Animal Nutrition 18 (2024) 72–83

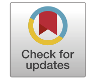
Contents lists available at ScienceDirect

Animal Nutrition

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

Original Research Article

Maternal supplementation with mulberry-leaf flavonoids improves the development of skeletal muscle in the offspring of chickens



Zhenwu Huang [b, c, †], Hongjian Dai [b, †], Simeng Li [d], Zhe Wang [b], Quanwei Wei [b], Zhonghua Ning [a], Yuming Guo [a], Fangxiong Shi [b], Zengpeng Lv [a, *]

[a] State Key Laboratory of Animal Nutrition and Feeding, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China
[b] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, 210095, China
[c] State Key Laboratory of Animal Nutrition and Feeding, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China
[d] School of Biotechnology, Aksu Vocational and Technical College, Aksu, 843000, China

A R T I C L E I N F O

Article history:
Received 2 June 2023
Received in revised form
16 February 2024
Accepted 10 April 2024
Available online 17 April 2024

Keywords:
Broiler breeder hen
Mulberry-leaf flavonoids
Offspring
Protein metabolism
Skeletal muscle

A B S T R A C T

The development of skeletal muscle is a crucial factor in determining the meat yield and economic benefits of broiler production. Recent research has shown that mulberry leaves and their extracts can be used to significantly improve the growth performance of livestock and poultry. The present study aims to elucidate the mechanisms involved in the regulation of skeletal muscle development in broiler offspring by dietary mulberry-leaf flavonoids (MLF) supplementation from the perspective of maternal effect theory. A total of 270 Qiling broiler breeder hens were randomly assigned to 3 treatments with different doses of MLF (0, 30, 60 mg/kg) for 8 weeks before collecting their fertilized eggs. The chicken offspring at 13 and 19 d of embryonic stage, and from 1 to 28 d old after hatching were included in this study. The results showed that maternal supplementation increased the breast muscle weight and body weight of the offspring at the embryo and chick stages ($P < 0.05$). This was followed by increased cross-sectional area of pectoral muscle fibres at 14 d ($P < 0.05$). Further determination revealed a tendency towards increased serum levels of insulin-like growth factor 1 (IGF-1) ($P = 0.092$) and muscle fibre count ($P = 0.167$) at 1 d post-hatching following maternal MLF treatment, while serum uric acid (UA) was decreased at 14 d after hatching ($P < 0.05$). Moreover, maternal MLF supplementation significantly up-regulated the mRNA expression of the myogenic regulatory factor *Myf5* in skeletal muscle at the both embryonic and growth stages ($P < 0.05$). The relative abundance of the downstream protein of BMPR2, Smad1 and p-Smad1/5/9 in the TGFβ signalling pathway was significantly increased by maternal MLF treatment. Meanwhile, the increased expression of the target protein p-mTOR in the breast muscle of the offspring chicks is in accordance with the improved growth rate of the breast and the body. In conclusion, maternal MLF supplementation can promote muscle protein metabolism and muscle fibre development of chick embryos through upregulation of *Myf5* expression and BMP/p-Smad1/5/9 axis, thereby improving growth performance of slow growing broiler.
© 2024 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

In the modern broiler production, there is a growing emphasis on optimizing the quantity and quality of skeletal muscle development in chicks. Skeletal muscle development is a very complex biological process that consists of both hyperplasia and hypertrophy (Velleman, 2007). Hyperplasia refers to the increasing number of fibres completed during the embryonic stage, which is mainly regulated by the myogenic regulatory factors (MRFs) and paired box transcription factors (Paxs) (Messina and Cossu, 2009; Zammit,

* Corresponding author.
E-mail address: lvzengpeng310@cau.edu.cn (Z. Lv).
† These authors contributed equally to this work.
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.2024.04.005
2405-6545/© 2024 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

2017). Consequently, the number of myofibres was determined before birth. Hypertrophy is characterised by the enlargement of muscle fibres during the process of muscle growth after hatching, which is closely linked to satellite cell differentiation and protein metabolism (Picard et al., 2003; Velleman, 2007). The protein synthesis and degradation in skeletal muscle are modulated by the mammalian target of rapamycin (mTOR) and ubiquitin-proteasome pathways (Bodine et al., 2001b; Egerman and Glass, 2014; Pallafacchina et al., 2002). The mitogen-activated protein kinase (MAPK) and transforming growth factor-β (TGFβ) signalling pathways are involved in the positive regulation of muscle protein synthesis (Ryu et al., 2019; Xing et al., 2021). Bone morphogenetic protein (BMP), as a member of the TGFβ superfamily, could promote skeletal muscle hypertrophy via activating the mTOR and the SMAD family member 1/5/8 (Smad1/5/8) pathway (Sartori and Sandri, 2015). Therefore, understanding the changes in these signalling pathways, especially due to maternal effects, is critical for regulating the muscle development of the offspring.

With the prohibition of growth-promoting antibiotics in feed, there is a growing interest to find new alternatives from the perspective of maternal nutrition (Gao et al., 2023; Liu et al., 2024). Growing evidence has shown that the growth performance of offspring can be improved by maternal supplementation with plant flavonoids, including genistein, daizein and quercetin (Amevor et al., 2022; Fan et al., 2018; Lv et al., 2018a,b). Our previous research revealed that epigenetic modification and nutrient composition alteration in breeding eggs were potential factors for the maternal effect (Fan et al., 2018; Jiang et al., 2022; Lv et al., 2018a). Plant flavonoids can both improve the reproduction performance of breeder hens and development of their offspring, which are conducive to realizing the optimum benefit of poultry production (Amevor et al., 2022; Lv et al., 2018a). However, the high cost of these plant extracts limits their application in animal feed. Therefore, it is of great significance to develop new plant extract feed additives for poultry production based on the above maternal nutrition theory.

Mulberry leaves are rich in nutrients and bioactive compounds, which could be used as a cost-effective feed material for livestock and poultry (Lin et al., 2017). A recent report has shown that fermented mulberry leaf powder could increase breast muscle yield and improve the slaughter performance of broilers (Ding et al., 2021). Similarly, another study reported that dietary mulberry leaf (0.6%) supplementation could improve muscle quality and increase the total fibre number of the longissimus dorsi in pigs (Liu et al., 2019). Recently, it was reported that mulberry leaf active components can activate the insulin receptor and TGFβ/Smads signalling pathways in mice (Zhang et al., 2019). Mulberry-leaf flavonoids (MLF) including rutin, isoquercitrin, kaempferol 3-(6-rhamnosylglucoside), and quercetin 3-(6-malonylglucoside), are the main active substances of mulberry leaves (Sugiyama et al., 2013). It has been demonstrated that MLF have multiple biological effects, such as antioxidation, immune regulation and promoting growth (Chen et al., 2020; Chen and Li, 2007; Lin et al., 2022). Our previous studies have found that dietary supplementation with MLF could improve liver lipid metabolism and ovarian function in aged breeder hens, consequently increase the egg-laying performance (Huang et al., 2022b). In addition, MLF could ameliorate the eggshell quality of aged hens by improving antioxidative capability and Ca deposition in the shell gland of uterus (Huang et al., 2022a). This suggests that MLF may prevent the ageing in the reproductive system of breeder hens. We assumed that the maternal MLF effect may regulate muscle protein metabolism and development, thereby improving growth performance of broilers. The aim of the present study was to clarify the possible regulatory mechanism involved in the maternal effect of MLF on the development of offspring skeletal muscle.

2. Materials and methods

2.1. Animal ethics statement

Animal management and experimental procedures were completely carried out following the Guidelines for Care and Use of Laboratory Animals of Nanjing Agricultural University (SYXK (SU) 2017-0007).

2.2. Animals and experimental design

Qiling breeder hens, used in the present study, are an important Chinese native breed. Qiling chickens are a slow growth broiler breed, and male broilers can be marketed at about 90 d with an individual weight of 1.5 to 1.6 kg. Based on our previous studies, 30 and 60 mg/kg MLF could improve reproductive performance and egg quality of aged breeder hens (Huang et al., 2022a,b). Thus, 270 healthy Qiling breeder hens (60-week-old) were randomly allocated into 3 groups supplemented with different MLF doses (0, 30, and 60 mg/kg), and with 6 replicates of 15 birds per replicate. All hens were raised in Tushan Breeding Farm (Changzhou, China) under a controlled environment. Mulberry-leaf flavonoids were purchased from Nantong Feiyu Biological Technology Co., Ltd. (Nantong, China) with a purity of 50%. During the experiment, the hens were fed with a restricted intake (108 g/d per hen) at 06:00 and were allowed water freely. The treatment of breeder hens lasted for 8 weeks, and all breeder hens were subjected to artificial insemination during the experiment. In the last three days of the formal experiment, we obtained 120 fertilized eggs from each treatment group for hatching in a local hatchery (Tushan, Changzhou, China) or in laboratory. In the hatchery, each group contained 6 replicates of 15 eggs, which were randomly set and incubated in the same incubator. In the laboratory, after each group of 30 eggs was tagged, they were randomized to the same incubator for hatching. Manufacturer guidelines for the incubation process were strictly followed and an appropriate incubation temperature and humidity were maintained during the whole incubation process.

