Low-protein diets supplemented with isoleucine alleviate lipid deposition in broilers through activating 5′ adenosine monophosphate-activated protein kinase and janus kinase 2/signal transducer and activator of transcription 3 signaling pathways

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ABSTRACT This study aimed to evaluate the effect of isoleucine (**Ile**) on growth performance, meat quality and lipid metabolism of broilers fed a low-protein diet (**LPD**). The 396 one-day-old male Cobb broilers were allocated to 4 treatment groups as follows: control diet (**CON**), LPD, LPD + 0.13% Ile (**LPD-LI**) and LPD + 0.26% Ile (**LPD-HI**), with nine replicates of 11 broilers each for 42 d. The Ile increased average daily gain, average daily feed intake, fiber density and the mRNA level of myosin heavy chain (***MyHC***)***-I*** in breast muscle, and decreased feed to gain ratio, shear force, fiber diameter and the mRNA level of *MyHC-IIb* in breast muscle, which were impaired by the LPD. Compared to the LPD group, broilers in LPD-LI and LPD-HI groups had lower serum lipid levels, liver fat content, abdominal adipose percentage and mRNA levels of peroxisome proliferator-activated receptor-γ, CCAAT/enhancer binding protein-α, ki-67, topoisomerase II alpha (***TOP2A***) and thioredoxin-dependent peroxidase 2 in abdominal adipose and liver X receptors-α, sterol regulatory element binding protein 1 (***SREBP1***), acetyl-CoA carboxylase (***ACC***) and fatty acid synthase (***FAS***) in liver, and higher mRNA levels of peroxisome proliferator activated receptor-α, carnitine palmitoyl-transferase 1 (***CPT-1***), and acyl-CoA oxidase 1 (***ACOX1***) in liver, which were equal to the CON levels. A LPD supplemented with Ile decreased enzyme activities of ACC and FAS in liver and glycerol-3-phosphate dehydrogenase and TOP2A in abdominal adipose, and increased enzyme activities of CPT-1 and ACOX1 in liver. Furthermore, Ile supplementation enhanced the mRNA level of leptin receptor and protein levels of phospho-5′ adenosine monophosphate-activated protein kinase (**AMPK**), mechanistic target of rapamycin, ribosomal protein 70 S6 kinase, janus kinase 2 (**JAK2**), and signal transducer and activator of transcription 3 (**STAT3**), and decreased the protein level of SREBP1 in the liver of broilers in LPD group. In conclusion, dietary supplementation with Ile to 0.83% could improve growth performance and meat quality and alleviate lipid deposition of broilers fed a LPD through activating AMPK and JAK2/STAT3 signaling pathways.

Key words: broiler, isoleucine, lipid metabolism, liver, low-protein diet

INTRODUCTION

In order to save protein resources and reduce nitrogen excretion, a low-protein diet (**LPD**) is proposed, with the dietary crude protein content reduced by 2% to 3% (Kidd et al., 2021). However, accumulating evidence demonstrated that a LPD depresses growth and deteriorates meat quality of broilers characterized by incremental drip loss and increased muscle fiber area (Chodová et al., 2021; Wang et al., 2022). More importantly, a LPD lacks adequate amounts of amino acids required for lipoprotein synthesis, thereby impeding lipid transport from the liver and disrupting the dynamic balance of lipid metabolism in the liver, blood, and peripheral tissues, eventually leading to excessive lipid deposition (Sigolo et al., 2017). Branched-chain amino acids (**BCAA**), including leucine, valine, and isoleucine (**Ile**), are important nutrient regulators of protein synthesis and strongly associated with glucose and lipid metabolism (Nie et al., 2018). Numerous studies have reported that BCAA supplemented in LPDs could

alleviate the lipid deposition in adipose tissue and liver of pigs (Li et al., 2017; Duan et al., 2018; Heng et al., 2020; Zhang et al., 2021). However, the effect and mechanism of BCAA supplemented in LPDs on regulating lipid metabolism in broilers remain largely unknown. Moreover, most studies of LPDs supplemented with BCAA have focused on either leucine or valine (Erwan et al., 2011; Nascimento et al., 2016), Ile has not attracted wide attention.

Ile, as the fourth co-limiting amino acid with valine for broilers fed corn-soybean meal-based diets, is critical to maintain growth performance, meat quality, and lipid metabolism (Dozier Iii et al., 2011). Broilers fed with Ile-deficient diet were reported to have lower body weight gain and feed intake and a higher feed to gain ratio (**F: G**) than with control diet (Kidd et al., 2004). In a previous study, increased Ile intake was found to promote accumulation of intramuscular fat (**IMF**) in finishing pigs by inhibiting phosphorylation of $5'$ adenosine monophosphate-activated protein kinase (**AMPK**)-acetyl CoA carboxylase (**ACC**) and stimulating the expression of lipogenesis-related genes in skeletal muscle (Luo et al., 2018). In addition, dietary Ile supplementation was reported to reduce the mass of white adipose tissue and ameliorate hepatic steatosis in obese mice by promoting the browning of white adipose tissue and regulating expression of genes associated with lipid metabolism (Ma et al., 2020). However, it remains unclear whether supplementation of a LPD with Ile can improve meat quality and alleviate lipid deposition in broilers.

The liver is the primary site of lipid metabolism in broilers (Cai et al., 2011). AMPK, the main sensor of cellular energy status, plays a pivotal role in the regulation of lipid, glucose, and protein metabolism in liver (Hu et al., 2019). Leptin is a hormone secreted by white adipose tissue and binds to the leptin receptor (**LEPR**) via the Janus kinase 2 (**JAK2**)/signal transducer and activator of transcription 3 (**STAT3**) pathway to regulate lipid metabolism, energy homeostasis, and immune responses (Park and Ahima, 2014). The effects of Ile on the AMPK/mechanistic target of rapamycin (**mTOR**) and Leptin/JAK2/STAT3 pathways in broilers are unknown. Therefore, the aim of the present study was to investigate the effects of Ile on the growth performance, meat quality, and lipid metabolism of broilers fed a LPD, and to determine whether the effect of Ile on lipid metabolism is linked to activation of the AMPK/mTOR and Leptin/JAK2/STAT3 pathways.

MATERIALS AND METHODS

Materials

The Ile (with a purity of 98%) was purchased from CJ (Shanghai) Trading Co., Ltd. (Shanghai, China).

