

Effects of high-dose folic acid on protein metabolism in breast muscle and performance of broilers

Saisai Liang,* Xinshuai Liu,* Jianfei Zhao,* Rui Liu,* Xinhua Huang,[†] Yanli Liu,* Xiaojun Yang,* and Xin Yang^{1,*}

*College of Animal Science and Technology, Northwest A&F University, Yangling, China; and[†]Nano Vitamin Engineering Research Center of Shaanxi Province, Xi'an, China

ABSTRACT Attaining the optimal feed conversion ratio is the unaltered goal for poultry breeding, meat yield is one of the vital reference indexes for that. Folic acid is involved in protein metabolism by acting as a transmitter of one carbon unit, and the detail mechanism for the high-dose folic acid on growth of broiler skeletal muscle is still unclarified. The present study was conducted to investigate the effect and regulatory mechanism of folic acid on deposition and metabolism of protein in broiler breast muscle. A total of 196 one-day-old AA broilers were randomly assigned to 2 treatment groups. The chicks were fed corn-soybean diet with folic acid levels of 1.3 mg/kg (**CON**) or 13 mg/kg (**FA**), respectively. The results showed that high dose of folic acid significantly increased the body weight gain, average daily gain, average daily feed intake, and feed conversion ratio of broilers during 1 to 42 d. Compared with control group, folic acid statistically augmented the breast muscle ratio of broilers at 42 d, abdominal fat percentage was also decreased in

FA group. Folic acid significantly increased the gene expression of folate receptor (**FR**) in duodenum and jejunum at 21 d, and its relative expression in jejunum of broilers at 42 d. Furthermore, relative expression of myogenin in broiler breast muscle was upregulated in folic acid group. Folic acid supplementation significantly enhanced the protein expression of phosphorylated serine/threonine kinase (**AKT**) and ribosomal protein S6 kinase 1 (**S6K1**) in the breast muscle of broilers at 21 d and 42 d. In conclusion, the results proved that high-dose folic acid activated the AKT/mammalian target of rapamycin (**mTOR**) pathway and increased the activity of phosphorylation of S6K1, thereby regulating the protein deposition in breast muscle. Meanwhile, the gene expression of the myogenic determinant factor was upregulated by folic acid and then promoted the growth of breast muscle. Consequently, the growth performance, meat production and feeding efficiency were improved of broilers by adding folic acid at 13 mg/kg.

Key words: breast muscle, broiler, folic acid, protein metabolism

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INTRODUCTION

Broiler breeding accounts for a large proportion of the meat supply in livestock production. The high-quality and high-efficiency supply of animal protein have especially become a concern of poultry farmers and consumers. Meanwhile, optimum performance is closely connected with meat production for broilers (Le Bihan-Duval et al., 1999). During the growth of broilers, nutritional manipulation is a direct and significant way to impact the protein deposition in breast and thigh (Mehri et al., 2016;

Attia et al., 2017). It has been documented that dietary level of amino acids and glucose closely influenced the tissue protein synthesis (Corzo et al., 2005; Abbasi et al., 2018; Attia et al., 2020). Li et al. (2011) found that leucine stimulated the mammalian target of rapamycin (**mTOR**) and influenced the protein synthesis in skeletal muscle. Many downstream signaling molecules of mTOR can simultaneously influence the protein metabolism (Pende et al., 2004; Ben-Sahra and Manning, 2017; Kim et al., 2022). Moreover, reports manifested that folic acid modulated the metabolism of amino acids and proteins (Brade et al., 1972; Yao et al., 2013), and activated the Akt pathway to stimulating the myogenesis in cells (Hwang et al., 2015). Mammalian target of rapamycin participates the regulation of Akt pathway (Villota-Narvaez et al., 2021). Most notably, skeletal muscle growth is also coordinated by myogenic regulatory factors (**MRFs**) (Fu et al., 2022). Apparently, growth and

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¹Corresponding author: yangx0629@163.com

protein deposition of muscle are influenced by multiple factors.

