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Original Research Article

Mulberry leaf powder regulates antioxidative capacity and lipid metabolism in finishing pigs

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ABSTRACT

This study evaluated the potential of mulberry leaf powder as an unconventional feed material for finishing pigs by assessing the growth performance, antioxidative properties, fatty acid profile, and lipid metabolism in 180 Xiangcun black pigs. Pigs with an initial body weight (BW) of 71.64 ± 1.46 kg were randomly assigned to 5 treatment groups, including the control diet and 4 experimental diets. The corn, soybean meal, and wheat bran in the control diet were partly replaced by 3%, 6%, 9%, or 12% mulberry leaf powder in experimental diets. There were 6 replicates (pens) of 6 pigs per replicate in each treatment. Blood and muscle samples were collected after the 50-day feed experiment. Compared with the control group, the 3%, 6%, and 9% mulberry diets had no adverse effect ($P > 0.05$) on the growth performance of pigs. The serum glutathione peroxidase activity and glutathione concentration increased linearly ($P < 0.05$) with the increase in dietary mulberry inclusion. There was no significant difference in the relative expression levels of antioxidant-related genes in muscle tissue between the control and mulberry groups. Inclusion of dietary mulberry powder increased ($P < 0.05$) the content of polyunsaturated fatty acids, especially in the longissimus dorsi (LD) muscle, up-regulated ($P < 0.05$) the relative mRNA expression level of uncoupling protein-3 in muscle tissue, but down-regulated ($P < 0.05$) the relative mRNA expression levels of hormone-sensitive lipase, acetyl CoA carboxylase α, lipoprotein lipase, and peroxisome proliferator-activated receptor γ in LD in a linear pattern. The nuclear respiratory factor 2 expression level in the LD muscle of pigs fed the 9% mulberry diet was higher ($P < 0.01$) than that in the other mulberry groups and control group. The inclusion of less than 12% dietary mulberry did not detrimentally affect the growth performance of Xiangcun black pigs, but enhanced the serum antioxidant property, increased the polyunsaturated fatty acid content, and inhibited lipid oxidation by regulating gene expression levels of lipid metabolism and mitochondrial uncoupling protein in muscle tissue. Mulberry leaves can be utilized as a forage crop in the diet of finishing pigs.

1. Introduction

The increasing demand for animal products has led to concomitant demand for sufficient and cheap livestock feed in many developing countries. Feeding strategies, which are based on native feed sources and cost-effective alternatives, have been improved to guarantee the sustainable development of animal husbandry. Many researchers have confirmed that unconventional feed materials can be used to partially replace cereal-based

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concentrates as livestock feed with no detrimental impact on animal production performance (Li et al., 2019; Yulistiani et al., 2015). Unconventional feed materials can have several beneficial functions, among which antimicrobial and antioxidant activities are the most important (Cheong et al., 2012; Li et al., 2019).

Mulberry trees (*Morus alba* L.) are deciduous plants with rapid growth which are planted in many countries. The cultivation area of mulberry in China is estimated to exceed 10^6 ha (Liu et al., 2001), and the biomass yield of fresh mulberry leaves is approximately 25 to 30 t/ha per year. Mulberry leaves have been used as feed for silk worms for hundreds of years. Based on their antioxidative, antibacterial, and antihyperlipidemic properties, mulberry leaves are also used in Chinese herbal medicine (Choi et al., 2013; Wang et al., 2012; Zou et al., 2012), e.g., to treat colds, fevers, headaches, coughs, hyperlipemia, diabetes, and rheumatic diseases (Yang et al., 2012). Furthermore, studies have demonstrated that mulberry leaves can be potential protein sources for cattle (Vu et al., 2011) and potential supplements of fermentable energy and protein for sheep (Cai et al., 2019; Yulistiani et al., 2015). The inclusion of mulberry leaves was shown to reduce the demand for expensive protein feeds in lamb diets (Salinas-Chavira et al., 2011). In a study of rumen and gastrointestinal digestibility of sheep, it was found that the digestible energy and crude protein values of mulberry leaves were similar to those of alfalfa hay (Doran et al., 2007).

The major active components of mulberry leaves, such as flavonoids and polyphenols, reportedly possess anti-inflammatory, antioxidant, antidiabetic, hypolipidemic properties, and a neuroprotective function (Chen et al., 2018; Choi et al., 2013; Zou et al., 2012). Mulberry leaves can reportedly be utilized as a new feed supplement to regulate the antioxidant capacity of laying hens (Lin et al., 2017). However, how mulberry leaf powder influences the antioxidative profile and lipid metabolism in pigs is rarely reported. In this study, we hypothesized that mulberry leaves could be used as a dietary supplement for finishing pigs to improve their antioxidative capacity and regulate their lipid metabolism, to improve animal health. The study objectives were to assess the growth performance, antioxidative capacity, fatty acid profile, and lipid metabolism of Xiangcun black pigs, which were fed various levels of mulberry leaf powder diets.

2. Material and methods

The experiment was conducted in accordance with the Chinese Guidelines for Animal Welfare and Experimental Protocols, and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

2.1. Preparation of mulberry leaf powder

After purchased from the Sericultural Research Institute of Hunan Province (Changsha, Hunan, China), green mulberry leaves were dried at 60 °C for 4 d in a heat drier room, where the moisture level was <8%. By using a grinder equipped with a sieve (mesh diameter: 1.5 mm), the dried leaves were crushed into powder, and then stored in a light-proof and well-sealed plastic bag in a 4 °C refrigerator. The nutrient content of mulberry powder was analyzed following the methods of the Association of Official Analytical Chemists (AOAC, 1990), and determined to be: 23.50% dry matter, 22.66% crude protein (CP), 4.93% ether extract, 12.06% crude fiber, 9.60% crude ash, and 15.27 MJ/kg digestible energy.