Animals and Experimental Design

The study protocol was approved by the Animal Care and Use Committee of Qingdao Agricultural University (Qingdao, China). A total of 396 one-day-old male Cobb 500 broilers were purchased from Yantai Dadi Animal Husbandry Co., Ltd. (Yantai, China). Broilers with similar initial body weights (46.24 ± 0.44 g) were randomly divided into 4 treatment groups composed of 9 replicated cages of 11 broilers each for a 42-d feeding period. The treatments consisted of a control (**CON**) diet, LPD, LPD + 0.13% Ile (**LPD-LI**), and LPD + 0.26% Ile (**LPD-HI**). The CON diet was formulated to meet all requirements recommended by the National Research Council (NRC, 1994; Table 1). The digestible Ile: lysine ratio was formulated at 0.74, meeting the amino acid requirement of broilers. Compared to the CON diet, the crude protein content of the LPD was reduced by 3%, while the levels of four limiting amino acids (lysine, methionine, threonine, and tryptophan) were the same. The Ile levels of the LPD-LI and LPD-HI groups were adjusted to that of the CON diet and 0.13% higher than that of the CON diet, respectively. All the diets were fed in mash form. During the experiment, feed and fresh water were provided ad libitum. Temperature was maintained at 33 to 35°C for the first week and then gradually reduced by 2°C every week to a final temperature of 24°C. Feed consumption of each group was measured daily

Table 1. Composition and nutrient levels of experimental diets (air-dry basis) %.

Item	Days 0 to 21		Days 21 to 42	
	CON[1]	LPD[1]	CON	LPD
Ingredients				
Corn	51.26	60.29	58.05	67.50
Soybean meal	38.20	29.53	32.95	24.22
Soybean oil	6.72	5.84	5.64	4.44
Limestone	1.26	1.28	1.43	1.41
CaHPO$_4$	1.68	1.77	1.13	1.22
NaCl	0.35	0.35	0.35	0.35
Vitamin premix[2]	0.10	0.10	0.10	0.10
Mineral premix[3]	0.15	0.15	0.15	0.15
Choline chloride	0.07	0.07	0.07	0.07
L-lysine hydrochloride	0.04	0.23	0.05	0.24
DL-methionine	0.17	0.21	0.08	0.12
L-threonine	0.00	0.13	0.00	0.13
L-tryptophan	0.00	0.05	0.00	0.05
Nutrient levels				
Metabolizable energy (MJ/kg)	13.14	13.12	13.09	13.07
Calcium (%)	0.98	0.98	0.91	0.90
Available phosphorus (%)	0.45	0.45	0.35	0.35
Crude protein (%)[4]	21.06	18.07	19.06	16.01
Lysine (%)[4]	1.12	1.11	1.00	1.02
Digestible lysine (%)	0.96	0.98	0.87	0.89
Methionine (%)[4]	0.52	0.51	0.38	0.40
Threonine (%)[4]	0.87	0.86	0.80	0.79
Tryptophan (%)[4]	0.31	0.32	0.26	0.27
Isoleucine (%)[4]	0.83	0.70	0.75	0.62
Digestible Isoleucine (%)	0.71	0.59	0.64	0.53

[1]CON, control diet; LPD, low-protein diet.

[2]Provided per kilogram of diet: vitamin A (trans-retinyl acetate), 8000 IU; vitamin D$_3$ (cholecalciferol), 3000 IU; vitamin E (all-rac-α-tocopherol acetate), 20 IU; vitamin K$_3$ (menadione), 2.0 mg; vitamin B$_1$ (thiamin), 4.2 mg; vitamin B$_2$ (riboflavin), 4.0 mg; vitamin B$_6$ (pyridoxine HCl), 4.5 mg; vitamin B$_{12}$ (cobalamin), 0.02 mg; nicotinic acid, 10 mg; calcium pantothenate, 11 mg; folic acid, 1.0 mg; biotin, 0.15 mg.

[3]Provided per kilogram of diet: Fe (as ferrous sulfate), 80 mg; Cu (as copper sulfate), 10 mg; Zn (as zinc sulfate), 75 mg; Mn (as manganese sulfate), 80 mg; Se (as sodium selenite), 0.30 mg; I (as potassium iodide), 0.40 mg.

[4]Analyzed value.

on a replicate basis and the average daily feed intake (**ADFI**) was calculated. The body weight per replicate of each group was measured on days 0, 21, and 42 and the average daily gain (**ADG**) and F:G were calculated.

Chemical Analysis

The crude protein and amino acid contents of the diets were determined in accordance with the methods described by the Association of Official Analytical Chemists (976.06 and 994.12, respectively). Amino acids, except tryptophan, methionine and cystine, were detected using the Hitachi L-8900 amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolyzing with 6 mol/L HCl at 110°C for 24 h. Tryptophan was detected using the Agilent 1200 Series high performance liquid chromatography (Aligent, Santa Clara, CA) after hydrolyzing with 4 mol/L LiOH at 110°C for 24 h. Methionine and cysteine were detected using the Hitachi L-8900 amino acid analyzer after cold performic acid oxidation and hydrolyzing with 6.8 mol/ L HCl at 110°C for 24 h.

Sample Collections

On day 42, after fasting for 12 h, one broiler per replicate was randomly selected and blood samples were collected from the jugular vein, centrifuged at $3,000 \times g$ and 4°C for 10 min, and the separated serum was stored at $-80°C$. After blood collection, the broilers were sacrificed by cervical dislocation and the abdominal and thoracic cavities were opened to harvest the left and right breast muscles, liver, and abdominal adipose tissue. The left breast muscle was stored at 4°C for meat quality assessment. Parts of the right breast muscle, liver, and abdominal adipose tissue were fixed in 4% neutral paraformaldehyde for histochemical analysis, while the remaining tissues were immediately frozen in liquid nitrogen and stored at $-80°C$ for further analysis.

Measurement of Meat Quality

After a 30-min blooming period, the meat color parameters, including lightness (**L***), redness (**a***) and yellowness (**b***) values, were measured at room temperature using a colorimeter (CR-410; standard observer, 2°; illuminant, D65; Konica Minota, Inc., Tokyo, Japan), as described by Zhang et al. (2022). The meat color parameters were immediately measured 3 times at different locations of the medial portion of each sample. Ultimate pH values were measured at 45 min (**pH$_{45min}$**) and 24 h (**pH$_{24h}$**) postmortem using a portable pH meter (Seven2Go; Mettler Toledo GmbH, Giessen, Germany) inserted 1 cm into the superior portion of the breast muscle, as described by Liu et al. (2021). Each measurement for each sample was performed with 3 replicates. Drip loss was calculated according to the equation: drip loss (%) = (initial weight − final weight)/(initial weight) × 100%, as described by Honikel (1998). Cooking loss was determined as described in a previous study

(Liu et al., 2016). Briefly, the breast muscle samples were placed into Ziploc plastic bags and heated to an internal temperature of 77 to 80°C, then cooled to room temperature, wiped with absorbent paper, and reweighed. Shear force was calculated as described by Castellini et al. (2002). The mid-portion of each heated sample was cut into sections measuring 1.25×2 cm along the direction of the fibers. The shear force of every section was measured 3 times using a digital meat tenderness meter (model C-LM3; Tenovo International Co., Ltd., Beijing, China).