Folic acid belongs to an essential water-soluble B vitamin for organism metabolism, and can only be obtained from the diet and exists in various active forms after absorbed by the body and plays an important role in the biosynthesis of nucleic acids and proteins (Liu and Ward, 2010; Ratajczak et al., 2021). For poultry, increase the protein level in the diet is also accompanied with the increase of the folic acid requirement, as folic acid is a necessary substance for the synthesis of uric acid during the protein metabolism (Scott and Weir, 1981). Wang et al. (2019) found that maternal folic acid supplementation enabled to affect metabolism of skeletal muscle protein by regulating relative genes of muscle growth and development in offspring lambs. In addition, our team previous study elucidated that perfusion of folic acid significantly reduced the weight and percentage of broiler abdominal fat, but no adverse effect was observed on body weight (Liu et al., 2019). Hereby, we wondered if the high dose of folic acid enables to manipulate the protein deposition in broiler chest, and the potential regulatory mechanism also need to be further elucidated.

The musculature of broiler accounts for about 40% of total body weight, while breast muscle holds half percent of the muscle tissue of the broiler. Therefore, with chicken breast muscle as the target tissue, current study initially adopted with the intervention of high level of folic acid and then explored the mechanism of folic acid on protein deposition and muscle generation. Then, mTOR signaling pathway, which closely related to protein synthesis, was also detected for further revealing the potential regulation mechanism of folic acid on protein metabolism. The results will also provide a theoretical basis for the rational and effective application of folic acid in broiler diet and a novelty method for augmenting the chicken production.

MATERIALS AND METHODS

Ethics Statement

The protocol for the animal experimental procedures was approved by the Institutional Animal Care and Use Committee of Northwest A&F University (Permit Number: NWAFAFAC 1008).

Experimental Design, Dietary Treatments, and Animal Husbandry

One hundred ninety-six hatched 1-day-old healthy male Arbor Acres broilers with similar body weight were obtained from the Xianyang Dacheng Poultry Co., Ltd. (Xianyang, China). The chicks were randomly assigned to 2 treatment groups, each group had 7 replicates and each replicate contained 14 birds. All chicks were reared in 2 layers metal cage (70 cm-width × 95 cm-length × 40 cm-height), 7 chickens were assigned randomly to each cage and the brooding temperature was

maintained at 35°C for 1 wk and gradually decreased to 27°C in the third week. For the first week, rearing room keeps 23 h of light per day, after that the daily lighting time was 18 h and 6 h in darkness. Broilers were fed with corn-soybean meal basal pellet diets with folic acid level at 1.3 mg/kg (CON) and 13 mg/kg (FA) for 42 d, respectively. All birds had free access to feed and water and the ingredients and nutrient levels of the basic diet are shown in Table 1. Experimental diets in control group were formulated according to the nutritional requirements of NRC (1994) for broilers.

Performance and Samples Collection

During the experimental period, body weight of broilers was recorded for each replicate at 1, 21, and 42 d of age and feed consumption was also monitored by week. Then body weight gain (BWG) in each treatment group, average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were calculated. At 21 and 42 d, one bird from each replicate, with the nearly average body weight, was killed. The entire muscle from the breast and leg and abdominal fat were separated and weighted, and then a small piece of chest muscle sample was frozen in liquid nitrogen for further detection. The relative weight of breast, leg, and abdominal fat were also calculated later.

The duodenum and jejunum were separated and the intestinal contents were removed with precooling saline. Then the mucosa was scraped using a glass microscope slide and stored at -80°C for further mRNA analysis.

Table 1. Ingredient and nutrient composition of experimental diets.