After hatching, the male and female chicks were separated by gender identification. A total of 120 male offspring chickens at 1 d old (40 chickens of each group) were then moved to brooder cages in a temperature-controlled room with continuous light. All chicks were given ad libitum access to the same basic diet and water. The diet compositions for the breeder hens and chicks are shown in (Table 1), which refers to the nutrient requirement of the China National Feeding Standard of Chicken (NY/T 33-2004). All routine procedures were the same as those used by the company. The offspring chicks were divided into 3 groups based on breeder hen treatment: (1) the offspring of breeder hens fed a basal diet (CON), (2) the offspring of breeder hens fed a basal diet supplemented with 30 mg/kg MLF (L-MLF) and (3) the offspring of breeder hens fed a basal diet supplemented with 60 mg/kg MLF (H-MLF). The selected male broilers were weighed immediately after birth, and body weight changes and feed intake were recorded at 14 and 28 d.

2.3. Sample collection

The nutrient levels of representative feed samples were analysed as follows: crude protein according to official method 990.03

(AOAC, 1995); available phosphorus according to official method 965.17 (AOAC, 1995); lysine according to official method 988.15 (AOAC, 1990); methionine, tryptophan and threonine according to official method 994.12 (AOAC, 1995); calcium according to official method 927.02 (AOAC, 1995). Metabolizable energy was calculated by using Tables of Feed Composition and Nutritive Values in China (2020).

On d 13 and 19 of incubation, 8 normal embryos per group were randomly selected for sampling. The embryo weight, breast muscle weight, and the length of the tibia were measured to evaluate embryonic development. Breast muscle after weighing was frozen rapidly with liquid nitrogen and stored at −80 °C for further analysis.

Another 8 offspring chickens from each group were selected and weighed at d 1 and 14 after hatching. The chickens were slaughtered through jugular exsanguination, and the serum was separated and stored at −20 °C. The breast muscles on the left were removed and weighed. The breast muscle (about 1 cm^2) was carefully collected, and then rapidly fixed in 4% paraformaldehyde. Another part of the muscle tissue was also collected and stored at −80 °C for further analysis.

2.4. The calculations of growth, feed utilisation and biological indices

Growth and biological indices of the offspring chicken embryo were calculated using the following formulae:

Relative embryo weight (%) = live embryo weight (g)/egg weight (g) × 100;

Relative embryo length (mm/g) = body length (mm)/egg weight (g) × 100;

Relative tibia length (mm/g) = tibia length (mm)/egg weight (g) × 100;

Relative breast muscle weight (%) = breast muscle weight (g)/embryo weight (g) × 100.

Growth, feed conversion and biological indices of the offspring chicks were calculated using the following formulae:

Average daily gain (g/d) = [final weight (g) − initial weight (g)]/number of days (d);

Average daily feed intake (g/d) = total feed intake (g)/number of days (d);

Feed conversion ratio (%) = feed intake (g)/[final weight of chick (g) − initial weight of chick (g)] × 100;

Relative tibia length (mm/g) = tibia length (mm)/live body weight (g);

Relative breast muscle weight (%) = breast muscle weight (g)/live body weight (g) × 100.

2.5. Serum biochemical and enzyme-linked immunosorbent assay (ELISA)

Enzyme labelling instrument (Spark, Tecan, Switzerland) and commercial kits (C012-2-1, C011-2-1, A045-4-2, A028-2-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to detect serum uric acid (UA), creatinine (CREA), total protein (TP), and albumin (ALB) contents of broiler chicken on d 1 and 14. The specific method of operation is performed as described in the kit directions.

The serum insulin-like growth factor 1 (IGF-1) and triiodothyronine (T3) content were determined using commercial ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

Table 1
Ingredients and nutrient levels of basic diet of breeder hens and broilers from 1 to 28 d old (DM basis, %).

Item	Breeder hens	Item	Broiler (1–28 d)
Ingredients[1]		Ingredients[2]	
Corn	54.99	Corn	53.28
Soybean meal	34.15	Soybean meal	38.57
Limestone	7.23	Soybean oil	3.70
Soybean oil	0.50	Dicalcium phosphate	1.98
Dicalcium phosphate	2.09	Mineral premix	0.50
NaCl	0.35	Vitamin premix	0.10
Mineral premix	0.30	Limestone	1.05
Choline chloride (50%)	0.12	Choline chloride (50%)	0.30
Met	0.17	NaCl	0.35
Vitamin premix	0.10	Met	0.17
Total	100.00	Total	100.00
Nutrient levels[3]		Nutrient levels[3]	
ME, MJ/kg	11.84	ME, MJ/kg	12.35
CP	16.10	CP	21.57
Available phosphorus	0.47	Available phosphorus	0.45
Lys	0.81	Lys	1.15
Met	0.63	Met	0.49
Thr	0.60	Ca	1.05
Try	0.18		

ME = metabolizable energy; CP = crude protein.
[1] Supplied the following per kilogram complete diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg; vitamin K_3, 2.65 mg; thiamine, 2 mg; riboflavin, 6 mg; vitamin B_{12}, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg; vitamin A, 12,500 IU; vitamin D_3, 2500 IU; vitamin E, 30 IU.
[2] Supplied the following per kilogram complete diet: Fe, 80 mg; Zn, 75 mg; Mn, 100 mg; Cu, 8 mg; I, 0.35 mg; Co, 0.2 mg; Se, 0.15 mg; vitamin A, 12,500 IU; vitamin D_3, 2500 IU; vitamin E, 80 IU; vitamin K, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; nicotinic acid, 50 mg; pantothenic acid, 20 mg; vitamin B_6, 4 mg; folic acid, 1.25 mg; vitamin B_{12}, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.
[3] Metabolizable energy is calculated value and the others are measured values.

The serum was diluted 1:4 (vol/vol) with 0.9% normal saline and all subsequent experimental procedures were performed accurately following the manufacturer's instructions. As indicated in the instructions of ELISA kits, the sensitivity for IGF-1 determination was 10 ng/mL, and the sensitivity for T3 determination was 1.0 nmol/L. The intra- and inter-assay coefficients of variation were <10% and <15%, respectively, for all ELISA kits. Data were analysed using samples from 7 chickens per group ($n = 7$).

2.6. Histomorphological analysis

Afterwards, breast muscles were fixed in 4% paraformaldehyde for at least 24 h, which were sequentially dehydrated with ethanol and xylene, embedded in paraffin, and dissected into 5-μm tissue sections using a microtome (Lecia Biosystems, Buffalo Grove, USA). Paraffin-embedded sections were immersed in 100% xylene and then sequentially soaked in gradient alcohol, and finally stained with hematoxylin and eosin (H&E). Olympus microscope (Olympus Optical, Beijing, China) was used to take images. Statistical analysis of muscle fibre density, diameter, and area was performed using Image Pro Plus and Image J (Media Cybernetics, Maryland, USA). Three visual fields were randomly selected for each tissue section when calculating diameter and area, and at least 50 muscular fibres were counted in each visual field. The data were analysed using samples from 8 chickens per group ($n = 8$).

2.7. Western blotting

The TP of breast muscle was extracted by RIPA lysate with 1% PMSF (Beyotime, Nanjing, China) at 4 °C. The protein concentration was detected using a BCA Protein Assay Kit (Beyotime, Nanjing, China). A total of 20 μg of proteins was electrophoresed using 4% to 15% SDS-PAGE precast gel (Feite Biotech Co., Ltd., Nanjing, China) and the gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). And the separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 70 V for 45 to 120 min by using the electrophoresis tank (Bio-Rad, Hercules, CA, USA). Then the PVDF membranes were blocked in TBS blocking buffer containing 5% non-fat dried milk for 1 h at room temperature. After washing 4 times with TBST buffer, the membranes were incubated overnight at 4 °C with the following primary antibodies respectively: SMAD family member 1 (Smad1), phospho-SMAD family member 1/5/9 (p-Smad1/5/9) (AF6451 and AF8313; Affinity Biosciences, USA); phospho-mitogen-activated protein kinase (p-MAPK), myogenic factor 5 (Myf5), myostatin (MSTN), mTOR, phosphorylated mammalian target of rapamycin (p-mTOR), BMP2, bone morphogenetic protein receptor 2 (BMPR2) (AP1165, A16227, A6913, A2445, AP0094, A0231 and A5666; ABclonal Technology, Wuhan, China); and MAPK (8690S, Cell Signaling Technology, Boston, USA). After washing with TBST buffer 4 times, the membranes were incubated with secondary antibodies, HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (AS014 and AS003; ABclonal Biotechnology, Wuhan, China), for 1 h at room temperature. Finally, the protein bands were detected using an enhanced chemiluminescence kit (Thermo Scientific, Wilmington, DE, USA), and were visualized using a Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference and the intensities of specific bands were quantified through Image J software. The data of Western blotting were analyzed using 4 randomly selected samples per group ($n = 4$).

