Effects of Long-term Feeding of Diets with Different Protein Levels on Lipid Metabolism-Related Gene Expression in Pigs (Postprint)

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Abstract

This study aimed to investigate the effects of long-term feeding of diets with different protein levels on the expression of genes related to lipid metabolism in pigs. Eighteen crossbred (Duroc × Landrace × Yorkshire) 28-day-old weaned piglets were randomly divided into three groups, with six replicates per group and one pig per replicate. The control group [high crude protein (HCP) group] was fed a diet meeting the NRC (2012) recommended nutritional requirements, while the experimental groups were formulated according to NRC (2012) standards, with supplementation of four essential amino acids—lysine (Lys), methionine (Met), threonine (Thr), and tryptophan (Try)—and dietary nitrogen levels reduced by 3% [medium crude protein (MCP) group] and 6% [low crude protein (LCP) group], respectively. The experimental period lasted 125 days. The results showed that: 1) In the liver, compared with the HCP group, the LCP group significantly decreased the expression of fatty acid synthesis-related genes acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), malic enzyme 1 (ME1), and ankyrin 1 (ANK1) ($P<0.05$); simultaneously significantly increased the expression of fatty acid transport-related genes peroxisome proliferator-activated receptor gamma (PPARγ) and fatty acid-binding protein (FABP) ($P<0.05$); whereas the MCP group showed no significant differences in the expression of fatty acid synthesis- and transport-related genes compared with the HCP group ($P>0.05$); compared with the HCP group, both MCP and LCP groups significantly decreased the expression of fatty acid oxidation and catabolism-related genes peroxisome proliferator-activated receptor alpha (PPARα) and carnitine palmitoyltransferase (CPT) ($P<0.05$). 2) In the longissimus dorsi muscle (LDM), the expression of fatty acid synthesis-related genes sterol regulatory element-binding protein (SREBP), FAS, and stearoyl-CoA desaturase (SCD) in the MCP group was significantly higher than in the other two groups ($P<0.05$); the expression of ACC and FABP in the LCP group was significantly

lower than in the other two groups (P<0.05); the expression of CPT gene and intramuscular fat (IMF) content in LDM were significantly lower in both LCP and MCP groups compared with the HCP group (P<0.05). 3) In both liver and LDM, no significant differences were observed in the expression of adipose triglyceride lipase (ATGL) and enoyl-CoA reductase (DECR) genes among all groups (P>0.05). These results indicate that, based on NRC (2012), appropriately reducing dietary protein level (by 3%) can promote the expression of fatty acid synthesis- and transport-related genes in LDM, but has no significant effect on the expression of fatty acid synthesis-related genes in the liver; appropriately reducing dietary protein level (by 3%) can decrease the expression of fatty acid oxidation and catabolism-related genes in both LDM and liver, but does not increase intramuscular fat content in LDM.

Full Text

Effects of Long-Term Feeding of Diets with Different Protein Levels on the Expression of Genes Related to Lipid Metabolism in Pigs

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Abstract: This experiment was conducted to investigate the effects of long-term feeding of diets with different protein levels on the expression of genes related to lipid metabolism in pigs. Eighteen cross-bred (Duroc × Landrace × Yorkshire) piglets weaned at 28 days of age were randomly assigned to three groups with six replicates per group and one pig per replicate. The control group [high crude protein (HCP) group] was fed a diet formulated according to NRC (2012) recommendations. The experimental groups were fed diets with crude protein levels reduced by 3% [medium crude protein (MCP) group] and 6% [low crude protein (LCP) group] relative to the NRC (2012) standard, supplemented with four essential amino acids (lysine, methionine, threonine, and tryptophan). The experimental period lasted 125 days. The results showed that: 1) In the liver, compared with the HCP group, the LCP group significantly decreased the expression of fatty acid synthesis-related genes including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), malic enzyme 1 (ME1), and ankyrin 1 (ANK1) ($P < 0.05$), while significantly increasing the expression of fatty acid transport-related genes peroxisome proliferator-activated receptor gamma (PPARγ) and fatty acid binding protein (FABP) ($P < 0.05$). However, there were no significant differences in the expression of fatty acid synthesis and transport-related genes between the MCP and HCP groups (P

