-mediated signaling and antimicrobial activity (He et al., 2024). Therefore, the changes in gut microbiota and serum metabolites observed after CDCA treatment in the present study may reflect a potential role of the gut microbiota-bile acid-host metabolism axis in improving maternal gestational homeostasis, although the specific causal relationships still require further verification.

2. Limitations

Several limitations should be considered when interpreting the findings of this thesis. First, the sample size of the sow experiments was relatively limited. In the protein-level trial, each treatment group included approximately 12-15 sows, while in the CDCA trial, each group included approximately 9-11 sows. These sample sizes were sufficient to detect several maternal metabolic, oxidative, and physiological responses, but the statistical power may have been limited for highly variable reproductive traits, such as total born piglets, stillbirth rate, and live-born rate. Reproductive performance under commercial farm conditions is easily affected by parity, genetic background, season, and management factors. Therefore, the absence of significant differences in some reproductive traits should be interpreted cautiously in the context of limited sample size and high biological variation. In addition, in the CDCA trial described in Chapter 5, the distribution of sows excluded because of abortion or failure to establish pregnancy was not completely balanced between the two groups, with three sows excluded from the CON group and one sow excluded from the CDCA group. Given the small sample size, this imbalance may have introduced some selection bias into the final groups included in the analysis, and should be considered when interpreting the effect of CDCA on litter size. At the same time, whether this observation suggests a potential role of CDCA in reducing early pregnancy loss or pregnancy failure remains to be tested in future studies with larger sample sizes.

Second, the sampling time points limited the ability to evaluate dynamic mechanisms. In the sow protein trial, maternal blood was mainly collected at GD 28, whereas placental samples were collected at farrowing. During mid- and late gestation, all sows were fed the same diet. Therefore, placental indicators measured at farrowing cannot fully reflect immediate placental changes that may have occurred during or shortly after the early-gestation protein intervention. It is also not possible to determine whether any early placental response was transient, gradually recovered, or continued to influence placental function later in gestation. Similarly, although the CDCA trial showed improvements in maternal metabolism and redox status during late gestation, placental samples were not collected. Thus, it remains unclear whether the placenta directly contributed to the long-term effects of early CDCA intervention. In addition, although CDCA supplementation increased total-born and live-born piglets, the gestational stage at which this reproductive benefit occurred cannot be determined. The greater litter size may have resulted from

improved embryo survival during early gestation, reduced fetal loss during mid- or late gestation, or a combination of these effects, because embryo and fetal numbers were not monitored throughout gestation.

Third, the mouse protein trial should be understood as a mechanistic amplification model rather than as a direct representation of commercial sow gestational diets. The nutritional imbalance caused by the HP mouse diet was much stronger than the 12%-15% commercial-range protein variation used in the sow trial. This model helped to identify potential biological pathways, such as placental oxidative stress, mitochondrial dysfunction, and altered nutrient transport, which may not be easily detected under commercial conditions. Although mice provide convenient tissue sampling, a clear genetic background, and some conserved molecular pathways related to placental development and function across mammals (Cox et al., 2009; Hemberger et al., 2020), there are clear differences between mice and pigs in placental structure, gestational physiology, and fetal development. In addition, mice were fed different protein levels throughout gestation, whereas the sow protein trial focused mainly on early-gestation intervention. Therefore, the two models differed in both intervention intensity and duration. For these reasons, the mouse model is more suitable for exploring potential mechanisms by which protein imbalance may affect placental function and fetal development, rather than for direct extrapolation to commercial sow nutrition.

Fourth, the HP treatment in the mouse study was accompanied by a reduced carbohydrate proportion. Therefore, the related findings cannot be attributed to protein alone. Because the diets were designed to be isocaloric, increasing the proportion of protein necessarily reduced the proportion of carbohydrate. Thus, the placental oxidative stress, mitochondrial dysfunction, altered nutrient transport, and fetal growth restriction observed in the HP group should be interpreted as effects of high-protein/low-carbohydrate macronutrient imbalance. Although this more extreme model was useful for amplifying and identifying potential pathways through which maternal nutritional imbalance affects placental function, future studies with independent manipulation of protein and carbohydrate levels are needed to separate their individual and interactive effects.

