Animal Nutrition 16 (2024) 147–157

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Flavonoids from mulberry leaves inhibit fat production and improve fatty acid distribution in adipose tissue in finishing pigs

Yingying Liu [a, b, c], Yinglin Peng [b, c], Chen Chen [b, c], Huibo Ren [b, c], Ji Zhu [b, c], Yuan Deng [b, c], Qingming Cui [b, c], Xionggui Hu [b, c], Jianhua He [a], Huali Li [b, c], Xinghui Zhu [d], Yulong Yin [a, e, *], Jun He [a], Yi Xiao [d, *]

[a] College of Animal Science and Technology, Hunan Agricultural University, Changsha, 410128, China
[b] Hunan Institute of Animal and Veterinary Science, Changsha, 410131, China
[c] Key Laboratory of Conservation and Genetic Analysis of Local Pig Breed Germplasm Resources, Changsha, 410131, China
[d] College of Information and Intelligence, Hunan Agricultural University, Changsha, 410128, China
[e] Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, 410125, China

ARTICLE INFO

Article history:
Received 15 May 2023
Received in revised form
5 November 2023
Accepted 23 November 2023
Available online 29 November 2023

Keywords:
Finishing pig
Mulberry leaf
Flavonoid
Lipid metabolism
Fatty acid
Lard

ABSTRACT

This study evaluated the effects of flavonoids from mulberry leaves (FML) on plasma biochemical indices, serum activities of lipid metabolism-related enzymes, fat morphology, fatty acid composition, and lipid metabolism in different adipose tissues of finishing pigs. We used 120 Chinese hybrid barrows of Berkshire and Bama mini-pigs with an average initial body weight of 45.11 ± 4.23 kg. The pigs were randomly assigned to five treatment groups and fed a control diet based on corn, soybean meal, and wheat bran or a control diet supplemented with 0.02%, 0.04%, 0.08%, or 0.16% FML. Each experimental group had six replicates (pens), with four pigs per pen. After a 7-d adaptation period, the feeding trial was conducted for 58 d. Blood and adipose tissue samples were collected from 30 pigs (one pig per pen) at the end of the test. The results showed that FML supplementation significantly decreased the feed intake to body gain ratio, the plasma concentrations of total cholesterol and free fatty acids, and the serum activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (linear or quadratic effects, $P < 0.05$), and decreased the plasma triglyceride concentration (quadratic, $P = 0.07$). Increasing FML supplementation increased the average daily gain and serum activities of lipoprotein lipase (linear and quadratic effects, $P < 0.05$) and adipose triglyceride lipase (linear, $P < 0.05$). Dietary FML supplementation decreased the adipocyte area in the dorsal subcutaneous adipose (DSA) tissue of finishing pigs (linear, $P = 0.05$) and increased the adipocyte area in the visceral adipose tissue (quadratic, $P < 0.01$). Increasing FML supplementation decreased the C20:1 content in DSA, abdominal subcutaneous adipose, and visceral adipose tissues of finishing pigs ($P < 0.05$) and increased the C18:3n3 and n-3 PUFA contents ($P < 0.05$). The lipid metabolism genes were regulated by the PPARγ-LXRα-ABCA1 signaling pathway, and their expressions differed in different adipose tissues. These findings suggest that FML improved growth performance, regulated lipid metabolism, inhibited fat production, and improved fatty acid distribution in the adipose tissue of finishing pigs, thereby improving pig fat's nutritional quality and health value.

1. Introduction

Pork is one of the most consumed meats worldwide; however, its production and quality face severe challenges, including feeding sustainability and a perceived negative image of fat (McGlone, 2013). Adipose tissue, the largest energy storage and supply site in the animal body, can be divided into subcutaneous, abdominal, visceral, intermuscular, and intramuscular fat. The order of fat

* Corresponding authors.
E-mail addresses: yinyulong@isa.ac.cn (Y. Yin), Xiaoyi@hunau.edu.cn (Y. Xiao).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2023.11.003

deposition in different parts of the animal body varies (Kolstad, 2001). Subcutaneous fat deposition is the earliest, followed by visceral fat (abdominal and perirenal fat) and fat deposition in the skeletal muscle tissue. Chinese people have eaten lard for thousands of years, and it still remains an important ingredient on the Chinese dining table. Approximately 40% of the fatty acids in lard are saturated fatty acids (SFA), and approximately 60% are unsaturated fatty acids (UFA) (Al-Rashood et al., 1996). Overeating SFA leads to lipid accumulation in the blood and increases the risk of cardiovascular diseases; however, UFA are healthy fats. Proper intake of UFA benefits cardiovascular, cerebrovascular, skin, and hormone secretion. From a health perspective, fat can be good or bad. The so-called "devil fat" is trans-fatty acids, which is more harmful than cholesterol and is the culprit in cardiovascular and cerebrovascular diseases (Denke, 2006). Encouragingly, very few trans-fatty acids are found in lard. Additionally, a well-balanced fatty acid intake can reduce the risk of cardiovascular and related diseases (Shahidi and Ambigaipalan, 2018).

Obesity and its related metabolic diseases caused by excessive accumulation of adipose tissue seriously threaten human health worldwide. Improving glycolipid metabolism using diet therapy/herbal remedies is an effective approach for ameliorating obesity (Kaila and Raman, 2008; Cao et al., 2022). Mulberry leaves are one of the earliest plant resources identified by the Ministry of Health of China as "food and medicine homology." They are rich in amino acids, carbohydrates, vitamins, calcium, iron, manganese, zinc, and other minerals. Moreover, they are rich in metabolites, including flavonoids, polyphenols, alkaloids, polysaccharides, steroids, and other natural active ingredients beneficial for animal health (Zhu et al., 2019; Li et al., 2020). Mulberry leaves have many physiological functions, such as blood sugar and lipid-reducing properties (Han et al., 2018), and antioxidant (Zeng et al., 2019), antiviral, and anticancer properties (Fallah et al., 2016; Thaipitakwong et al., 2018; Zhang et al., 2018; Liu et al., 2019). Furthermore, *Morus* spp. extracts have no toxic effects and have nutraceutical potential (Rodrigues et al., 2019). Over the years, mulberry leaves have gained considerable attention as traditional medicines and have been widely used as adjuvants for weight loss (Chan et al., 2016). The reported benefits of mulberry leaves include inhibiting adipogenesis, improving the lipid profile, and reducing fat deposit and body weight (Sheng et al., 2017; Liu et al., 2021). These effects are likely due to abundant functional components such as flavonoids (Sheng et al., 2019). Mulberry leaves contain a range of flavonoids, including flavone glycosides and flavonoid derivatives, collectively referred to as flavonoids from mulberry leaves (FML). These compounds constitute approximately 10–30 g/kg of the dry weight of mulberry leaves (Jia et al., 1999), and rutin and isoquercetin are primary components (Ju et al., 2018). In recent studies, FML administration decreased lipid accumulation, alleviated liver steatosis and hepatic injury in a non-alcoholic fatty liver disease rat model (Hu et al., 2020), reversed the whitening of brown adipose tissue (BAT), and induced a dramatic shift in the gut microbiota of high-fat diet (HFD)-fed mice (Zhong et al., 2020). Similarly, rutin activated BAT in genetically obese and HFD-induced obese mice (Yuan et al., 2017). Additionally, studies have shown that FML can enhance growth performance and exhibit hypolipidemic effects in rodents and other animals (Kong et al., 2019; Hassan et al., 2020), improve antioxidant capacity, and regulate lipid metabolism in the skeletal muscles of pigs (Liu et al., 2022). Based on these findings, we hypothesized that FML could improve lipid metabolism in adipose tissue by regulating the distribution of fatty acids. Although mulberry leaves have been suggested as a potential intervention to improve lipid traits in finishing pigs, direct experimental or clinical evidence to validate this hypothesis is limited. Therefore, we aimed to investigate whether the FML administration could influence the fatty acid profile and lipid metabolism in finishing pigs.

2. Materials and methods

2.1. Animal ethics statement

The animal experiment conducted in this study was approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University and was conducted in accordance with the Chinese Guideline for Ethical Review of Animal Welfare (ACC2019006, Changsha, China).

2.2. Preparation of flavonoids from mulberry leaves

Mulberry leaves were obtained from the Sericultural Research Institute of Hunan Province (Changsha, China), dried, and crushed into powder. Next, flavonoids were extracted from the leaves using 60% ethanol at 80 °C for 3 h and purified using AB-8 macroporous resin (Sun et al., 2014); a yield of 1.90% total flavonoid was obtained.