2.2. Study animals

A total of 180 Xiangcun black pigs (a Chinese native breed; finishing barrows) obtained from a local commercial farm was used.

2.3. Experimental design

The experimental animals with an average initial body weight (BW) of 71.64 ± 1.46 kg were randomly allocated to 5 treatment groups, including the control diet and 4 experimental diets. The corn, soybean meal, and wheat bran in the control diet were partly replaced by 3%, 6%, 9%, or 12% mulberry leaf powder in the experimental diets. There were 6 replicates (pens) of 6 pigs per replicate in each treatment. All the diets (Table 1) were formulated to meet the recommendations of the Chinese National Feeding Standard of Swine (2004) and contained similar levels of CP. Diets were fed to pigs in pellet form. The animals had ad libitum access to drinking water and feed throughout the experiment. Pigs were fed three times per day at 08:00, 13:00, and 18:00. The trial lasted for 50 d after a 7-d adaptation period. At the beginning and the end of the experiment, pigs were weighed. Feed consumption was recorded daily to determine the average daily gain (ADG), average daily feed intake (ADFI), and the feed intake-to-body gain (F:G) ratio.

2.4. Sample collection

At the end of the experimental period, 30 pigs (6 pigs per treatment) were selected for sample collection and slaughter. Blood samples were collected via jugular venipuncture, and centrifuged

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

Item	Mulberry inclusion level, %				
	0	3	6	9	12
Ingredients					
Corn	67.52	66.77	65.65	64.72	63.80
Soybean meal	18.00	17.33	16.50	15.73	14.90
Wheat bran	12.00	10.50	9.60	8.40	7.29
Mulberry powder	0.00	3.00	6.00	9.00	12.00
$CaHPO_4$	0.50	0.60	0.60	0.65	0.66
$CaCO_3$	0.68	0.50	0.35	0.20	0.05
Salt	0.30	0.30	0.30	0.30	0.30
Premix[2]	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00
Chemical composition					
Digestible energy, MJ/kg	13.72	13.74	13.73	13.73	13.73
Crude protein	14.45	14.47	14.47	14.47	14.45
Crude fiber	2.34	3.01	3.53	4.01	4.34
Total calcium	0.55	0.56	0.55	0.56	0.56
Total phosphorus	0.47	0.48	0.48	0.48	0.48
Available phosphorus	0.20	0.21	0.21	0.22	0.22
Fatty acids composition (% of total fatty acids)					
C14:0	0.64	0.57	0.51	0.46	0.41
C16:0	22.12	22.57	22.18	22.19	23.40
C16:1	0.11	0.15	0.11	0.14	0.10
C17:0	0.19	0.20	0.24	0.18	0.25
C18:0	10.06	10.03	9.46	9.16	10.09
C18:1	1.44	1.40	1.37	1.36	1.34
C18:2	60.69	57.32	57.84	58.12	54.40
C18:3	0.20	0.21	0.28	0.16	0.20
C20:0	0.44	0.43	0.52	0.59	0.61
C20:1	4.12	7.12	7.49	7.63	9.18

[1] Basal diet formulated according to the Chinese National Feeding Standard of Swine.

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

at 3,000 × *g* at 4 °C for 15 min to get the supernatants (serum), which was then stored at −20 °C. According to standard commercial procedures, pigs were then electrically stunned, exsanguinated, dehaired, eviscerated, and split down the midline. Muscle tissue samples (about 50 g) of longissimus dorsi muscle (LD) and biceps femoris muscle (BF) from the right side of the carcass were collected within 20 min of slaughter and frozen at −20 °C immediately. Other samples (1.0 cm thick) of LD and BF were quickly frozen in liquid nitrogen and stored at −80 °C.

2.5. Analysis of serum antioxidative parameters

The total antioxidant capacity (T-AOC), contents of glutathione (GSH) and malondialdehyde (MDA), and activities of total superoxide dismutase (T-SOD) and glutathione peroxidase (GPx) in serum were detected according to the manufacturer's instructions of Nanjing Jiancheng commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), by using colorimetric methods with a spectrophotometer (Biomate 5, Thermo Electron Corporation, Rochester, NY).

2.6. Analysis of fatty acid composition

Lipids from the freeze-dried samples of LD and BF (approximately 0.5 g) were extracted in a solution of chloroform and methanol (1:1, vol/vol), and subsequently methylated to fatty acid methyl esters using KOH/methanol (Demirel et al., 2004). The fatty acid methyl esters were analyzed using an Agilent 7890 A gas chromatographer equipped with a SP-2560 column (100 m × 250 μm × 0.2 μm) (Agilent Technologies Inc., Santa Rosa, CA) according to the method described by Liu et al. (2015). By comparison of their retention times with those of the standards (Sigma Chemical Co., St. Louis, MO), individual fatty acid peaks were identified. Results are expressed as a percentage of the total fatty acids. The contents of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA), and ratios of PUFA to SFA and Σ*n*-6 to Σ*n*-3 were calculated, as well as the lipid quality indices, i.e., atherogenicity index (AI) and thrombogenicity index (TI), which were evaluated (Σ g/100 g) according to Ulbricht and Southgate (1991):

$$AI = (4 \times [C14:0] + [C16:0])/(n\text{-}6\ PUFA + n\text{-}3\ PUFA + MUFA)$$

$$TI = ([C14:0] + [C16:0] + [C18:0])/(0.5 \times MUFA + 0.5 \times n\text{-}6\ PUFA + 3 \times n\text{-}3\ PUFA + n\text{-}3\ PUFA/n\text{-}6\ PUFA)$$

where the brackets indicate the concentrations.

