

# Quercetin improves immune function in Arbor Acre broilers through activation of NF- $\kappa$ B signaling pathway

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**ABSTRACT** Quercetin, the main component of flavonoids, has a wide range of biological actions. Quercetin can be made into a variety of additives for practice, because of the stable chemical structure and water-soluble derivatives. This study was intended to explore the effects of quercetin on immune function and its regulatory mechanism in Arbor Acre broiler to provide a practical basis for improving poultry immune function and figure out the optimum supplementation as functional feed additives. A total of 240 one-day-old healthy Arbor Acre broilers, similar in body weight, were randomly allotted to 4 treatments with 6 replicates, 10 broilers in each replicate and fed with diets containing quercetin at 0, 0.02, 0.04, and 0.06% for 6 wk. Blood and immune organs (spleen, thymus, and bursa) were collected from chickens at the end of the experiment. Growth performance, immune organs indexes, contents of serum immune molecules, splenic T lymphocyte proliferative responses, and expression of immune related genes were evaluated. The results showed that dietary quercetin had no significant

effect ( $P > 0.05$ ) on growth performance of broilers. Compared with control, 0.06% quercetin supplementation in diet significantly increased spleen index and thymus index ( $P < 0.05$ ). It also increased the secretion of immune molecules including immunoglobulin A (IgA), interleukin-4 (IL-4) ( $P < 0.001$ ), immunoglobulin M (IgM) ( $P = 0.007$ ), complement component 4 (C4) ( $P = 0.001$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) ( $P < 0.05$ ). On the other hand, 0.02% quercetin supplementation significantly increased complement component 3 (C3) ( $P < 0.05$ ). Additionally, both 0.04 and 0.06% quercetin supplementation significantly increased expression of TNF- $\alpha$ , TNF receptor associated factor-2 (TRAF-2), TNF receptor superfamily member 1B (TNFRSF1B), nuclear factor kappa-B p65 subunit (NF- $\kappa$ Bp65), and interferon- $\gamma$  (IFN- $\gamma$ ) mRNA ( $P < 0.05$ ), and expression of NF- $\kappa$ B inhibitor- $\alpha$  (I $\kappa$ B- $\alpha$ ) mRNA were significantly decreased ( $P < 0.05$ ). Thus, quercetin improved immune function via NF- $\kappa$ B signaling pathway triggered by TNF- $\alpha$ .

**Key words:** quercetin, broiler, immune organs, immune molecules, gene expression

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## INTRODUCTION

Poor disease resistance in commercial chicken production is the major problem which directly affects the economic benefits (Subah and Praveen, 2016; Sadiq and Mohammed, 2017; Zahoor et al., 2018; Broom and Kogut, 2019). However, the presence of various viruses, bacteria, parasites, microbial toxins, and nutrient deficiencies may lead to the emergence of immunosuppressive diseases. Immunosuppressive diseases may damage the immune organs and affect the activity of immune cells by interfering with the antigen present, inhibiting or blocking the formation of immune antibody

(immunoglobulin), thus causing a reduction in the normal immune response (Sharma et al., 2000; Hoerr, 2010; Wang et al., 2014). Clinically common immunosuppressive diseases in chicken include infectious bursa disease, Marek's disease, and chicken infectious anemia (Umar et al., 2017). In these diseases, both humoral and cellular immune responses are threatened.

Quercetin is one of the most prominent dietary flavonoid which is ubiquitously present in foods including vegetables, fruit, tea, and wine as well as in countless food supplements, and it is claimed to exert beneficial health effects (Boots et al., 2008; Alfredo et al., 2019). Studies showed that quercetin has stable chemical structure and its derivative is water soluble. It can be absorbed in vivo and used for a variety of additives in production practice (Materska, 2008; Mishurov et al., 2016). The literatures showed that quercetin has many pharmacological activities, including antioxidation, anti-inflammation, antibacterial, scavenging free radicals and improving immune functions. Moreover,

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quercetin is nontoxic, noncarcinogenic, and harmless to the body (Bravo and Anaconda, 2001; Comalada et al., 2005; Davis et al., 2008, 2009; Anand et al., 2016).