Ingredient (%)	1–21 d	22–42 d
Corn	56.12	54.94
Soybean meal	27.64	22.69
Flour	6.00	8.00
Cotton meal	3.00	3.00
Corn protein flour	2.00	4.00
Soybean oil	0.84	3.51
Sodium chloride	0.30	0.30
Lysine	0.54	0.51
Threonine	0.10	0.06
DL-Methionine	0.18	0.11
Choline chloride	0.10	0.10
Stone powder	1.39	1.58
Dicalcium phosphate	1.56	0.96
Premix*	0.24	0.24
In total	100	100
Calculated nutrient levels		
Metabolizable energy (kcal/kg)	2900	3100
Crude protein	21.00	16.50
Total phosphorus	0.65	0.54
Nonphytate phosphorus	0.50	0.40
Calcium	0.92	0.85
Total lysine	1.28	1.15
Total threonine	0.88	0.79
Total methionine	0.51	0.44

*Provided per kg of diet: manganese 83.2 mg, copper 10.0 mg, zinc 93.6 mg, iron 122.4 mg, iodine 0.40 mg, cobalt 0.15 mg, selenium 0.39 mg, vitamin A 8400 IU, vitamin D₃ 3000 IU, vitamin E 54.9 mg, vitamin K₃ 2.7 mg, thiamine 1.93 mg, riboflavin 7.92 mg, pantothenic acid 15.73 mg, nicotinamide 50.31 mg, pyridoxine 4.7 mg, biotin 0.20 mg, cobalamin 0.04 mg, control group folic acid 1.3 mg, high-dose group folic acid 13.0 mg.

Table 2. Gene primer sequences for RT-qPCR.

Genes	Accession no.	Sequences (5'–3')	Product size (bp)
<i>FR</i>	XM_015280910	F: CATCCAGGATATGTGCTTGTATGA R: CAGCCCTTGTGCCAGTTCTC	180
<i>PCFT</i>	NM_001205066	F: GCTGGCCACCCAGTACCTAT R: GCCTCTAATGCCTTGC GGTA	156
<i>RFC</i>	NM_001006513	F: TTTCTGGTTCCCATCGCTATTTTC R: AGACCAGTGCCAGCAGTGAAAAAGT	205
<i>AKT</i>	XM015274151	F: GAAGTGCTGGAGGACAACGA R: CCTGGTTGTAGAAGGGCAGG	103
<i>S6K1</i>	NM_001030721.1	F: TGACCTTGGCATGGAACATT R: TACCTTTCATAGCCACCTT	130
<i>MYOD</i>	NM_204214.2	F: AGGAGGATGCATACTACCCAGT R: CCCATGCTTTGGGTCATTTGG	179
<i>MYOG</i>	NM_204184.1	F: AGCCTTCGAGGCTCTGAAAC R: GAGGTGATCTGCGGGGTTG	271
<i>β-actin</i>	XM_029084520.1	F: ATTGTCCACGCAAATGCTTC R: AAATAAAGCCATGCCAACTCGTC	173

Abbreviations: AKT, serine/threonine kinase; FC, folate receptor; MyoD, myocyte differentiation factor; MyoG, myogenin; PCFT, proton coupled folate transporter; RFC, reduced folate carrier; S6K1, ribosomal protein S6 kinase.

Quantitative RT-PCR Analysis of Gene Expression

The total RNA of breast muscle sample and intestinal mucosa samples were extracted using the Trizol Reagent kit (Invitrogen Co., Carlsbad, CA). The concentration and quality of RNA was detected using a spectrophotometer (NanoDrop-2000; Thermo Fisher Scientific, Waltham, MA) at 260 and 280 nm. Then the cDNA was synthesized using the Primer Script RT Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Primer sequences of β -actin, proton coupled folate transporter (*PCFT*), reduced folate carrier (*RFC*), folate receptor (*FR*), serine/threonine kinase (*AKT*), ribosomal protein S6 kinase (*S6K1*), myocyte differentiation factor (*MyoD*), and myogenin (*MyoG*) are listed in Table 2. Primers were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). A total volume of 20 mL of reaction system included 10 mL of SYBR Promerx Ex Taq, 0.4 mL upstream primers (10 mM/L), 0.4 mL downstream primers (10 mM/L), 2 mL cDNA, and 7.2 mL DEPC H₂O. The reactions of real-time PCR were carried out at 95°C for 15 s, followed by 40 cycles at 95°C for 15 s, and at 60°C for 60 s. Gene expression was finally normalized to β -actin and the relative expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method.