2.3. Study animals and experimental design

We used 120 crossbred F1 barrows of Berkshire and Bama minipigs with an average initial body weight of 45.11 ± 4.23 kg. All pigs were randomly allocated to five treatment groups, with six replicates (pens) per group and four pigs per pen. Next, the pigs were fed a control diet based on corn, soybean meal, and wheat bran or a control diet supplemented with 0.02%, 0.04%, 0.08%, or 0.16% FML, meeting the recommendations of the Chinese National Feeding Standard of Swine (NY/T 65-2004). Nutrient levels in the experimental diet were analyzed using the methods of the Association of Official Analytical Chemists (2005), or calculated according to the Chinese National Feeding Standard of Swine (NY/T 65-2004) (Table 1). Briefly, crude protein, crude fat, and ash values were determined, using the method 954.01 of Kjeldahl, method 920.39 of Soxhlet extraction, and method 942.05 of ashing, respectively, following the procedures outlined by the Association of Official Analytical Chemists (2005). Moreover, digestible energy and other nutrients were calculated according to the Chinese National Feeding Standard of Swine (NY/T 65-2004). Pellet feed was

Table 1
Ingredients and nutrient levels of experimental basal diet (%, as-fed basis).[1].

Ingredients	Content
Corn	61.00
Soybean meal	22.00
Wheat bran	14.00
Lysine	0.10
$CaHPO_4$	0.70
Limestone	0.90
Salt	0.30
Premix[2]	1.00
Nutrient levels	
Digestible energy, MJ/kg	13.60
Crude protein	16.06
Crude fat	2.54
Ash	5.23
Calcium	0.61
Total phosphorus	0.54
Available phosphorus	0.24

[1] Adapted from Liu et al. (2022). Basal diet formulated following the Chinese National Feeding Standard of Swine (NY/T 65-2004).

[2] Supplied per kilogram of diet: 400 mg $FeSO_4 \cdot 7H_2O$; 359 mg $ZnSO_4 \cdot 7H_2O$; 80 mg choline chloride; 19.8 mg $CuSO_4 \cdot 5H_2O$; 10.2 mg $MnSO_4 \cdot H_2O$; 0.56 mg $NaSeO_3$; 0.20 mg KI; 15 mg vitamin B_2; 5 mg vitamin K; 2 mg vitamin B_1; 30 μg vitamin B_{12}; 5400 IU vitamin A; 110 IU vitamin D_3; 18 IU vitamin E.

provided thrice daily, and the pigs had ad libitum access to drinking water and were fed throughout the 58-d experiment after a 7-d adaptation period. Each pen was equipped with a stainless-steel feeder and a nipple drinker. The feed consumption of each pen was recorded daily, and the pigs were weighed at the end of the experiment to determine the average daily gain (ADG), average daily feed intake (ADFI), and feed intake to body gain ratio (F:G).

2.4. Sample collection

At the end of this test, one pig per pen was randomly selected for sample collection and slaughtered after fasting for 12 h. To obtain blood samples, jugular venipuncture was performed, and two samples were collected in 10-mL heparin and heparin-free tubes. After centrifugation at 3,000 × *g* and 4 °C for 15 min, plasma and serum samples were obtained. Subsequently, the pigs were electrically stunned, exsanguinated, dehaired, eviscerated, and split down at the midline following standard commercial slaughtering procedures. Immediately after, samples of 1.0 cm^3 section from the dorsal subcutaneous adipose (DSA), abdominal subcutaneous adipose (ASA), and visceral adipose tissues on the right side of the carcass were cut and flashed frozen using liquid N_2 and stored at −80 °C for quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. One sample of a 1.0-cm^3 section from the DSA tissue was collected for Western blot (WB) analysis. Moreover, approximately 100 g of the DSA, ASA, and visceral adipose tissues were collected and stored at −20 °C to determine the fatty acid profile. Finally, samples of 1.0 cm^3 section from the DSA, ASA, and visceral adipose tissues were fixed in 4% paraformaldehyde for histological analysis.

2.5. Analysis of plasma biochemical indices

Plasma concentrations of glucose, triglyceride, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), free fatty acid (FFA), and insulin were detected using a CX-4 Automatic Biochemical Analyzer (Beckman Coulter Inc., Brea, CA, USA) and commercial kits from Leadman Biochemistry Technology Company (Beijing, China), following the manufacturer's instructions.

2.6. Analysis of serum enzyme activities

The activities and concentrations of several enzymes in the serum were analyzed using colorimetric methods with a spectrophotometer (Biomate 5; Thermo Electron Corporation, Rochester, NY, USA). Specifically, the activities of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and lipoprotein lipase (LPL) and concentrations of lecithin-cholesterol acyltransferase and adipose triglyceride lipase (ATGL) were measured using commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

2.7. Morphological study of adipose tissues

Adipocyte areas in the DSA, ASA, and visceral adipose tissues were measured using the classic method of Oil Red O (ORO) staining (Yin et al., 2018). Briefly, the adipose tissues were fixed in a solution containing formalin (10%) and calcium (2%), and incubated with ORO working solution at room temperature (18–28 °C) for 5 min. Finally, the sections were cleared in 60% isopropyl alcohol for 5 min and counterstained with Mayer's hematoxylin for 15 s.

2.8. Analysis of fatty acid composition

To determine the fatty acid composition of the DSA, ASA, and visceral adipose tissues, lipids were extracted from approximately 0.5 g of adipose tissue using chloroform. Lipid methyl esters were obtained by saponification with 2 mL hexane, 40 μL methyl acetate, and 100 μL sodium methoxide solution (Postaue et al., 2020). Next, the fatty acids were analyzed using an Agilent 7890A Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) with a chromatographic column, according to the method previously described by Liu et al. (2015). Afterward, individual fatty acid peaks were identified by comparing their retention times with those of standards. The results were expressed as a percentage of total fatty acids, and SFA, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were calculated, along with the ratios of PUFA to SFA and Σn-6 PUFA to Σn-3 PUFA.

2.9. Quantitative real-time PCR analysis

Using qRT-PCR, we measured mRNA expression levels of genes (Table 2) involved in lipid metabolism in DSA, ASA, and visceral adipose tissues, including acetyl-CoA carboxylase α (*ACCα*), fatty acid synthase (*FAS*), 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*), peroxisome proliferator-activated receptor γ (*PPARγ*), sterol regulatory element-binding proteins 1 (*SREBP1*), liver X receptor α (*LXRα*), and ATP-binding cassette subfamily A member 1 (*ABCA1*) (Liu et al., 2015; Yan et al., 2020). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control.

2.10. Analysis of PPARγ-pathway proteins

The protein expression levels of PPARγ, SREBP1, LXRα, ABCA1, and GAPDH in DSA tissue were determined using Western blot, as previously described (Liu et al., 2018). The primary antibodies and dilutions used are listed in Table 3. The secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) was diluted to 1:6000. Finally, the protein bands were visualized using chemiluminescent reagents, and the density of protein bands was detected using Alpha Imager 2200 software (Alpha Innotech Corporation, San Leandro, CA, USA).

Table 2
Primers used for quantitative real-time PCR.

Genes	Accession no.	Sequence (5′—3′)	Size, bp
ABCA1	NM_001317080.1	F: AATTGTGCCCTAAGTATCGTCA R: GGACCCTGCTATTCGTACAAC	192
ACCα	NM_001114269.1	F: ATCCCTCCTTGCCTCTCCTA R: ACTTCCCGTTCAGATTTCCG	208
FAS	NM_001099930.1	F: GTCCTGCTGAAGCCTAACTC R: TCCTTGGAACCGTCTGTG	206
GAPDH	NM_001206359.1	F: CCACGGTCCATGCCATCACT R: CAGGTCAGATCCACAACCGACAC	214
HMGCR	NM_001122988.1	F: TTTGCCCTCAGCTCCAACTCA R: CCAACTCCAATCACAAGGCAT	119
LXRα	NM_001101814.1	F: AGCTCCGTCCACAAAAGCG R: ATAGCGAGCCCCTTTGATGACA	164
PPARγ	NM_214379.1	F: ATAAAGTCCTTCCCGCTGACC R: GACACCCCTGAAAGATGCGAA	190
SREBP1	NM_214157.1	F: GCAAGGCCATCGACTACATCCG R: CTACCACCTCCGGCTTCACAC	174

ABCA1 = ATP binding cassette subfamily A member 1; F = forward; R = reverse; *ACCα* = acetyl CoA carboxylase α; *FAS* = fatty acid synthase; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *HMGCR* = 3-hydroxy-3-methylglutaryl coenzyme A reductase; *LXRα* = liver X receptor α; *PPARγ* = peroxisome proliferator activated receptor γ; *SREBP1* = sterol regulatory element binding proteins 1.

Table 3
Antibodies and dilution used for Western blot analysis.

Antibody	Catalog number	Dilution
Rabbit polyclonal anti-PPARγ	ab209350	1:500
Rabbit polyclonal anti-Phospho-PPARγ (ser273)	bs-4888R	1:500
Rabbit polyclonal anti-SREBP1	NB100-2215	1:500
Rabbit polyclonal anti-SREBP1 (pS439)	CPA4833	1:500
Rabbit polyclonal anti-LXRα	ab231942	4 μg/mL
Rabbit polyclonal anti-ABCA1	NB400-105	1:500
Rabbit polyclonal anti-GAPDH	10494-1-AP	1:3000

ABCA1 = ATP binding cassette subfamily A member 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; LXRα = liver X receptor α; PPARγ = peroxisome proliferator activated receptor γ; SREBP1 = sterol regulatory element binding proteins 1.