2.8. RNA extraction and detection and real-time PCR analysis

Total RNA of myoblasts from the chicken embryo or broiler chicken chest muscle after treatment was isolated with the RNAios Plus kit (9109, TaKaRa Company, Dalian, China), and the quality and concentration of total RNA were identified by an ND-2000 microspectrophotometer (Thermo Scientific, Wilmington, USA). After cDNA synthesis using HiScript II First Strand cDNA Synthesis Kit (R323-01, Vazyme Biotech Co., Ltd., Nanjing, China), the cDNA was diluted 1:4 using RNase free water. The real-time PCR was carried out using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) on the QuantStudio 7 Real-Time PCR System (Thermo Scientific, Wilmington, DE, USA). The reaction procedure was as follows: denaturing at 95 °C for 30 s, and 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. All operations were done according to the instructions of the kit. The primer pairs used are presented in Table 2 and were synthesized by Sangon Biotech (Shanghai, China). *GAPDH* was used as the housekeeping gene. Relative mRNA expression levels of each target gene were normalized to the control using the $2^{-\Delta\Delta Ct}$ method. The ratio of the target gene to *GAPDH* represents the amount of relative gene expression. Eight chicken samples per group were used for detection and the average level of two repeats was used for statistical analysis ($n = 8$).

2.9. Statistical analysis

All data were analysed by one-way ANOVA using SPSS (version 21.0, SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used to compare the differences among groups, and results were given as means and standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

3. Results

3.1. Embryonic development and growth performance

To assess the effect of maternal MLF supplementation on embryonic development of chick offspring, we analyzed the growth performance of the embryos at 13 and 19 d of embryonic age, presented in Table 3. Compared with CON group, maternal supplementation with 30 mg/kg MLF increased the length of tibia and relative length of tibia in offspring embryos on d 13 ($P < 0.05$). And 60 mg/kg MLF treatment increased relative weight of breast muscle and relative length of tibia of offspring on d 13 of embryonic age ($P < 0.05$). At d 19 of embryonic age, maternal supplementation with 30 mg/kg MLF increased embryo weight and weight of breast muscle ($P < 0.05$), and 60 mg/kg MLF treatment increased the weight of breast muscle and relative weight of breast muscle ($P < 0.05$). Maternal MLF supplementation did not affect the relative embryo weight, length of the embryo, or relative length of the embryo at d 13 or 19 of incubation.

3.2. Gene expressions in embryonic breast muscle

Myogenic regulatory factors are important positive regulators of muscle fibre differentiation and formation. As shown in Figs. 1 and 2, we examined the expression of the relative mRNA in the breast muscle at d 13 and 19 of incubation. Compared with the control, maternal supplementation with 30 mg/kg MLF increased the expression of *Myf5* and estrogen receptor alpha (*ERα*) at d 13 of embryonic age ($P < 0.05$, Fig. 1A). In addition, maternal supplementation with 30 mg/kg MLF increased the expression of *Myf5* at

Table 2
Primer sequences used in real-time PCR.

Gene	GenBank ID	Primer sequence (5′ → 3′)	Product size, bp
ERα	NM_205183.2	R: CAGGCCTGGCAACTCTTTCT F: CGGGCGTGGTGACATTAAAC	167
ERβ	NM_204794.2	R: CCAGGATGAAGGGTGTGCAA F: GCAACGAATCGCTGACACTC	192
MSTN	NM_001001461	F: GCTTTTGATGAGACTGGACGAG R: AGCGGGTAGCGACAACATC	173
Atrogin-1	NM_001030956	F: CACGGAAGGAGCAGTATGGT R: AGGTCTCTGGGTTGTTGGCT	124
MURF1	XM_424369	F: CGACATCTACAAGCAGGAGT R: TGAGCACCGAAGACCTT	163
IGF-1R	NM_205032.1	F: CTGTGTCCGACAAATGGGGA R: TGACGGTCAGTTTCGGGAAG	169
MyoD	NM_204214	F: ATCACCAAATGACCCAAAGC R: GGGAACAGGGACTCCCTTCA	149
Myf5	NM_001030363	F: TGAGGAACGCCATCAGGT R: GCGAGTCCGCCATCACAT	141
MyoG	NM_204184	F: GGAGGCTGAAGAAGGTGAA R: BTGCTGGTTGAGGCTGCTGA	152
Pax7	NM_205313.1	F: CACCTACAGCACCACCAGTTA R: TGTTCTCCCAGCTTCATCCT	135
m-TOR	XM_417614	F: GAAGTCCTGCGCGAGCATAAG R: TTTGTGTCCATCAGCCTCCAGT	92
GAPDH	NM_203405	F: GGTGAAAGTCGGAGTCAACGG R: CGATGAAGGGATCATTGATGGC	244

ERα = estrogen receptor alpha; *ERβ* = estrogen receptor beta; *MSTN* = myostatin; *Atrogin-1* = muscle atrophy f-box (MAFbx); *MURF1* = muscle RING finger 1; *IGF-1R* = insulin-like growth factor 1 receptor; *MyoD* = myogenic differentiation antigen; *Myf5* = myogenic factor 5; *MyoG* = myogenin; *Pax7* = paired box protein pax-7; *m-TOR* = mammalian target of rapamycin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.
F represents forward; R represents reverse.

d 19 of embryonic age ($P < 0.05$, Fig. 2A). No significant differences in the expression of *MSTN*, paired box protein pax-7 (*Pax7*), myogenic differentiation antigen (*MyoD*) and myogenin (*MyoG*) were observed among the three groups at d 13 or 19 of embryonic age.

3.3. Protein abundance in embryonic breast muscle

As presented in Fig. 1, compared with CON group, maternal supplementation with 30 mg/kg MLF increased the relative protein abundance of p-Smad1/5/9 in breast muscle at d 13 of embryonic age, and maternal 60 mg/kg supplementation increased the relative protein abundance of Smad1 ($P < 0.05$, Fig. 1D). On d 19 of embryonic age, compared with the control, maternal 60 mg/kg supplementation increased the relative protein abundance of Smad1, p-Smad1/5/9, Myf5 and BMPR2 ($P < 0.05$, Fig. 2D, F and G). The relative protein abundance of p-Smad1/5/9 was also higher than the CON group when maternal supplementation with 30 mg/kg MLF ($P < 0.05$, Fig. 3D). Whereas, it had no detectable difference in the protein abundance of MSTN at d 13 and 19 of embryonic age.

3.4. Chicken development, growth performance and breast muscles histomorphology

We evaluated the effects of maternal MLF on chicken development, growth performance, and breast muscle histomorphology. The data are shown in Table 4, Figs. 3 and 4. Maternal supplementation with MLF increased average daily gain including d 1 to 28, and maternal supplementation with 30 mg/kg MLF increased average daily gain including d 1 to 14 ($P < 0.05$, Table 4). However, maternal MLF supplementation did not change the average daily feed intake or feed conversion rate of offspring during the whole experimental period.

Organ development of offspring chicken at 1 and 14 d were also recorded. Compared with the control, maternal supplementation with MLF increased the length of tibia and relative length of tibia at 1 d after hatching ($P < 0.05$). At d 1 after hatching, maternal supplementation with 60 mg/kg MLF increased the weight of breast muscle ($P < 0.05$). Maternal supplementation with 30 mg/kg MLF increased the relative weight of breast muscle at 14 d ($P < 0.05$). H&E staining was performed to observe the effects of maternal MLF supplementation on the breast muscle morphology of chicken offspring (Figs. 3A and 4A), and the number of muscle fibres per unit area showed an increasing trend at 1 d of age ($P = 0.167$, Fig. 3B). Interestingly, maternal supplementation with MLF increased the cross-sectional area of breast muscle of offspring chicken at 14 d ($P < 0.05$, Fig. 4B).

3.5. Chicken serum biochemical parameters and growth hormones

Biochemical serum parameters and growth hormone contents of offspring chicken are presented in Table 5. Maternal supplementation with MLF increased serum ALB content at d 1 post-hatch ($P < 0.05$), Moreover, at 14 d post-hatch, compared with the control, maternal supplementation with 30 mg/kg MLF decreased serum UA content ($P < 0.05$), and maternal supplementation with 60 mg/kg MLF increased serum CREA content.

3.6. Gene expressions in offspring chicken breast muscle

As illustrated in Figs. 3C and 4C, we performed real-time PCR to detect the transcription expression of genes related to MRF, protein synthesis, and proteolysis regulation in the breast muscle of chicken offspring. At 1 d after hatching, maternal supplementation with 60 mg/kg increased the expression of *Myf5* ($P < 0.05$, Fig. 3C). In addition, we observed that maternal supplementation with 30 mg/kg MLF increased the expression of muscle atrophy f-box (*MAFbx*, also called *Atrogin-1*) of breast muscle at 14 d after hatching ($P < 0.05$, Fig. 4C). Noteworthy, the relative expression of insulin-like growth factor 1 receptor (*IGF-1R*) and *mTOR* also had an increasing trend at 1 d after hatching ($P = 0.066$, $P = 0.101$, Fig. 3C). However, no differences were observed in the expression of *MyoG*, *MyoD*, *MSTN*, and muscle RING finger 1 (*MURF1*) at 1 and 14 d after hatching.

Table 3
Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on the development of offspring embryos.