Fat Content Measurement and Histological Analysis

The liver and breast muscle tissues were cut into small sections, which were freeze-dried and ground into powder. The liver fat and IMF contents were measured using the Soxhlet extraction method with petroleum and ether. The IMF content was converted to the percentage of wet meat weight in accordance with the following equation: IMF (%) = (fat weight/wet weight of breast muscle) × 100% (Luo et al., 2018).

The breast muscle and abdominal adipose tissues were stained with hematoxylin and eosin, as described by Bai et al. (2015). Briefly, the breast muscle and abdominal adipose were cut into sections, which were fixed with Carnoy's solution at $4 \pm 1°C$ for 24 h, then dehydrated with ethanol/xylene, and embedded in paraffin. The paraffin-embedded sections were cut into 4 μm-thick slices that were mounted on glass slides and stained with hematoxylin and eosin. For each sample, 3 sections were randomly photographed to calculate the muscle fiber diameter, muscle fiber density, and area of adipocytes.

Oil red O staining was conducted as described by Li et al. (2019). Briefly, frozen liver samples (5 μm) were fixed with 1% calcium chloride and 4% formaldehyde for 1 h, then treated with 60% isopropanol, and stained with oil red O solution and Mayer's hematoxylin solution for 15 min. The integrated optical density was measured under a microscope (Olympus Corporation, Tokyo, Japan) using the IAS−−2000D image analysis system (Quality Engineering Associates, Inc., Acton, MA). Three images of each section were randomly captured for each sample.

Measurement of Biochemical Parameters and Enzyme Activities

Commercial enzyme-linked immunosorbent kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) were used to measure serum levels of triglyceride (**TG**; cat. no. YJ90993), total cholesterol (**TC**; cat. no. ML036921), high-density lipoprotein cholesterol (**HDL-C**; cat. no. ML036973), low-density lipoprotein cholesterol (**LDL-C**; cat. no. ML036983), free fat acid (**FFA**; cat. no. YJ68293), leptin (cat. YJ45283) and insulin (cat. no. YJ09873), enzyme activities of ACC (cat. no. ML09632), fatty acid synthase (**FAS**;

cat. no. ML02302), carnitine palmitoyl-transferase 1 (**CPT-1**; cat. no. ML25412) and acyl-CoA oxidase 1 (**ACOX1**; cat. no. ML36214) in liver and enzyme activities of glycerol-3-phosphate dehydrogenase (**G3PDH**; cat. no. ML41236) and topoisomerase II alpha (**TOP2A**; cat. no. ML85214) in abdominal adipose according to the manufacturer's instructions.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from the liver, abdominal adipose, and breast muscle samples using TRIzol reagent (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) in accordance with the manufacturer's instructions. The concentration and quality of RNA were measured using SpectraMax QuickDrop Micro-Volume Spectrophotometer (Molecular Devices, LLC, San Jose, CA). Samples with A260 nm/A280 nm values of 1.8 to 2.0 were considered acceptable. Then, the isolated RNA was reverse-transcribed into cDNA using the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (Hunan Aikerui Biological Engineering Co., Ltd., Hunan, China) in accordance with the manufacturer's instructions. All cDNA samples were stored at $-20°C$ until further use. Relative gene expression was quantified by real-time

PCR using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with TB Green Premix Ex Taq polymerase (Takara Bio, Inc., Shiga, Japan) and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primers for PCR analysis are listed in Table 2.

Western Blot Analysis

Liver tissue proteins were extracted using radio immunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and quantified using the bicinchoninic acid assay (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of proteins from each group were separated by electrophoresis and then transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany), which were blocked with 5% skimmed milk and then incubated overnight at 4°C with primary antibodies against β-actin (Beyotime Institute of Biotechnology), AMPK (Cell Signaling Technology, Inc., Danvers, MA, USA), p-AMPK (Thr172; Cell Signaling Technology, Inc.), mTOR (Cell Signaling Technology, Inc.), p-mTOR (Ser2448; Cell Signaling Technology, Inc.), ribosomal protein 70 S6 kinase (**p70S6K**; Cell

Table 2. Primers used for real time-PCR.

Gene[1]	Primer sequence (5' to 3')	Product size (bp)	GenBank no.
MyHC-I	F: AACGCCGCAACAACCT	330	U85023.1
	R: TTCTTCTTCATCCGCTCC		
MyHC-IIa	F: CCACCAATCCATACGACT	216	NM 204228.1
	R: CTGGCTCTGCTTGCTCT		
MyHC-IIb	F: TGTGAGTCAAGGCGAGAT	347	NM_001013396.1
	R: CCAGAGCACCTACAGCAT		
LXRα	F: GTGCAGAGAGTGACGAGCTT	175	NM_204542
	R: AGAGGTTTACGTGCGTGGAG		
SREBP-1	F: TCACCGCTTCTTCGTGGAC	220	AY029224
	R: CTGAAGGTACTCCAACGCATC		
ACC	F: AATGGCAGCTTTGGAGGTGT	137	NM_205505.2
	R: TTCTGTTTGGGTGGGAGGTG		
FAS	F: AACTCTCTGCCATCTCCCGA	165	NM_205155.4
	R: TAGCCTGGTAGCCAGTTCGT		
PPARα	F: AATCATAAAGGAGTTTAAGTGACCG	264	NM_001001464.1
	R: GCTGGTGAAAGGGTGTCTGT		
CPT-1	F: GGCTCTGGCAGGAGCTACA	108	XM_040700878.1
	R: AGTAGGTCAGGACACGCTCA		
ACOX1	F: ATGTCACGTTCACCCCATCC	133	NM_001006205
	R: AGGTAGGAGACCATGCCAGT		
PPARγ	F: CAGTGGATCTGTCTGCGATG	172	NM_001001460
	R: CTTTGGCAATCCTGGAGCTTG		
C/EBPα	F: ATGGAGCAAGCCAACTTCTAC	230	NM_001031459
	R: GCCAGGAACTCGTCGTTGAA		
KI67	F: AGGATGGAAGCAAGTCACCTGGAT	129	XM_004020367
	R: CTTCTGAACGGGGACTGGAATCTT		
TOP2A	F: TAAGCCCACTAGCACGGTTG	142	NM_204791.3
	R: CCAATCCCTTCTGCCCCATT		
TPX2	F: TGGAGGGTGGGCCAATC	57	NM_204437.1
	R: TTGGCTGTGTGAGTTCCTTCAC		
LEPR	F: TCCCCCAGTGACCCGACCTCT	201	NM_204323
	R: CGCCTCATTTCCCAGCTCCCA		
GAPDH	F: GGGCCGCCATCACTATCTT	148	NM_204305.1
	R: TAACACGCTTAGCACCACCC		

[1]*MyHC*, myosin heavy chain; *LXRα*, liver X receptors-α; *SREBP1*, sterol regulatory element binding protein 1; *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *PPARα*, peroxisome proliferator activated receptor-α; *CPT-1*, carnitine palmitoyl-transferase 1; *ACOX1*, acyl-CoA oxidase 1; *KI67*, ki-67; *TOP2A*, topoisomerase II alpha; *TPX2*, thioredoxin-dependent peroxidase 2; *PPARγ*, peroxisome proliferator-activated receptor-γ; *C/EBPα*, CCAAT/enhancer binding protein-α; *LEPR*, leptin receptor; *GAPGH*, glyceraldehyde-3-phosphate dehydrogenase.