> 0.05). Both MCP and LCP groups significantly decreased the expression of fatty acid oxidation-related genes PPARα and carnitine palmitoyltransferase (CPT) compared with the HCP group ($P < 0.05$). 2) In the longissimus dorsi muscle (LDM), the MCP group showed significantly higher expression of fatty acid synthesis-related genes sterol regulatory element binding protein (SREBP), FAS, and stearoyl-CoA desaturase (SCD) than the other two groups ($P < 0.05$). The expression of ACC and FABP in the LCP group was significantly lower than in the other two groups ($P < 0.05$). The expression of CPT gene and intramuscular fat (IMF) content in LDM were significantly lower in both LCP and MCP groups compared with the HCP group ($P < 0.05$). 3) In both liver and LDM, there were no significant differences in the expression of adipose triglyceride lipase (ATGL) and 2,4-dienoyl-CoA reductase (DECR) genes among groups ($P > 0.05$). In conclusion, appropriately reducing dietary crude protein level (by 3%) can promote the expression of fatty acid synthesis and transport-related genes in LDM, but has no significant effect on the expression of fatty acid synthesis-related genes in the liver. Appropriate reduction of dietary protein level (by 3%) can decrease the expression of fatty acid oxidation-related genes in both LDM and liver, but does not increase IMF content in LDM.

Keywords: diet; protein; finishing pigs; lipid; metabolism; gene

Introduction

Pork is a primary source of dietary protein for humans, and China is a major producer and consumer of pork. Dietary protein level is one of the most important economic and environmental factors affecting the swine industry. Currently, most dietary protein levels far exceed the requirements for animal growth and development, which not only increases production costs and reduces nitrogen utilization efficiency but also intensifies environmental pressure from pig farming. Dietary protein plays crucial nutritional and physiological roles in animal growth and development. Previous studies have shown that low-protein diets supplemented with essential amino acids can achieve similar nitrogen absorption and body weight (BW) as standard diets [1-3], while other research indicates that reducing dietary protein level can decrease growth performance but increase fat content and improve meat quality [4-5]. Tian et al. [6] reported that reducing dietary protein level from 20% to 17% significantly decreased growth performance while improving intestinal digestion and absorption in weaned piglets. Madeira et al. [4] found that reducing dietary protein from 16% to 13% increased backfat thickness and improved pork quality traits including intramuscular fat (IMF) content and juiciness, but reduced growth performance in finishing pigs. They also noted that low-protein diets supplemented with arginine did not affect muscle IMF content, whereas those supplemented with leucine increased IMF content. However, other studies have shown that arginine supplementation affects pork quality by reducing total fat deposition, increasing muscle IMF content, and improving muscle antioxidant capacity [7-8]. Tous et al. [3] reported that

reducing dietary protein level from 12% to 9.8% in 62–97 kg pigs did not affect growth performance but increased muscle IMF content, and that lysine supplementation improved pork quality without affecting growth performance.

As living standards improve, pork quality has become a primary concern for consumers. Fat and fatty acid composition are important determinants of pork eating quality, with IMF content playing a particularly crucial role. Current feeding strategies to improve fat distribution in pigs involve reducing dietary protein and supplementing with amino acids [9-11]. IMF content is a key factor affecting sensory quality traits such as taste and flavor, as well as influencing drip loss and juiciness [12-14]. In recent decades, selection for lean pigs has reduced IMF by nearly 1%, which is a major cause of deteriorating meat quality [15], leading to increasing attention on IMF content [16-18]. Numerous studies have focused on promoting IMF deposition, with reducing dietary protein being a typical approach [19-22]. Therefore, this experiment investigated the effects of reducing dietary protein levels across growth stages on the expression of genes related to lipid metabolism, providing a theoretical basis for reducing production costs and nitrogen emissions while improving pork quality.