Item	Treatment groups			*P*-value
	CON	L-MLF	H-MLF	
Day 13				
Embryo weight, g	7.51 ± 0.78	7.99 ± 0.19	7.11 ± 0.95	0.561
Embryo length, mm	41.7 ± 1.8	42.7 ± 0.9	41.9 ± 1.7	0.439
Breast muscle weight, g	0.50 ± 0.03	0.58 ± 0.04	0.54 ± 0.05	0.304
Tibia length, mm	15.4 ± 0.9[b]	17.0 ± 0.9[a]	16.0 ± 0.9[b]	0.006
Relative embryo weight, %	12.4 ± 1.5	13.5 ± 0.7	12.3 ± 1.8	0.154
Relative embryo length, mm/g	69.1 ± 3.6	72.2 ± 4.2	72.2 ± 4.3	0.280
Relative breast muscle weight, %	6.64 ± 0.16[b]	7.09 ± 0.37[ab]	7.54 ± 0.14[a]	0.026
Relative tibia length, mm/g	25.5 ± 1.3[b]	28.8 ± 2.1[a]	27.4 ± 2.3[a]	0.007
Day 19				
Embryo weight, g	26.7 ± 1.0[b]	28.9 ± 2.1[a]	28.1 ± 0.8[ab]	0.031
Embryo length, mm	70.2 ± 2.7	68.6 ± 2.8	69.7 ± 4.8	0.374
Breast muscle weight, g	0.59 ± 0.02[b]	0.66 ± 0.07[a]	0.73 ± 0.08[a]	0.033
Tibia length, mm	24.5 ± 2.3	25.7 ± 0.7	25.3 ± 1.1	0.403
Relative embryo weight, %	52.2 ± 2.4	52.6 ± 3.2	51.8 ± 1.7	0.975
Relative embryo length, mm/g	137.4 ± 7.7	138.8 ± 3.3	132.4 ± 4.3	0.192
Relative breast muscle weight, %	2.19 ± 0.04[b]	2.27 ± 0.21[ab]	2.64 ± 0.26[a]	<0.001
Relative tibia length, mm/g	53.2 ± 1.4	55.8 ± 4.9	54.8 ± 3.9	0.291

CON, the offspring embryos of breeder hens fed with a normal diet; L-MLF, the offspring embryos of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the offspring embryos of breeder hens fed a basal diet supplemented with 60 mg/kg MLF.
Mean values ± SEM are used to represent data ($n = 8$). $P < 0.05$ was considered a significant difference, and different letters represent statistically significant differences among the groups.

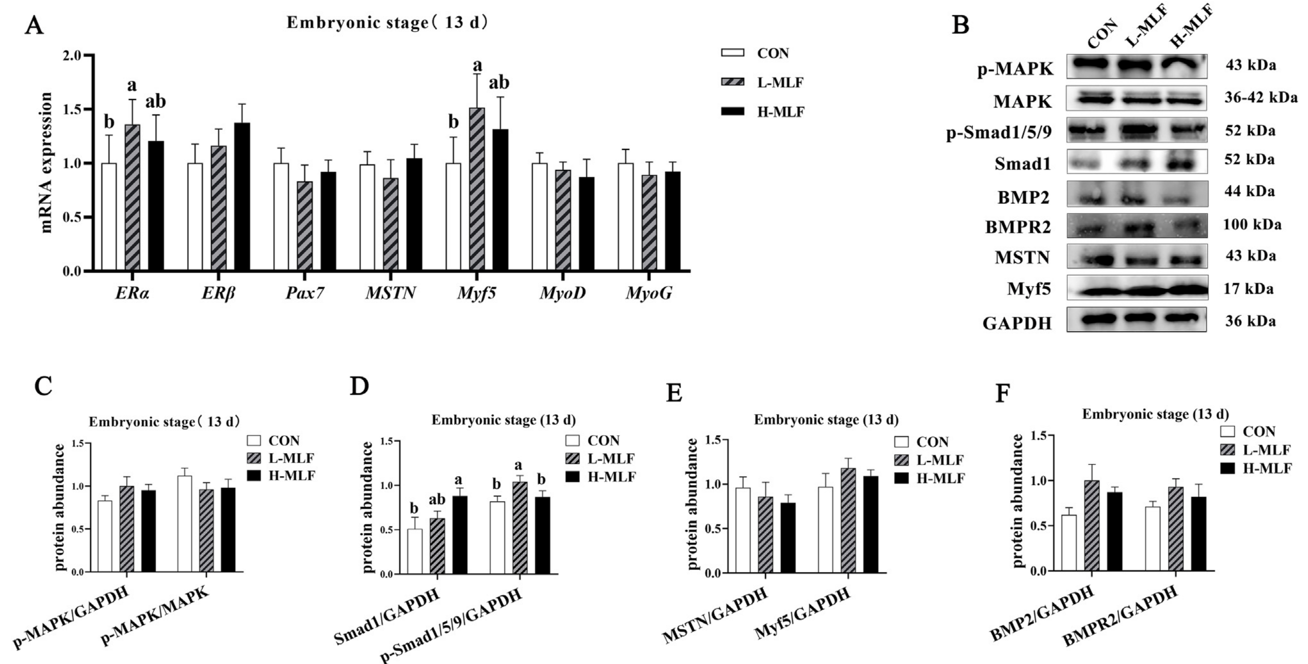


Fig. 1. Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on the relative mRNA expression and protein abundance in the breast muscle of offspring embryo at 13 d. (A) Relative mRNA expression of genes related with myogenic regulatory factors ($n = 8$). (B to F) Western blot analysis of p-MAPK, MAPK, p-Smad1/5/9, Smad1, BMP2, BMPR2, MSTN and Myf5 in the breast muscle ($n = 4$). *ERα* = estrogen receptor alpha; *ERβ* = estrogen receptor beta; *Pax7* = paired box protein pax-7; *MSTN* = myostatin; *Myf5* = myogenic factor 5; *MyoD* = myogenic differentiation antigen; *MyoG* = myogenin; MAPK = mitogen-activated protein kinase; p-MAPK = phospho-mitogen-activated protein kinase; Smad1 = SMAD family member 1; p-Smad1/5/9 = phospho-SMAD family member 1/5/9; BMP2 = bone morphogenetic protein 2; BMPR2 = bone morphogenetic protein receptor 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. CON, the offspring embryos of breeder hens fed with a normal diet; L-MLF, the offspring embryos of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the offspring embryos of breeder hens fed a basal diet supplemented with 60 mg/kg MLF. [a,b]Different letter represents statistically significant differences among the groups ($P < 0.05$).

3.7. Protein abundance in offspring chicken breast muscle

As shown in Figs. 3 and 4, we examined the protein abundance levels of breast muscle protein synthesis-related pathways in the offspring chicken at d 1 and 14 after hatching. Compared with the control, maternal supplementation with MLF increased the protein abundance of Samd1, and p-Smad1/5/9 at 1 d after hatching ($P < 0.05$, Fig. 3F), as did the p-mTOR/mTOR ratio ($P < 0.05$, Fig. 3E). Furthermore, maternal supplementation with 30 mg/kg MLF increased the protein abundance of BMPR2 and maternal supplementation with 60 mg/kg MLF increased the protein abundance of Myf5 at 1 d after hatching ($P < 0.05$, Fig. 3G and H). On d 14 post-