The hypocholesterolaemic-to-hypercholesterolaemic fatty acids ratio (h:H ratio) was calculated following Fernández et al. (2007):

$$h/H = ([C18:1] + [C18:2] + [C18:3] + [C20:3] + [C20:4])/([C14:0] + [C16:0])$$

where the brackets indicate the concentrations.

2.7. Analysis of quantitative real-time PCR

Total RNA isolation and reverse transcription, cDNA synthesis, and quantitative real-time PCR analysis were performed as described in detail by Liu et al. (2016). In brief, total RNA was extracted from LD and BF samples (approximately 100 mg) with TRIzol Reagent (Invitrogen-Life Technologies, Carlsbad, CA). By using 1% agarose gel electrophoresis and staining with 10 μg/mL ethidium bromide, RNA was quantified to have an optical density $(OD)_{260}$-to-OD_{280} ratio between 1.8 and 2.0. Then, the first-strand cDNA was synthesized according to the manufacturers' instructions. Primers for the selected genes (Table 2) were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA). A real-time PCR was performed using the SYBR Green detection kit (TaKaRa) and the ABI prism 7900HT (Applied Biosystems, USA). The amplification of β-actin in each sample was used to normalize the mRNA levels of the selected genes. We calculated the relative expression ratio (R) of mRNA using $R = 2^{-\Delta\Delta Ct\ (sample\text{-}control)}$, where −ΔΔCt (sample − control) = (Ct gene of interest − Ct β-actin) for the sample − (Ct gene of interest − Ct β-actin) for the control (Livak and Schmittgen, 2001).

2.8. Statistical analysis

ANOVA of Statistical Packages for Social Science 18.0 (SPSS 18.0) software was used to test the data between the 5 treatment groups, and orthogonal polynomial contrasts were used to evaluate the linear and quadratic effects of increasing dietary mulberry inclusion on the detected traits in the experimental animals. Probability (*P*) values < 0.05 were considered to be significant, and 0.05 < *P* < 0.10 were considered to indicate trends.

3. Results

3.1. Growth performance

There was no significant difference in the initial BW (71.38 to 71.96 kg) of experimental pigs among groups. Inclusion of mulberry leaves from 3% to 12% quadratically decreased ($P < 0.01$) the final BW (98.38 ± 1.66 kg to 93.60 ± 1.58 kg) and ADG (540.00 ± 32 g to

Table 2
Primers used for quantitative real-time PCR.

Gene name	Sequence (5′–3′)	Size, bp
SOD1	F: GAGACCTGGGCAATGTGACT	189
	R: CCAAACGACTTCCAGCATTT	
GPX1	F: AGCCCAACTTCATGCTCTTC	159
	R: CATTGCGACACACTGGAGAC	
NFE2L2	F: GAAAGCCCAGTCTTCATTGC	190
	R: TTGGAACCGTGCTAGTCTCA	
GCLC	F: CAAACCATCCTACCCTTTGG	172
	R: ATTGTGCAGAGAGCCTGGTT	
HSL	F: GCAGCATCTTCTTCCGCACA	195
	R: AGCCCTTGCGTAGAGTGACA	
ACCα	F: ATCCCTCCTTGCCTCTCCTA	208
	R: ACTTCCCGTTCAGATTTCCG	
LPL	F: CTCGTGCTCAGATGCCCTAC	148
	R: GGCAGGGTGAAAGGGATGTT	
PPARγ	F: TGACCATGGTTGACACCG	381
	R: AAGCATGAACTCCATAGTGG	
FATP1	F: GGAGTAGAGGGCAAAGCAGG	208
	R: AGGTCTGGCGTGGGTCAAAG	
PGC-1α	F: GCCCAGTCTGCGGCTATTT	265
	R: GTTCAGCTCGGCTCGGATTT	
Nrf2	F: GCCCCTGGAAGCGTTAAAC	59
	R: GGACTGTATCCCCAGAAGGTTGT	
UCP2	F: CTTCTGCGGTTCCTCTGTGT	641
	R: CATAGGTCACCAGCTCAGCA	
UCP3	F: GAGATGGTGACCTATGATGT	260
	R: CGCAAAAAGGAAGGTGTGAA	
β-actin	F: TGCGGGACATCAAGGAGAAG	216
	R: AGTTGAAGGTGGTCTCGTGG	

SOD1 = superoxide dismutase 1; F = forward; R = reverse; *GPX1* = glutathione peroxidase 1; *NFE2L2* = nuclear factor erythroid 2-like 2; *GCLC* = glutamate cysteine ligase catalytic subunit; *HSL* = hormone-sensitive lipase; *ACCα* = acetyl CoA carboxylase α; *LPL* = lipoprotein lipase; *PPARγ* = peroxisome proliferator-activated receptor γ; *FATP1* = fatty acid transport protein 1; *PGC-1α* = peroxisome proliferator-activated receptor γ coactiva-tor-1α; *Nrf2* = nuclear respiratory factor 2; *UCP2* = uncoupling protein-2; *UCP3* = uncoupling protein-3.

432.80 ± 24 g) of Xiangcun black pigs, but quadratically increased ($P < 0.01$) the F:G ratio (3.81 to 4.43). No change in the ADFI was observed. Compared with the control group, the 3%, 6%, and 9% mulberry diets had no effect ($P > 0.05$) on the growth performance of pigs. However, the 12% mulberry diet decreased ($P < 0.05$) the final BW (93.60 ± 1.58 kg) and ADG (432.80 ± 24 g), and increased ($P = 0.05$) the F:G ratio (4.43) of Xiangcun black pigs.