1.1 Experimental Animals and Design

Eighteen cross-bred (Duroc × Landrace × Yorkshire) piglets weaned at 28 days of age [initial body weight = (9.57 ± 0.64) kg] were randomly assigned to three groups with six replicates per group and one pig per replicate. Pigs were housed individually in pens. The control group [high crude protein (HCP) group] was fed a diet formulated according to NRC (2012) recommendations. The experimental groups were fed diets with nitrogen levels reduced by 3% [medium crude protein (MCP) group] and 6% [low crude protein (LCP) group] relative to the NRC (2012) standard, supplemented with four essential amino acids (lysine, methionine, threonine, and tryptophan). After a 7-day pre-trial period, the formal experiment began with phased feeding until the end of the finishing period. During the piglet stage (10–30 kg), the LCP, MCP, and HCP groups were fed diets with crude protein levels of 14%, 17%, and 20%, respectively, for 45 days. During the growing stage (30–60 kg), they were fed diets with crude protein levels of 12%, 15%, and 18%, respectively, for 30 days. During the finishing stage (60–90 kg), they were fed diets with crude protein levels of 10%, 13%, and 16%, respectively, for 50 days (Table 1). After the feeding trial, pigs were slaughtered and samples were collected. Diet composition and nutrient levels are shown in Table 2 . Pigs had free access to feed and water throughout the experiment.

Table 1 Dietary crude protein levels and feeding duration

Items	Dietary crude protein level/%	Feeding time/d
	LCP group	MCP group
Piglets (10–30 kg)	14	17

Items	Dietary crude protein level/%	Feeding time/d
Growing pig (30-60 kg)	12	15
Finishing pig (60-90 kg)	10	13

Table 2 Composition and nutrient levels of experimental diets (air-dry basis), %

Items	Piglets (10-30 kg)	Growing pig (30-60 kg)	Finishing pig (60-90 kg)
	LCP	MCP	HCP
Ingredients			
Corn	68.00	62.00	56.00
Soybean meal	22.00	28.00	34.00
Whey powder	5.00	5.00	5.00
Fish meal	2.00	2.00	2.00
Wheat bran	-	-	-
Soybean oil	0.50	0.50	0.50
Lysine	0.40	0.40	0.40
Methionine	0.10	0.10	0.10
Threonine	0.10	0.10	0.10
Tryptophan	0.03	0.03	0.03
Calcium hydrogen phosphate	0.80	0.80	0.80
Limestone	0.80	0.80	0.80
NaCl	0.27	0.27	0.27
Premix[1])	1.00	1.00	1.00
Total	100.00	100.00	100.00
Nutrient levels[2])			
DE/(MJ/kg)	14.23	14.23	14.23
CP	14.00	17.00	20.00
Ca	0.70	0.70	0.70
TP	0.62	0.62	0.62

[1]) The premix provided the following per kilogram of diet: VA 10,800 IU, VD_3 4,000 IU, VE 40 IU, VK_3 4 mg, VB_1 6 mg, VB_2 12 mg, VB_6 6 mg, VB_{12} 0.05 mg, biotin 0.2 mg, folic acid 2 mg, niacin 50 mg, D-calcium pantothenate 25 mg, Fe (as ferrous sulfate) 100 mg, Cu (as copper sulfate) 150 mg, Mn (as manganese oxide) 40 mg, Zn (as zinc oxide) 100 mg, I (as potassium iodide) 0.5 mg, Se (as sodium selenite) 0.3 mg.

[2]) DE was a calculated value, while the others were measured values.

1.2 Slaughter and Sample Collection

Prior to slaughter, finishing pigs were stunned by electric shock. Immediately after death, tissues and organs were separated and placed on ice. Approximately 80 g of longissimus dorsi muscle (LDM) was collected in sealed bags and stored at -80 °C for IMF content analysis. Three grams of LDM were divided into three 1.5 mL EP tubes, immediately frozen in liquid nitrogen, and subsequently transferred to -80 °C storage. Liver tissue (approximately 3 g, with consistent sampling location) was rinsed with precooled phosphate-buffered saline (PBS) to remove surface blood, blotted dry with absorbent paper, divided into three 1.5 mL EP tubes, immediately frozen in liquid nitrogen, and finally transferred to -80 °C storage.