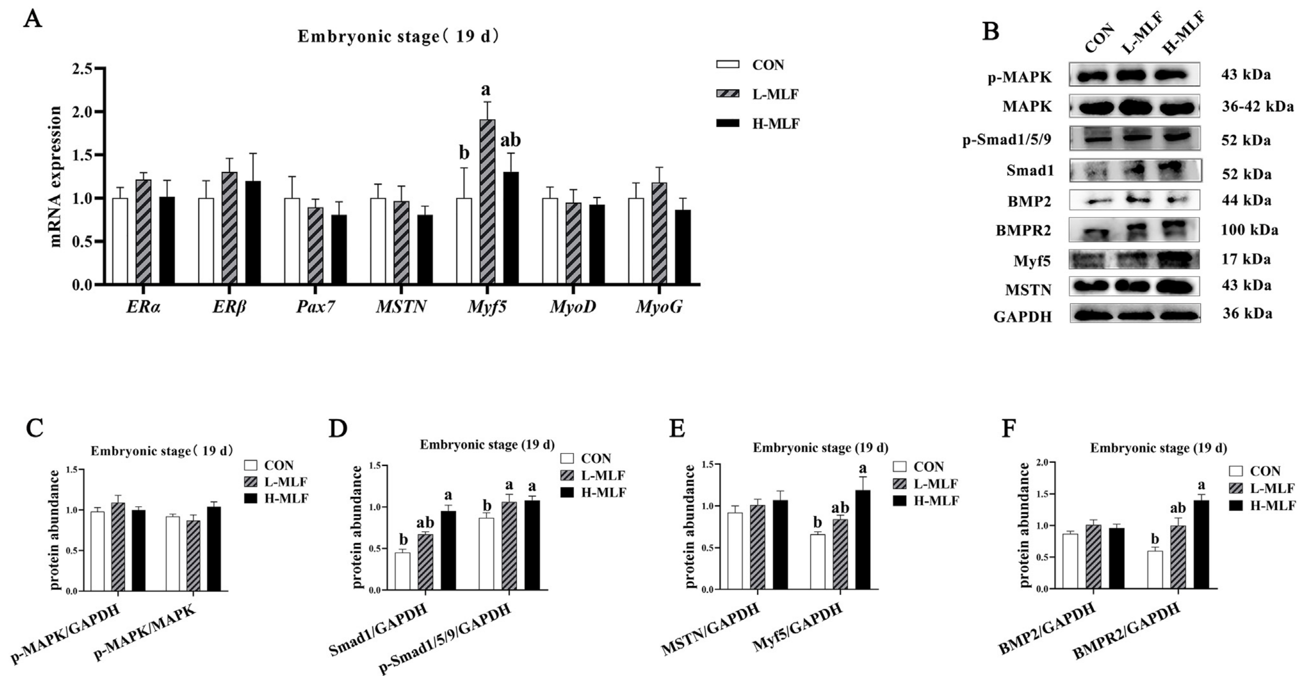


Fig. 2. Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on the relative mRNA expression and protein abundance in the breast muscle of offspring embryo at 19 d. (A) Relative mRNA expression of genes related with myogenic regulatory factors ($n = 8$). (B to F) Western blot analysis of p-MAPK, MAPK, p-Smad1/5/9, Smad1, BMP2, BMPR2, MSTN and Myf5 in the breast muscle ($n = 4$). *ERα* = estrogen receptor alpha; *ERβ* = estrogen receptor beta; *Pax7* = paired box protein pax-7; *MSTN* = myostatin; *Myf5* = myogenic factor 5; *MyoD* = myogenic differentiation antigen; *MyoG* = myogenin; MAPK = mitogen-activated protein kinase; p-MAPK = phospho-mitogen-activated protein kinase; Smad1 = SMAD family member 1; p-Smad1/5/9 = phospho-SMAD family member 1/5/9; BMP2 = bone morphogenetic protein 2; BMPR2 = bone morphogenetic protein receptor 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. CON, the offspring embryos of breeder hens fed with a normal diet; L-MLF, the offspring embryos of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the offspring embryos of breeder hens fed a basal diet supplemented with 60 mg/kg MLF. [a,b]Different letter represents statistically significant differences among the groups ($P < 0.05$).

hatch, compared with the control, maternal supplementation with MLF increased the protein abundance of p-Smad1/5/9 ($P < 0.05$, Fig. 4F). Moreover, maternal supplementation with 30 mg/kg MLF significantly increased the protein abundance of Smad1 in the breast muscle at 14 d after hatching ($P < 0.05$, Fig. 4F). However it had no detectable difference in the protein abundance of MSTN and p-MAPK/MAPK ratio at 1 and 14 d after hatching.

4. Discussion

Maternal supplementation with phytoflavonoids could improve the growth and development of poultry offspring (Amevor et al., 2022; Lv et al., 2018b). Herein, we found that maternal supplementation with MLF in aged breeder hens could significantly improve tibial growth and skeletal muscle development of offspring chick embryos. Maternal effect on the development of slow growth broilers extended to at least 28 d after hatching. Maternal supplementation with MLF could increase breast muscle weight, tibia length and average daily gain of offspring broilers. Consistent with our previous findings that maternal supplementation with genistein could improve the muscle development of offspring broilers, which related to regulating growth hormone secretion (Lv et al., 2018a). The change of reproductive function of breeder hens can affect the deposition of nutrients in eggs and the subsequent development of chicks (Nangsuay et al., 2016; Yilmaz-Dikmen and Sahan, 2009). Interestingly, our recent studies revealed that dietary supplementation with MLF could improve the reproductive function and vitellogenin synthesis of aged breeder hens, which might be involved in regulating offspring development (Huang et al., 2022a,b). Previous research has revealed that dietary plant flavonoids can be deposited into breeding eggs efficiently, which may affect the development directly (Saitoh et al., 2004). However, further research is needed to identify the change in composition of the breeding egg after dietary MLF supplementation.

Secondary muscle fibres of chickens begin to form in the embryonic stage at d 13, and the number of muscle fibres will not change after hatching (Gao et al., 2014; Yablonka-Reuveni, 1995). The developmental process is closely linked to the proliferation, differentiation and fusion of myoblasts and satellite cells, which are mainly regulated by MRF and Pax, including the transcription factors *MyoD*, *Myf5*, and *MyoG* (Miller and Stockdale, 1986; Velleman, 2007). MSTN is a negative regulator for muscle development (Buckingham and Relaix, 2007; Dou et al., 2018). In the present study, maternal supplementation with MLF significantly increased the expression of *Myf5* in the breast skeletal muscle during embryonic period and at d 1 after hatching, and thus the positive effect on muscle fibre development could be confirmed. However, maternal MLF treatment exerted a dose effect on the development of offspring chicks. Specially, low dose MLF supplementation had a more significant effect on offspring muscle fibre development during the embryonic stage. Supplementation with high doses of MLF appeared to have better efficacy than low doses at chick stage. The number of muscle fibres after hatching is the most important indicator for the potential of skeletal muscle development. The histological analysis suggested an increase of breast muscle fibre numbers of 1-d-old chicks in the present experiment. The up-regulated expression of *Myf5* induced by maternal MLF effect might be a regulatory factor involved in the development of offspring myofibrils.

As outlined previously, skeletal muscle growth requires a higher rate of protein synthesis than that of degradation. The TP