3.2. Antioxidative parameters

The concentrations of GSH and MDA, and activities of T-SOD, GPx, and T-AOC in serum of Xiangcun black pigs affected by dietary mulberry inclusion are shown in Table 3. The activity of serum GPx and the concentration of GSH enhanced linearly ($P < 0.05$) with the increase of dietary mulberry inclusion. The highest values of GPx activity and GSH concentration were observed in the 9% mulberry group and 12% mulberry group, respectively.

The relative mRNA expression levels of the antioxidant-related key genes, superoxide dismutase 1 (*SOD1*), glutathione peroxidase 1 (*GPX1*), nuclear factor erythroid 2-like 2 (*NFE2L2*), and glutamate cysteine ligase catalytic subunit (*GCLC*) were detected in LD and BF tissues (Table 4), aiming to excavate further the antioxidation function of mulberry diet in skeletal muscle. There was no significant difference in the expression level of antioxidant-related genes between the control group and mulberry groups. The expression level of *SOD1* mRNA in LD tissue of pigs fed the 3% mulberry diets was greater ($P = 0.09$) than in the 6% mulberry diet. The 6% mulberry group had a higher ($P = 0.09$) expression level of *GPX1* mRNA in BF than that of the control group.

3.3. Muscular fatty acid profile

A large change was observed in the fatty acid profile of LD and BF tissues in response to the level of dietary mulberry inclusion. As shown in Table 5, the mulberry inclusion level increasing from 0 to 12% linearly decreased ($P < 0.05$) the concentrations of C16:0 and C16:1 fatty acids, SFA level and indices of AI and TI, and quadratically decreased ($P < 0.01$) the concentration of C14:0 in LD. Conversely, as the mulberry inclusion increased, the concentrations of C18:2*n*-6, C18:3*n*-3, C20:3*n*-6 and C20:4*n*-6 fatty acids, PUFA concentration, and ratios of PUFA to SFA and h to H rose linearly ($P < 0.05$), and the concentration of C17:0 rose quadratically ($P < 0.05$).

As shown in Table 6, the increase in dietary mulberry quadratically increased ($P < 0.05$) the concentration of C17:0 and linearly increased ($P < 0.05$) the C18:3*n*-3 concentration, but linearly decreased ($P < 0.05$) the ratio of $\sum n$-6 to $\sum n$-3 in BF. No obvious difference was detected in the other fatty acids in BF ($P > 0.05$).

Collectively, only the concentrations of C18:0, C18:1, and C20:0 fatty acids in LD were not influenced by mulberry inclusion, while in BF, the concentrations of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2*n*-6, C20:0, C20:3*n*-6, and C20:4*n*-6 fatty acids were similar among groups. The influence of dietary mulberry level on fatty acid traits in LD muscle was greater than in BF muscle.

3.4. Expression levels of lipid metabolism-related genes

We then determined the relative mRNA expression level of the lipid-related genes, hormone-sensitive lipase (*HSL*), acetyl CoA carboxylase α (*ACCα*), lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor γ (*PPARγ*), and fatty acid transport protein 1 (*FATP1*) in skeleton muscle tissues by RT-PCR analysis (Table 7). Dietary mulberry inclusion down-regulated ($P < 0.05$) the relative mRNA expression levels of *HSL*, *ACCα*, *LPL*, and *PPARγ* in LD in a linear pattern. Notably, the *PPARγ* expression level in BF of pigs fed the 12% mulberry diet was greater ($P < 0.05$) than the other treatment groups. The *FATP1* expression levels in BF of the 3%, 6%, and 12% mulberry groups were down-regulated ($P < 0.01$), compared with that of the 9% mulberry group and the control group.

3.5. Expression levels of mitochondrial respiratory-chain related genes

We also determined the mRNA expression levels of mitochondrial respiratory chain related genes, peroxisome proliferator-activated receptor γ coactiva-tor-1α (*PGC-1α*), nuclear respiratory factor 2 (*Nrf2*), uncoupling protein-2 (*UCP2*), and uncoupling protein-3 (*UCP3*), in LD and BF muscle tissues (Table 8). Dietary mulberry inclusion up-regulated quadratically ($P < 0.05$) the mRNA expression level of *UCP3* in LD. The *Nrf2* expression level in LD of the 9% mulberry group was greater ($P < 0.01$) than those of all the other groups. Similarly, in BF, dietary mulberry inclusion up-regulated (linearly and quadratically, $P < 0.05$) the mRNA expression level of *UCP3*. The *PGC-1α* expression level in BF in the 9% mulberry group was greater ($P < 0.01$) than that of the other mulberry groups and the control group. The *UCP2* expression level in the 6% mulberry group was greater ($P < 0.05$) than that of the other groups.

4. Discussion

Morus alba L. (family: Moraceae), commonly known as the white mulberry, is native to China but is currently planted in many countries in the world (Gao et al., 2018). All the parts of this plant, including the leaves, root bark, stem and fruits, have been used in traditional Chinese medicine (Pel et al., 2017). Mulberry leaves, specifically, have been used as one of the ingredients in traditional Chinese medicine for the treatment of diabetes (Wilson and Islam, 2015; Zhang et al., 2014), atherosclerosis (Chan et al., 2013; Sugimoto et al., 2009), and as an immune enhancer because of their antioxidant potential (Bharani et al., 2010; Yimam et al., 2015). Previous studies have reported that a moderate content of mulberry leaves in the diet does not detrimentally impact the growth

Table 3
Effect of mulberry powder on serum antioxidative parameters of Xiangcun black finishing pigs.