2.11. Statistical analysis

The treatment and control group data were compared using one-way ANOVA in SPSS (IBM Corp. Released 2011, Version 20.0. Armonk, NY, USA). Orthogonal polynomial comparisons were conducted to determine the linear and quadratic effects of increasing dietary FML supplementation on the measured traits. The pen was the experimental unit for growth performance, and the pig was used for other determinations. The results are expressed using mean values and SEM. Results with $P \leq 0.05$ are considered significant, and results with $0.05 < P < 0.10$ are considered trends.

3. Results

3.1. Growth performance

As reported by our previous study (Liu et al., 2022), elevating the FML level from 0.02% to 0.16% resulted in increased final body weight (linear and quadratic, $P < 0.05$, 74.98 ± 1.66 kg to 81.31 ± 1.91 kg) with no change in ADFI (2.34 ± 0.05 kg). Additionally, FML increased ADG (linear and quadratic, $P < 0.05$, 0.52 ± 0.03 kg to 0.62 ± 0.04 kg) and decreased F:G (linear and quadratic, $P < 0.05$, 4.54 ± 0.53 to 3.78 ± 0.37), with the highest value of ADG (0.62 ± 0.04 kg) and the lowest value of F:G (3.78 ± 0.37) in 0.16% FML group.

3.2. Plasma biochemical indices

Dietary FML decreased (linear and quadratic, $P < 0.05$) the plasma concentrations of total cholesterol and FFA in experimental pigs (Table 4). The lowest values of total cholesterol and FFA were observed in the 0.08% and 0.04% FML groups, respectively. Moreover, glucose concentration increased (quadratic, $P = 0.08$) with increasing FML levels. However, the triglyceride concentration decreased (quadratic, $P = 0.07$) with increasing FML levels.

3.3. Serum enzyme activities

Increasing dietary FML supplementation decreased (linear and quadratic, $P < 0.01$) the serum activity of HMGCR in finishing pigs and increased the activities of LPL (linear and quadratic, $P < 0.05$) and ATGL (linear, $P < 0.05$) (Table 5). The lowest activities of HMGCR and LPL were observed in the 0.08% FML and control group, respectively.

3.4. Adipocyte area in adipose tissues

Dietary FML decreased (linear, $P = 0.05$) the adipocyte area in the DSA tissue of finishing pigs and increased (quadratic, $P < 0.01$) the adipocyte area in the visceral adipose tissue (Figs. 1 and 2), while had no significant effect ($P > 0.05$) on the adipocyte area in the ASA tissue of the studied pigs.

3.5. Fatty acid profile in adipose tissues

As shown in Table 6, increasing dietary FML supplementation decreased (linear, $P < 0.05$) the C20:1 content and increased (linear, $P < 0.05$) the C18:3n3 and n-3PUFA contents in the DSA tissue of finishing pigs.

Increasing dietary FML supplementation decreased (linear and quadratic, $P < 0.05$) the C20:1 content and ∑n-6/∑n-3 ratio in the ASA tissue of finishing pigs and increased (linear, $P < 0.05$) the C18:2n6, C18:3n3, C20:4n6, PUFA, n-3PUFA, and n-6PUFA contents, and the PUFA/SFA ratio (Table 7). The highest values of C18:3n3, C20:4n6, and n-3PUFA contents and the PUFA/SFA ratio were all observed in the 0.08% FML group, while the lowest content of C20:1 was observed in the 0.16% FML group.

Increasing dietary FML supplementation decreased the C16:1 (quadratic, $P < 0.05$) and C20:1 contents (linear, $P < 0.05$) in the visceral adipose tissue of finishing pigs, and increased (linear and quadratic, $P < 0.05$) the C18:3n3, C20:4n6, n-3PUFA, and PUFA contents (Table 8). Additionally, the C18:2n6, n-6PUFA contents, and the PUFA/SFA ratio were increased (linear, $P < 0.05$).

3.6. Expression levels of lipid metabolism-related genes in adipose tissues

Dietary FML upregulated (linear and quadratic, $P < 0.05$) the mRNA expression levels of *ABCA1* and *SREBP1* in the DSA tissues of finishing pigs (Table 9). The highest values of *ABCA1* and *SREBP1* expression levels were observed in the 0.16% and 0.08% FML groups, respectively. And the highest values of *HMGCR* and *PPARγ* expression levels were observed in the 0.04% FML group.

Dietary FML upregulated (linear and quadratic, $P < 0.01$) the *PPARγ* expression level in the ASA tissue of finishing pigs (Table 10).

Table 4
Dietary supplementation of flavonoids from mulberry leaves (FML) affected plasma chemical indexes of pigs ($n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
Glu, mmol/L	5.51	6.62	6.19	6.03	6.04	0.198	0.54	0.08
Triglyceride, mmol//L	0.66	0.57	0.57	0.47	0.65	0.051	0.47	0.07
Total cholesterol, mmol//L	2.38	2.27	2.11	2.05	2.12	0.043	<0.01	<0.01
HDL, mmol//L	0.72	0.71	0.74	0.62	0.77	0.055	0.96	0.71
LDL, mmol//L	1.13	1.04	0.90	1.07	1.00	0.060	0.27	0.19
HDL/total cholesterol ratio	0.30	0.31	0.35	0.30	0.36	0.023	0.15	0.35
HDL/LDL ratio	0.66	0.71	0.82	0.58	0.78	0.079	0.65	0.89
FFA, μmol//L	499.32	496.55	436.57	465.35	466.61	11.154	0.03	0.02
Insulin, mIU/L	27.42	26.73	32.18	29.57	28.35	1.541	0.38	0.23

Glu = glucose; HDL = high density lipoprotein; LDL = low density lipoprotein; FFA = free fatty acid.

Table 5
Dietary supplementation of flavonoids from mulberry leaves (FML) affected serum enzyme activities of pigs ($n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
HMGCR, U/L	118.18	111.28	92.69	83.61	96.54	6.699	<0.01	<0.01
G6PD, U/L	18.16	19.55	19.70	22.62	21.73	1.895	0.09	0.23
IDH, U/L	68.79	69.46	78.84	72.27	77.40	5.670	0.26	0.51
LPL, U/L	512.70	627.86	632.55	591.36	649.71	31.425	0.04	0.05
LCAT, mIU/mL	51.63	51.15	55.89	56.83	58.34	4.754	0.19	0.43
ATGL, mIU/mL	461.35	458.59	493.88	535.38	506.43	24.688	0.04	0.11

HMGCR = 3-hydroxy-3-methylglutaryl coenzyme A reductase; G6PD = glucose-6-phosphate dehydrogenase; IDH = isocitrate dehydrogenase; LPL = lipoprotein lipase; LCAT = lecithin-cholesterol acyltransferase; ATGL = adipose triglyceride lipase.

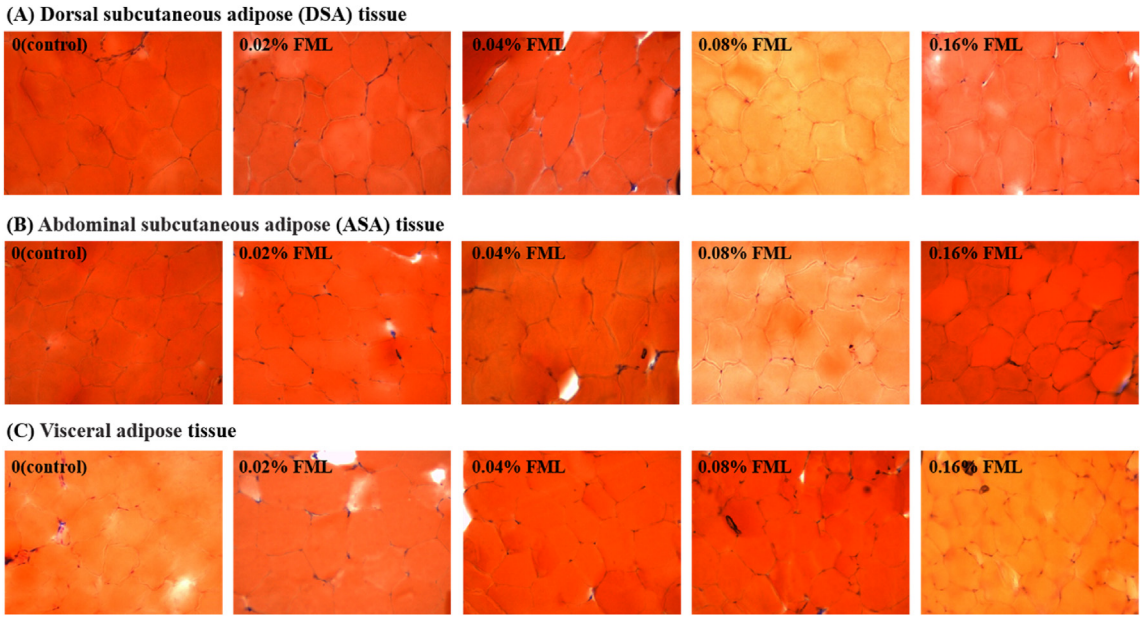

Fig. 1. Representative frozen section of adipose tissues (Oil Red O staining, 200× magnification) of pigs ($n = 6$). (A) Dorsal subcutaneous adipose tissue, (B) abdominal subcutaneous adipose tissue, (C) visceral adipose tissue. FML = flavonoids from mulberry leaves.