1.3 Determination of IMF Content

After removing fascia, LDM samples were minced. Forty grams of muscle sample were placed in a petri dish, spread evenly, and frozen overnight at -80 °C. Samples were then lyophilized in a freeze dryer and weighed. Three grams of lyophilized sample were measured using a filter cartridge, and IMF content was determined by Soxhlet extraction using a fat analyzer (FOSS-2055 SOXTEC). The calculation formula was as follows:

IMF content (%) = 100 × (weight of oil cup -weight of aluminum cup) / sample weight.

1.4 Measurement of Gene Expression Related to Lipid Metabolism

Genes related to lipid metabolism and meat quality included peroxisome proliferator-activated receptor gamma (PPARγ), peroxisome proliferator-activated receptor alpha (PPARα), fatty acid synthase (FAS), sterol regulatory element binding protein (SREBP), fatty acid binding proteins (FABP), carnitine palmitoyltransferase (CPT), stearoyl-CoA desaturase (SCD), adipose triglyceride lipase (ATGL), acetyl-CoA carboxylase (ACC), malic enzyme 1 (ME1), 2,4-dienoyl-CoA reductase (DECR), and ankyrin 1 (ANK1). Total RNA was extracted from 100 mg of liver tissue or LDM samples using TRIzol reagent (TaKaRa, Japan) and dissolved in RNase-free water. Total RNA (1 μg) was reverse-transcribed to cDNA using a reverse transcription kit (TaKaRa, Japan) according to the manufacturer' s instructions. Primers were designed using Primer Premier 5.0 based on porcine gene sequences from GenBank (Table 3).

Real-time quantitative PCR standard curves were constructed to determine optimal reaction conditions. The reaction system (20 μL) contained 2 μL cDNA, 10 μL SYBR Green 2× mix, 0.8 μL each of forward and reverse primers (100 nmol/L), and 6.4 μL ddH_2O. Reaction conditions were: pre-denaturation at 95 °C for 3 min; 39 cycles of denaturation at 95 °C for 15 s, annealing for 30 s, and extension at 72 °C for 30 s. After screening housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin was selected as the internal reference gene for real-time quantitative PCR due to its more stable expression. Gene expression was calculated using the $2^{-}\Delta\Delta$Ct method.

Table 3 Primer sequences for real-time qPCR

Genes | Primer sequences (5' $→$3') | Size/bp | Accession No. | Tm/℃ |

|—-|————————-|——|———|—| | SREBP | F: GCGACGGTGCCTCTGGTAGT R: CGCAAGACGGCGGATTTA | 55-68 | AF102873 | | | ACC | F: ATGTTTCGGCAGTCCCTGAT R: GTGGACCAGCTGACCTTGA | | EF618729 | | | FAS | F: AGCCTAACTCCTCGCTGCAAT R: TCCTTGGAACCGTCTGTGTTC | | AY183428 | | | ME1 | F: ATCCAACCAGCAAAGCAGA R: CACAACACCAAGGGCAACT | | XM001924333.4 | | | SCD | F: TCTGGGCGTTTGCCTACTATCT R: TCTTTGACGGCTGGGTGTTT | | AY487829 | | | FABP | F: GCCTTCACCCAGACCTGATA R: GGCTTGAGATCACTGCTTCC | | XM001928667.1 | | | PPARγ | F: AAGACGGGGTCCTCATCTCC R: CGCCAGGTCGCTGTCATCT | | NM214379 | | | PPARα | F: CAGGAAAGTCAAGAGCACCACAG R: TCGGGACAATACATCCAACA | | AJ416020 | | | CPT | F: TCAAGAGCCTGAGGAAACCC R: CAAATGATAGCAGCCACAAAGAG | | NM_{001044526}.1 | | | ATGL | F: ATGGTGGGCGACTAACT R: TGCCTGCTGTCTGTGAG | | AY181062 | | | DECR | F: AGTTCAGCCTGCGCAACCTC R: AGGGCACCATCATGGCTG | | EF583921 | | | β-actin | F: CACGCCATCCTGCGTCTGGA R: AGCACCGTGTTGGCGTAGAG | | NM001190232.2 | |

1.5 Statistical Analysis

Experimental data were analyzed using Prism 6 software by one-way ANOVA. Data are expressed as mean ± standard error (mean ± SE). Differences were considered significant at $P < 0.05$.