Item	Mulberry inclusion level, %					SEM	*P*-value		
	0	3	6	9	12		ANOVA	Linear	Quadratic
T-SOD, U/mL	95.04	101.89	98.18	92.19	106.83	4.37	0.17	0.36	0.48
GPx, U/mL	987.48^{b}	979.60^{b}	980.71^{b}	1,042.36^{a}	1,014.80^{ab}	17.19	0.06	0.04	0.12
T-AOC, U/mL	1.49	1.49	1.77	1.33	1.27	0.17	0.30	0.27	0.24
GSH, mg/L	7.55^{b}	10.45^{ab}	10.44^{ab}	11.11^{ab}	13.45^{a}	1.29	0.06	<0.01	0.02
MDA, nmol/mL	3.04	3.93	3.82	3.75	2.99	0.74	0.83	0.86	0.50

T-SOD = total superoxide dismutase; GPx = glutathione peroxidase; T-AOC = total antioxidant capacity; GSH = glutathione; MDA = malonaldehyde.
[a, b] Within a row, values with different superscript letters differ ($P < 0.05$). $n = 6$.

Table 4
Antioxidant-related genes expression in skeletal muscle of Xiangcun black finishing pigs fed the diets with various levels of mulberry powder.

Item	Mulberry inclusion level, %					SEM	*P*-value		
	0	3	6	9	12		ANOVA	Linear	Quadratic
Longissimus dorsi muscle									
SOD1	1.07^{ab}	1.12^{a}	0.78^{b}	0.95^{ab}	0.82^{ab}	0.10	0.09	0.05	0.13
GPX1	1.07	1.29	1.08	1.21	1.48	0.15	0.33	0.13	0.25
NFE2L2	1.03	0.77	0.89	0.98	0.74	0.10	0.21	0.27	0.54
GCLC	1.03	0.93	0.83	0.96	0.81	0.09	0.34	0.13	0.29
Biceps femoris muscle									
SOD1	1.05	0.92	1.12	0.81	1.00	0.11	0.32	0.56	0.81
GPX1	1.02^{b}	1.14^{ab}	1.53^{a}	1.22^{ab}	1.32^{ab}	0.10	0.09	0.12	0.11
NFE2L2	1.03	0.79	1.15	0.96	1.03	0.12	0.37	0.71	0.92
GCLC	1.04	0.88	0.92	0.79	0.96	0.12	0.67	0.50	0.45

SOD1 = superoxide dismutase 1; *GPX1* = glutathione peroxidase 1; *NFE2L2* = nuclear factor erythroid 2-like 2; *GCLC* = glutamate cysteine ligase catalytic subunit.
[a, b] Within a row, values with different superscript letters differ ($P < 0.05$). $n = 6$.

Table 5
Fatty acid profile in longissimus dorsi muscle of Xiangcun black finishing pigs fed the diets with various levels of mulberry powder (%, dry matter basis).

Item	Mulberry inclusion level, %					SEM	*P*-value		
	0	3	6	9	12		ANOVA	Linear	Quadratic
Fatty acid composition									
C14:0	1.63^{ab}	1.68^{a}	1.59^{abc}	1.51^{bc}	1.43^{c}	0.05	0.02	<0.01	<0.01
C16:0	28.81^{a}	28.44^{ab}	27.84^{abc}	27.00^{bc}	26.38^{c}	0.51	0.01	<0.01	<0.01
C16:1	4.08	4.07	4.01	3.51	3.60	0.19	0.10	0.01	0.05
C17:0	0.20^{c}	0.22^{bc}	0.25^{abc}	0.29^{a}	0.27^{ab}	0.02	0.01	<0.01	<0.01
C18:0	13.98	13.25	13.91	13.67	12.86	0.41	0.27	0.16	0.30
C18:1	41.36	42.4	41.69	42.57	42.92	0.88	0.71	0.23	0.49
C18:2*n*-6	7.10^{c}	7.35^{bc}	7.89^{bc}	8.51^{ab}	9.23^{a}	0.43	0.01	<0.01	<0.01
C20:0	0.21	0.19	0.20	0.19	0.19	0.01	0.66	0.22	0.44
C20:1	0.96^{a}	0.68^{ab}	0.83^{ab}	0.93^{a}	0.61^{b}	0.10	0.07	0.18	0.39
C18:3*n*-3	0.51^{b}	0.49^{b}	0.57^{b}	0.90^{a}	0.85^{a}	0.09	<0.01	<0.01	<0.01
C20:3*n*-6	0.21^{b}	0.22^{b}	0.24^{ab}	0.23^{b}	0.30^{a}	0.02	0.07	0.01	0.03
C20:4*n*-6	1.21^{b}	1.24^{b}	1.26^{b}	1.14^{b}	1.79^{a}	0.14	0.02	0.04	0.02
Partial sums of fatty acids									
SFA	44.83^{a}	43.79^{a}	43.78^{a}	42.66^{ab}	41.13^{b}	0.77	0.02	<0.01	<0.01
MUFA	46.40	47.15	46.53	47.01	47.13	0.83	0.95	0.60	0.87
PUFA	9.03^{b}	9.30^{b}	9.97^{b}	10.78^{ab}	12.17^{a}	0.59	<0.01	<0.01	<0.01
PUFA:SFA ratio	0.20^{c}	0.21^{bc}	0.23^{bc}	0.25^{ab}	0.30^{a}	0.02	<0.01	<0.01	<0.01
∑*n*-6:∑*n*-3 ratio	16.86^{ab}	18.48^{a}	18.02^{a}	11.76^{b}	16.08^{ab}	1.74	0.07	0.16	0.38
Indices									
h:H ratio[1]	1.66^{c}	1.72^{bc}	1.76^{bc}	1.88^{ab}	1.99^{a}	0.06	<0.01	<0.01	<0.01
AI[2]	0.64^{a}	0.63^{ab}	0.61^{ab}	0.57^{bc}	0.54^{c}	0.02	<0.01	<0.01	<0.01
TI[3]	1.53^{a}	1.48^{ab}	1.46^{ab}	1.36^{bc}	1.29^{c}	0.05	<0.01	<0.01	<0.01