Western Blot Analysis

The muscle samples were lysed in a RIPA lysis buffer kit with a protease inhibitor and phosphatase inhibitor (Heart Biological Technology, Hong Kong, China). The detailed procedures of Western blot analysis refer to the previous method (Zhang et al., 2021). Proteins were detected by the following primary antibodies: GAPDH (Proteintech, Wuhan, China, 60004-1-Ig), total mTOR (Cell Signaling Technology, Shanghai, China, 2972), S6K1 (Cell Signaling Technology, Shanghai, China, 9202S), phosphorylated (p-)S6K1 (Cell Signaling

Technology, Shanghai, China, 9234S/L), AKT (Cell Signaling Technology, Shanghai, China, 9272), and p-AKT (Cell Signaling Technology, Shanghai, China, 9271). Antibodies were incubated overnight at 4°C, membranes were washed 3 times with Tris buffered saline Tween (**TBST**) buffer, and protein bands were visualized after binding of a secondary antibody conjugated with HRP (Bioss, Beijing, China) by ECL reagents (Affinity Biosciences, Beijing, China). The glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was used as an internal control. Western-blot were quantified by Image-J software analysis of 3 blots.

Statistical Analysis

Significant differences between control group and folic acid group were tested using *t-test* and all data were analysis by SPSS 20.0 and shown as mean \pm SEM. *P* < 0.05 was indicated by * and considered statistically significant. The experimental unit was the replicate.

Table 3. Effects of high-dose folic acid on growth performance of broilers.

Period	Items	CON ¹	FA	<i>P</i> -value
1–21 d	BWG (g)	542.11 \pm 6.68	607.51 \pm 8.31*	<0.001
	ADFI (g)	44.84 \pm 0.58	47.79 \pm 0.44*	0.002
	ADG (g)	27.11 \pm 0.33	30.38 \pm 0.42*	<0.001
	FCR	1.66 \pm 0.03*	1.57 \pm 0.02	0.019
22–42 d	BWG (g)	1,749.23 \pm 26.30	1,959.77 \pm 30.81*	<0.001
	ADFI (g)	155.55 \pm 3.33	169.01 \pm 2.53*	0.007
	ADG (g)	83.30 \pm 1.25	93.32 \pm 1.47*	<0.001
	FCR	1.87 \pm 0.23	1.82 \pm 0.33	0.202
1–42 d	BWG (g)	2,318.45 \pm 27.89	2,597.65 \pm 35.01*	<0.001
	ADFI (g)	100.19 \pm 1.77	108.40 \pm 1.30*	0.003
	ADG (g)	55.20 \pm 0.66	61.85 \pm 0.83*	<0.001
	FCR	1.76 \pm 0.02*	1.69 \pm 0.02	0.019

¹Abbreviations: BWG, body weight gain; CON, basal diet; FA, basal diet with high level of folic acid; FCR, feed conversion ratio.

*Means within a row without common superscript differ significantly (*P* < 0.05) between groups.

Table 4. Effects of high-dose folic acid on chest muscle, leg muscle, and abdominal fat ratio.

Period	Items	CON ¹	FA	<i>P</i> -value
21 d	Breast muscle percentage (%)	17.10 ± 0.26	17.66 ± 0.25	0.075
	Thigh muscle percentage (%)	12.10 ± 0.17	12.32 ± 0.12	0.250
	Abdominal fat percentage (%)	0.78 ± 0.04*	0.68 ± 0.03	0.039
42 d	Breast muscle percentage (%)	23.18 ± 0.66	25.34 ± 0.57*	0.028
	Thigh muscle percentage (%)	14.03 ± 0.34	14.55 ± 0.44	0.379
	Abdominal fat percentage (%)	1.08 ± 0.05*	0.82 ± 0.06	0.004

¹Abbreviations: CON, basal diet; FA, basal diet with high level of folic acid.

*Means within a row without common superscript differ significantly ($P < 0.05$) between groups.