The highest value of *PPARγ* expression level was observed in pigs fed the 0.16% FML diet.

Increasing dietary FML supplementation upregulated (linear and quadratic, $P < 0.05$) the mRNA expression levels of *ACCα* and *PPARγ* in the visceral adipose tissue of finishing pigs (Table 11). The highest values of *ACCα* and *PPARγ* were observed in the 0.16% and 0.08% FML groups, respectively.

3.7. Abundance of PPARγ-pathway proteins

The protein abundance of total or phosphorylated (P)-PPAR, PPARγ, P-SREBP1, SREBP1, LXRα, and ABCA1 in the DSA tissue of finishing pigs increased (linear or quadratic, $P < 0.05$) with increasing FML inclusion (Fig. 3). Additionally, increasing dietary FML supplementation increased (linear and quadratic, $P < 0.05$) the P-PPARγ/PPARγ ratio, with the highest value observed in the 0.16% FML group. And the highest values of P-SREBP1/SREBP1 ratio, and protein abundances of LXRα and ABCA1 were observed in the 0.08%, 0.02%, and 0.04% FML groups, respectively.

4. Discussion

Currently, the pork industry faces significant challenges related to sustainability and the perceived negative image of fat (Font-i-Furnols and Guerrero, 2014). Pork accounts for approximately 38% of the total amount of meat produced worldwide and is the most commonly consumed meat in European, American, and Asian countries (Szűcs and Vida, 2017). China is rich in pig breeds, mostly lard-type breeds, such as Bama miniature pigs. The Bama miniature pig (*Suscrofa domestica*), located in Bama County, Guangxi Province, China, is a potential animal model for studying lipid metabolism. Due to their long feeding history, genetic stability, and excellent meat quality, many studies in genetics and nutrition have used hybrid offspring of Bama mini-pigs and Berkshire pigs. Furthermore, lard is a popular table seasoning in some countries, especially China. Excessive consumption of lard can harm human health (Dunshea and D' Souza, 2003). Obtaining healthy lard with no side effects has been the focus of research and exploration in food nutrition. FML, the main component of mulberry leaves, regulates lipid metabolism in animals. However, its ability to modulate the formation of pig fat products, such as lard, has not been studied. To our knowledge, our study is the first to demonstrate that the growth performance of Chinese hybrid barrows of Berkshire and Bama mini-pigs could be improved by including FML (0.02% to 0.16%) in their diet. We further investigated the effects of FML supplementation on the blood lipid index concentration, lipid-related enzyme activity, adipose tissue histomorphology, fatty acid composition and content in different adipose tissues, and their regulatory mechanisms. We established that FML reduced blood lipids and improved the distribution of fatty acids in the adipose

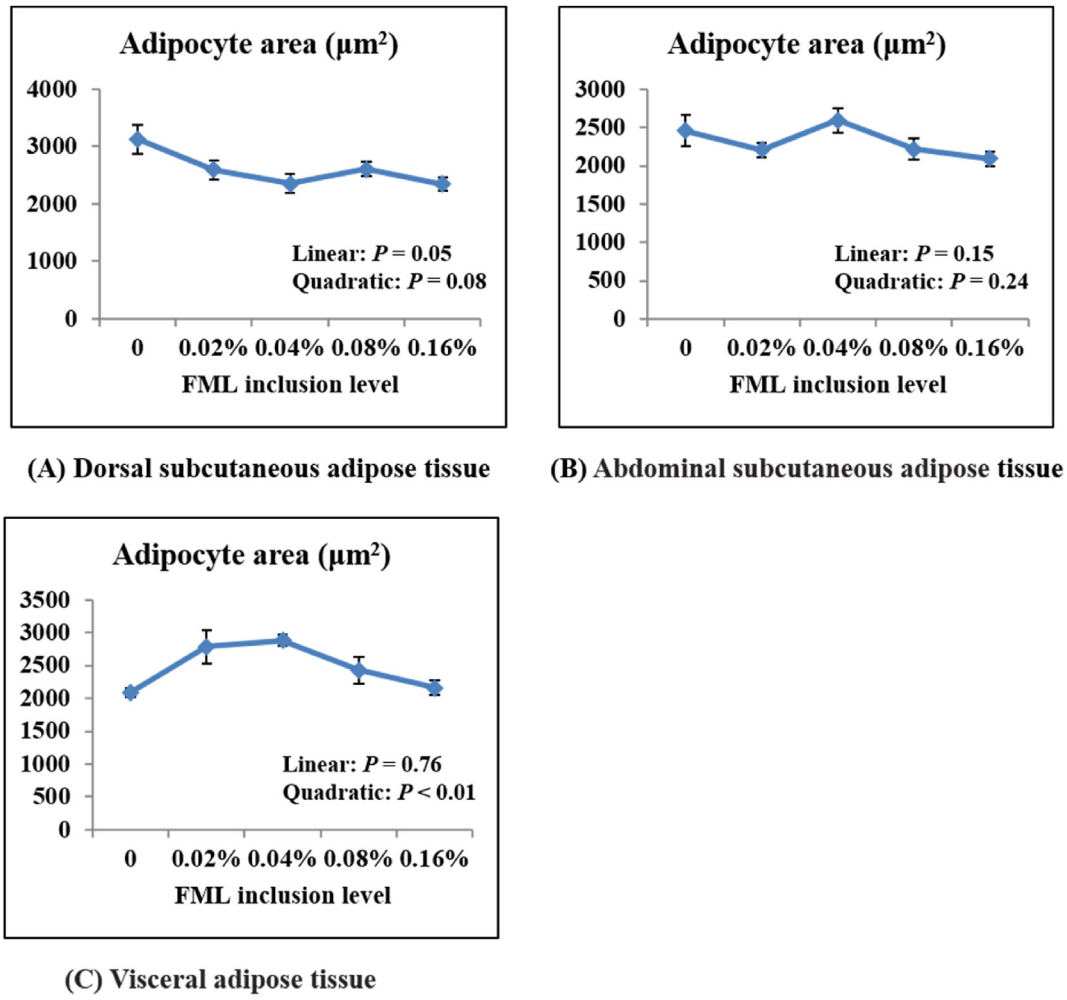

Fig. 2. Dietary supplementation of flavonoids from mulberry leaves (FML) affected morphology traits in adipose tissues of pigs ($n = 6$). (A) Dorsal subcutaneous adipose tissue, (B) abdominal subcutaneous adipose tissue, (C) visceral adipose tissue. FML = flavonoids from mulberry leaves.

Table 6
Dietary supplementation of flavonoids from mulberry leaves (FML) affected fatty acid percentages in dorsal subcutaneous adipose tissue of pigs (%, $n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
C8:0	0.17	0.11	0.17	0.16	0.14	0.017	0.88	0.98
C10:0	0.09	0.09	0.10	0.09	0.10	0.003	0.19	0.42
C12:0	0.17	0.12	0.14	0.14	0.14	0.011	0.71	0.64
C14:0	1.46	1.34	1.46	1.40	1.48	0.027	0.63	0.45
C16:0	26.64	26.48	27.22	25.94	27.21	0.241	0.72	0.82
C16:1	1.62	1.31	1.60	1.68	1.81	0.062	0.08	0.08
C17:0	0.79	0.62	0.62	0.68	0.74	0.030	0.84	0.10
C18:0	16.70	18.29	17.45	16.30	16.77	0.299	0.38	0.42
C18:1n9	38.33	38.28	38.06	38.46	37.15	0.309	0.32	0.46
C18:2n6	10.69	10.13	10.19	11.52	11.47	0.332	0.21	0.34
C20:0	0.27	0.30	0.26	0.24	0.28	0.009	0.58	0.85
C20:1	1.53	1.53	1.39	1.30	1.20	0.057	0.02	0.06
C18:3n3	0.48	0.46	0.47	0.55	0.54	0.015	0.02	0.12
C20:2	0.82	0.78	0.72	0.78	0.73	0.023	0.23	0.43
C20:4n6	0.22	0.23	0.24	0.27	0.25	0.009	0.10	0.25
SFA	46.30	47.35	47.43	45.72	46.86	0.440	0.87	0.93
MUFA	41.48	41.12	41.05	41.44	40.16	0.315	0.30	0.51
PUFA	12.21	11.60	11.61	13.23	12.99	0.361	0.21	0.36
PUFA/SFA ratio	0.27	0.25	0.25	0.29	0.28	0.010	0.32	0.50
n-3PUFA	0.48	0.46	0.47	0.55	0.54	0.015	0.02	0.12
n-6PUFA	10.91	10.36	10.42	11.79	11.72	0.339	0.20	0.34
∑n-6/∑n-3 ratio	22.45	22.36	22.32	21.96	21.81	0.170	0.16	0.36