Signaling Technology, Inc.), p-p70S6K (Thr389; Cell Signaling Technology, Inc.), sterol regulatory element binding protein 1 (**SREBP1**; Wanlei Biotechnology Co., Ltd., Shenyang, China), JAK2 (Cell Signaling Technology, Inc.), p-JAK2 (Tyr1007/1008; Cell Signaling Technology, Inc.), STAT3 (Santa Cruz Biotechnology, Inc., Dallas, TX), and p-STAT3 (Tyr705; Santa Cruz Biotechnology, Inc.). After washing 3 times with $1 \times$ Tris-buffered saline with 0.1% Tween 20 detergent (**TBST**), the membranes were incubated with a horse-radish peroxidase-conjugated secondary antibody (Beyotime Institute of Biotechnology) for 1 h at room temperature, then washed 5 times with $1 \times$ TBST. The signals were visualized with an enhanced chemilumines-cence kit (Beyotime Institute of Biotechnology) and imaged with a CanoScan LiDE 100 scanner (Canon, Inc., Tokyo, Japan). Relative protein levels were deter-mined using ImageJ software.

Statistical Data Analysis

All data were analyzed by one-way analysis of vari-ance using IBM SPSS Statistics for Windows, version 20.0. (IBM Corporation, Armonk, NY). The results are presented as the mean $\pm$ SEM and compared using Tur-key's multiple range test. Statistical significance was defined as $P < 0.05$.

RESULTS

Growth Performance

As shown in Table 3, there were no significant differ-ences in the ADG, ADFI, and F:G among the 4 groups from d 0 to 21. From d 21 to 42 and d 0 to 42, ADG and ADFI were significantly greater ($P < 0.001$) in the CON, LPD-LI, and LPD-HI groups than the LPD group, while there were no significant differences among the CON, LPD-LI, and LPD-HI groups. The F:G was significantly greater ($P < 0.001$) in the LPD group than the other

Table 3. Effect of a LPD supplemented with isoleucine (Ile) on growth performance of broilers.

Item[1]	CON[2]	LPD	LPD-LI	LPD-HI	SEM	P-value
Days 0−21						
ADG (g)	35.86	35.02	35.69	35.23	0.420	0.175
ADFI (g)	50.32	49.48	50.27	49.97	0.584	0.464
F:G	1.40	1.41	1.41	1.42	0.023	0.948
Days 21−42						
ADG (g)	77.51[a]	66.59[b]	77.87[a]	77.74[a]	0.962	<0.001
ADFI (g)	134.88[a]	124.73[b]	137.28[a]	137.88[a]	1.637	<0.001
F:G	1.74[b]	1.87[a]	1.77[b]	1.78[b]	0.020	<0.001
Days 0−42						
ADG (g)	56.63[a]	50.61[b]	56.61[a]	56.30[a]	0.521	<0.001
ADFI (g)	92.48[a]	86.48[b]	93.44[a]	93.18[a]	0.801	<0.001
F:G	1.63[b]	1.71[a]	1.65[b]	1.66[b]	0.014	<0.001

[1]ADFI, average daily feed intake; ADG, average daily gain; F:G, feed to gain ratio.
[2]CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile.
[a,b]Mean values within a row with no common superscript differ signifi-cantly ($P < 0.05$).

groups, with no significant differences among the CON, LPD-LI, and LPD-HI groups.

Meat Quality

Compared with the CON and LPD groups, the L* value ($P = 0.017$) and drip loss ($P = 0.026$) were signifi-cantly lower in the LPD-HI group, with no significant differences between the LPD-LI and LPD-HI groups (Table 4). Meanwhile, the a* value was significantly lower ($P = 0.001$) and the pH_{24h} value ($P = 0.014$) and the IMF content ($P = 0.012$) were significantly higher in the LPD-HI group than the other groups, but there were no significant differences among the CON, LPD, and LPD-LI groups. Shear force was significantly increased ($P = 0.048$) in the LPD group compared to the other groups, with no significant differences among the CON, LPD-LI, and LPD-HI groups.

Hematoxylin and eosin staining of the breast muscle showed that the diameter of the muscle fibers was signif-icantly increased ($P < 0.001$), while the density was sig-nificantly decreased ($P < 0.001$) in the LPD group compared to the other groups, with no significant differ-ences among the CON, LPD-LI, and LPD-HI groups (Table 4 and Figure 1A). Compared to the other groups, relative expression of myosin heavy chain (**MyHC**)-*I* was significantly decreased ($P = 0.038$) and that of *MyHC-IIb* was significantly increased ($P = 0.027$) in the LPD group, but there were no significant differences among the CON, LPD-LI, and LPD-HI groups (Figure 1B).

Lipid-Related Metabolite and Hormone Levels in Serum

As shown in Table 5, the TG ($P = 0.041$) and insulin ($P = 0.006$) levels were higher in the LPD group than the CON and LPD-HI groups, with no significant differ-ences among the CON, LPD-LI and LPD-HI groups. The TC level was higher ($P = 0.009$) in the LPD group than the CON, LPD-LI and LPD-HI groups, while there were no significant differences among the CON, LPD-LI, and LPD-HI groups. Meanwhile, the HDL-C ($P = 0.006$), leptin ($P = 0.003$), and FFA ($P = 0.026$) levels were lower in the LPD group than the other groups, but there were no significant differences among the CON, LPD-LI, and LPD-HI groups.

Lipid Metabolism in Liver and Abdominal Adipose Tissues

The liver fat content and integrated optical density were significantly higher ($P < 0.001$) in the LPD group compared to the other groups, while there were no signif-icant differences among the CON, LPD-LI, and LPD-HI groups (Table 6 and Figure 2A). Compared to the LPD group, Ile supplementation significantly downregulated gene expressions of liver X receptors-α (**LXRα**; $P = 0.015$), *SREBP1* ($P = 0.016$), *ACC* ($P < 0.001$) and

Table 4. Effect of a LPD supplemented with isoleucine (Ile) on meat quality of broilers.