RESULTS

Effects of Folic Acid on the Growth Performance of Broiler Chickens

The results of adding a high dose of folic acid on performance of broiler is presented in Table 3. Compared with control group, folic acid significantly increased the BWG, ADFI, and ADG during the experimental period. Broilers in high-dose folic acid group had higher FCR

during 1 to 21 d and 1 to 42 d, and no significant change was observed between groups during 21 to 42 d.

As shown in Table 4, for 21-day-old broilers, no significant difference was found about these parameters except for the abdominal fat percentage. Whereas, breast muscle percentage of 42-day-old broilers was significantly increased in high-dose folic acid group. When compared with control group, folic acid also induced the reduction of abdominal fat percentage of broilers at 42 d. Meanwhile, folic acid had no significant effect on thigh muscle percentage. On the other hand, as shown in Supplemental Table S1, high level of folic acid increased the breast and thigh weight at 21 and 42 d, abdominal fat weight was decreased at 42 d.

Regulation of Folic Acid on Expression of Folic Acid Transporters in Intestine

Folic acid is mainly absorbed at the foregut, hence, we tested the mRNA expression of folate transporters in duodenum and jejunum (Figure 1). It was shown that the relative expression of *FR* in duodenum and jejunum were upregulated for 21-day-old broilers in folic acid group. There were no differences between the control and folic acid group about the expression *PCFT* and *RFC* in foregut at 21 d. At 42 d, in the duodenum, changes in the 3 transporters were not observed between