Previous studies have shown that dietary supplementation of quercetin improved the laying performance by modulating intestinal environment and liver superoxide dismutase content in laying hens (Liu et al., 2014). Particularly, animal model and human in vitro studies have shown that flavonoids alleviate inflammation by modulating the production of pro-inflammatory and anti-inflammatory molecules in cells of innate and adaptive immune system (e.g., macrophages and T cells) in response to stimuli that upregulate inflammatory processes (Kumazawa et al., 2006; Gonzalez-Gallego et al., 2010). The process of immunomodulation involves nuclear factor-kappa B (NF- $\kappa$ B) signaling pathway, which is activated by TNF- $\alpha$  (Kumazawa et al., 2006). The NF- $\kappa$ B family of eukaryotic transcription factors plays an important role in regulation of immune response, inflammation, and oncogenesis. Activated NF- $\kappa$ B is translocated to the nucleus, where it modulates the expression of immuno-related genes (Chen et al., 2001; Badr et al., 2009). However, the immune mechanism of dietary quercetin in broilers has not been fully elucidated yet. Therefore, we examined the effects of quercetin on growth performance, immune organs indexes, contents of serum immune molecules, and genes expression in immune organs to elucidate the immunomodulatory properties of dietary quercetin and mechanism of action in broilers.

## MATERIALS AND METHODS

### Experimental Design, Diets, and Management

Housing, management, and care of the birds conformed to the guidelines of Animal Care and Use Committee of Northeast Agricultural University and Agricultural Animal in Agricultural Research and Teaching of Heilongjiang Province (HEI Animal Management Certificate No. 11928).

A total of 240 Arbor Acre (AA) broilers (1-day-old, healthy and similar in body weight) from a commercial company (Yinong Poultry Limited Company, Harbin, China) were purchased and randomly divided into 4 groups with 6 replicates per group and 10 broilers per replicate. The broilers were housed in wire cages (4-stacked cages, width: 52.6 cm, depth: 42.3 cm, height: 38.1 cm). A 16L: 8D lighting was provided during the 6-wk experimental period, and temperature was maintained at 32 to 34°C in the starting 3 D and decreased by 2 to 3°C per week to a final temperature of 24°C. Humidity of the experimental room varied from 60 to 65%. Experimental diets and water were available ad libitum. Chickens were inspected daily for any health-related problems.

**Table 1.** Composition and nutrient level of basal diet (as-fed basis).

Items	Content (1 to 3 wk)	Content (4 to 6 wk)
Ingredients (%)		
Corn	57.50	62.30
Soybean meal	34.50	30.00
Vegetable oil	3.00	3.00
Fish meal	1.00	1.00
Methionine	0.20	0.20
Dicalcium phosphate	1.62	1.70
Limestone	1.55	1.17
Sodium chloride	0.30	0.30
Multivitamin premix <sup>1</sup>	0.03	0.03
Mineral premix <sup>1</sup>	0.20	0.20
Choline	0.10	0.10
Total	100.00	100.00
Nutrient levels <sup>2</sup>		
Metabolizable energy (ME), MJ/kg	12.33	12.50
CP (%)	21.75	19.72
Total lysine (%)	1.18	1.04
Methionine (%)	0.91	0.86
Ca (%)	1.07	0.96
Total P (%)	0.70	0.68
Available P (%)	0.46	0.45

<sup>1</sup>Provided per kilogram of diet: vitamin A, 15,000 IU; vitamin D<sub>3</sub>, 3,200 IU; vitamin E, 10 IU; vitamin K, 0.5 mg; vitamin B<sub>1</sub>, 1.8 mg; vitamin B<sub>2</sub>, 3.6 mg; vitamin B<sub>6</sub>, 3.5 mg; vitamin B<sub>12</sub>, 0.01 mg; biotin, 0.15 mg; folic acid, 0.55 mg; niacin, 30 mg; pantothenic acid, 10 mg; Cu (CuSO<sub>4</sub> · 5H<sub>2</sub>O), 8 mg; I (KI), 0.35 mg; Fe (FeSO<sub>4</sub> · 7H<sub>2</sub>O), 80 mg; Mn (MnSO<sub>4</sub> · H<sub>2</sub>O), 60 mg; Se (NaSeO<sub>3</sub>), 0.15 mg; Zn (ZnO), 40 mg.

<sup>2</sup>Based on composition of ingredients provided by NRC (1994).

Corn soybean meal-based diets were formulated to meet or exceed NRC (1994) nutrient recommendations. The composition of the basal diet is shown in Table 1, with 4 levels of quercetin in feed: 0, 0.02, 0.04, and 0.06%. Quercetin dihydrate powder with 97% purity was purchased from Sigma-Aldrich Company (St. Louis, MO) and mixed with basal diet and was offered in mash form (5 mm) after grinding.

### Growth Performance

At 42 D of age, broilers were weighed after feed deprivation for 12 h and growth performance in terms of final body weight (BW), total feed intake (FI), and feed to weight gain ratio (F:G) per replicate (cage) were recorded weekly, and performance was calculated during 6-wk experimental period.