2.1 Effects of Dietary Protein Level on IMF Content in LDM

As shown in Figure 1 [Figure 1: see original paper], IMF content in LDM was significantly higher in the HCP group than in the LCP and MCP groups ($P < 0.05$), while there was no significant difference between the LCP and MCP groups ($P > 0.05$).

Figure 1 Effects of dietary protein level on IMF content in LDM of pigs

2.2 Effects of Dietary Protein Level on Expression of Lipid Metabolism-Related Genes in Liver

As shown in Table 4 , in the liver, SREBP, ACC, ME1, SCD, and ANK1 showed consistent expression trends, while FABP showed the opposite trend. Compared with the LCP group, the MCP and HCP groups showed increased expression of SREBP, ACC, FAS, ME1, and SCD, with FAS, ACC, and ME1 being significantly higher ($P < 0.05$). ANK1 expression in the HCP group was significantly higher than in the LCP group ($P < 0.05$), but showed no significant difference between the MCP group and the other two groups ($P > 0.05$). In the liver, PPARγ and FABP showed similar expression trends, with the LCP group being significantly higher than the MCP and HCP groups ($P < 0.05$), but no significant difference between the MCP and HCP groups ($P > 0.05$). PPARα and CPT expression showed no significant difference between the LCP and MCP groups ($P > 0.05$), but both were significantly lower than in the HCP group ($P < 0.05$). Dietary protein level had no significant effect on hepatic expression of SREBP, SCD, ATGL, and DECR genes ($P > 0.05$).

Table 4 Effects of dietary protein level on expressions of genes related to lipid metabolism in hepatic tissue of pigs

Items	LCP group	MCP group	HCP group	P-value
SREBP	0.56 ± 0.09	0.88 ± 0.28	1.00 ± 0.12	
ACC	0.50 ± 0.02	0.91 ± 0.10	1.00 ± 0.09	< 0.0001
FAS	0.50 ± 0.05	1.12 ± 0.16	1.00 ± 0.07	< 0.0001
ME1	0.69 ± 0.05	1.00 ± 0.08	1.00 ± 0.01	< 0.0001
SCD	0.62 ± 0.07	0.86 ± 0.22	1.00 ± 0.04	
ANK1	0.54 ± 0.15	0.70 ± 0.08	1.00 ± 0.08	
PPARγ	1.28 ± 0.09	0.81 ± 0.01	1.00 ± 0.03	
FABP	1.66 ± 0.14	1.02 ± 0.05	1.00 ± 0.09	
PPARα	0.80 ± 0.07	0.71 ± 0.02	1.00 ± 0.04	
CPT	0.69 ± 0.07	0.67 ± 0.06	1.00 ± 0.08	
ATGL	0.92 ± 0.17	0.99 ± 0.05	1.00 ± 0.15	
DECR	1.12 ± 0.11	1.07 ± 0.06	1.00 ± 0.05	

Values in the same row with different superscripts differ significantly ($P < 0.05$), while those with the same or no superscript do not differ significantly ($P > 0.05$).

2.3 Effects of Dietary Protein Level on Expression of Lipid Metabolism-Related Genes in LDM

As shown in Table 5 , in LDM, SREBP, ACC, FAS, SCD, FABP, and PPARα showed similar expression trends, with highest expression in the MCP group compared to LCP and HCP groups. Specifically, SREBP, FAS, and SCD expression in the MCP group was significantly higher than in the other two groups

($P < 0.05$). ACC and FABP expression in the LCP group was significantly lower than in the MCP and HCP groups ($P < 0.05$), but showed no significant difference between the MCP and HCP groups ($P > 0.05$). ME1 expression in the HCP group was significantly higher than in the LCP group ($P < 0.05$), while showing no significant difference between the MCP group and the other two groups ($P > 0.05$). ANK1 expression showed no significant difference between the LCP and MCP groups ($P > 0.05$), but both were significantly higher than in the HCP group ($P < 0.05$). There were no significant differences in PPARα and PPARγ expression among the three groups ($P > 0.05$). CPT expression in the HCP group was significantly higher than in the other two groups ($P < 0.05$), but showed no significant difference between the MCP and LCP groups ($P > 0.05$). Dietary protein level had no significant effect on ATGL and DECR gene expression in LDM ($P > 0.05$).