SFA = saturated fatty acids; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

Table 7
Dietary supplementation of flavonoids from mulberry leaves (FML) affected fatty acid percentages in abdominal subcutaneous adipose tissue of pigs (%, $n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
C8:0	0.24	0.47	0.24	0.14	0.17	0.042	0.11	0.21
C10:0	0.14	0.09	0.13	0.13	0.13	0.005	0.57	0.54
C12:0	0.22	0.19	0.12	0.11	0.14	0.021	0.08	0.13
C14:0	1.72	1.63	1.67	1.62	1.69	0.034	0.80	0.75
C16:0	27.53	27.46	27.43	26.49	27.13	0.219	0.26	0.52
C16:1	2.50	2.23	2.52	2.43	2.92	0.096	0.13	0.11
C17:0	0.67	0.73	0.60	0.67	0.68	0.023	0.74	0.87
C18:0	14.04	14.97	13.85	13.74	13.18	0.279	0.14	0.24
C18:1n9	40.80	40.20	40.34	40.71	40.76	0.416	0.89	0.91
C18:2n6	9.54	9.76	10.11	11.33	10.67	0.267	0.04	0.12
C20:0	0.20	0.20	0.17	0.17	0.21	0.006	0.64	0.18
C20:1	1.20	1.17	1.18	1.01	0.92	0.040	<0.01	0.02
C18:3n3	0.46	0.47	0.50	0.56	0.54	0.013	<0.01	0.01
C20:2	0.61	0.61	0.63	0.64	0.57	0.013	0.52	0.58
C20:4n6	0.26	0.30	0.27	0.33	0.31	0.008	0.02	0.06
SFA	44.76	45.74	44.52	43.07	43.32	0.408	0.05	0.14
MUFA	44.50	43.59	44.05	44.14	44.60	0.473	0.83	0.83
PUFA	10.86	11.14	11.52	12.85	12.09	0.289	0.04	0.11
PUFA/SFA ratio	0.24	0.24	0.26	0.30	0.28	0.007	<0.01	0.03
n-3PUFA	0.46	0.47	0.50	0.56	0.54	0.013	<0.01	0.01
n-6PUFA	9.80	10.06	10.38	11.65	10.98	0.272	0.04	0.11
∑n-6/∑n-3 ratio	21.47	21.30	20.75	20.71	20.38	0.166	0.01	0.05

SFA = saturated fatty acids; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

Table 8
Dietary supplementation of flavonoids from mulberry leaves (FML) affected fatty acid percentages in visceral adipose tissue of pigs (%, $n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
C8:0	0.21	0.21	0.25	0.22	0.20	0.021	0.92	0.85
C10:0	0.15	0.12	0.15	0.16	0.14	0.007	0.94	1.00
C12:0	0.23	0.16	0.19	0.23	0.25	0.020	0.47	0.44
C14:0	1.54	1.41	1.43	1.42	1.51	0.032	0.83	0.31
C16:0	28.11	27.40	27.55	27.04	28.36	0.237	0.87	0.24
C16:1	1.47	1.13	1.28	1.21	1.53	0.051	0.54	0.02
C17:0	0.62	0.62	0.59	0.67	0.68	0.028	0.37	0.56
C18:0	20.86	22.67	21.18	21.54	20.95	0.301	0.65	0.46
C18:1n9	36.90	34.56	36.02	35.39	34.12	0.471	0.15	0.36
C18:2n6	7.44	9.03	8.76	9.66	9.83	0.333	0.02	0.05
C20:0	0.25	0.28	0.25	0.23	0.25	0.009	0.49	0.76
C20:1	1.17	1.12	1.17	0.98	0.87	0.052	0.04	0.08
C18:3n3	0.36	0.43	0.42	0.46	0.48	0.016	0.01	0.04
C20:2	0.48	0.51	0.55	0.56	0.51	0.014	0.33	0.16
C20:4n6	0.22	0.25	0.23	0.25	0.27	0.007	0.01	0.04
SFA	51.96	52.88	51.58	51.50	52.41	0.408	0.90	0.94
MUFA	39.54	36.81	38.46	37.58	36.52	0.513	0.14	0.34
PUFA	8.50	10.32	9.96	10.93	11.09	0.358	0.02	0.05
PUFA/SFA ratio	0.16	0.20	0.19	0.21	0.21	0.007	0.02	0.06
n-3PUFA	0.36	0.43	0.42	0.46	0.48	0.016	0.01	0.04
n-6PUFA	7.66	9.32	8.98	9.91	10.10	0.338	0.02	0.05
∑n-6/∑n-3 ratio	21.20	21.93	21.21	21.49	21.02	0.158	0.46	0.45

SFA = saturated fatty acids; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

tissue and could thus be an interesting source for supplying these beneficial fatty acids to humans.

The triglyceride, total cholesterol, HDL, and LDL levels are commonly used indicators of blood lipid metabolism. Studies on pigs, rats, chickens, and other animals have shown that adding mulberry leaf extract to the diet reduces blood total cholesterol and triglyceride concentrations and inhibits serum lipase activity. Song et al. (2016) found that mulberry leaf extract significantly decreased the serum concentrations of total cholesterol, triglyceride, LDL-cholesterol, and HDL-cholesterol in HFD-fed mice. Similarly, Folium Mori extract decreased body weight and triglyceride, total cholesterol, and LDL levels, improving insulin resistance in type 2 diabetes mellitus rats (Cai et al., 2016). These findings are consistent with our study, in which dietary FML decreased the plasma concentrations of total cholesterol and FFA in finishing pigs, and the triglyceride concentration decreased with increasing FML levels. Furthermore, previous studies have demonstrated the potential of mulberry leaf extract as a complementary mealtime glucose option for patients with type 2 diabetes mellitus because it significantly lowers postprandial glucose levels (Riche et al., 2017). Additionally, an ethanol extract from mulberry leaves, 1-deoxynojirimycin, lowered blood glucose levels in db/db mice (Hu et al., 2017). In our study, the glucose concentration increased with the increasing FML levels. This difference may be because FML activates the AMPK-PGC-1 pathway, improving glucose uptake and mitochondrial function (Meng et al., 2020a).

Table 9
Dietary supplementation of flavonoids from mulberry leaves (FML) modulated the mRNA expression levels of lipid metabolism-related genes in dorsal subcutaneous adipose tissue of pigs ($n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
ABCA1	1.17	1.49	1.83	1.66	2.19	0.132	<0.01	<0.01
ACCα	0.04	0.06	0.06	0.07	0.07	0.013	0.14	0.29
FAS	0.59	0.79	0.76	0.76	0.62	0.153	0.93	0.51
HMGCR	0.09	0.11	0.15	0.10	0.10	0.010	0.52	0.03
LXRα	8.04	9.71	9.18	7.40	8.99	0.454	0.82	0.80
PPARγ	0.95	1.10	1.65	0.80	1.60	0.135	0.12	0.30
SREBP1	0.25	0.40	0.31	0.43	0.41	0.039	0.01	0.04

ACCα = acetyl CoA carboxylase α; *ABCA1* = ATP binding cassette subfamily A member 1; *HMGCR* = 3-hydroxy-3-methylglutaryl coenzyme A reductase; *LXRα* = liver X receptor α; *FAS* = fatty acid synthase; *PPARγ* = peroxisome proliferator activated receptor γ; *SREBP1* = sterol regulatory element binding proteins 1.

Table 10
Dietary supplementation of flavonoids from mulberry leaves (FML) modulated the mRNA expression levels of lipid metabolism-related genes in abdominal subcutaneous adipose tissue of pigs ($n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
ABCA1	1.18	0.61	0.74	1.18	0.82	0.088	0.72	0.34
ACCα	0.12	0.10	0.12	0.18	0.12	0.041	0.55	0.81
FAS	0.78	0.98	0.98	0.72	0.88	0.265	0.94	0.94
HMGCR	0.09	0.08	0.07	0.11	0.07	0.010	0.83	0.92
LXRα	6.92	5.36	5.93	7.82	5.41	0.340	0.75	0.94
PPARγ	0.65	0.78	0.88	0.89	1.15	0.090	<0.01	<0.01
SREBP1	0.44	0.36	0.41	0.37	0.30	0.042	0.07	0.19

ACCα = acetyl CoA carboxylase α; *ABCA1* = ATP binding cassette subfamily A member 1; *HMGCR* = 3-hydroxy-3-methylglutaryl coenzyme A reductase; *LXRα* = liver X receptor α; *FAS* = fatty acid synthase; *PPARγ* = peroxisome proliferator activated receptor γ; *SREBP1* = sterol regulatory element binding proteins 1.

Table 11
Dietary supplementation of flavonoids from mulberry leaves (FML) modulated the mRNA expression levels of lipid metabolism-related genes in visceral adipose tissue of pigs ($n = 6$).