### Collection of Samples

At 42 D of age, after feed deprivation for 12 h, 2 broilers per replicate similar in body weight were randomly selected, weighed, and killed by rapid decapitation (n = 12 per group). Blood samples from jugular vein (10 mL) were obtained and kept in ice. Immune organs namely spleen, thymus, and bursa of fabricius were dissected, washed, rinsed in saline water for splenic T lymphocyte proliferative responses, and stored in sterile tubes at -80°C for RNA extraction.

**Table 2.** Sequences of primers for quantitative real-time PCR.

Target gene <sup>1</sup>	Primer sequences (5'→3')	Gene Bank No.	Product size (bp)
$\beta$ -Actin	F: CACCACAGCCGAGAGAGAAAT R: TGACCATCAGGGAGTTCATAGC	L08165	135
TNF- $\alpha$	F: GAGCGTTGACTTGGCTGTC R: AAGCAACAACCAGCTATGCAC	NM_204267	129
TRAF-2	F: CCAGAACACGAAAGCAAGTG R: ATGAGGGAATGGCAGGAAG	XM_015279623.1	112
TNFRSF1B	F: TGCCTACTCACAGCCAACTG R: AGATGCTGCTCCTCCTGTTC	NM_204439.3	126
NF- $\kappa$ Bp65	F: GTGTGAAGAAACGGGAAGTGG R: GGCACGGTTGTCATAGATGG	NM_205129	125
I $\kappa$ B- $\alpha$	F: GGCAGATGTGAACAAGGTGA R: TATCTGCAGGTCAGCTGTGG	NM_001001472.2	118
IFN- $\gamma$	F: GCTCCCGATGAACGACTTGA R: TGTAAGATGCTGAAGAGTTCATTCCG	NM_205149	150

<sup>1</sup>TNF- $\alpha$  = tumor necrosis factor alpha; TRAF-2 = TNF receptor associated factor-2; TNFRSF1B = TNF receptor superfamily member 1B; NF- $\kappa$ Bp65 = nuclear factor kappa-B p65 subunit; I $\kappa$ B- $\alpha$  = NF- $\kappa$ B inhibitor-alpha; IFN- $\gamma$  = interferon- $\gamma$ .

### Immune Organs Indexes

Immune organs including spleen, thymus, and bursa of fabricius obtained in sterile atmosphere were weighed to calculate the immune organ index using the formula: Immune organ index = (immune organ weight in g/body weight in g)  $\times$  100.

### Blood Immune Parameters

Serum was obtained after centrifuging the blood at 4°C, 3,000  $\times$  g for 15 min and stored at -20°C until analysis of hormones. Contents of IgA, IgM, C3, C4, IL-4, and TNF- $\alpha$  were determined using ELISA assay kit following the instructions provided by the manufacturer's (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jinagsu, P. R. China).

### Cell Immune Parameter

The sterile spleen was placed in a culture dish with PBS for 5 min. Then, spleen was gently torn up in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 medium. Splenocyte deposit was obtained by percolating with a 200-mesh sieve and centrifugation at 1,200  $\times$  g for 6 min. Afterwards, Tris-NH<sub>4</sub>Cl was added 3 times into splenocyte deposit and vortex oscillation, incubated at 37°C for 5 min, and centrifuged at 1,200  $\times$  g for 10 min, and the supernatant was removed. Splenocyte suspension was acquired by resuspending with 2 mL of RPMI1640 medium, and the concentration was maintained at 5  $\times$  10<sup>6</sup>/mL.

Splenocyte suspension (100  $\mu$ L) was added in 96-well cell culture plates, which was divided into 2 groups with 3 replicates (samples were run in triplicates). One group contains 100  $\mu$ L of RPMI1640 medium, and another includes 100  $\mu$ L of ConA (10  $\mu$ g/mL). 96-well cell culture plates were cultured in CO<sub>2</sub> incubators (37°C, 5% CO<sub>2</sub>, saturation humidity) for 24 h. Then, 10  $\mu$ L of methyl thiazolyl tetrazolium (MTT) (5 mg/mL) was added to each well before 4 h of termination of the culture. Cell

culture plates were centrifuged, and supernatant was removed. Then, 150  $\mu$ L of dimethylsulphoxide (DMSO) was injected in cell culture plates in a sterile atmosphere at room temperature for 20 min using Tecan Sunrise (Infinite 200 PRO, Austria).