Fifth, the mechanistic analysis of placental function could be further strengthened. This thesis measured placental oxidative stress, mitochondrial function, and selected nutrient transport-related indicators, but did not systematically examine placental nutrient-sensing pathways, especially the mTOR pathway. mTOR responds to amino acids, energy status, and insulin/IGF-related metabolic signals, and regulates cell metabolism, growth, and proliferation. Among its functions, mTORC1 is considered an important positive regulator of placental amino acid transport and can influence the activity of transport systems such as System A and System L (Roos et al., 2009). Previous studies have shown that abnormal placental mTOR signaling is associated with fetal growth restriction or fetal overgrowth (Roos et al., 2007; Jansson et al., 2013), and maternal low-protein intake can also reduce placental mTORC1 activity (Rosario et al., 2011). Therefore, whether the changes in placental nutrient transport-related genes observed in this study are related to mTOR signaling still needs further investigation.

Sixth, the qPCR analysis in the mouse experiment had several methodological limitations. GAPDH and β -actin were initially examined as candidate reference genes. GAPDH showed lower variation in Ct values than β -actin and similar Ct values across the dietary treatment groups and was therefore selected for normalization. Although these observations support the relative stability of GAPDH in the present dataset, only two candidate genes were examined, its stability was not formally evaluated using a dedicated algorithm, and only one reference gene was used for normalization. This limitation warrants particular consideration because GAPDH is involved in glycolysis and the carbohydrate proportion differed among the experimental diets. In addition, primer amplification efficiencies were not determined using serial-dilution standard curves. Therefore, the assumptions underlying the comparative $\Delta\Delta$ Ct method were not fully verified, which may have affected the precision of the estimated expression fold changes. Future studies should validate the amplification efficiency of each primer pair and use at least two reference genes demonstrated to be stable under the specific experimental conditions.

Seventh, the relationships among gut microbiota, serum metabolites, and maternal phenotypes in the CDCA trial remain mainly associative. Although CDCA supplementation altered maternal inflammation, oxidative stress, insulin-related metabolism, serum metabolites, and gut microbiota, the present study cannot fully establish a causal chain linking changes in gut microbiota to changes in metabolites and then to improved maternal phenotypes. Therefore, the specific pathways through which CDCA improves maternal gestational homeostasis require further validation. Furthermore, the metabolomic analysis in the CDCA trial was limited to serum. Metabolomic profiling of the placenta and liver could help identify tissue-specific responses and clarify how maternal metabolic changes relate to placental function and fetal development. In addition, targeted bile acid profiling in serum and faeces or intestinal contents could determine whether CDCA supplementation altered the broader bile acid pool and provide complementary information on circulating bile acid exposure, intestinal microbial transformation, and enterohepatic recycling. Combining these measurements with microbial analyses and functional assays would help identify other bile acids and bile acid-related pathways that may contribute to the observed effects of CDCA. In particular, it remains necessary to determine whether these effects are mediated through FXR/TGR5 bile acid receptor signaling, gut microbiota-host metabolic interactions, the Nfe2l2 antioxidant pathway, or placental functional regulation. Future studies could combine fecal microbiota transplantation, receptor inhibition, in vitro cell experiments, and placental functional assays to verify these causal mechanisms.

Overall, the main limitation of this thesis is that there remains a gap between phenotypic associations and causal mechanistic validation. This study has established an integrated link among maternal nutritional input, maternal metabolic homeostasis, placental functional adaptation, and fetal development. However, this regulatory axis still needs to be further validated through larger-scale sow trials, longitudinal sampling, functional measurements of placental transport, and causal mechanistic experiments.