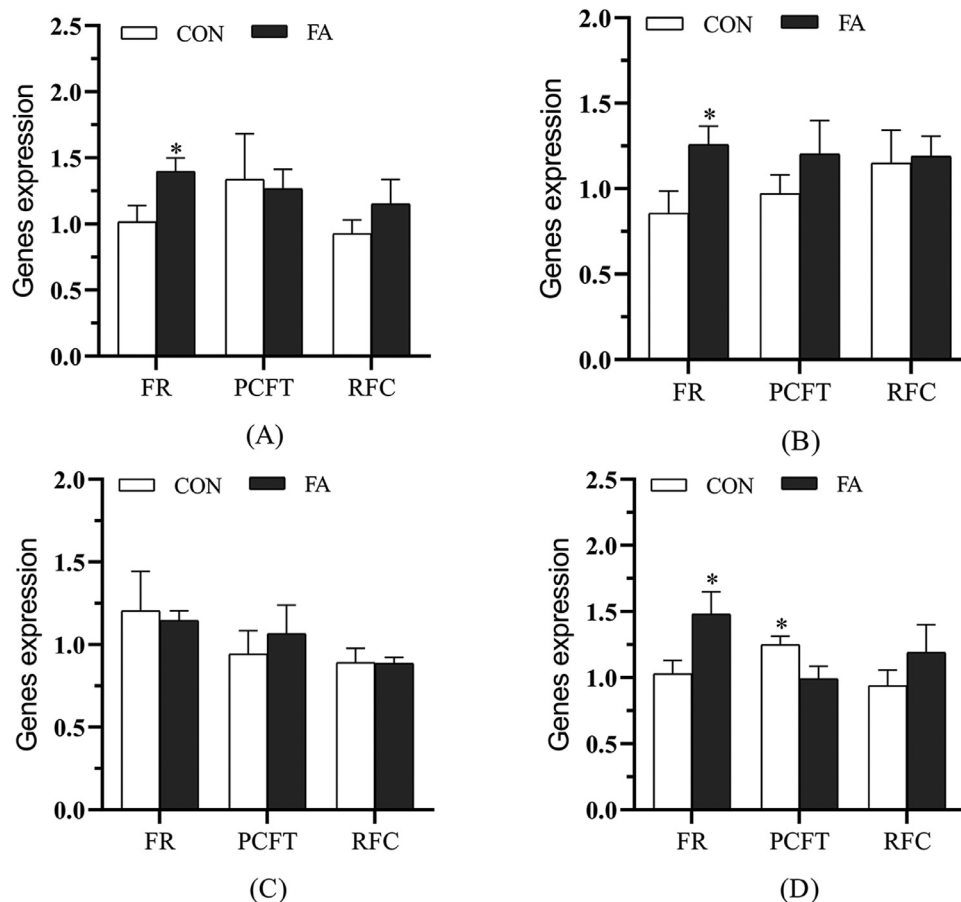


Figure 1. Effects of high-dose folic acid on expression of folic acid transporters in broiler duodenum and jejunum at 21 d (A, B) and 42 d (C, D). Data are shown as mean ± SEM. * Means statistically significant differences between groups ($P < 0.05$). Abbreviations: CON, control group; FA, high-dose folic acid group; FC, folate receptor; PCFT, proton coupled folate transporter; RFC, reduced folate carrier.

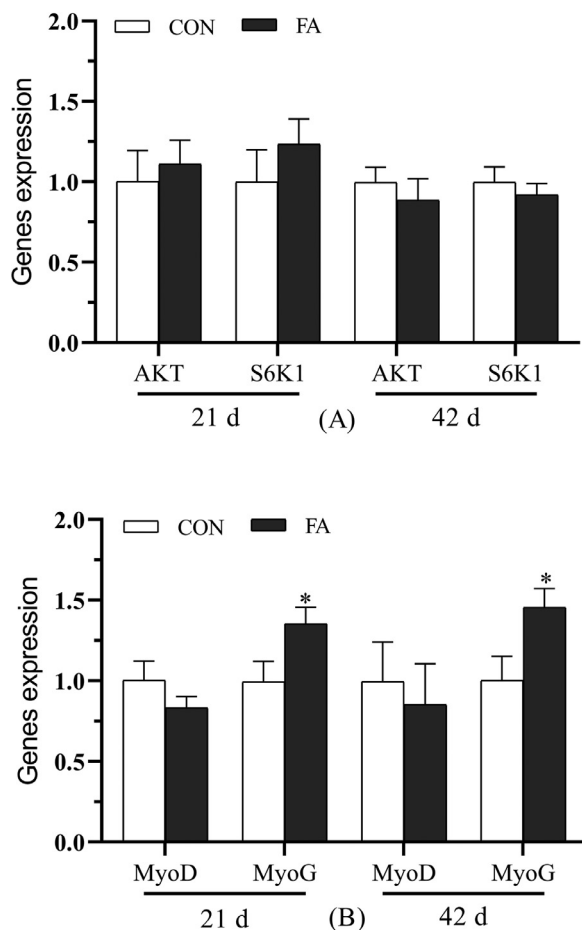


Figure 2. Effects of high-dose folic acid on gene expression of protein synthesis (A) and myogenic regulatory factors (B) in breast muscle. Data are shown as mean \pm SEM. *Means statistically significant differences between groups ($P < 0.05$). Abbreviations: AKT, serine/threonine kinase; CON, control group; FA, high-dose folic acid group; MyoD, myocyte differentiation factor; MyoG, myogenin; S6K1, ribosomal protein S6 kinase.

groups. The mRNA expression of *FR* in jejunum was increased, but *PCFT* expression was down-regulated in the high-dose folic acid group. There was no difference on the *RFC* expression between groups.

Influence of Folic Acid on Gene Expression of Protein Synthesis and Myogenic Regulatory Factors

The protein synthesis gene and myogenic regulatory factors are vital regulatory factors in the process of protein deposition in the muscle and the detection results are presented in Figure 2. At 21 and 42 d, high dose of folic acid had no influence on the relative expression of *AKT*, *S6K1* and *MyoD* in broiler breast muscle, while the expression of *MyoG* was remarkably increased.

Effects of Folic Acid on the Protein Synthesis by Regulating the mTOR Pathway

In order to clarify the possible regulatory mechanism of folic acid on protein synthesis on breast muscle, we

measured the protein level of involved key factors in mTOR pathway. Results are exhibited in Figure 3. Folic acid increased the protein abundance of p-AKT and p-S6K1 at 21 d and 42 d. No differences were observed on protein abundance of AKT, S6K1, and mTOR between groups.