Item	FML inclusion level					SEM	*P*-value	
	0	0.02%	0.04%	0.08%	0.16%		Linear	Quadratic
ABCA1	1.05	1.33	0.89	1.57	1.18	0.131	0.34	0.60
ACCα	0.31	0.33	0.23	0.41	0.60	0.075	0.02	0.01
FAS	0.10	0.09	0.11	0.13	0.12	0.037	0.46	0.76
HMGCR	0.16	0.18	0.15	0.21	0.17	0.021	0.37	0.66
LXRα	7.11	6.84	5.37	8.11	6.80	0.483	0.73	0.62
PPARγ	0.72	0.85	1.01	1.22	1.09	0.105	<0.01	0.01
SREBP1	0.31	0.19	0.20	0.28	0.22	0.029	0.37	0.26

ACCα = acetyl CoA carboxylase α; *ABCA1* = ATP binding cassette subfamily A member 1; *HMGCR* = 3-hydroxy-3-methylglutaryl coenzyme A reductase; *LXRα* = liver X receptor α; *FAS* = fatty acid synthase; *PPARγ* = peroxisome proliferator activated receptor γ; *SREBP1* = sterol regulatory element binding proteins 1.

LPL plays a key role in triglyceride metabolism in animals and is vital for lipid digestion and utilization when activated. It can hydrolyze chylomicron particles, LDL, and very low-density lipoproteins in triglyceride to FFAs and monoacylglycerols, which various tissues can use. Hence, it has an evident effect in eliminating excess triglyceride from the body. HMGCR is the rate-limiting enzyme in cholesterol metabolism, which regulates the body's cholesterol levels; its inhibition can control the amount of endogenous cholesterol. In our study, increasing dietary FML supplementation decreased the serum activity of HMGCR in finishing pigs and increased the activities of LPL and ATGL. This suggests that FML increases lipolytic enzyme activity and decreases cholesterol-producing enzyme activity to reduce blood lipids.

Lipid metabolism is reflected in the lipid profile of blood and lipid accumulation in tissues. Fat deposition in the tissues primarily involves the increase in the number and volume of adipocytes. Subcutaneous and visceral adipose tissues exhibit distinct developmental and metabolic patterns, particularly regarding fatty acid composition. Typically, immature adipose tissue, such as subcutaneous fat, is located externally, and unsaturated fatty tissues, such as perirenal fat, are located internally (Jiang et al., 2013). Adipose tissue is primarily composed of fatty acids and triglyceride. The types and proportions of fatty acids in the adipose tissue are influenced by various factors, including dietary intake (Martins et al., 2012), breed, and genotype. In our study, dietary FML supplementation decreased the adipocyte area in DSA tissue, but did not affect ASA tissue. Additionally, the adipocyte area in the visceral adipose tissue increased with 0.02% and 0.04% FML administration and decreased with 0.16% FML administration. Some studies found that FML reduced the body weight and liver index of type 2 diabetes mellitus rats (Duan et al., 2022) and reduced fat deposition in obese mice (Lim et al., 2013); however, they did not investigate the effects of flavonoids on subcutaneous or visceral fat. Further research is needed to confirm these findings and determine the mechanisms underlying these effects.

Furthermore, we tested the fatty acid content in the three adipose tissues to verify the above findings. The fatty acid profiles were similar in all three adipose tissues, with oleic (C18:1n9) and palmitic acids (C16:0) accounting for approximately 70% of the total fatty acids in DSA, ASA, and visceral adipose tissues. Additionally, stearic (C18:0) and linoleic acids (C18:2n6c) accounted for approximately 25% of the total fatty acids in each tissue. This result is consistent with Lorenzo et al. (2012) and Jiang et al. (2018). Oleic acid (C18:1n9), the most abundant fatty acids, is essential for human heart health and lowers LDL (so-called bad cholesterol) levels. Its practical application recently suggests that the demand for foods with nutritional characteristics is increasing, and it has aroused interest in developing a new generation of lipids, the so-called healthy lipids. FML supplementation decreased C20:1, and increased C18:3n3 and n-3PUFA contents in DSA, ASA, and visceral adipose tissues. Additionally, FML supplementation increased the C20:4n6, n-3PUFA, n-6PUFA, and PUFA contents and the PUFA/SFA ratios in the ASA and visceral adipose tissues. PUFA is an important component of the lipid structure of biofilms, which maintains and stabilizes their structure and function. Of particular interest are the health benefits associated with the consumption of n-3PUFA. These fatty acids have shown positive effects on cardiovascular diseases, diabetes, cancer, depression, and various mental illnesses (Saini and Keum, 2018; Shahidi and Ambigaipalan, 2018). C20:4n6, C18:2n6, and C18:3n3 are the three essential PUFAs in the human body. Therefore, adding an appropriate proportion of FML to the diet can improve the composition of fatty acids in the adipose tissues of finishing pigs, specifically by increasing the content of PUFA, especially n-3PUFA in adipose tissues, and reducing the content of SFA.

Lipid metabolism is influenced by the balance between lipogenesis and lipolysis, which are critical for adipose tissue accumulation. Several genes related to lipid metabolism, including *ACCα*, *FAS*, *PPARγ*, *SREBP1*, *LXRα*, and *ABCA1*, have been identified as potential regulators. The nuclear receptor superfamily of transcription factors, including PPAR and LXR, regulates cholesterol homeostasis in the body (Li and Glass, 2004). As a member of the PPAR superfamily, PPARγ is widely expressed in animal adipose tissue with tissue specificity (Madeira et al., 2014; Chen et al., 2017).

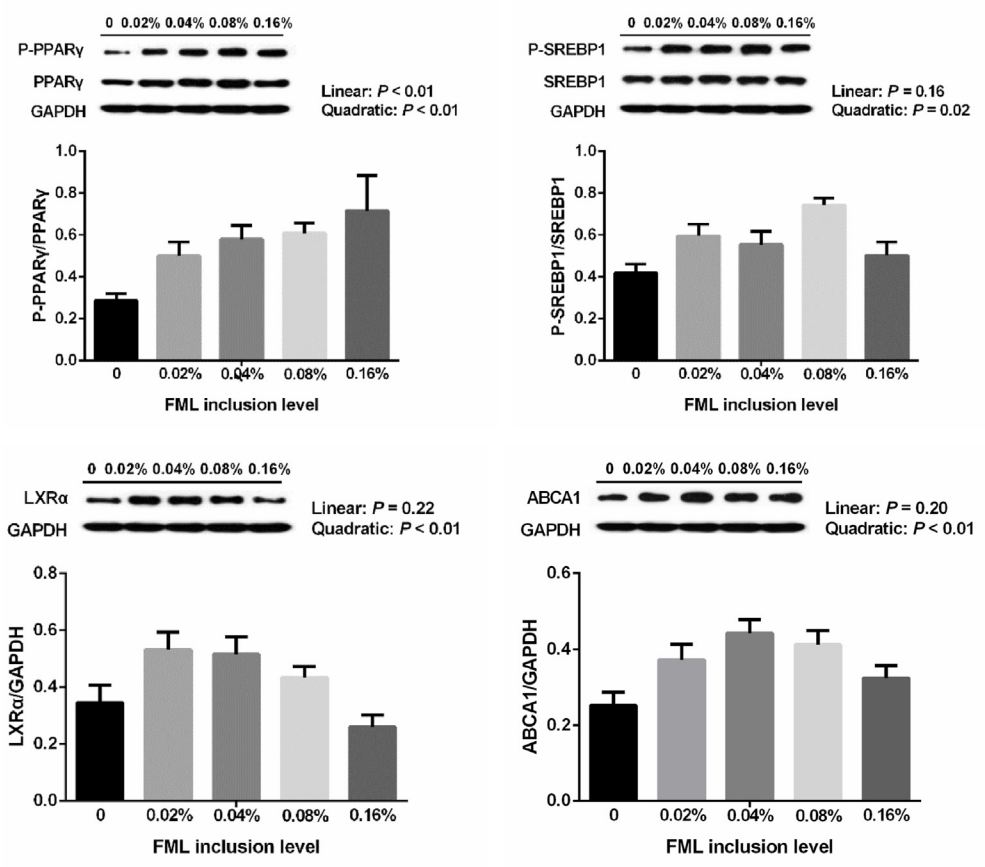

Fig. 3. Representative Western blot analysis of total and phosphorylated protein in dorsal subcutaneous adipose (DSA) tissue of pigs (n = 6). PPARγ = peroxisome proliferator activated receptor γ; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; FML = flavonoids from mulberry leaves; SREBP1 = sterol regulatory element binding proteins 1; LXRα = liver X receptor α; ABCA1 = ATP binding cassette subfamily A member 1.