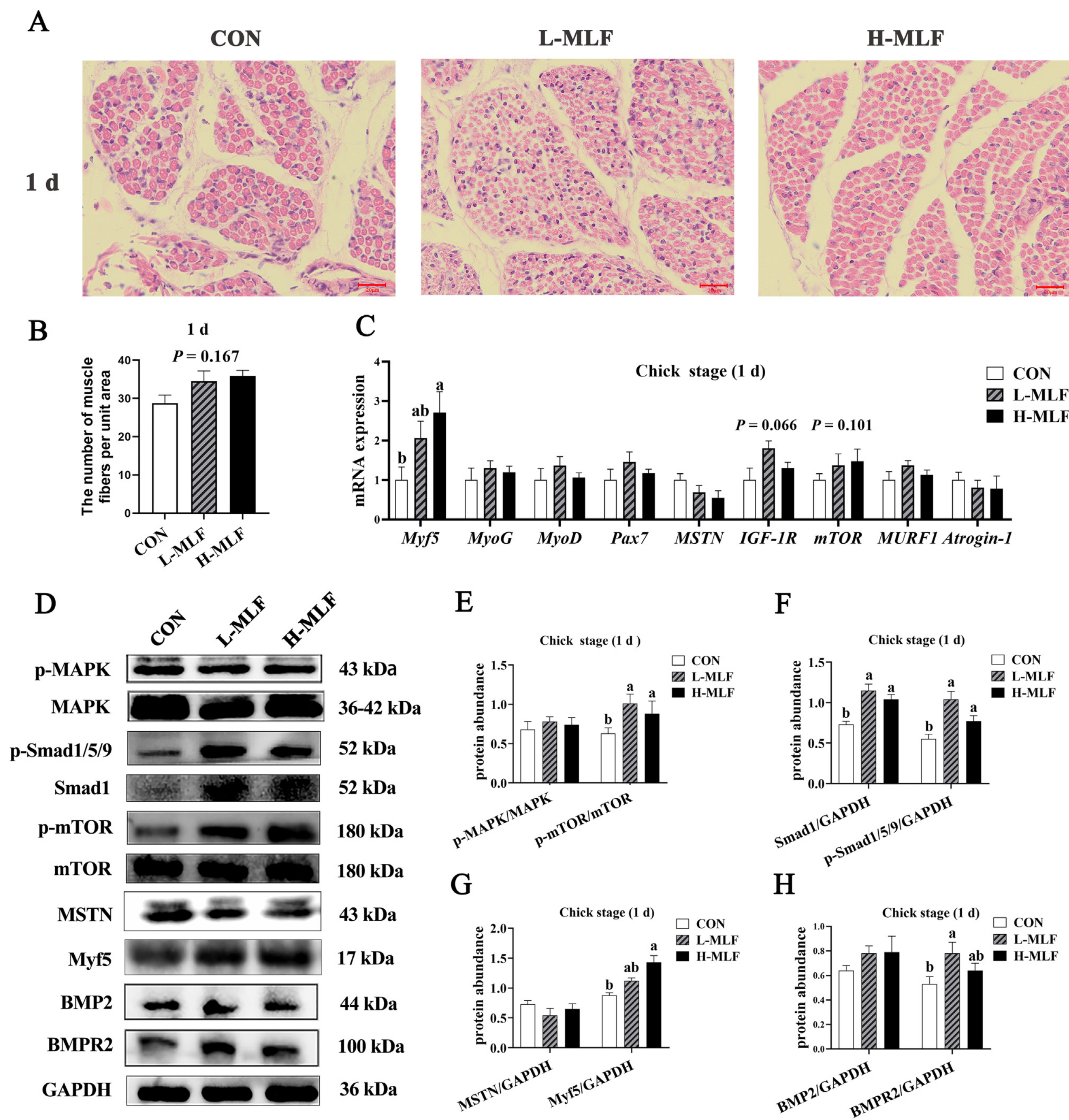


Fig. 3. Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on the skeletal muscle development of chicken offspring at 1 d post-hatch. (A) The representative images of hematoxylin and eosin (H&E) staining on the breast muscle. Scale bar = 20 μm. (B) The number of muscle fibres per unit area in the chicken offspring at 1 d after hatching. (C) Relative mRNA expression of genes related with myogenic regulatory factors, protein synthesis and degradation at 1 d after hatching ($n = 8$). (D to H) Western blot analysis of p-MAPK, MAPK, p-mTOR, mTOR, Smad1, p-Smad1/5/9, BMP2, BMPR2, MSTN and Myf5 in the breast muscle ($n = 4$). *Myf5* = myogenic factor 5; *MyoG* = myogenin; *MyoD* = myogenic differentiation antigen; *Pax7* = paired box protein pax-7; *MSTN* = myostatin; *IGF-1R* = insulin-like growth factor 1 receptor; *mTOR* = mammalian target of rapamycin; *MURF1* = muscle RING finger 1; *Atrogin-1* = muscle atrophy f-box (MAFbx); MAPK = mitogen-activated protein kinase; p-MAPK = phospho-mitogen-activated protein kinase; p-mTOR = phosphorylated mammalian target of rapamycin; Smad1 = SMAD family member 1; p-Smad1/5/9 = phospho-SMAD family member 1/5/9; BMP2 = bone morphogenetic protein 2; BMPR2 = bone morphogenetic protein receptor 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. CON, the chicken offspring of breeder hens fed with a normal diet; L-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 60 mg/kg MLF. [a,b]Different letter represents statistically significant differences among the groups ($P < 0.05$).

and ALB in the serum are important indicators of protein synthetic capacity in the body (Hu et al., 2021). In the present study, maternal MLF effect could increase the content of ALB in the serum of offspring chicks at d 1 post-hatching, which indicated that the protein synthesis capacity was increased. The serum UA and CREA are the end-metabolites of nucleic acid and protein catabolism (Donsbough et al., 2010; Ospina-Rojas et al., 2013), the content of which could directly reflect the level of protein catabolism in the body. In the present study, when maternal supplementation with MLF, serum CREA levels of offspring chicken were

Table 4
Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on the development of chicken offspring.

Item	Treatment groups			*P*-value
	CON	L-MLF	H-MLF	
Growth performance				
Average daily gain, g/d				
1–14 d	13.2 ± 0.3[b]	14.3 ± 0.3[a]	14.0 ± 0.4[ab]	0.026
14–28 d	17.6 ± 1.1	19.1 ± 0.7	18.8 ± 0.8	0.243
1–28 d	16.7 ± 0.5[b]	18.0 ± 0.3[a]	18.2 ± 0.4[a]	0.013
Average daily feed intake, g/d				
1–14 d	31.2 ± 0.5	32.0 ± 0.6	31.7 ± 0.4	0.152
14–28 d	53.8 ± 1.5	54.2 ± 1.0	54.3 ± 1.7	0.638
1–28 d	43.8 ± 0.9	44.0 ± 1.3	44.4 ± 0.8	0.824
Feed conversion ratio, %				
1–14 d	2.36 ± 0.38	2.25 ± 0.19	2.26 ± 0.11	0.096
14–28 d	2.94 ± 0.13	2.85 ± 0.25	2.87 ± 0.37	0.147
1–28 d	2.62 ± 0.20	2.50 ± 0.17	2.43 ± 0.12	0.208
Organ development				
Day 1				
Breast muscle weight, g	0.70 ± 0.02[b]	0.75 ± 0.04[ab]	0.77 ± 0.01[a]	0.025
Tibia length, mm	25.8 ± 1.3[b]	28.6 ± 0.5[a]	29.1 ± 0.6[a]	<0.001
Relative breast muscle weight, %	1.76 ± 0.16	1.85 ± 0.09	1.80 ± 0.11	0.379
Relative tibia length, mm/g	0.57 ± 0.04[b]	0.64 ± 0.06[a]	0.66 ± 0.02[a]	<0.001
Day 14				
Breast muscle weight, g	20.1 ± 2.4	21.8 ± 3.2	20.9 ± 1.7	0.248
Relative breast muscle weight, %	7.46 ± 0.34[b]	8.59 ± 0.19[a]	8.18 ± 0.18[ab]	0.047

CON, the chicken offspring of breeder hens fed with a normal diet; L-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 60 mg/kg MLF.
Mean values ± SEM are used to represent data ($n = 8$). $P < 0.05$ was considered a significant difference, and different letters represent statistically significant differences among the groups.

increased at 14 d after hatching, along with the decrease of UA levels, which suggested protein metabolism was affected. Insulin-like growth factors and BMP are considered two of important bioactive factors for the assessment of early development in animals, and both play an essential role in bone and skeletal muscle development (Cussonneau et al., 2021; Ohba et al., 2012). Insulin-like growth factor 1 is the key growth factor of regulating protein anabolic and catabolic process in the skeletal muscle (Yoshida and Delafontaine, 2020). In agreement with the above results, the serum level of IGF-1 and the mRNA expression of *IGF-1R* at 1 d of age had an increasing trend after maternal MLF treatment. These findings pushed us to analyse the down-stream signalling factors by further detection.

It was reported that IGF-1 can increase skeletal muscle protein synthesis via activating PI3K/AKT/mTOR pathways (Yoshida and Delafontaine, 2020). Dietary MLF for breeder hens significantly increased the level of p-mTOR/mTOR in breast muscle of chicks at 1 d after hatching, indicating that the up-regulated expression of p-mTOR was an important effector molecule to promote the development of offspring. A number of studies have considered the relative breast muscle weight and relative tibial length during the incubation as important parameters for embryonic development (Akil and Zakaria, 2015; Öznurlu et al., 2021; Piestun et al., 2015). And BMP is involved in osteoblast differentiation and bone formation regulation (Gomez-Puerto et al., 2019). In the present study, maternal MLF supplementation could significantly increasing the relative tibia length of chicken offspring, and also specifically activated BMPR2 in the breast muscle. It suggested that maternal MLF appears to be a good promoter of offspring bone development. Similar to our results, a previous study showed that maternal supplementation with genistein increased the width of the proliferative zone of the tibial growth plate during the embryonic and growth phases of the offspring (Lv et al., 2018a). Quercetin, a main component of the MLF, has been reported that could activate the BMP2 pathway in mice to promote osteoblast proliferation and differentiation, with the activation of upstream estrogen receptors (ER) and downstream p-Samd1 thought to be involved in producing the overall physiological effects (Pang et al., 2018). Further study indicated that the BMP are involved in the process of muscle hypertrophy through the activation of Smad1/5 to the mTOR signalling pathway (Sartori and Sandri, 2015; Winbanks et al., 2013). Accordingly, the present study revealed that the protein expression of p-mTOR, Smad1, and p-Smad1/5/9 could be markedly up-regulated by maternal MLF supplementation, which can further elucidate the molecular mechanism in improving the protein synthesis of offspring skeletal muscle. Interestingly, this effect persists throughout the embryonic stage to chick stage and extends to 14 d after hatching.

The development of skeletal muscle is dependent upon a balance of protein synthesis and catabolism. Protein synthesis must be greater than protein degradation for the enlargement of muscle fibres to happen. Histological data suggested that maternal supplementation with MLF significantly increased the cross-sectional area of breast muscle fibres of offspring chicken at 14 d after hatching. The ubiquitin-proteasome pathway was reported to modulate protein degradation in animals (Bodine et al., 2001a). The *Atrogin-1* and *MURF1* are the two most important genes in the ubiquitin-proteasome pathway (Glass, 2005). In the present study, maternal MLF supplementation could up-regulate the mRNA expression of *Atrogin-1* at offspring 14 d of age. It may be related to positive feedback regulation of skeletal muscle protein metabolism, which was also consistent with the increased CREA in the serum. The enhanced skeletal muscle hypertrophy process in the offspring can reflect that maternal MLF feeding has a greater impact on protein anabolism than catabolism. Therefore, maternal MLF supplementation could promote skeletal muscle hypertrophy by enhancing the metabolism of muscle protein of chicks during the embryonic and post-hatch stage.