Table 5 Effects of dietary protein level on expressions of genes related to lipid metabolism in LDM of pigs

Items	LCP group	MCP group	HCP group	P-value
SREBP	1.04 ± 0.08	1.49 ± 0.18	1.00 ± 0.09	
ACC	0.74 ± 0.10	1.11 ± 0.05	1.00 ± 0.10	
FAS	0.74 ± 0.20	1.74 ± 0.26	1.00 ± 0.19	
ME1	0.80 ± 0.04	0.89 ± 0.02	1.00 ± 0.07	
SCD	0.96 ± 0.30	2.54 ± 0.33	1.00 ± 0.17	
ANK1	1.97 ± 0.11	1.85 ± 0.18	1.00 ± 0.14	
PPARγ	1.24 ± 0.04	1.05 ± 0.11	1.00 ± 0.09	
FABP	0.69 ± 0.10	1.14 ± 0.06	1.00 ± 0.05	
PPARα	0.96 ± 0.10	1.15 ± 0.12	1.00 ± 0.09	
CPT	0.64 ± 0.12	0.56 ± 0.06	1.00 ± 0.03	
ATGL	1.00 ± 0.03	0.93 ± 0.04	1.00 ± 0.02	
DECR	0.92 ± 0.09	0.96 ± 0.06	1.00 ± 0.05	

3.1 Effects of Dietary Protein Level on Growth Performance of Pigs

Under current swine production conditions, reducing dietary protein level can decrease both production costs and nitrogen emissions due to ecological and economic pressures. Some studies have shown that low-protein diets can reduce nitrogen excretion and ammonia emission without affecting growth performance [23-25], while others have reported that reducing dietary protein level negatively affects growth performance [21,26-27]. Additional research has demonstrated that reducing dietary protein level affects growth performance in piglets and growing pigs but not in finishing pigs when essential amino acids (lysine, methionine, threonine, tryptophan) are supplemented [6,28]. Another study from our research group showed that growth performance decreased with reduced dietary protein levels in weaned piglets, and this effect persisted into the growing stage, though continuous feeding of low-protein diets did not affect average daily

gain, average daily feed intake, or feed-to-gain ratio in finishing pigs [29]. Our research also indicated that reducing dietary protein level during the early post-weaning period is detrimental to piglets in overcoming weaning stress and affects their growth performance [6]. The negative effects of low-protein diets on piglet performance may be attributed to the special physiological state during weaning, which involves nutritional, environmental, and psychological stress. After weaning, changes in energy sources, intestinal damage, alterations in gut microbiota, and loss of maternal antibodies cause low immunity and affect physiological growth. Even with balanced lysine, methionine, threonine, and tryptophan, low-protein diets may be deficient in other essential amino acids, failing to meet nutritional requirements and thereby reducing growth performance. As feeding duration extended, growth performance continued to decline with reduced protein levels, possibly because low-protein diets decreased feed intake, which hindered intestinal repair, adaptation to post-weaning changes, and tolerance to weaning stress, consequently affecting digestive and absorptive functions in growing pigs. Since feed intake is a key determinant of daily gain, Brillouet [31] demonstrated that reduced daily gain in piglets affects subsequent growth. However, when feeding continued to the finishing stage, low-protein diets still had persistent negative effects on growth performance, whereas medium-protein diets did not significantly affect finishing pig performance. This indicates that excessively low protein is detrimental to pig growth, while moderately reduced protein does not affect finishing pigs. These findings further confirm that continuous feeding of low-protein diets is unfavorable for early growth, and excessively low dietary protein levels are detrimental to overall pig growth.

3.2 Effects of Dietary Protein Level on Hepatic Lipid Metabolism-Related Gene Expression

Muscle fat content and composition affect pork quality. Animals acquire fat through two pathways: dietary fat absorbed as glycerol and fatty acids after small intestine digestion, and endogenous synthesis in the liver from carbohydrate and amino acid metabolic intermediates. The liver contains abundant lipid metabolism-related enzymes and is a major site for fatty acid synthesis, playing an important role in lipid metabolism. Therefore, hepatic lipid metabolism influences intramuscular lipid metabolism and consequently affects meat quality.