### Expression of Immuno-Related Genes in Spleen

Gene expressions (TNF- $\alpha$ , TRAF-2, TNFRSF1B, NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , and IFN- $\gamma$ ) were measured using real-time quantitative RT-PCR (7500 Real-Time PCR System, Singapore). The primer sequences for broilers (Table 2) were designed to span an intron to avoid genomic DNA contamination using Primer 5.0. Total RNA was isolated from spleen using Trizol (Invitrogen, San Diego, CA); the quantity and quality of the isolated RNA were determined as described by Wang et al. (2010). Total cDNA was synthesized following the manufacturer's instructions. Briefly, total 20  $\mu$ L of reaction mixture containing 5  $\mu$ L of total RNA, 4  $\mu$ L of 5  $\times$  Prime Script Buffer, 1  $\mu$ L of Prime Script RT Enzyme MixI, 1  $\mu$ L of Oligo dT Prime (50  $\mu$ mol/L), 1  $\mu$ L of Random 6 mers (10  $\mu$ Mmol/L), and 8  $\mu$ L of RNase Free dH<sub>2</sub>O (TaKaRa Biotechnology, Co., Ltd. Dalian, P. R. China) was incubated in the following conditions: reverse transcription at 37°C for 15 min, inactivation of reverse transcriptase at 85°C for 5 min, until temperature was decreased to 4°C. Successful cDNA synthesis was confirmed by amplifying the  $\beta$ -actin amplicon using PCR. cDNA was amplified using a 20  $\mu$ L PCR reaction system containing 2  $\mu$ L of cDNA, 10  $\mu$ L of 2  $\times$  SYBR Green PCR Mix, 0.4  $\mu$ L of 50  $\times$  ROX Reference DyeII (TaKaRa Biotechnology, Co., Ltd. Dalian, P. R. China), 0.8  $\mu$ L of PCR Forward Primer, 0.8  $\mu$ L of PCR Reverse Primer (Sangon Biological Engineering Technology & Service Co., Ltd. Shanghai, P. R. China), and 6  $\mu$ L of ddH<sub>2</sub>O. The PCR conditions were as follows: initial denaturation at 95°C for 30 s, followed by PCR reaction at 40 cycles of 95°C for 5 s and 60°C for 34 s,

**Table 3.** Effect of quercetin on growth performance in Arbor Acre broilers.<sup>1</sup>

Items <sup>2</sup>	Quercetin				SEM	P-value
	0	0.02%	0.04%	0.06%		
BW (kg/bird)	3.62	3.25	3.66	3.57	0.092	0.163
FI (kg/bird)	7.99	6.32	7.31	7.61	0.333	0.385
F:G (kg:kg)	2.19	1.95	1.99	2.13	0.071	0.220

<sup>1</sup>Data are means of 6 replicates per treatment.

<sup>2</sup>BW = body weight; FI = feed intake; F: G = feed to weight gain ratio.

**Table 4.** Effect of quercetin on immune organs in Arbor Acre broilers.<sup>1</sup>

Items	Quercetin				SEM	P-value
	0	0.02%	0.04%	0.06%		
Spleen index (%) <sup>2</sup>	0.93 <sup>b</sup>	0.93 <sup>b</sup>	1.06 <sup>a,b</sup>	1.17 <sup>a</sup>	.0184	0.008
Thymus index (%) <sup>3</sup>	2.34 <sup>b</sup>	2.62 <sup>a,b</sup>	2.96 <sup>a,b</sup>	3.28 <sup>a</sup>	.0755	0.016
Bursa of fabricius index (%) <sup>4</sup>	1.14	0.98	1.27	1.41	.0541	0.117

<sup>1</sup>Data are means of 6 replicates per treatment.

<sup>2</sup>Spleen index = spleen weight/broiler weight × 100.

<sup>3</sup>Thymus index = thymus weight/broiler weight × 100.

<sup>4</sup>Bursa of fabricius index = bursa of fabricius weight/broiler weight × 100.

<sup>a,b</sup>Mean values within the same row sharing a common superscript letter differ significantly ( $P < 0.05$ ).

and melting curve analysis at 95°C for 15 s, 60°C for 2 s, and 95°C for 15 s. The PCR products were verified by electrophoresis on 1% agarose gel and DNA sequencing. Standard curves were generated using pooled cDNA from the assayed samples, and the comparative cycle threshold method ( $2^{-\Delta\Delta Ct}$ ) was used for quantifying mRNA levels (Livak and Schmittgen, 2001).