3. Perspectives

At present, large-scale commercial pig farms usually use relatively uniform gestational diets, with feed allowance mainly adjusted according to sow body condition and stage of gestation. However, the findings of this thesis suggest that the effects of dietary protein level on maternal metabolism, placental function, and fetal growth may depend on gestational stage and may also show a threshold pattern. Therefore, future nutritional management of gestating sows should not focus only on the amount of nutrients supplied in the diet, but should also give greater attention to maternal metabolic status, placental functional adaptation, and nutrient utilization efficiency.

In terms of nutritional strategy, future work should place more emphasis on appropriate protein supply and amino acid balance, rather than simply increasing CP level. An appropriate protein level should meet the needs of pregnancy maintenance, placental development, fetal growth, and maternal metabolic homeostasis, while avoiding excessive amino acid catabolism, nitrogen metabolic burden, and nitrogen excretion. Diet formulation that considers digestible amino acid patterns, the energy-to-protein ratio, parity, body condition, and gestational stage may be more effective than simply increasing CP concentration. Together with the present finding that the diet formulated to contain 12% CP was sufficient to maintain reproductive performance in multiparous sows during early gestation, these observations support further evaluation of lower-CP, amino acid-balanced feeding strategies. Such an approach may improve nutrient utilization efficiency, reduce feed costs, and decrease environmental emissions. Previous studies have shown that moderately low-protein diets during mid- and late gestation can reduce nitrogen excretion and decrease emissions of ammonia and hydrogen sulfide, thereby lowering environmental burden while reducing feed costs (Kim et al., 2023; Zhang et al., 2025). Therefore, future protein nutrition management in sows should balance reproductive performance, feed cost, and environmental sustainability, while still meeting amino acid requirements and maintaining maternal metabolic homeostasis.

The findings of this thesis also support the need for stage specific and parity specific nutritional management. Gilts require amino acids not only for pregnancy, but also for their own continued growth, and their responses to dietary protein may differ from those of mature multiparous sows. Nutrient requirements also change across gestation. Therefore, using a single gestational diet and adjusting only feed allowance may not fully match the changing nutritional needs of sows at different stages of pregnancy. Phase feeding and dynamic adjustment of amino acid to energy ratios therefore deserve further evaluation.

Metabolism-targeted nutritional interventions may become an important direction for improving sow health and reproductive efficiency during gestation. In this thesis, the CDCA supplementation trial suggests that targeting gut microbiota-host metabolic interactions, redox balance, and inflammatory status during early gestation may produce benefits on maternal health in late gestation. In addition to CDCA, future studies may further explore bile acid related metabolic regulators, antioxidant nutrients, probiotics or postbiotics, short- or medium-chain fatty acids, and functional amino acid combinations. However, before these strategies can be

applied in production, it will be necessary to clarify their optimal dosage, supplementation window, safety, long-term effects, and consistency of response in sows with different parity and body condition.

In addition, pigs have translational value in studies of digestive physiology, metabolic characteristics, and placental development, and are often used as a reference model for human nutrition and pregnancy-related research (Ferenc et al., 2014). In recent years, high-protein and low-carbohydrate dietary patterns have become increasingly popular in humans, but their potential effects during pregnancy remain unclear (Trichopoulou et al., 2007; Clifton et al., 2014). Therefore, the findings of this thesis on high-protein/low-carbohydrate nutritional imbalance, maternal metabolic homeostasis, and placental functional adaptation during pregnancy may provide useful reference information not only for precision nutrition in sows, but also for understanding the possible maternal-fetal metabolic effects of high-protein dietary patterns in human pregnancy. However, because pigs, mice, and humans differ in placental structure, gestational physiology, and nutritional exposure, these findings still need to be further validated by human studies and clinical evidence.

Overall, future nutritional management of gestating sows should aim to improve maternal health, placental function, and fetal development by optimizing protein supply and the energy-to-amino acid ratio, reducing oxidative and inflammatory stress, and incorporating metabolism-targeted interventions, while meeting basal nutritional requirements. Precision nutrition strategies based on parity, body condition, gestational stage, and metabolic biomarkers may become an important approach to improving reproductive efficiency, maternal-fetal health, and sustainable pig production.