Item[1]	CON[2]	LPD	LPD-LI	LPD-HI	SEM	P-value
L*	53.23[a]	53.91[a]	52.92[ab]	51.90[b]	0.596	0.017
a*	5.23[a]	5.26[a]	5.24[a]	5.14[b]	0.028	0.001
b*	2.24	2.22	2.23	2.27	0.034	0.488
pH$_{45min}$	6.80	6.78	6.82	6.81	0.023	0.345
pH$_{24h}$	6.56[b]	6.52[b]	6.57[b]	6.65[a]	0.039	0.014
Drip loss (%)	2.55[a]	2.58[a]	2.51[ab]	2.39[b]	0.064	0.026
Cooking loss (%)	14.53	14.74	13.68	14.44	0.970	0.711
Shear force (N)	8.64[b]	9.06[a]	8.62[b]	8.66[b]	0.175	0.048
IMF (%)	2.35[b]	2.33[b]	2.36[b]	2.48[a]	0.052	0.012
Fiber diameter (μm)	69.44[b]	86.87[a]	68.40[b]	64.37[b]	3.094	<0.001
Fiber density (number/mm^2)	455.04[a]	418.34[b]	456.67[a]	457.84[a]	8.265	<0.001

[1]L*, lightness; a*, redness; b*, yellowness; pH$_{45min}$, postmortem pH at 45 min; pH$_{24h}$, post-mortem pH at 24 h; IMF, intramuscular fat.
[2]CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile.
[a,b]Mean values within a row with no common superscript differ significantly ($P < 0.05$).
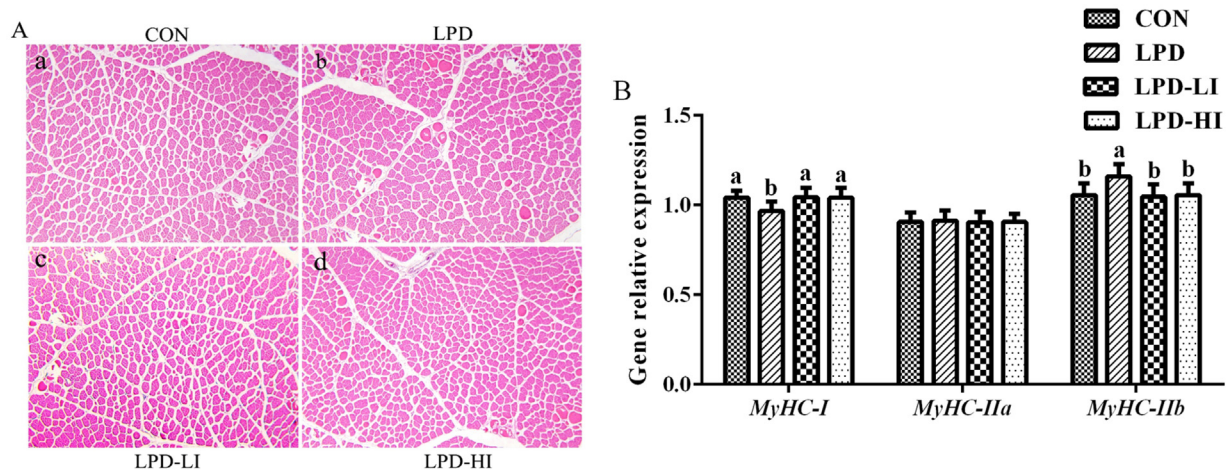

Figure 1. Effect of a LPD supplemented with isoleucine (Ile) on muscle fiber of broilers. (A) Histological analysis of breast muscle by staining with hematoxylin and eosin. (B) Relative *MyHC* expressions. *MyHC*, myosin heavy chain; CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile. [a,b]Bars with no common superscript differ significantly ($P < 0.05$).

FAS ($P < 0.001$), and enzyme activities of ACC ($P = 0.014$) and FAS ($P = 0.001$), while the gene expression levels of *PPARα* ($P = 0.003$), *CPT-1* ($P = 0.005$) and *ACOX1* ($P = 0.002$) and the enzyme activities of CPT-1 ($P = 0.011$) and ACOX1 ($P = 0.010$) were significantly upregulated, with no significant differences among the CON, LPD-LI, and LPD-HI groups (Table 6 and Figure 2B, C).

The abdominal adipose percentage ($P < 0.001$) and adipocyte area ($P = 0.003$) in the abdominal adipose tissue were higher in the LPD group than the other groups,

but there were no significant differences among the CON, LPD-LI, and LPD-HI groups (Table 7 and Figure 3A). Relative expression levels of peroxisome proliferator-activated receptor-γ (***PPARγ***; $P = 0.023$), CCAAT/enhancer binding protein-α (***C/EBPα***; $P = 0.006$), ki67 (***KI67***; $P < 0.001$), *TOP2A* ($P < 0.001$) and thioredoxin-dependent peroxidase 2 (***TPX2***; $P < 0.001$) and enzyme activities of G3PDH ($P = 0.002$) and TOP2A ($P = 0.005$) were significantly increased in the LPD group compared to the CON group, while Ile supplementation inhibited ($P < 0.05$) this trend and the

Table 5. Effect of a LPD supplemented with isoleucine (Ile) on serum lipid-related metabolite and hormone levels in broilers.

Item[1]	CON[2]	LPD	LPD-LI	LPD-HI	SEM	P-value
TG (mmol/L)	1.17[b]	1.22[a]	1.20[ab]	1.16[b]	0.022	0.041
TC (mmol/L)	3.08[b]	3.22[a]	3.11[b]	3.11[b]	0.039	0.009
LDL-C (mmol/L)	0.54	0.56	0.56	0.56	0.021	0.640
HDL-C (mmol/L)	1.87[a]	1.77[b]	1.85[a]	1.89[a]	0.032	0.006
Leptin (μg/L)	1.77[a]	1.69[b]	1.76[a]	1.76[a]	0.020	0.003
FFA (μmol/L)	368.30[a]	356.30[b]	367.58[a]	365.07[a]	4.008	0.026
Insulin (mU/L)	17.63[b]	19.04[a]	18.44[ab]	17.66[b]	0.403	0.006

[1]TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FFA, free fat acid.
[2]CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile.
[a,b]Mean values within a row with no common superscript differ significantly ($P < 0.05$).

Table 6. Effect of a LPD supplemented with isoleucine (Ile) on hepatic lipid metabolism in broilers.

Item[1]	CON[2]	LPD	LPD-LI	LPD-HI	SEM	P-value
Liver fat content (%)	12.90[b]	16.61[a]	12.97[b]	12.80[b]	0.343	< 0.001
Integrated optical density	2.43[b]	5.46[a]	2.63[b]	2.48[b]	0.224	< 0.001
Enzyme activity (IU/g)						
ACC	6.93[b]	7.94[a]	6.83[b]	6.79[b]	0.364	0.014
FAS	35.52[b]	47.26[a]	39.57[b]	38.74[b]	2.360	0.001
CPT-1	4.38[a]	3.36[b]	4.39[a]	4.42[a]	0.335	0.011
ACOX1	3.93[a]	3.02[b]	3.94[a]	4.14[a]	0.319	0.010

[1]ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; CPT-1, carnitine palmitoyl-transferase 1; ACOX1, acyl-CoA oxidase 1.

[2]CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile.

[a,b]Mean values within a row with no common superscript differ significantly (P < 0.05).

expression levels of these genes and the activities of these enzymes were equal to those of the CON group (Table 7 and Figure 3B, C).