## Statistical Analysis

All the data were analyzed by 1-way analysis of variance using SPSS 22.0 statistical package for Windows (2010, SPSS Inc., Chicago, IL 60606-6307). Differences among treatments were examined using Duncan's multiple range test. Calculated  $\Delta Ct$  (corrected sample) = mean value of target gene - mean value of internal reference gene,  $\Delta\Delta Ct$  =  $\Delta Ct$  - mean value of control group. Differences with treatment means with a probability of  $P < 0.05$  were accepted as statistically significant.

## RESULTS

### Growth Performance

There were no significant differences in ADG, ADFI, and F: G ( $P > 0.05$ ) (Table 3).

### Immune Organ Indexes

Compared with control, spleen index and thymus index were significantly increased with increasing quercetin, and the best result was obtained at 0.06% quercetin supplementation ( $P < 0.05$ ). However,

bursa of fabricius index was not significantly affected by quercetin supplementation ( $P > 0.05$ ) (Table 4).

### Blood Immune Molecules

IgA and C4 contents were significantly increased in dose-dependent manner ( $P < 0.01$ ). IgM and IL-4 contents were significantly increased by 0.06% quercetin supplementation, compared with control ( $P < 0.01$ ). On the other hand, TNF- $\alpha$  content was significantly increased by 0.04 and 0.06% quercetin supplementation ( $P < 0.05$ ). However, C3 content was increased by 0.02% quercetin supplementation ( $P > 0.05$ ) (Table 5).

### Splenic T Lymphocyte Proliferative Responses

Splenic T lymphocyte proliferative responses (SLPR) were significantly increased with increasing quercetin supplementation ( $P < 0.01$ ). However, SLPR of 0.04% quercetin supplementation was significantly higher than those of quercetin with 0.02 and 0.06% quercetin supplementation (Figure 1).

### Immuno-Related Genes in Spleen

In this study, we focused on the expression of genes which are influenced by quercetin supplementation in spleen. In addition, the selected genes, based on their role in inflammation and their function in the signal pathway of NF- $\kappa$ B which is activated by TNF- $\alpha$ , are TNF- $\alpha$ , TRAF-2, TNFRSF1B, NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , and IFN- $\gamma$ .

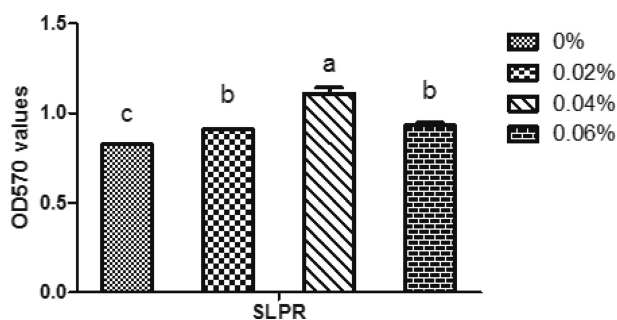
**Table 5.** Effect of quercetin on immune molecules of blood in Arbor Acre broilers.<sup>1</sup>

Items <sup>2</sup>	Quercetin				SEM	P-value
	0	0.02%	0.04%	0.06%		
IgA (mg/mL)	4.84 <sup>c</sup>	5.35 <sup>b,c</sup>	5.72 <sup>b</sup>	6.67 <sup>a</sup>	0.1737	<0.001
IgM (mg/mL)	3.24 <sup>b</sup>	3.03 <sup>b</sup>	3.46 <sup>a,b</sup>	3.87 <sup>a</sup>	0.0964	0.007
C3 (ng/L)	658.72 <sup>b</sup>	802.65 <sup>a</sup>	694.32 <sup>b</sup>	689.50 <sup>b</sup>	20.36	0.045
C4 (ng/L)	236.86 <sup>c</sup>	275.88 <sup>b,c</sup>	307.46 <sup>a,b</sup>	336.62 <sup>a</sup>	10.50	0.001
IL-4 (ng/L)	139.74 <sup>b</sup>	130.07 <sup>b</sup>	131.55 <sup>b</sup>	177.37 <sup>a</sup>	5.118	<0.001
TNF- $\alpha$ (ng/L)	209.18 <sup>b</sup>	248.50 <sup>a,b</sup>	285.53 <sup>a</sup>	278.73 <sup>a</sup>	10.41	0.026

<sup>1</sup>Data are means of 6 replicates per treatment.

<sup>2</sup>IgA = immunoglobulin A; IgM = immunoglobulin M; C3 = complement 3; C4 = complement 4; IL-4 = interleukin-4; TNF- $\alpha$  = tumor necrosis factor alpha.