Peroxisome proliferator-activated receptors (PPAR) and SREBP are important transcription factors regulating lipid metabolism [10,32]. PPARα and PPARγ belong to the PPAR superfamily and regulate free fatty acid (FFA) oxidation and absorption through mitochondrial and peroxisomal β-oxidation, participating in lipid metabolism regulation [33-34]. SREBP acts as a candidate gene for lipid synthesis, regulating the expression of FAS, ME1, ACC, and other genes [33,35-39]. Our results showed that low-protein diets decreased hepatic expression of SREBP, FAS, ME1, and ACC, with FAS, ME1, and ACC being significantly lower than in the other two groups, while no significant differences were observed between the medium- and high-protein groups. Since SREBP,

FAS, ME1, and ACC are involved in regulating *de novo* FFA synthesis, low-protein diets affect hepatic FFA synthesis by reducing *de novo* synthesis [33,40]. The lack of significant differences between medium- and high-protein groups indicates that appropriately reducing dietary protein level does not affect hepatic expression of fatty acid synthesis-related genes.

PPARγ regulates adipocyte differentiation, transport, and expression of proteins related to fat deposition, while FABP promotes fatty acid and other lipid mediator transport through cell membrane structures [41-43] and facilitates transport of fatty acids and lipophilic molecules from the cytoplasm to nuclear receptors [44-45]. Our results showed that PPARγ and FABP, genes involved in fatty acid transport, exhibited opposite expression trends to fatty acid synthesis genes (SREBP, FAS, ME1, ACC). The LCP group showed significantly higher PPARγ and FABP expression than the other two groups, suggesting that excessively reducing dietary protein promotes hepatic fatty acid transport by upregulating the transcription factor PPARγ and increasing FABP expression [46]. These results demonstrate that low-protein diets reduce hepatic fatty acid synthesis while increasing fatty acid transport, whereas appropriately reduced protein diets do not significantly differ from high-protein diets in hepatic fatty acid synthesis and transport processes.

PPARα regulates mitochondrial and peroxisomal fatty acid oxidation, ketogenesis, and gluconeogenesis by controlling expression of lipid metabolism genes, thereby affecting lipid metabolism rate [47-48]. The significantly higher hepatic expression of PPARα and CPT in the HCP group compared to the LCP and MCP groups indicates that low-protein diets are detrimental to hepatic fatty acid oxidation and metabolism. Therefore, appropriately reducing dietary protein level does not affect hepatic fatty acid synthesis and transport gene expression but decreases fatty acid oxidation, suggesting that moderate protein reduction promotes hepatic fatty acid anabolism while reducing catabolism, thereby increasing fatty acid transport from liver to other tissues.

3.3 Effects of Dietary Protein Level on Lipid Metabolism in LDM

IMF affects sensory quality traits such as meat texture, color, and flavor. Muscle IMF content is closely related to muscle lipid metabolism, depending on fatty acid synthesis, degradation, transport, and deposition [49]. Therefore, studying muscle lipid metabolism provides a theoretical basis for improving pork quality. Our results showed that the MCP group had the highest expression of SREBP, ACC, FAS, SCD, and FABP in LDM. Specifically, SREBP, FAS, and SCD expression in the MCP group was significantly higher than in the LCP and HCP groups, while ACC and FABP expression was significantly higher than in the LCP group but not different from the HCP group. These results indicate that appropriately reducing dietary protein level promotes fatty acid synthesis and transport in LDM. ME1 expression in the HCP group was significantly higher than in the LCP group, with no significant difference between the MCP group and the other two groups, suggesting that low-protein diets reduce *de*

novo fatty acid synthesis in both liver and LDM, while medium-protein diets have no significant effect on *de novo* synthesis in LDM. Previous studies have shown that reducing dietary protein level in growing and finishing pigs promotes expression of fatty acid synthesis genes such as ACC, FAS, and ME1 [32,40], indicating that long-term excessive protein reduction is detrimental to muscle fatty acid synthesis. Doran et al. [50] reported that low-protein diets induce SCD expression in muscle, promoting IMF deposition. However, our results showed that although appropriately reduced protein induced expression of fatty acid synthesis genes like SCD in LDM, it simultaneously decreased IMF content.