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; h:H ratio = hypocholesterolaemic-to-hypercholesterolaemic fatty acids ratio; AI = atherogenicity index; TI = thrombogenicity index.
[a, b, c] Within a row, values with different superscript letters differ ($P < 0.05$). $n = 6$.
[1] h:H ratio = ([C18:1] + [C18:2] + [C18:3] +[C20:3] + [C20:4])/([C14:0 + [C16:0]), where the brackets indicate the concentrations.
[2] AI = (4 × [C14:0] + [C16:0])/(*n*-6 PUFA + *n*-3 PUFA + MUFA), where the brackets indicate the concentrations.
[3] TI = ([C14:0] + [C16:0] + [C18:0])/(0.5 × MUFA + 0.5 × *n*-6 PUFA + 3 × *n*-3 PUFA + *n*-3 PUFA/*n*-6 PUFA), where the brackets indicate the concentrations.

performance of finishing pigs, but improves meat quality by balancing muscle pH, enriching intramuscular fat, and regulating fatty acid profile and glucose metabolic enzyme activities (Li et al., 2012; Song et al., 2016). Mulberry leaf powder has been shown to improve nutrient digestibility as well as the development of rumen papillae and stratum basale of fattening Hu sheep (Ouyang et al., 2019). In the present study, we noted that mulberry leaves did not adversely impact the growth performance of finishing pigs but did positively affect their antioxidant capacity and lipid metabolism.

Oxidative damage occurs when the antioxidant capacity of cells and extracellular space is overwhelmed by exogenous or endogenous reactive oxygen species (ROS). Oxidative damage is a noticeable problem in animal production, because it can affect enzyme activation, signal transduction, and gene expression, and eventually disrupt the redox balance of cells (Shin et al., 2013). Growing-finishing pigs may easily suffer from oxidative stress due to their rapid growth and oxidative metabolism related to the production of large quantities of free radicals and other active oxygen metabolites. Oxidative stress can compromise the antioxidant status, such as increasing oxidative stress levels and lipid peroxidation, or reducing plasma concentrations of antioxidants. These changes can consequently reduce the health of animals and simultaneously adversely influence productive performance. GPx is part of the enzymatic antioxidant system that can eliminate the peroxides produced during the reactions of molecules with ROS (Liu et al., 2018). The elevated serum GSH content and GPx activity in the pigs fed mulberry diets in the present study indicated that the antioxidant ability of extracellular enzyme system was increased. Similarly, Lin et al. (2017) reported that the addition of mulberry leaves could enhance the abilities of scavenging free radicals and ROS of laying hens and reducing the MDA concentration to prevent lipid peroxidation. Zeng et al. (2019) also found that a 15% dietary supplement of mulberry leaf reduced the growth performance but

Table 6
Fatty acid profile in biceps femoris muscle of Xiangcun black finishing pigs fed the diets with various levels of mulberry powder (%, dry matter basis).

Item	Mulberry inclusion level, %					SEM	*P*-value		
	0	3	6	9	12		ANOVA	Linear	Quadratic
Fatty acid composition									
C14:0	1.25	1.29	1.54	1.27	1.30	0.09	0.27	0.83	0.39
C16:0	24.05	22.18	24.21	24.09	22.17	0.90	0.30	0.50	0.65
C16:1	3.19	3.00	2.78	3.16	2.96	0.23	0.76	0.70	0.76
C17:0	0.23^{b}	0.30^{ab}	0.39^{a}	0.32^{ab}	0.32^{ab}	0.03	0.05	0.10	0.02
C18:0	11.38	11.14	12.72	11.27	10.99	0.71	0.50	0.75	0.53
C18:1	45.12	44.05	43.13	44.04	42.99	1.38	0.84	0.33	0.60
C18:2*n*-6	10.62	12.00	11.14	11.37	13.27	0.88	0.31	0.10	0.22
C20:0	0.16	0.18	0.21	0.19	0.18	0.03	0.75	0.64	0.43
C20:1	0.87^{ab}	0.94^{a}	0.65^{b}	0.87^{ab}	0.68^{b}	0.08	0.06	0.09	0.24
C18:3*n*-3	0.75^{c}	1.11^{b}	0.87^{bc}	1.03^{bc}	1.51^{a}	0.11	<0.01	<0.01	<0.01
C20:3*n*-6	0.31	0.51	0.35	0.42	0.54	0.07	0.13	0.10	0.26
C20:4*n*-6	2.44	3.85	2.44	2.49	3.84	0.60	0.24	0.45	0.64
Partial sums of fatty acids[1]									
SFA	37.08	35.09	39.06	37.14	34.97	1.45	0.31	0.61	0.49
MUFA	49.17	48.00	46.57	48.07	46.63	1.48	0.74	0.29	0.53
PUFA	14.12	17.47	14.81	15.31	19.15	1.57	0.14	0.10	0.21
PUFA:SFA ratio	0.39	0.51	0.38	0.43	0.56	0.06	0.16	0.13	0.22
∑*n*-6:∑*n*-3 ratio	18.22^{a}	14.65^{ab}	17.85^{a}	13.89^{ab}	12.21^{b}	1.61	0.05	0.01	0.05
Indices									
h:H ratio[1]	2.35	2.63	2.29	2.43	2.68	0.15	0.27	0.32	0.46
AI[2]	0.46	0.42	0.5	0.47	0.42	0.03	0.32	0.72	0.50
TI[3]	1.1	0.98	1.19	1.09	0.94	0.08	0.19	0.43	0.34

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; h:H ratio = hypocholesterolaemic-to-hypercholesterolaemic fatty acids ratio; AI = atherogenicity index; TI = thrombogenicity index.
[a, b, c] Within a row, values with different superscript letters differ ($P < 0.05$). $n = 6$.
[1] h:H ratio = ([C18:1] + [C18:2] + [C18:3] +[C20:3] + [C20:4])/([C14:0 + [C16:0]), where the brackets indicate concentrations.
[2] AI = (4 × [C14:0] + [C16:0])/(*n*-6 PUFA + *n*-3 PUFA + MUFA), where the brackets indicate concentrations.
[3] TI = ([C14:0] + [C16:0] + [C18:0])/(0.5 × MUFA +0.5 × *n*-6 PUFA + 3 × *n*-3 PUFA + *n*-3 PUFA/*n*-6 PUFA), where the brackets indicate concentrations.