DISCUSSION

Growth performance is the invariable concern and a critical index for evaluating the economic value of livestock and poultry. Deposition of protein in muscle is a decisive process of gaining weight for animals (Geiger et al., 2018). A previous study found that perfusion administration of 10 times the dose of folic acid to broiler chickens effectively reduced abdominal fat, but no change of body weight and feed conversion ratio were observed (Liu et al., 2019). This might be due to the fact that perfusion was only administered for 11 d. Wang et al. (2011) reported that appropriate amount of folic acid had potential to promote the protein synthesis in lactating sows. For ruminants, rumen-protected folic acid increased the activity of digestive enzymes in the gut, and hence growth performance (Li et al., 2020). Folic acid is involved in several metabolic processes. In the present study, ADG and FCR were improved during 1 to 42 d and breast muscle rate was also increased at 42 d. Consistent with previous studies, abdominal fat was decreased in the present study (Liu et al., 2019). The increase of muscle weight may indicate that folic acid has potential to regulate the process of protein deposition in breast muscle. On the other hand, changes in body weight were likely due to the increase of muscle mass.

To further explore the pathway of folic acid on protein deposition, we firstly confirmed the absorption of folate in intestine. Although *RFC* can be express in the intestinal, the low pH of the intestinal tract is a major impediment for its function (Sirotnak and Tolner, 1999; Wang et al., 2005). In contrast to the low affinity of *RFC* for folic acid, *PCFT* shows higher affinity for reduced folate and folic acid, under acidic ambient of the small intestine (Qiu et al., 2006; Zhao and Goldman, 2007). The mRNA expression of *RFC* and *PCFT* was not affected by folic acid supplementation, especially in duodenum. Whereas, relative expression of *FR* was most affected by folic acid treatment and highly expressed in the upper small intestine. Folate receptors deliver folic acid into cells via endocytosis (Zhao and Goldman, 2007). This effectively ensured that more folic acid is metabolized and utilized by broiler.

The process of skeletal muscle myogenesis is closely regulated by myogenic regulatory factors (MRFs), which means that muscle protein synthesis is inseparable from that (Kevin et al., 2012; Zammit, 2017; Hwang et al., 2019). *MyoD* is a myogenic regulatory factor that can activate the myogenic differentiation program along with the downstream myogenin (*MyoG*) (Zammit, 2017; Bhattamisra et al., 2021). Previous