Teye et al. [51] reported that low-protein diets improve pork quality traits including tenderness, juiciness, and IMF content, while increasing fatty acid oxidation. Our study obtained opposite results: reducing dietary protein level did not affect expression of fatty acid oxidation genes ATGL and DECR in liver and muscle but decreased CPT expression in LDM. This suggests that low-protein diets reduce long-chain fatty acid oxidation rather than affecting triglyceride hydrolysis or unsaturated fatty acid oxidation in liver and muscle. The lack of significant differences in PPARα expression among the three protein levels and inconsistent expression trends between PPARγ and FABP indicate that PPAR regulation of fatty acid oxidation and transport is tissue-specific, and other pathways may co-regulate fatty acid metabolism in muscle.

Zhao et al. [49] reported that IMF content depends on the rates of fatty acid synthesis, transport, and degradation. Our results showed that the MCP group had higher expression of fatty acid synthesis genes (SREBP, ACC, FAS, SCD) and transport protein FABP in LDM, with no significant changes in oxidation genes ATGL and DECR, while CPT expression was significantly lower than in the HCP group. However, IMF content was significantly lower than in the HCP group. Reducing dietary protein decreased ANK1 expression in liver but increased it in muscle. Ankyrins (ANK) are an important family of structural proteins, with ANK1 being a functional candidate gene related to meat quality, particularly affecting IMF and water-holding capacity [52-53]. Increased ANK1 expression in muscle induced by low-protein diets may reduce aggregation of lipid droplets (a major IMF component) by enhancing cytoskeletal protein hydrolysis and rearrangement, thereby decreasing IMF content in LDM. This suggests that ANK1 may be a determinant of IMF deposition. These findings indicate that IMF deposition may be regulated not only by fatty acid synthesis, transport, and degradation but also closely related to metabolism of the three major nutrients: protein, carbohydrates, and lipids [54-56]. Hamill et al. [56] proposed that in muscle tissues with high IMF content, many fatty acid oxidation pathway genes are downregulated, and high IMF content inhibits fatty acid turnover in muscle, thereby affecting fat deposition.

Reducing dietary protein level affects growth performance in piglets and growing pigs. Even though long-term feeding of MCP diets did not affect finishing pig growth performance, it reduced IMF content in LDM. The early growth stage, particularly in weaned piglets, is not suitable for dietary protein reduction, as

feeding low-protein diets during the initial post-weaning period may lead to decreased meat quality in finishing pigs. Gondret et al. [57-58] also reported that long-term protein reduction decreases IMF content and suggested that IMF accumulation depends on the balance between fatty acid synthesis in adipocytes and fatty acid oxidation in muscle fibers—essentially, fatty acid turnover. Therefore, long-term moderate reduction of dietary protein level can increase fatty acid synthesis and transport while decreasing degradation in LDM, yet fails to increase IMF content, indicating that IMF improvement involves not only lipid metabolism but also other nutrient metabolic pathways. Even though medium-protein diets increase fatty acid synthesis, reducing protein level throughout the piglet-to-finishing stage may affect protein and fatty acid metabolism, failing to meet body requirements for protein and fatty acids and leading to conversion of fatty acids and carbohydrates to amino acids, which affects muscle fat deposition. During the finishing stage, appropriately reducing dietary protein level without affecting growth performance may allow for modulation of dietary nutrients and ratios, such as adding specific amino acids, to increase IMF content and improve meat quality. Therefore, using dietary protein reduction to regulate pork quality requires consideration of not only crude protein level but also the magnitude of reduction, types of supplemented amino acids, timing, and duration to achieve both reduced nitrogen emissions and feeding costs while improving pork quality.

In summary: 1) Appropriately reducing dietary protein level (by 6%) had no significant effect on the expression of fatty acid synthesis-related genes in the liver. 2) Reducing dietary protein level throughout the piglet-to-finishing stage promoted expression of fatty acid synthesis and transport-related genes in LDM but significantly decreased IMF deposition in porcine LDM, which is unfavorable for meat quality improvement.

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