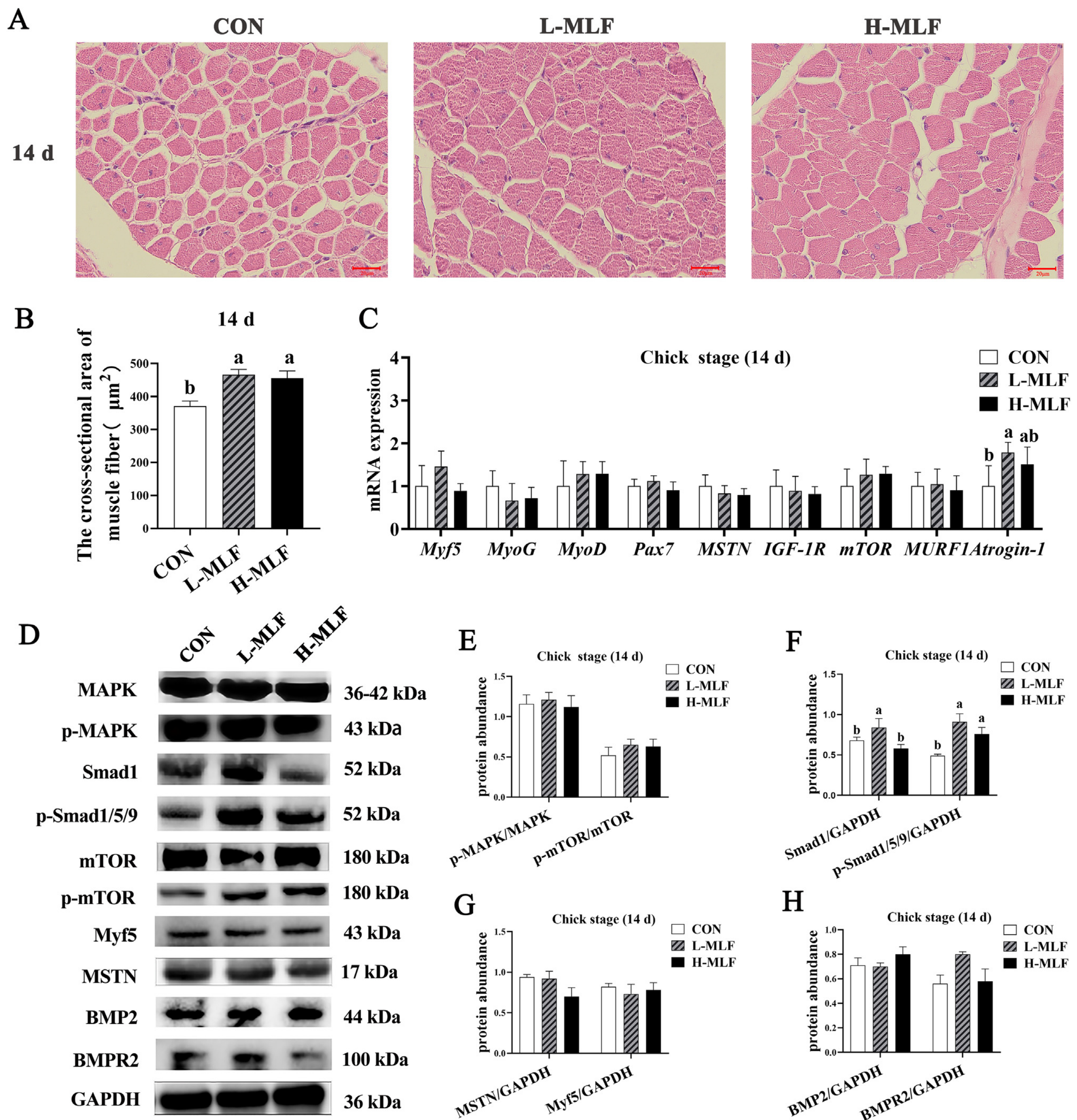


Fig. 4. Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on the skeletal muscle development of chicken offspring on 14 d post-hatch. (A) The representative images of hematoxylin and eosin (H&E) staining on the breast muscle. Scale bar = 20 μm. (B) The cross-sectional area in the chicken offspring at 14 d after hatching. (C) Relative mRNA expression of genes related with myogenic regulatory factors, protein synthesis and degradation at 14 d after hatching ($n = 8$). (D to H) Western blot analysis of p-MAPK, MAPK, p-mTOR, mTOR, Smad1, p-Smad1/5/9, BMP2, BMPR2, MSTN and Myf5 in the breast muscle ($n = 4$). *Myf5* = myogenic factor 5; *MyoG* = myogenin; *MyoD* = myogenic differentiation antigen; *Pax7* = paired box protein pax-7; *MSTN* = myostatin; *IGF-1R* = insulin-like growth factor 1 receptor; *mTOR* = mammalian target of rapamycin; *MURF1* = muscle RING finger 1; *Atrogin-1* = muscle atrophy f-box (MAFbx); MAPK = mitogen-activated protein kinase; p-MAPK = phospho-mitogen-activated protein kinase; p-mTOR = phosphorylated mammalian target of rapamycin; Smad1 = SMAD family member 1; p-Smad1/5/9 = phospho-SMAD family member 1/5/9; BMP2 = bone morphogenetic protein 2; BMPR2 = bone morphogenetic protein receptor 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. CON, the chicken offspring of breeder hens fed with a normal diet; L-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 60 mg/kg MLF. [a,b]Different letter represents statistically significant differences among the groups ($P < 0.05$).

Table 5
Effects of maternal mulberry-leaf flavonoids (MLF) supplementation on serum biochemical of chicken offspring.

Item	Treatment groups			*P*-value
	CON	L-MLF	H-MLF	
Day 1				
IGF-1, ng/mL	307.3 ± 5.4	314.7 ± 6.2	320.9 ± 3.1	0.092
T3, nmol/L	17.4 ± 0.2	16.9 ± 0.4	17.5 ± 0.4	0.433
UA, μmol/L	468.8 ± 74.7	493.1 ± 51.3	476.2 ± 56.4	0.759
CREA, μmol/L	47.7 ± 5.6	43.9 ± 6.9	32.8 ± 5.3	0.168
TP, g/L	20.6 ± 1.0	23.4 ± 0.9	23.2 ± 1.9	0.301
ALB, g/L	12.1 ± 0.6^{b}	14.6 ± 0.3^{a}	14.3 ± 1.0^{a}	0.021
Day 14				
IGF-1, ng/mL	310.9 ± 3.5	324.6 ± 17.0	344.8 ± 6.4	0.251
T3, nmol/L	20.4 ± 0.4	19.7 ± 0.6	18.9 ± 0.5	0.534
UA, μmol/L	471.0 ± 32.0^{a}	339.1 ± 37.2^{b}	436.9 ± 78.2^{ab}	0.049
CREA, μmol/L	6.9 ± 1.3^{b}	10.7 ± 1.2^{ab}	13.6 ± 3.6^{a}	0.026
TP, g/L	27.3 ± 2.2	28.6 ± 1.5	28.5 ± 1.1	0.974
ALB, g/L	14.8 ± 0.9	16.5 ± 1.1	15.3 ± 0.8	0.448

IGF-1 = insulin-like growth factor 1; T3 = triiodothyronine; UA = uric acid; CREA = creatinine; TP = total protein; ALB = albumin.
CON, the chicken offspring of breeder hens fed with a normal diet; L-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 30 mg/kg MLF; H-MLF, the chicken offspring of breeder hens fed a basal diet supplemented with 60 mg/kg MLF.
Mean values ± SEM are used to represent data ($n = 7$). $P < 0.05$ was considered a significant difference, and different letters represent statistically significant differences among the groups.

5. Conclusion

In conclusion, maternal MLF supplementation could improve the development of the skeletal muscle in slow-growth broilers, which is conducive to the offspring growth performance. This intergenerational effect is highly related to the activation of the BMP/p-Smad1/5/9 axis of chicks after hatching, which increases skeletal muscle protein metabolism. Our findings provide a reference for the application of dietary flavonoids from the perspective of maternal effect.

Author contributions

Zhenwu Huang: writing-original draft, conceptualization, methodology, investigation, visualization. **Hongjian Dai:** software, formal analysis, investigation. **Simeng Li:** investigation, writing - review & editing. **Zhe Wang:** methodology, formal analysis. **Quanwei Wei:** resources, visualization. **Zhonghua Ning:** writing - review & editing. **Yuming Guo:** funding acquisition. **Fangxiong Shi:** writing - review & editing, supervision, project administration. **Zengpeng Lv:** funding acquisition, resources, writing - review & editing, supervision. All authors contributed to the article and approved the submitted version.

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, and 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 study was supported by National Natural Science Foundation of China (No. 32202724), Beijing Natural Science Foundation (No. 6222036), and China Agriculture Research System program (CARS-40 and CARS-41-G11).

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.005.