Table 7
Relative mRNA expression levels of lipid metabolic genes in skeletal muscle of Xiangcun black finishing pigs fed the diets with various levels of mulberry powder.

Item	Mulberry inclusion level, %					SEM	*P*-value		
	0	3	6	9	12		ANOVA	Linear	Quadratic
Longissimus dorsi muscle									
HSL	1.13^{a}	1.03^{a}	0.77^{ab}	1.05^{a}	0.65^{b}	0.13	0.05	0.03	0.09
ACCα	1.20^{a}	0.87^{ab}	0.65^{b}	0.86^{ab}	0.51^{b}	0.13	<0.01	<0.01	<0.01
LPL	1.09^{a}	0.96^{ab}	0.63^{b}	0.81^{ab}	0.61^{b}	0.14	0.08	0.01	0.04
PPARγ	1.13^{a}	0.84^{ab}	0.63^{bc}	0.78^{bc}	0.44^{c}	0.11	<0.01	<0.01	<0.01
FATP1	1.10	1.04	0.77	0.98	0.78	0.13	0.26	0.09	0.23
Biceps femoris muscle									
HSL	1.14	1.03	1.20	1.40	1.32	0.12	0.26	0.07	0.19
ACCα	1.05	0.91	0.93	1.00	1.16	0.15	0.76	0.48	0.38
LPL	1.07	0.72	0.81	0.83	0.91	0.13	0.44	0.65	0.26
PPARγ	1.02^{b}	1.04^{b}	0.98^{b}	0.99^{b}	1.54^{a}	0.15	0.05	0.05	0.02
FATP1	1.54^{a}	0.69^{b}	0.78^{b}	1.42^{a}	0.69^{b}	0.12	<0.01	0.08	0.10

HSL = hormone-sensitive lipase; *ACCα* = acetyl CoA carboxylase α; *LPL* = lipoprotein lipase; *PPARγ* = peroxisome proliferator-activated receptor γ; *FATP1* = fatty acid transport protein 1.
[a, b, c] Within a row, values with different superscript letters differ ($P < 0.05$). $n = 6$.

increased the T-AOC and GPx, and tended to strengthen the T-SOD activity in serum of finishing pigs.

In the present study, we analyzed the oxidation-related gene expression levels in muscle tissues in an attempt to explain the underlying mechanism. Unexpectedly, however, the results showed no significant difference in the gene mRNA levels related to the oxidative capacity of mulberry diets and the control diet. This result differed from the previous statement, which indicated that the indexes in serum were in accordance with the antioxidant capacity of muscle (Ma et al., 2010; Zhang et al., 2017). Such associated mechanisms require further exploration.

Although the high content of PUFA in pork is a satisfactory health characteristic, the influence of PUFA on the oxidative stability, shelf life, and processing of pork is not desirable. Therefore, intramuscular or intermuscular fat and meat quality should be balanced via the use of feed additives or supplements. Jeon (2012) reported that the addition of mulberry leaf silage to beef cattle diets increased the unsaturated fatty acids (USFA) content in the LD muscle of beef cattle. Martínez et al. (2005) also confirmed that the inclusion of mulberry leaves in diets could increase the content of USFA in the muscle, decrease the content of SFA, and especially increase the content of the *n*-3 and *n*-6 groups of USFA in meat rabbits. In the present study, extensive changes in fatty acid composition in muscle tissues were observed. It was worth noting that this effect was more obvious in LD muscle than in BF muscle, indicating that mulberry may have slightly different influences on different muscle types. The concentrations of PUFA in LD muscle, such as C18:2*n*-6, C18:3*n*-3, C20:3*n*-6, and C20:4*n*-6 fatty acids,

Table 8
Relative mRNA expression levels of mitochondria respiratory chain related genes in skeletal muscle of Xiangcun black finishing pigs fed the diets with various levels of mulberry powder.

Item	Mulberry inclusion level, %					SEM	*P*-value		
	0	3	6	9	12		ANOVA	Linear	Quadratic
Longissimus dorsi muscle									
PGC-1α	0.85	0.81	0.68	0.85	0.89	0.12	0.80	0.74	0.59
Nrf2	1.53^{b}	1.43^{bc}	1.47^{bc}	1.96^{a}	1.05^{c}	0.15	<0.01	0.44	0.15
UCP2	0.93	0.70	0.91	0.79	1.12	0.11	0.11	0.20	0.08
UCP3	0.99^{ab}	0.80^{b}	0.77^{b}	1.11^{ab}	1.23^{a}	0.13	0.08	0.07	0.03
Biceps femoris muscle									
PGC-1α	1.29^{b}	0.85^{b}	1.24^{b}	1.78^{a}	0.91^{b}	0.16	<0.01	0.78	0.63
Nrf2	1.25^{a}	0.53^{b}	0.83^{b}	0.71^{b}	0.62^{b}	0.11	<0.01	0.01	0.01
UCP2	0.99^{b}	0.92^{b}	1.66^{a}	1.18^{b}	1.21^{b}	0.15	0.01	0.20	0.11
UCP3	0.65^{b}	0.54^{b}	0.58^{b}	0.71^{b}	1.39^{a}	0.11	<0.01	<0.01	<0.01