4. General conclusion

Overall, this thesis supports the view that maternal metabolic homeostasis is an important regulatory axis linking dietary protein input, placental functional adaptation, fetal development, and pregnancy outcomes. In multiparous sows, changes in dietary protein level within a commercial range during early gestation mainly induced maternal metabolic responses, but did not markedly alter litter outcomes. This suggests that, when basal protein and amino acid supply is adequate, reproductive traits related to litter size show a certain degree of robustness to moderate changes in dietary protein. In contrast, the mouse model further showed that sustained and stronger high-protein/low-carbohydrate macronutrient imbalance may exceed placental adaptive capacity, disturb placental redox homeostasis, mitochondrial function, and nutrient transport, and eventually impair fetal growth. These findings suggest that the placenta may act as a key buffering and amplifying site through which maternal nutritional disturbance is transformed from a metabolic response into abnormal fetal development. Early-gestation CDCA supplementation was associated with improved maternal metabolic status and reproductive performance, and beneficial effects remained detectable during late gestation. Compared with the supply of a single nutrient, maternal metabolic homeostasis may

represent a key integrative mechanism that links nutritional input, placental functional adaptation, fetal development, and reproductive performance. Therefore, future gestational nutrition strategies for sows should place greater emphasis on precise protein and amino acid supply, maintenance of maternal metabolic balance, and metabolism-targeted interventions, in order to improve sow reproductive efficiency, promote maternal-fetal health, and support sustainable pig production.

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Appendices

Scientific publications

Publications

1. **Zhao Y[#]**, Cai L[#], He L, Gao G, Schroyen M, Pi Y, Sun W, Ming D, Li Y, Li X*. Maternal high-protein diet impairs fetal growth through placental dysfunction in mice. *Placenta*. 2026 Mar 24;178:30-38.
2. **Zhao Y[#]**, He L[#], Ming D, Gao G, Schroyen M, Pi Y, Sun W, Li Y, Li X. Effects of dietary protein levels during early gestation on reproductive performance, oxidative stress, and placental nutrient transport in sows. *Italian Journal of Animal Science*. 2026 Dec 31;25(1):468-78.
3. **Zhao Y**, Wang X, Ma Q, Liu E, Zhang D, Liu H, Cai L, Wang J, Feng T, Schroyen M, and Chen M*. Persistent Benefits of Early Gestational Chenodeoxycholic Acid Supplementation on Late Pregnancy in Sows via Sustained Modulation of the Gut-Metabolism Axis. *Journal of nutrition*. 2026 Jun 10:101664.
4. Cai L[#], **Zhao Y[#]**, Chen W, Li Y, Han Y, Zhang B, Pineda L, Li X, Jiang X. Effect of an organic acid blend as an antibiotic alternative on growth performance, antioxidant capacity, intestinal barrier function, and fecal microbiota in weaned piglets. *Journal of Animal Science*. 2024 Jan 3;102:skae149.
5. Chen M[#], **Zhao Y[#]**, Li S, Chang Z, Liu H, Zhang D, Wang S, Zhang X, Wang J. Maternal Malic Acid May Ameliorate Oxidative Stress and Inflammation in Sows through Modulating Gut Microbiota and Host Metabolic Profiles during Late Pregnancy. *Antioxidants*. 2024 Feb 19;13(2):253.
6. Chen M, **Zhao Y**, Ji H, Li L, Liu H, Wang S, Zhang D, Yin J, Wang J, Zhang X. Chenodeoxycholic Acid Improves Embryo Implantation and Metabolic Health through Modulating Gut Microbiota-Host Metabolites Interaction during Early Pregnancy. *Antioxidants*. 2023 Dec 19;13(1):8.
7. **Ying Zhao**, Long Cai, Martine Schroyen, Dongxu Ming, Wenjuan Sun, Yu Pi, Yanpin Li and Xilong Li. Matrix value of a novel phytase in weaned piglets fed Ca and P-deficient diet. (To be submitted)