AMPK/mTOR and Leptin/JAK2/STAT3 Signaling Pathways in Liver

As shown in Figure 4, dietary supplementation with Ile significantly increased the downregulated relative gene expression of $LEPR$ ($P < 0.001$) and relative protein expression levels of AMPK/β-actin ($P < 0.001$), p70S6K/β-actin ($P < 0.001$), p-AMPK/AMPK ($P = 0.009$), p-p70S6K/p70S6K ($P < 0.001$), and p-STAT3/STAT3 ($P < 0.001$) in the LPD group, and decreased ($P < 0.001$) the upregulated relative protein expression level of SREBP1/β-actin, whereas there were no significant differences among the CON, LPD-LI, and LPD-HI groups. Meanwhile, dietary Ile supplementation significantly decreased ($P < 0.001$) the upregulated relative protein expression of mTOR/β-actin and increased ($P < 0.001$) the downregulated relative protein expression of p-JAK2/JAK2 in the LPD group, but were higher ($P < 0.001$) than in the CON group. Relative protein expressions of p-mTOR/mTOR were higher ($P < 0.001$) in the LPD-LI and LPD-HI groups compared to the LPD group, but lower ($P < 0.001$) than in the CON group.

DISCUSSION

Considering the economic and environmental advantages of a LPD, to meet the critical amino acid requirements for optimum performance of broilers fed a LPD is crucial (Attia et al., 2020). Mixed BCAA are reportedly vital to the regulation of growth performance and meat
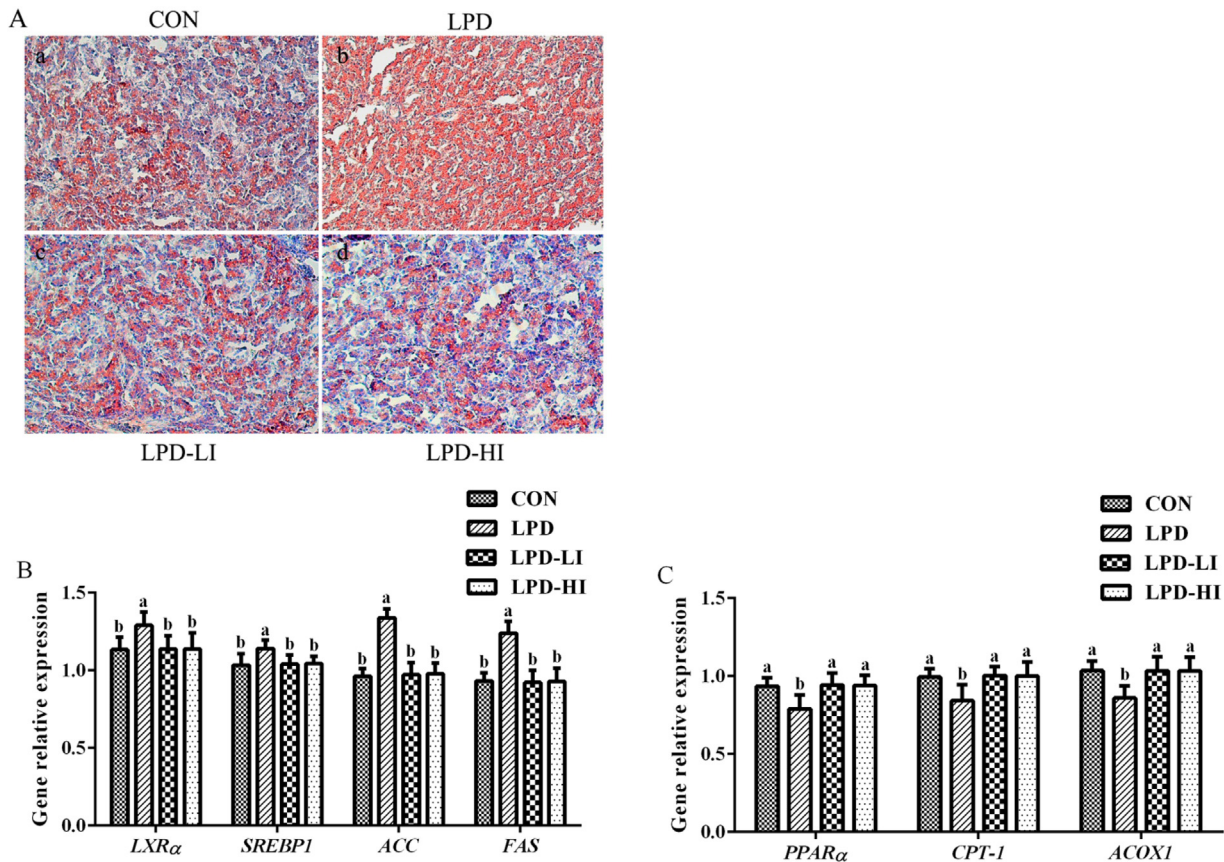

Figure 2. Effect of a LPD supplemented with isoleucine (Ile) on hepatic lipid metabolism in broilers. (A) Histological analysis of liver sections by staining with oil red O. (B) Key genes related to lipogenesis. (C) Key genes related to lipolysis. $LXR\alpha$, liver X receptors-α; $SREBP1$, sterol regulatory element-binding protein 1; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; $PPAR\alpha$, peroxisome proliferator activated receptor-α; $CPT-1$, carnitine palmitoyl-transferase 1; $ACOX1$, acyl-CoA oxidase 1; CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile. [a,b]Bars with no common superscript differ significantly (P < 0.05).

Table 7. Effect of a LPD supplemented with isoleucine (Ile) on lipid metabolism in abdominal adipose tissue of broilers.

Item[1]	CON[2]	LPD	LPD-LI	LPD-HI	SEM	P-value
Abdominal adipose percentage (%)	1.41[b]	2.49[a]	1.53[b]	1.44[b]	0.105	< 0.001
Adipocyte area (μm^2)	226.70[b]	325.90[a]	263.48[b]	244.99[b]	23.066	0.003
Enzyme activity (IU/g)						
G3PDH	1.86[b]	2.55[a]	2.08[b]	1.96[b]	0.159	0.002
TOP2A	2.81[b]	3.44[a]	2.78[b]	2.85[b]	0.186	0.005

[1]G3PDH, glycerol-3-phosphate dehydrogenase; TOP2A, topoisomerase II alpha.
[2]CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile.
[a,b]Mean values within a row with no common superscript differ significantly ($P < 0.05$).