<sup>a-c</sup>Mean values within the same row sharing a common superscript letter differ significantly ( $P < 0.05$ ).



**Figure 1.** Effect of quercetin on splenic T lymphocyte proliferative responses (SLPR) in Arbor Acre broilers compared with those without quercetin. Values are means of OD<sub>570</sub> using Tecan Sunrise. Data are presented as means  $\pm$  SE ( $n = 12$ ). Different superscript letters indicate significant differences among the means ( $P < 0.05$ ).

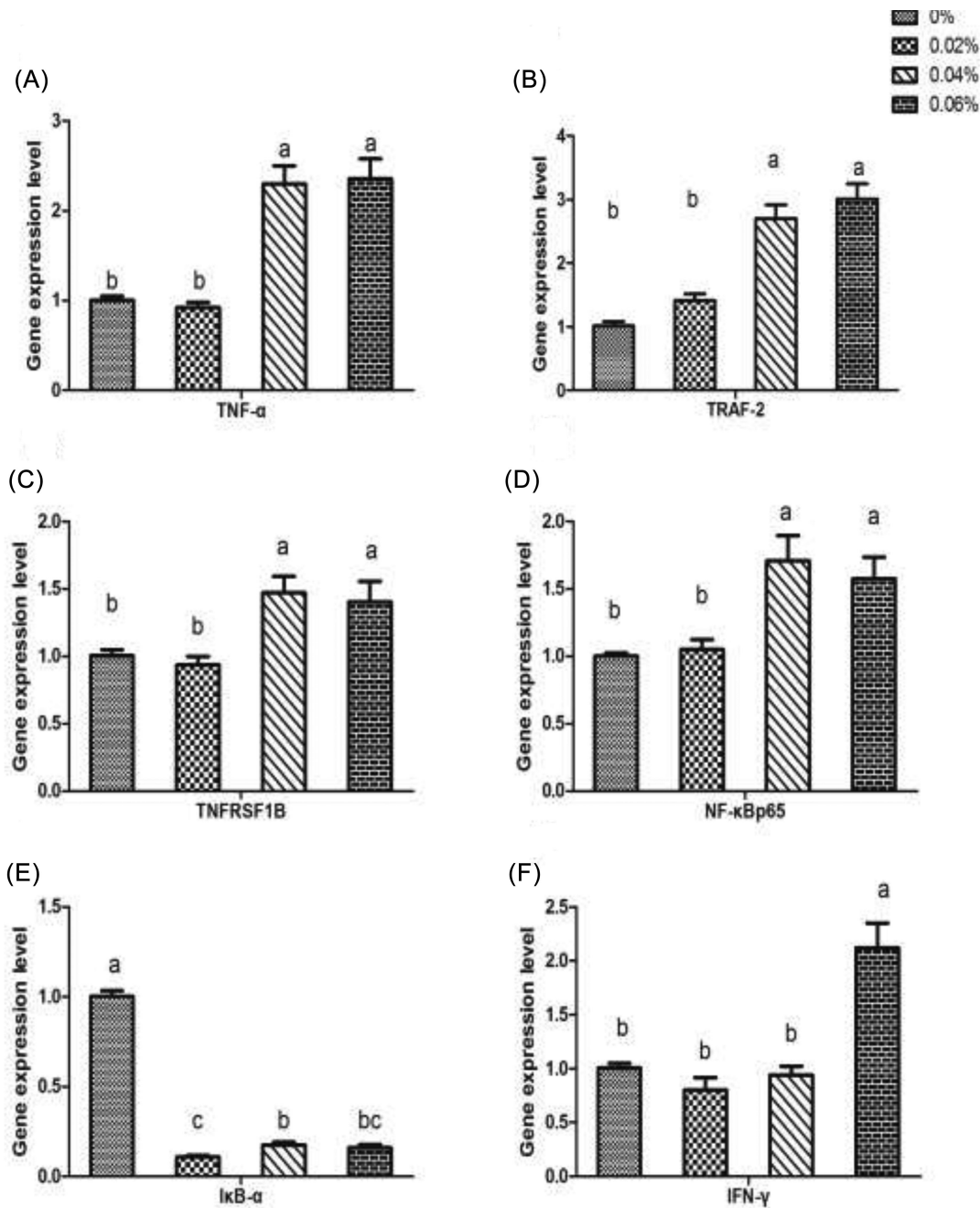
Quercetin supplementation at 0.04 and 0.06% significantly increased the expression of TNF- $\alpha$ , TRAF-2, TNFRSF1B, and NF- $\kappa$ B p65 compared to control ( $P < 0.01$ ,  $P < 0.05$ ). On the contrary, we observed a significant decrease in I $\kappa$ B- $\alpha$  expression of quercetin supplementation compared to control ( $P < 0.01$ ). Nevertheless, compared with control, we observed a significant increase in the expression of IFN- $\gamma$  under the 0.06% quercetin supplementation ( $P < 0.01$ ) (Figure 2).