Similar to PPARγ, LXRα is a member of ligand-activated nuclear transcription factor superfamily. PPARγ and LXRα regulate adipose differentiation, transport, and lipid deposition in cells and have become new targets of drugs for hyperlipidemia. PPARγ can indirectly participate in the up-regulation of ABCA1 expression and cholesterol reverse transport by enhancing LXRα transcription. Several studies have shown that nuclear receptors PPAR and LXR, along with LPL and ABCA1, play a significant role in regulating lipid metabolism and efflux in humans and hamsters (Forcheron et al., 2002; Beaven and Tontonoz, 2006; Srivastava and He, 2010; Saurav et al., 2012). In our study, the gene expression levels of *ABCA1* and *PPARγ* in the DSA tissues of experimental pigs were upregulated with increasing FML supplementation. Additionally, Western blot revealed that the protein abundances of LXRα and ABCA1 and the ratio of P-PPARγ/PPARγ increased significantly. Therefore, considering these findings and the decrease in adipocyte area in the DSA tissue, we inferred that FML might synergistically upregulate *ABCA1* expression based on PPAR and LXR targets on the PPARγ-LXRα-ABCA1 signaling pathway, thus reducing lipid deposition in the DSA tissue. Recent in vitro research using the 3T3-L1 adipocyte IR model showed that flavonoids from mulberry leaf extract could alleviate glycolipid metabolic abnormalities (Meng et al., 2020b). However, unlike the DSA tissue, we observed increased *PPARγ* and *LXRα* expression levels and decreased *ABCA1* expression levels in the ASA tissue with FML supplementation. The above phenomenon is inconsistent with the PPAR γ-LXR α-ABCA1 signaling pathway in the DSA tissue, suggesting that FML has no effect on adipocyte area in the ASA tissue. Activated LXRα can induce the transcription of SREBP-1, an important nuclear transcription factors, mainly responsible for regulating the expression of genes related to FAS, triglyceride, and glucose metabolism to maintain the dynamic balance of blood lipid (Walker et al., 2011; Peng et al., 2017). Activated SREBP-1 enhanced the expression of cholesterol and fatty acid synthesis-related genes (Hu et al., 2019). Additionally, overexpression of the SREBP-1c protein can increase the expression and activity of FAS, resulting in abnormal lipid deposition in liver tissue, thus forming a fatty liver (Zarate et al., 2017). In our study, the expression levels of *SREBP1* in the visceral adipose tissues of the FML groups were lower than those of the control group; however, the expression level of *ACCα* increased. Combined with the fatty acid distribution in visceral adipose tissue, the adipocyte area in visceral adipose tissue first increased and decreased with an increase in the FML dose, suggesting that different FML doses may have different effects on gene expression and related signaling pathways in this tissue.

5. Conclusions

FML supplementation improved the growth performance, regulated the biochemical indices and enzyme activities related to lipid metabolism in the blood of finishing pigs, increased beneficial fatty acids, such as C18:3n3, n-3PUFA, and reduced the ratio of n-6PUFA to n-3PUFA in adipose tissue, thus improving the nutritional value of pig fat. Additionally, the expressions of related genes and proteins were affected by the PPARγ-LXRα-ABCA1 signaling pathway. Overall, this study suggests that FML is an effective and feasible diet/herbal therapy to improve lipid metabolism, inhibit obesity in pigs, and improve the health value of pig fat.

Author contributions

Yingying Liu and **Yi Xiao** carried out the animal experiments and formal analysis, and drafted the manuscript. **Yulong Yin** and **Yi Xiao** designed the study and revised the manuscript. **Yi Xiao**, **Xinghui Zhu**, and **Chen Chen** helped with the data collection and formal analysis. **Huibo Ren**, **Ji Zhu**, **Yuan Deng**, **Qingming Cui**, **Xionggui Hu**, and **Huali Li** participated in the animal trial. **Yinglin Peng**, **Jianhua He**, and **Jun He** reviewed the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

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

Acknowledgements

This work was supported by the Hunan Province Key R&D Program Project (2020NK2033), Natural Science Foundation of Hunan Province (2022JJ30317), Innovation Platform and Talent Plan Program of Hunan Province (2022NK4145), National Natural Science Foundation of China (31972600).