quality in pigs fed a LPD (Zhang et al., 2022). However, it is unknown whether a LPD supplemented with Ile promotes growth performance and meat quality. In the present study, growth performance was depressed in broilers fed a LPD, similar to a report by Liu et al. (2022). However, Ile supplementation significantly improved growth performance of broilers fed a LPD, partially in agreement with a previous study that reported significantly increased ADG and decreased F:G of broilers fed a diet supplemented with Ile (Corrent and Bartelt, 2011). Chicken meat is an excellent source of animal protein for human nutrition, thus meat quality is especially important to the consumer (Tang et al., 2021). The pH is an important parameter of meat quality. Within limits, pH is negatively correlated with the L* value, a* value, drip loss, and shear force (Le Bihan-Duval et al., 2008). The results of the present study showed that the pH$_{24h}$ value was higher, while the L* value, a* value, drip loss, and shear force were lower in the LPD-HI group, similar to report by Xu et al. (2020) that high Ile intake significantly increased the pH$_{24h}$ value and tended to decrease the a* value and drip loss of pork. To elucidate the possible mechanism affected by Ile supplementation on improving meat quality, the fiber characteristics and expression profiles of *MyHC* isoforms in breast muscle were examined in the present study. It is reported that there is a close relationship between fiber characteristics and shear force (An et al., 2010). In the present study, Ile supplementation decreased the fiber diameter and increased fiber density in broilers fed a LPD, which is a possible reason for the decreased shear force caused by Ile. Muscle fibers of broilers are classified
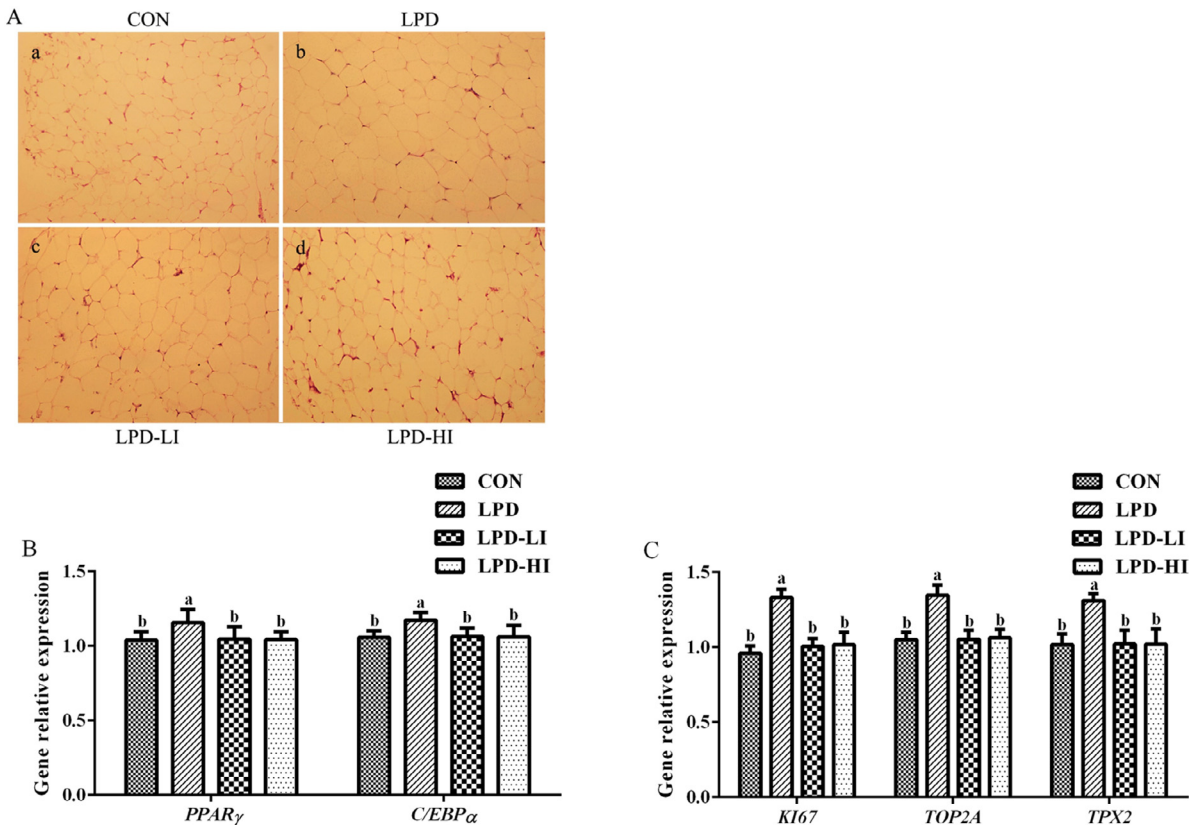

Figure 3. Effect of a LPD supplemented with isoleucine (Ile) on lipid metabolism in abdominal adipose tissue of broilers. (A) Histological analysis of abdominal adipose tissue sections by staining with hematoxylin and eosin. (B) Key genes related to preadipocyte differentiation. (C) Key genes related to adipocyte proliferation. *PPARγ*, peroxisome proliferator-activated receptor-γ; *C/EBPα*, CCAAT/enhancer binding protein-α; *KI67*, ki67; *TOP2A*, topoisomerase II alpha; *TPX2*, thioredoxin-dependent peroxidase 2; CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile. [a,b]Bars with no common superscript differ significantly ($P < 0.05$).
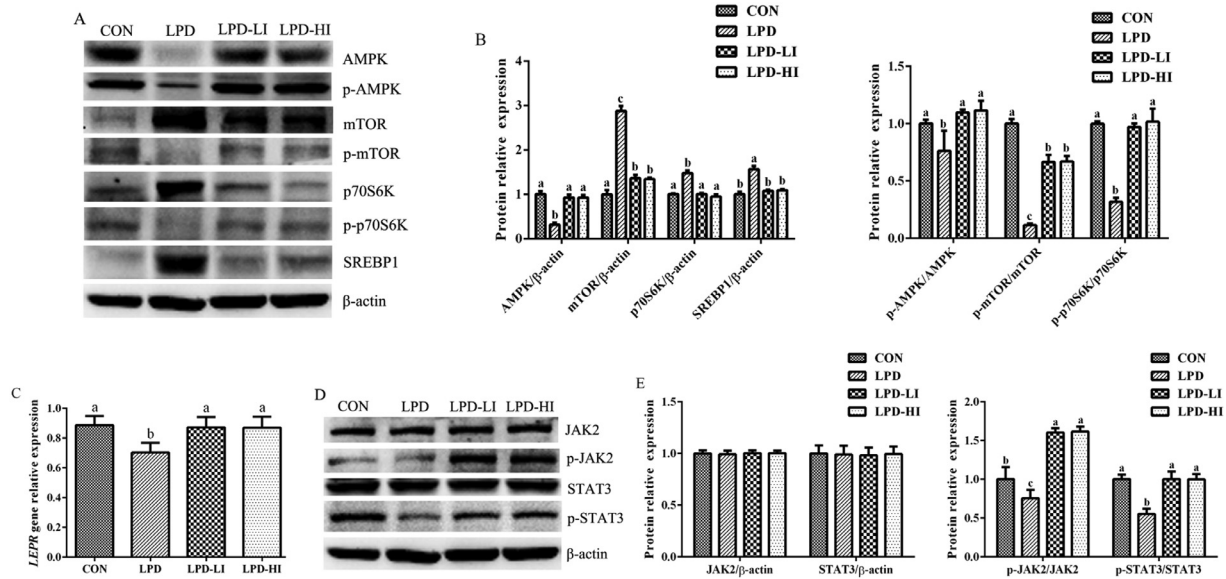
Figure 4. Effect of a LPD supplemented with isoleucine (Ile) on the AMPK/mTOR and Leptin/JAK2/STAT3 signaling pathways in the liver of broilers. (A) Western blot analysis of proteins associated with the AMPK/mTOR signaling pathway. (B) Relative expression level of proteins associated with the AMPK/mTOR signaling pathway. (C) *LEPR* gene expression level. (D) Western blot analysis of proteins associated with the JAK2/STAT3 signaling pathway. (E) Relative expression level of proteins associated with the JAK2/STAT3 signaling pathway. AMPK, 5′ adenosine monophosphate-activated protein kinase; mTOR, mechanistic target of rapamycin; p70S6K, ribosomal protein 70 S6 kinase; SREBP1, sterol regulatory element-binding protein 1; *LEPR*, leptin receptor; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3; CON, control diet; LPD, low-protein diet; LPD-LI, low-protein diet + 0.13% Ile; LPD-HI, low-protein diet + 0.26% Ile. [a,b,c]Bars with no common superscript differ significantly ($P < 0.05$).