References

Akil R, Zakaria AH. Egg laying characteristics, egg weight, embryo development, hatching weight and post-hatch growth in relation to oviposition time of broiler breeders. Anim Reprod Sci 2015;156:103–10.
Amevor FK, Cui Z, Du X, Ning Z, Deng X, Xu D, et al. Synergy between dietary quercetin and vitamin E supplementation in aged Hen's diet improves hatching traits, embryo quality, and antioxidant capacity of chicks hatched from eggs subjected to prolonged storage. Front Physiol 2022;13:873551.
AOAC. Official methods of analysis. 15th ed. Gaithersburg, MD: AOAC International; 1990.
AOAC. Official methods of analysis. 16th ed. Gaithersburg, MD: AOAC International; 1995.
Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 2001a;294:1704–8.
Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 2001b;3:1014–9.
Buckingham M, Relaix F. The role of Pax genes in the development of tissues and organs: pax3 and Pax7 regulate muscle progenitor cell functions. Annu Rev Cell Dev Biol 2007;23:645–73.
Chen G, Shui S, Chai M, Wang D, Su Y, Wu H, et al. Effects of paper mulberry (broussonetia papyrifera) leaf extract on growth performance and fecal microflora of weaned piglets. BioMed Res Int 2020;2020:6508494.
Chen J, Li X. Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice. Asia Pac J Clin Nutr 2007;16(Suppl 1):290–4.
China Feed Database. Tables of feed composition and nutritive values in China (in Chinese), https://www.chinafeeddata.org.cn/admin/Login/slcfb; 2020 [Accessed 10 June 2021].
Cussonneau L, Boyer C, Brun C, Deval C, Loizon E, Meugnier E, et al. Concurrent BMP signaling maintenance and TGF-β signaling inhibition is a hallmark of natural resistance to muscle atrophy in the hibernating bear. Cells 2021;10:1873.
Ding Y, Jiang X, Yao X, Zhang H, Song Z, He X, et al. Effects of feeding fermented mulberry leaf powder on growth performance, slaughter performance, and meat quality in chicken broilers. Animals 2021;11:3294.
Donsbough AL, Powell S, Waguespack A, Bidner TD, Southern LL. Uric acid, urea, and ammonia concentrations in serum and uric acid concentration in excreta as indicators of amino acid utilization in diets for broilers. Poultry Sci 2010;89:287–94.
Dou T, Li Z, Wang K, Liu L, Rong H, Xu Z, et al. Regulation of myostatin expression is associated with growth and muscle development in commercial broiler and DMC muscle. Mol Biol Rep 2018;45:511–22.
Egerman MA, Glass DJ. Signaling pathways controlling skeletal muscle mass. Crit Rev Biochem Mol Biol 2014;49:59–68.
Fan H, Lv Z, Gan L, Guo Y. Transcriptomics-related mechanisms of supplementing laying broiler breeder hens with dietary daidzein to improve the immune function and growth performance of offspring. J Agric Food Chem 2018;66:2049–60.
Gao J, Lv Z, Li C, Yue Y, Zhao X, Wang F, et al. Maternal zinc supplementation enhanced skeletal muscle development through increasing protein synthesis and inhibiting protein degradation of their offspring. Biol Trace Elem Res 2014;162:309–16.
Gao M, Wang J, Lv Z. Supplementing genistein for breeder hens alters the growth performance and intestinal health of offspring. Life 2023;13:1468.
Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 2005;37:1974–84.
Gomez-Puerto MC, Iyengar PV, García de Vinuesa A, Ten Dijke P, Sanchez-Duffhues G. Bone morphogenetic protein receptor signal transduction in human disease. J Pathol 2019;247:9–20.
Hu H, Bai X, Xu K, Zhang C, Chen L. Effect of phloretin on growth performance, serum biochemical parameters and antioxidant profile in heat-stressed broilers. Poultry Sci 2021;100:101217.
Huang Z, Dai H, Jiang J, Ye N, Zhu S, Wei Q, et al. Dietary mulberry-leaf flavonoids improve the eggshell quality of aged breeder hens. Theriogenology 2022a;179:177–86.
Huang Z, Lv Z, Dai H, Li S, Jiang J, Ye N, et al. Dietary mulberry-leaf flavonoids supplementation improves liver lipid metabolism and ovarian function of aged breeder hens. J Anim Physiol Anim Nutr 2022b;106:1321–32.
Jiang J, Qi L, Wei Q, Shi F. Maternal stevioside supplementation improves intestinal immune function of chicken offspring potentially via modulating gut microbiota and down-regulating the promoter methylation level of suppressor of cytokine signaling 1 (SOCS1). Anim Nutr 2022;10:329–46.
Lin WC, Lee MT, Chang SC, Chang YL, Shih CH, Yu B, et al. Effects of mulberry leaves on production performance and the potential modulation of antioxidative status in laying hens. Poultry Sci 2017;96:1191–203.
Lin Z, Gan T, Huang Y, Bao L, Liu S, Cui X, et al. Anti-inflammatory activity of mulberry leaf flavonoids in vitro and in vivo. Int J Mol Sci 2022;23:7694.
Liu Y, Li S, Huang Z, Dai H, Shi F, Lv Z. Dietary collagen peptide-chelated trace elements supplementation for breeder hens improves the intestinal health of chick offspring. J Sci Food Agric 2024;104:174–83.

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 Anim Nutr 2019;103:1934–45.
Lv Z, Fan H, Zhang B, Ning C, Xing K, Guo Y. Dietary genistein supplementation in laying broiler breeder hens alters the development and metabolism of offspring embryos as revealed by hepatic transcriptome analysis. Faseb J 2018a;32:4214–28.
Lv Z, Fan H, Zhang B, Xing K, Guo Y. Dietary genistein supplementation for breeders and their offspring improves the growth performance and immune function of broilers. Sci Rep 2018b;8:5161.
Messina G, Cossu G. The origin of embryonic and fetal myoblasts: a role of Pax3 and Pax7. Genes Dev 2009;23:902–5.
Miller JB, Stockdale FE. Developmental regulation of the multiple myogenic cell lineages of the avian embryo. J Cell Biol 1986;103:2197–208.
Ministry of Agriculture of the People's Republic of China. China national feeding standard of chicken (NY/T 33-2004). Beijing: China Agriculture Press; 2004.
Nangsuay A, Meijerhof R, van den Anker I, Heetkamp MJW, Morita VS, Kemp B, et al. Effects of breeder age, broiler strain, and eggshell temperature on development and physiological status of embryos and hatchlings. Poultry Sci 2016;95: 1666–79.
Ohba S, Hojo H, Chung UI. Bioactive factors for tissue regeneration: state of the art. Muscles Ligaments Tendons J 2012;2:193–203.
Ospina-Rojas IC, Murakami AE, Moreira I, Picoli KP, Rodrigueiro RJ, Furlan AC. Dietary glycine+serine responses of male broilers given low-protein diets with different concentrations of threonine. Br Poultry Sci 2013;54:486–93.
Öznurlu Y, Özaydın T, Sur E, Özparlak H. The effects of in ovo administered bisphenol A on tibial growth plate histology in chicken. Birth Defects Res 2021;113:1130–9.
Pallafacchina G, Calabria E, Serrano AL, Kalhovde JM, Schiaffino S. A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proc Natl Acad Sci USA 2002;99:9213–8.
Pang XG, Cong Y, Bao NR, Li YG, Zhao JN. Quercetin stimulates bone marrow mesenchymal stem cell differentiation through an estrogen receptor-mediated pathway. BioMed Res Int 2018;2018:4178021.
Picard B, Jurie C, Cassar-Malek I, Hocquette JF, Rescan PY. Myofibre typing and ontogenesis in farm animal species. Productions Animales -Paris- Institut National de la Recherche Agronomique 2003;16:117–23.
Piestun Y, Yahav S, Halevy O. Thermal manipulation during embryogenesis affects myoblast proliferation and skeletal muscle growth in meat-type chickens. Poultry Sci 2015;94:2528–36.
Ryu Y, Lee D, Jung SH, Lee KJ, Jin H, Kim SJ, et al. Sabinene prevents skeletal muscle atrophy by inhibiting the MAPK-MuRF-1 pathway in rats. Int J Mol Sci 2019;20: 4955.
Saitoh S, Sato T, Harada H, Matsuda T. Biotransformation of soy isoflavone-glycosides in laying hens: intestinal absorption and preferential accumulation into egg yolk of equol, a more estrogenic metabolite of daidzein. Biochim Biophys Acta 2004;1674:122–30.
Sartori R, Sandri M. Bone and morphogenetic protein signalling and muscle mass. Curr Opin Clin Nutr Metab Care 2015;18:215–20.
Sugiyama M, Katsube T, Koyama A, Itamura H. Varietal differences in the flavonol content of mulberry (Morus spp.) leaves and genetic analysis of quercetin 3-(6-malonylglucoside) for component breeding. J Agric Food Chem 2013;61:9140–7.
Velleman SG. Muscle development in the embryo and hatchling. Poultry Sci 2007;86:1050–4.
Winbanks CE, Chen JL, Qian H, Liu Y, Bernardo BC, Beyer C, et al. The bone morphogenetic protein axis is a positive regulator of skeletal muscle mass. J Cell Biol 2013;203:345–57.
Xing T, Zhao ZR, Zhao X, Xu XL, Zhang L, Gao F. Enhanced transforming growth factor-beta signaling and fibrosis in the pectoralis major muscle of broiler chickens affected by wooden breast myopathy. Poultry Sci 2021;100:100804.
Yablonka-Reuveni Z. Development and postnatal regulation of adult myoblasts. Microsc Res Tech 1995;30:366–80.
Yilmaz-Dikmen B, Sahan U. The relationship among age, yolk fatty acids content, and incubation results of broiler breeders. Poultry Sci 2009;88:185–90.
Yoshida T, Delafontaine P. Mechanisms of IGF-1-mediated regulation of skeletal muscle hypertrophy and atrophy. Cells 2020;9:1970.
Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. Semin Cell Dev Biol 2017;72:19–32.
Zhang L, Su S, Zhu Y, Guo J, Guo S, Qian D, et al. Mulberry leaf active components alleviate type 2 diabetes and its liver and kidney injury in db/db mice through insulin receptor and TGF-β/Smads signaling pathway. Biomed Pharmacother 2019;112:108675.