## DISCUSSION

In this study, quercetin supplementation has no significant effect on growth performance in AA broilers. Several polyphenolic compounds and flavonoids including quercetin, silymarin, hesperidin, soybean isoflavone, alfalfa flavonoids, and thymol have been previously used as dietary supplements in broilers, but contradictory results were found. Quercetin is one of the major flavonoids in sea buckthorn leaves. Previous experiments in our lab showed that 0.5% sea buckthorn leaves significantly increased the average daily weight gain; however, F:G ratio was significantly decreased by 15.17% when compared with control. Therefore, 0.5% the sea buckthorn leaves is beneficial to the growth performance of AA broilers (Chen et al., 2011). Later studies indicated that the average daily intake of AA

broiler was significantly decreased by 0.1% flavones of sea buckthorn leaves without affecting the growth performance (Zhao et al., 2012). Flavones of sea buckthorn fruits (FSBF) quadratically improved ADFI, ADG, and final BW ( $P = 0.002$ ,  $P = 0.019$ , and  $P = 0.018$ , respectively). This result suggested that adding FSBF in to the diets improved the growth performance of broilers (Ma et al., 2015). According to the findings of Golomytis et al. (2014), supplementation of quercetin at the levels of 0.5 and 1.0 g/kg feed had no significant effects on body weight and cumulative feed intake, but poor feed conversion ratios were obtained with increasing quercetin levels in broilers. Similar results were reported by Schiavone et al. (2007), who showed that silymarin supplementation at the dose rate of 40 and 80 ppm had no effects on growth performance of broilers. On the other hand, results of present study were not supported by the findings of Jiang et al. (2007), who reported that addition of synthetic soybean isoflavone from 10 to 80 mg/kg feed significantly increased weight gain and feed intake, while significantly decreased feed to gain ratio of chickens. Alfalfa flavonoids significantly improved average daily gain and feed to gain ratio of broiler chickens (Ouyang et al., 2016). Therefore, we speculated that the difference in results may be caused by doses and structure of various flavonoids (Espin et al., 2017).

In extensive broiler production system, chickens are continuously exposed to wide range of stresses, which decreases growth performance, disturbs/breaks immune homeostasis, and suppresses the expression of immune-related genes. Immune system is a network which is spread throughout the body and may automatically fight against foreign harmful substances. The structure of the immune system is complex, including immune organs, immune cells, and immune molecules (Work et al., 2015). Among the system, immune molecules play very important roles in regulating immune reactions. Immunoglobulins are globulins similar to antibodies that have antibody activity or chemical structure; the degree of humoral immunity is based on the immune globulin (Yasuma et al., 2016). In the primary response, IgM are first produced and are more abundant than



**Figure 2.** Effect of 3 different levels of quercetin on expression of immuno-related genes in spleen of Arbor Acre broilers compared with those without quercetin. Values are means of 6 replicates of 2 samples each. All data are presented as means  $\pm$  SE. Different superscript letters indicate significant differences among the means ( $P < 0.05$ ). TNF- $\alpha$  = tumor necrosis factor alpha; TRAF-2 = TNF receptor associated factor-2; TNFRSF1B = TNF receptor superfamily member 1B; NF- $\kappa$ Bp65 = nuclear factor kappa-B p65 subunit; I $\kappa$ B- $\alpha$  = NF- $\kappa$ B inhibitor-alpha; IFN- $\gamma$  = interferon- $\gamma$ .

IgG; IgA is the main Ig in external secretions (Zhang et al., 2015). In the present study, dietary quercetin enhanced humoral immune response in a dose-dependent manner. These results are in line with those of Liang et al. (2011), who reported that 10 mg/kg of flavonoids from *Scutellaria baicalensis* significantly increased content of IgA and IgG in broilers. Normally, the components of complement system are activated by antigen-antibody complexes to play an anti-inflammatory role in the presence of inactive precursors in plasma. Previous studies showed that total

flavonoids of hippophae significantly increased the contents of serum C3 (Su et al., 2009). Our data showed that 0.02% quercetin supplementation significantly increased the contents of serum C3; on the contrary, the contents of serum C<sub>4</sub> were significantly increased by 0.06% quercetin supplementation. In the immune responses, IL-4 plays a relevant role through a specific binding with interleukin-4 receptor, which is on the surface of target cells (LaPorte et al., 2008). The content of serum IL-4 in poultry is restricted by a series of conditions, such as breeding environment, heat stress, and