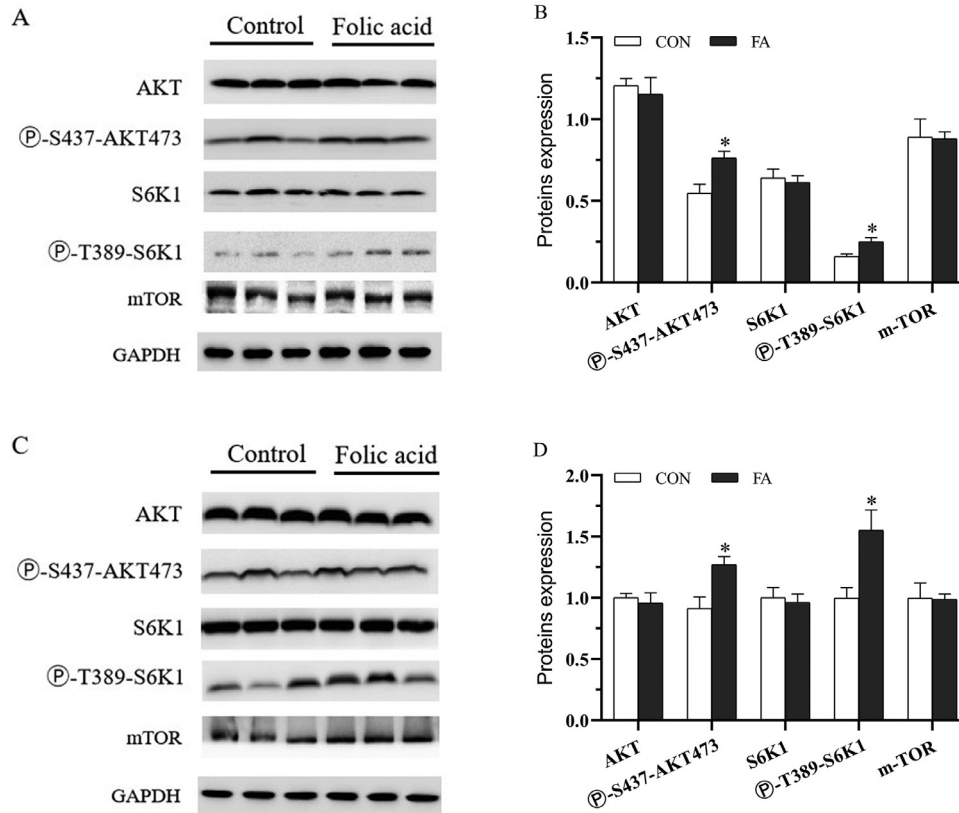


Figure 3. Folic acid regulates the protein synthesis in breast muscle via AKT/ mTOR pathway at 21 d (A, B) and 42 d (C, D). Data are shown as mean \pm SEM. * Means statistically significant differences between groups ($P < 0.05$). Abbreviations: AKT, serine-threonine kinase; CON, control group; FA, high-dose folic acid group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; S6K1, ribosomal protein S6 kinase 1.

studies indicated that folic acid increased the mRNA expression of MRFs and induced the myogenic differentiation in C2C12 cell (Hwang et al., 2018). Hasty et al. (1993) also reported that MyoG protein played a central regulatory role in myocyte differentiation. Our results showed that folic acid significantly upregulate the expression of *MyoG* in breast muscle, which further illustrated that folic acid enabled to enhance the protein deposition in broiler chest muscle.

Protein metabolism is regulated by various nutrients and signal pathways. Mammalian target of rapamycin can be affected by nutrients (amino acid) and hormones (insulin), mTOR also involves in the protein synthesis (Wang and Proud et al., 2006; Ilha et al., 2018). It has been reported that amino acids can bind with mTOR, and activate the mTOR pathway, finally affecting the protein synthesis efficiency of piglets (Wang et al., 2017). Furthermore, numerous studies declared the crucial effect of AKT on myogenic differentiation (Jiang et al., 1999; Sumitani et al., 2002; Briata et al., 2012), and stimulation of AKT/mTOR pathway can facilitate the protein synthesis in skeletal muscle (Cai et al., 2016; Sassoli et al., 2018). S6K1 and eIF4E-binding protein (4EBP1) are the downstream factors of AKT/mTOR pathway, and its activity can be improved by folic acid in a dose-dependent manner (Pende et al., 2004; Hwang et al., 2015). In mammalian cell models, S6K1 is a predominantly regulator of cell and body size (Ruvinsky and Meyuh, 2006; Bonucci et al., 2020) and

phosphorylates ribosomal protein S6 kinase accelerates the protein synthesis by enhancing the translation initiation (Wilson et al., 2009; Fidalgo et al., 2022). In the current study, although no differences were found on mRNA expression and protein abundance of AKT and S6K1 between groups, higher protein expression of p-AKT and p-S6K1 was observed in the folic acid supplementation group. These results demonstrated that folic acid upregulated the protein synthesis in broiler breast muscle by stimulating the AKT/mTOR pathway, and then the chest weight percentage was increased.

CONCLUSIONS

In conclusion, this study showed that adding folic acid at 13 mg/kg improved the broiler performance and promoted the muscle protein deposition, which partly due to the positive effect of folic acid on the expression of myogenic determinants family. On the other hand, folic acid activated the AKT/mTOR pathway to increase the activity of its downstream signal molecules and finally affected the protein metabolism process. In addition, it would be also important to investigate extra potential mechanism of folic acid metabolites in protein deposition, especially under the excess folic acid intake for meat-yield domestic animals. Basing on our experiment, the use of other optimal dosages that less than 10 times folic acid is also important to study.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101935.

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