References

Al-Rashood KA, Abou-Shaaban RRA, Abdel-Moety EM, Raul A. Compositional and thermal characterization of genuine and randomized lard: a comparative study. J Am Oil Chem Soc 1996;73(3):303–9.
Association of Official Analytical Chemists (AOAC). In: George J, Latimer W, editors. Official methods of analysis. 18th ed. Gaithersburg, MD: AOAC International; 2005.
Beaven SW, Tontonoz P. Nuclear receptors in lipid metabolism: targeting the heart of dyslipidemia. Annu Rev Med 2006;57:313–29.
Cai S, Sun W, Fan Y, Guo X, Xu G, Xu T, et al. Effect of mulberry leaf (*Folium Mori*) on insulin resistance via IRS-1/PI3K/Glut-4 signalling pathway in type 2 diabetes mellitus rats. Pharm Biol 2016;54(11):2685–91.
Cao S, Tang W, Diao H, Li S, Yan H, Liu J. Reduced meal frequency decreases fat deposition and improves feed efficiency of growing-finishing pigs. Animals 2022;12(19):2557.
Chan EW, Lye PY, Wong SK. Phytochemistry, pharmacology, and clinical trials of *Morus alba*. Chin J Nat Med 2016;14(1):17–30.
Chen X, Luo Y, Wang R, Zhou B, Huang Z, Jia G, et al. Effects of fatty acid transport protein 1 on proliferation and differentiation of porcine intramuscular preadipocytes. Anim Sci J 2017;88(5):731–8.
Duan Y, Dai H, An Y, Cheng L, Shi L, Lv Y, et al. Mulberry leaf flavonoids inhibit liver inflammation in type 2 diabetes rats by regulating TLR4/MyD88/NF-κB signaling pathway. Evid-Based Compl Alt 2022;2022:3354062.
Dunshea FR, D'souza DN. A review—fat deposition and metabolism in the pig. Manipulating pig production 2003;IX:127–50.
Denke MA. Dietary fats, fatty acids, and their effects on lipoproteins. Curr Atherosclerosis Rep 2006;8(6):466–71.
Fallah S, Karimi A, Panahi G, Gerayesh Nejad S, Fadaei R, Seifi M. Human colon cancer HT-29 cell death responses to doxorubicin and *Morus Alba* leaves flavonoid extract. Cell Mol Biol 2016;62(3):72–7.
Font-i-Furnols M, Guerrero L. Consumer preference, behavior and perception about meat and meat products: an overview. Meat Sci 2014;98(3):361–71.
Forcheron F, Cachefo A, Thevenon S, Pinteur C, Beylot M. Mechanisms of the triglyceride- and cholesterol-lowering effect of fenofibrate in hyperlipidemic type 2 diabetic patients. Diabetes 2002;51(12):3486–91.
Han T, Wang W, Cao X. Purification and activity research of hypoglycemic components from the extract of mulberry (*Morus alba* L.) leaves. Sep Sci Plus 2018;1(7):520–5.
Hassan F, Arshad MA, Li M, Rehman MS, Loor JJ, Huang J. Potential of mulberry leaf biomass and its flavonoids to improve production and health in ruminants: mechanistic insights and prospects. Animals 2020;10(11):2076.
Hu J, Hong W, Yao KN, Zhu XH, Chen ZY, Ye L. Ursodeoxycholic acid ameliorates hepatic lipid metabolism in LO2 cells by regulating the AKT/mTOR/SREBP-1 signaling pathway. World J Gastroenterol 2019;25(12):1492–501.
Hu XQ, Thakur K, Chen GH, Hu F, Zhang JG, Zhang HB, et al. Metabolic effect of 1-deoxynojirimycin from mulberry leaves on db/db diabetic mice using liquid chromatography-mass spectrometry based metabolomics. J Agric Food Chem 2017;65(23):4658–67.
Hu Y, Xu J, Chen Q, Liu M, Wang S, Yu H, et al. Regulation effects of total flavonoids in *Morus alba* L. on hepatic cholesterol disorders in orotic acid induced NAFLD rats. Bmc Complement Med 2020;20(257):1–12.
Jia ZS, Tang MC, Wu JM. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 1999;64(4):555–9.
Jiang Q, Li C, Yu Y, Xing Y, Xiao D, Zhang B. Comparison of fatty acid profile of three adipose tissues in Ningxiang pigs. Anim Nutr 2018;4(3):256–9.
Jiang T, Mueller CJ, Busboom JR, Nelson ML, O'Fallon J, Tschida G. Fatty acid composition of adipose tissue and muscle from Jersey steers was affected by finishing diet and tissue location. Meat Sci 2013;93(2):153–61.
Ju WT, Kwon OC, Kim HB, Sung GB, Kim HW, Kim YS. Qualitative and quantitative analysis of flavonoids from 12 species of Korean mulberry leaves. J Food Sci Technol 2018;55(5):1789–96.
Kaila B, Raman M. Obesity: a review of pathogenesis and management strategies. Can J Gastroenterol 2008;22(1):61–8.
Kolstad K. Fat deposition and distribution measured by computer tomography in three genetic groups of pigs. Livest Prod Sci 2001;67(3):281–92.
Kong L, Yang C, Dong L, Diao Q, Si B, Ma J, et al. Rumen fermentation characteristics in pre- and post-weaning calves upon feeding with mulberry leaf flavonoids and *Candida tropicalis* individually or in combination as a supplement. Animals 2019;9(11):990.
Li AC, Glass CK. PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. J Lipid Res 2004;45(12):2161–73.
Li D, Ma B, Xu X, Chen G, Li T, He N. MMHub, a database for the mulberry metabolome. Database (Oxford) 2020; 2020. p. 1–8.
Lim HH, Yang SJ, Kim Y, Lee M, Lim Y. Combined treatment of mulberry leaf and fruit extract ameliorates obesity-related inflammation and oxidative stress in high fat diet-induced obese mice. J Med Food 2013;16(8):673–80.
Liu J, Cao S, Liu M, Chen L, Zhang H. A high nutrient dense diet alters hypothalamic gene expressions to influence energy intake in pigs born with low birth weight. Sci Rep 2018;8(1):5514.
Liu Y, Li F, He L, Tan B, Deng J, Kong X, et al. Dietary protein intake affects expression of genes for lipid metabolism in porcine skeletal muscle in a genotype-dependent manner. Br J Nutr 2015;113(7):1069–77.
Liu Y, Li Y, Peng Y, He J, Xiao D, Chen C, et al. Dietary mulberry leaf powder affects growth performance, carcass traits and meat quality in finishing pigs. J Anim Physiol An N 2019;103(6):1934–45.
Liu Y, Li Y, Xiao Y, Peng Y, He J, Chen C, et al. Mulberry leaf powder regulates antioxidative capacity and lipid metabolism in finishing pigs. Anim Nutr 2021;7(2):421–9.
Liu Y, Xiao Y, Xie J, Peng Y, Li F, Chen C, et al. Dietary supplementation with flavonoids from mulberry leaves improves growth performance and meat quality, and alters lipid metabolism of skeletal muscle in a Chinese hybrid pig. Anim Feed Sci Technol 2022;285:115211.
Lorenzo JM, Montes R, Purrinos L, Cobas N, Franco D. Fatty acid composition of Celta pig breed as influenced by sex and location of fat in the carcass. J Sci Food Agric 2012;92(6):1311–7.
Madeira MS, Pires VM, Alfaia CM, Luxton R, Doran O, Bessa RJ, et al. Combined effects of dietary arginine, leucine and protein levels on fatty acid composition and gene expression in the muscle and subcutaneous adipose tissue of cross-bred pigs. Br J Nutr 2014;111(9):1521–35.
Martins AP, Lopes PA, Madeira MS, Martins SV, Santos NC, Moura TF, et al. Differences in lipid deposition and adipose membrane biophysical properties from lean and obese pigs under dietary protein restriction. Biochem Biophys Res Commun 2012;423(1):170–5.
McGlone JJ. The future of pork production in the world: towards sustainable, welfare-positive systems. Animals 2013;3(2):401–15.
Meng Q, Qi X, Fu Y, Chen Q, Cheng P, Yu X, et al. Flavonoids extracted from mulberry (*Morus alba* L.) leaf improve skeletal muscle mitochondrial function by activating AMPK in type 2 diabetes. J Ethnopharmacol 2020a;248:112326.
Meng Q, Qi X, Chao Y, Chen Q, Cheng P, Yu X, et al. IRS1/PI3K/AKT pathway signal involved in the regulation of glycolipid metabolic abnormalities by Mulberry (*Morus alba* L.) leaf extracts in 3T3-L1 adipocytes. Chin Med 2020b;15:1.
Ministry of Agriculture of the People's Republic of China. Chinese national feeding standard of swine (NY/T 65-2004). Beijing, China: China Agriculture Press; 2004.
Peng CH, Yang MY, Yang YS, Yu CC, Wang CJ. *Antrodia cinnamomea* prevents obesity, dyslipidemia, and the derived fatty liver via regulating AMPK and SREBP signaling. Am J Chin Med 2017;45(1):67–83.
Postaue N, de Mello BTF, Cardozo-Filho L, da Silva C. Use of the product from low pressure extraction (crambe seed oil and methyl acetate) for synthesis of methyl esters and triacetin under supercritical conditions. Eur J Lipid Sci Technol 2020;122(6):2000004.
Riche DM, Riche KD, East HE, Barrett EK, May WL. Impact of mulberry leaf extract on type 2 diabetes (Mul-DM): a randomized, placebo-controlled pilot study. Compl Ther Med 2017;32:105–8.
Rodrigues EL, Marcelino G, Silva GT, Figueiredo PS, Garcez WS, Corsino J, et al. Nutraceutical and medicinal potential of the Morus species in metabolic dysfunctions. Int J Mol Sci 2019;20(2):301.
Saini RK, Keum YS. Omega-3 and omega-6 polyunsaturated fatty acids: dietary sources, metabolism, and significance - a review. Life Sci 2018;203:255–67.
Saurav A, Kaushik M, Mohiuddin SM. Fenofibric acid for hyperlipidemia. Expet Opin Pharmacother 2012;13(5):717–22.
Shahidi F, Ambigaipalan P. Omega-3 polyunsaturated fatty acids and their health benefits. Annu Rev Food Sci Technol 2018;9:345–81.

Sheng Y, Liu J, Zheng S, Liang F, Luo Y, Huang K, et al. Mulberry leaves ameliorate obesity through enhancing brown adipose tissue activity and modulating gut microbiota. Food Funct 2019;10(8):4771–81.
Sheng Y, Zheng S, Ma T, Zhang C, Ou X, He X, et al. Mulberry leaf alleviates streptozotocin-induced diabetic rats by attenuating NEFA signaling and modulating intestinal microflora. Sci Rep 2017;7(1):12041.
Song H, Lai J, Tang Q, Zheng X. Mulberry ethanol extract attenuates hepatic steatosis and insulin resistance in high-fat diet-fed mice. Nutr Res 2016;36(7):710–8.
Srivastava RA, He S. Anti-hyperlipidemic and insulin sensitizing activities of fenofibrate reduces aortic lipid deposition in hyperlipidemic Golden Syrian hamster. Mol Cell Biochem 2010;345(1–2):197–206.
Sun LH, Xiang FF, Wang ZG, Li A, Du YY, Huang GY, et al. Extraction and purification of flavonoids from mulberry leaves. China Feed 2014;19:22–7 (in Chinese).
Szűcs I, Vida V. Global tendencies in pork meat-production, trade and consumption. Applied Studies in Agribusiness and Commerce 2017;11(3–4):105–11.
Thaipitakwong T, Numhom S, Aramwit P. Mulberry leaves and their potential effects against cardiometabolic risks: a review of chemical compositions, biological properties and clinical efficacy. Pharm Biol 2018;56(1):109–18.
Walker AK, Jacobs RL, Watts JL, Rottiers V, Jiang K, Finnegan DM, et al. A conserved SREBP-1/phosphatidylcholine feedback circuit regulates lipogenesis in metazoans. Cell 2011;147(4):840–52.
Yan H, Cao S, Li Y, Zhang H, Liu J. Reduced meal frequency alleviates high-fat diet-induced lipid accumulation and inflammation in adipose tissue of pigs under the circumstance of fixed feed allowance. Eur J Nutr 2020;59:595–608.
Yin J, Li Y, Han H, Chen S, Gao J, Liu G, et al. Melatonin reprogramming of gut microbiota improves lipid dysmetabolism in high-fat diet-fed mice. J Pineal Res 2018;65(4):e12524.
Yuan X, Wei G, You Y, Huang Y, Lee HJ, Dong M, et al. Rutin ameliorates obesity through brown fat activation. Faseb J 2017;31(1):333–45.
Zarate R, El Jaber-Vazdekis N, Tejera N, Perez JA, Rodriguez C. Significance of long chain polyunsaturated fatty acids in human health. Clin Transl Med 2017;6(1):25.
Zeng Z, Jiang J, Yu J, Mao X, Yu B, Chen D. Effect of dietary supplementation with mulberry (*Morus alba* L.) leaves on the growth performance, meat quality and antioxidative capacity of finishing pigs. J Integr Agric 2019;18(1):143–51.
Zhang Q, Zhang F, Thakur K, Wang J, Wang H, Hu F, et al. Molecular mechanism of anti-cancerous potential of Morin extracted from mulberry in Hela cells. Food Chem Toxicol 2018;112:466–75.
Zhong Y, Song B, Zheng C, Zhang S, Yan Z, Tang Z, et al. Flavonoids from mulberry leaves alleviate lipid dysmetabolism in high fat diet-fed mice: involvement of gut microbiota. Microorganisms 2020;8(60):1–17.
Zhu W, Zhong Z, Liu S, Yang B, Komatsu S, Ge Z, et al. Organ-specific analysis of *Morus alba* using a gel-free/label-free proteomic technique. Int J Mol Sci 2019;20(2):365.