PGC-1α = peroxisome proliferator-activated receptor γ coactiva-tor-1α; *Nrf2* = nuclear respiratory factor 2; *UCP2* = uncoupling protein-2; *UCP3* = uncoupling protein-3.
[a, b, c] Within a row, values with different superscript letters differ ($P < 0.05$). $n = 6$.

increased linearly, and ratios of PUFA to SFA and h to H also increased, indicating an improved nutritional value of the meat (Duan et al., 2014). Moreover, AI and TI are also health indicators. In this study, the low values of AI and TI in LD muscle in pigs fed mulberry leaves indicated that fat composition was “healthier”, which is opposite to the ratio of h to H (Welter et al., 2016). Collectively, the indices (ratios of PUFA to SFA and h to H, AI, and TI) of meat in pigs fed the mulberry-supplemented diets were more favorable.

Oxidative reactions can activate lipid peroxidation; therefore, reducing oxidative stress can prevent lipid peroxidation (Abdel-Wahhab et al., 2005). Prior researches have stated that mulberry leaf and its extracts can modify glycometabolism and lipometabolism in other species such as the rat (Wilson and Islam, 2015) and broilers (Islam et al., 2015). The bioactive substances in mulberry leaves play important coordinating roles in fat metabolism and deposition. Such compounds include anthocyanin (Chang et al., 2013), 1-deoxynojirimycin (Tsuduki et al., 2013), and quercetin derivatives (Sun et al., 2015), which can inhibit fatty acid synthesis and reduce lipid accumulation in fatty tissues of the liver, kidney, mesentery and epididymis. In vitro testing has also confirmed mulberry leaves to be an excellent source of inhibitory phytochemicals to combat lipid accumulation (Li et al., 2018). *Folium Mori* extract has been shown to possess prominent antihyperglycemic and antihyperlipidemic functions by activating the IRS-1/PI3K/Glut-4 signaling pathway in skeletal muscles of type 2 diabetes mellitus rats (Cai et al., 2016). In the present study, dietary mulberry inclusion down-regulated the mRNA expression levels of adipogenesis genes, such as *ACCα*, and *PPARγ*, and lipolysis genes, such as *HSL* and *LPL*, in LD in a linear pattern. We propose that the anabolism and catabolism of lipids were suppressed simultaneously in the LD muscle of finishing pigs fed the mulberry leaf diet as a result of the high levels of bioactive compounds contained in the leaves.

As a redox-sensitive transcription factor, Nrf2 interacts with Kelch-like ECH associated protein 1 (Keap 1) in the cytoplasm (McMahon et al., 2006). When electrophilic insults or ROS signaling are targeting the Nrf2-Keap1 complex, Keap1 separates from Nrf2 (Na and Surh, 2008). Then, the latter reacts with antioxidant response elements to modulate the expression of downstream antioxidant genes, such as *HO-1* and *GST*, to achieve antioxidant/detoxifying effects. A previous study by Lin et al. (2017) exhibited that dietary supplementation of 0.5% mulberry leaf resulted in significantly greater mRNA levels of antioxidant-related genes, such as *HO-1*, *GST*, and *Nrf2*, which can potentially enhance the production performance and egg quality of laying hens, and in the meantime regulate their antioxidant status. In the current study, the *Nrf2* expression level in the LD muscle of the 9% mulberry group was greater than that of the other mulberry groups and the control group. Unlike in the LD muscle, we found that dietary mulberry inclusion linearly down-regulated the mRNA expression level of *Nrf2*, but up-regulated the mRNA expression level of *UCP3* in BF muscle. In recent years, the discovery of uncoupling protein confirmed that mitochondria can reduce the production of ROS by uncoupling. *UCP3*, an important member of the mitochondrial vector protein family and a candidate gene for obesity, mediates the decoupling of oxidation and phosphorylation of ADP. *UCP3* is activated by superoxide and the lipid peroxidation product 4-hydroxy-2-nonenal, thus providing a negative feedback loop for mitochondrial ROS production (Sánchez-Pérez et al., 2018). Pohl et al. (2018) demonstrated that *UCP3* is a specific marker for adult cardiomyocytes, which relies on fatty acid beta-oxidation. Mulberry leaf powder in the present study resulted in a significantly elevated content of PUFA, which could improve beneficial health characteristics of meat, but make it easier to oxidize. The plant additive effectively reduced this process, which was evidenced by a sharp rise in *UCP3* mRNA expression level in muscle tissue. Lipids are especially apt to be oxidized; therefore, it can be supposed that inclusion of mulberry leaves protected the USFA from oxidative damage. The components of antioxidant phenols and flavonoids are the main contributing factors of the demonstrated and effective biological activities of mulberry leaves (Gundogdu et al., 2011). Such leaves can prevent cellular damage and lipid peroxidation by chelating metal ions and scavenging free radicals (Andallu et al., 2014).

5. Conclusions

The inclusion of mulberry at < 12% in the diet did not impact growth performance but improved oxidative stability in finishing pigs. Mulberry leaf inclusion also affected the expression of genes involved in lipid metabolism and mitochondrial uncoupling in porcine skeletal muscle. These changes can beneficially regulate the pork fatty acid profile and prevent lipid oxidation, which has a positive impact on the health of consumers.

Author contributions

Y. Liu and **Y. Li** carried out the animal experiments and data analysis, and drafted the manuscript. **F. Li** and **Y. Yin** designed the study and revised the manuscript. **Y. Xiao** helped with the data collection and analysis. **C. Chen** and **D. Xiao** participated in the animal trial. **J. He** and **Y. Peng** reviewed the manuscript.

Conflict of 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.

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