inflammatory response. Previous experiments have shown that soybean isoflavones significantly increased the content of serum IL-4 and upregulated IL-4 expression by increasing DNA restriction activity of phosphatase-1 (Han et al., 2002). There are dual effects between IL-4 and TNF- $\alpha$  on resistance to systemic gram-negative infection (Giampietri et al., 2000). Similarly, our results showed that quercetin significantly increased the contents of serum IL-4 and TNF- $\alpha$ . Consequently, it revealed that quercetin significantly influenced the secretion of cytokines in AA broilers.

Reduced weight of the immune organs represents immunosuppression, while increase in weight of immune organs was a manifestation of the enhancement of immunity (Iftikhar et al., 2012). Nevertheless, quercetin did not significantly affect spleen index, thymus index, and bursa of fabricius index of Cobb chickens in former study (Hager-Theodorides et al., 2014). On the contrary, we found that 0.06% quercetin supplementation significantly increased the spleen and thymus index. The inconsistent result may be related to the dose and different breeds of broiler.

Lymphocytes respond to antigen challenge by proliferating and expanding the antigen-specific lymphocyte clones, and producing lymphokines, thus amplifying immune responses (Singh et al., 2016). Therefore, it is an important index to evaluate cellular immunity. In this study, quercetin significantly increased the splenic lymphocyte proliferative response. It suggested that quercetin can promote the proliferation of splenic lymphocytes, thereby improving cellular immune function.

NF- $\kappa$ B plays a fundamental role in the adaptive immune response and is involved in the regulation of embryonic development, lymphopoiesis, and osteogenesis (Karin and Benneriah, 2000; Li and Verma, 2002). The NF- $\kappa$ B pathway converges with the classical pathway at I $\kappa$ B kinase complex activation. Resulting cytokine expression may activate NF- $\kappa$ B in a positive feed forward loop (Hellweg, 2015). Previous studies of TNF- $\alpha$  signaling suggested that it may independently activate the NF- $\kappa$ B pathway and diverge early in the TNF- $\alpha$  signaling cascade (Antwerp et al., 1996). TNFR2 is the receptor of TNF- $\alpha$ , and the structure has the base sequence of TRAF; thereby, it may combine with TRAF to activate NF- $\kappa$ B pathway, induce gene transcription, and promote cell survival, proliferation, and differentiation (Depuydt et al., 2005). TNFRSF is a protein superfamily of cytokine receptors characterized by binding TNFs via an extracellular cysteine-rich domain (Locksley et al., 2001; Hehlhans and Pfeffer, 2005). With the exception of nerve growth factor, all TNFs are homologous to the archetypal TNF- $\alpha$  (Gravestain and Borst, 1998). In addition, most TNF receptors require specific adaptor proteins such as TRADD, TRAF, RIP, and FADD for downstream signaling. The transcription factor NF- $\kappa$ B is composed of 2 subgroups, including 5 proteins (p105, p50, p100, p52, and p65). In the absence of TNF stimulation, the NF- $\kappa$ B is as-

sociated with its inhibitory subbase I kappa B (I $\kappa$ B) in the cytoplasm (Albert and Baldwin, 2003). In the absence of I $\kappa$ B, the NF- $\kappa$ B signaling pathway was activated, anti-inflammatory cytokine (IFN- $\gamma$ ) was produced, and the expression of genes in upstream or downstream was increased. It has also been previously shown that the structure of the flavonoids affects NF- $\kappa$ B activation (Shin et al., 2011). Our study showed that quercetin significantly increased expressions of TNF- $\alpha$ , TRAF-2, TNFRSF1B, NF- $\kappa$ Bp65, and IFN- $\gamma$ . On the contrary, quercetin significantly decreased I $\kappa$ B- $\alpha$  expression. Considered together, our data indicated that decreased I $\kappa$ B- $\alpha$  resulting from NF- $\kappa$ B signaling pathway activated by quercetin, thus promoting the cytokine production and facilitating downstream signaling NF- $\kappa$ B translocation to nucleus.

This study demonstrated that quercetin regulated the immune response by increasing secretion of cytokines, and promoting development of immune organs and the formation of splenic lymphocytes. The result revealed that dietary supplementation of quercetin improved the immune function in AA broilers via activating the NF- $\kappa$ B signaling pathway.

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