into 3 types based on enzyme activity (Huo et al., 2022). Muscles with a higher proportion of *MyHC-I* fibers have higher pH and IMF content (Hou et al., 2020). In contrast, muscles with a higher proportion of *MyHC-IIb* fibers exhibit a quite opposite trend because of the increased content of glycogen and activities of glycolytic enzymes (Ismail and Joo, 2017). In the present study, Ile supplementation increased expression of *MyHC-I* and decreased expression of *MyHC-IIb*, which could explain the effect of Ile on the pH_{24h} value and IMF content.

The abdomen is the main site of lipid deposition in broilers (Li et al., 2020). In the present study, the abdominal adipose percentage and adipocyte area were increased in broilers fed a LPD, in agreement with the findings of a previous study (Sharma et al., 2022). Dietary Ile supplementation markedly decreased lipid deposition in the abdomen as reported in a previous study of broilers (Maynard et al., 2022). The formation of adipose tissue includes the differentiation of preadipocytes to adipocytes and the proliferation of adipocytes (Bai et al., 2017). The *PPARγ* and *C/EBPα* are the main regulators of preadipocyte differentiation, while *KI67*, *TOP2A*, and *TPX2* are markers of adipocyte proliferation. G3PDH, an indirect marker of triacylglycerol synthesis, is closely associated with preadipocyte differentiation (Sledzinski et al., 2013). In this study, Ile decreased the gene expressions of *PPARγ*, *C/EBPα*, *KI67*, *TOP2A*, and *TPX2*, and the enzyme activities of G3PDH and TOP2A, suggesting that Ile reduced lipid deposition in the abdomen by inhibiting preadipocyte differentiation and adipocyte proliferation.

In avian species, the liver accounts for about 95% of de novo synthesis of fatty acids, indicating that liver is the most important organ for intermediary metabolism of lipids and energy (Wan et al., 2021). Compared to the more vigorous lipid deposition in the liver of broilers fed a LPD, the fat content and integrated optical density in the liver of broilers fed a LPD supplemented with Ile were closer to those of the CON group. Consistently, dietary supplementation with Ile significantly reduced serum lipids levels, further suggesting that Ile alleviates lipid deposition. Lipid deposition in liver is reportedly dependent on the dynamic balance between fatty acid synthesis and β-oxidation (Li et al., 2019). *LXRα*, as the primary regulator of hepatic lipid metabolism, regulates fatty acid synthesis via activation of *SREBP1*, which binds to the promoter regions of major genes associated with lipogenesis, including *ACC* and *FAS*, to enhance expression (Schultz et al., 2000). The results of the present study showed that Ile downregulated the increased gene expressions of *LXRα, SREBP1, ACC*, and *FAS*, and decreased the elevated enzyme activities of ACC and FAS in broilers fed a LPD, resulting in decreased fatty acid synthesis. *PPARα*, as a member of the nuclear receptor superfamily, serves an important role in fatty acid β-oxidation (Huang et al., 2013). Activation of *PPARα* is reported to upregulate the expression levels of genes related to lipolysis (*CPT-1* and *ACOX1*) and enhance fatty acid β-oxidation (Li et al., 2021). In this study, decreased gene expressions of *PPARα, CPT-I*, and *ACOX1* and downregulated enzyme activities of CPT-1 and ACOX1 in broilers fed a LPD were drastically increased by Ile. Genes and enzymes related to both lipogenesis and lipolysis are regulated by the AMPK/mTOR and Leptin/JAK2/STAT3 signaling pathways. Activation of hepatic AMPK suppresses fatty

acid synthesis and stimulates fatty acid β-oxidation via inhibition of the activities of mTOR and p70S6K by phosphorylation at Ser2448 and Thr389 and reducing protein expression of SREBP1 (Wang et al., 2017). Leptin suppresses food intake, reduces blood TG content, and increases energy expenditure (Wang et al., 2020). The binding of leptin and LEPR phosphorylates JAK2 and activates protein kinase sites that recruit STAT3 and tyrosine phosphorylation by receptor-associated JAK2. Phosphorylated STAT3 dimerizes and translocates into the nucleus, where it binds to the promoters of target genes to regulate lipid metabolism (Chen et al., 2019). In the present study, Ile supplementation upregulated the decreased serum leptin level and expressions of *LEPR* at the mRNA level and p-AMPK/AMPK, p-mTOR/mTOR, p-p70S6K/p70S6K, p-JAK2/JAK2, and p-STAT3/STAT3 at the protein level, and downregulated the increased protein relative expression of SREBP1. These modulation patterns were in line with the regulatory roles of the AMPK/mTOR and Leptin/JAK2/STAT3 signaling pathways, suggesting that Ile could alleviate the LPD-induced lipid deposition through activation of these two pathways.

In summary, the results of the present study demonstrated that dietary supplementation with Ile improved the growth performance and meat quality of broilers fed a LPD. Furthermore, Ile supplementation activated the AMPK/mTOR and Leptin/JAK2/STAT3 signaling pathways, decreased lipogenesis and promoted lipolysis in the liver, and inhibited differentiation and proliferation of abdominal adipocytes, thereby alleviating the lipid deposition induced by a LPD.

AUTHOR CONTRIBUTIONS

Shengnan Ma: Data collection, Data analyses, Writing-Original Draft. Kai Zhang: Data collection & Data analyses. Shuyan Shi: Investigation. Xuemin Li: Formal analysis. Chuanyan Che: Investigation. Peng Chen: Visualization; Huawei Liu: Conceptualization, Writing-Review & Editing, Funding acquisition. All authors have read and approved the published version of the manuscript.

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DISCLOSURES

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