

# Evaluation of potential antioxidant and anti-inflammatory effects of *Antrodia cinnamomea* powder and the underlying molecular mechanisms via Nrf2- and NF- $\kappa$ B-dominated pathways in broiler chickens

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**ABSTRACT** *Antrodia cinnamomea*, a precious and unique medical fungus existing exclusively in Taiwan, exhibits antioxidant and immunomodulatory properties. This study was conducted to evaluate the beneficial effects of *A. cinnamomea* powder (ACP) and to further illuminate its underlying antioxidant and immunomodulation molecular mechanisms in broilers. The functional compounds of ACP—crude triterpenoids, crude polysaccharides, and total phenolic content—were assayed, respectively. Two-hundred-forty one-day-old broilers (Ross 308) were assigned to 4 treatment groups receiving dietary supplementation with ACP at 0, 0.1, 0.2, and 0.4% for 35 days. Each group had 4 replicate pens, with 15 birds per pen. During 1 to 21- and 22 to 35-day periods, chickens on ACP-supplemented diet demonstrated increased body weight gain, compared to those on the control diet, resulting in increased weight gain throughout the entire experimental period with an increased tendency in feed consumption yet no significant difference in FCR. Blood antioxidant potentiality, superoxide dismutase (SOD), increased in birds fed the supplemented diet at both 21 and 35 d, accompanied by higher catalase (CAT) activity at 21 days. In vivo peripheral blood mononu-

clear cells (PBMC) exposed to lipopolysaccharide (LPS) and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) capability showed that the diminished cell viability caused by both challenge factors was improved in ACP-supplemented groups. Antioxidant genes dominated by Nrf2 genes, such as HO-1 and GCLC, were up-regulated in 35-day-old birds. Inflammatory-related genes, such as IL-1 $\beta$  and IL-6, ruled mainly by NF- $\kappa$ B, were rather down-regulated by 0.2% ACP addition at 21 and 35 days. Protein expression of Nrf2 and NF- $\kappa$ B in the liver supported the mRNA results, demonstrating that all ACP-supplemented groups showed significantly higher Nrf2 expression, whereas the NF- $\kappa$ B was inhibited. In conclusion, preferable microbial balance may putatively indicate the improvement of immunomodulatory-related capacity by ACP. Furthermore, ACP could induce the Nrf2-dependent pathway and decrease the NF- $\kappa$ B-dominated inflammatory signaling pathway. Antioxidant and immune capacity in terms of antioxidant enzymes and cell tolerance also was elevated by ACP. Concomitantly, body weight increasing with ACP supplementation as compared to the corresponding control group further implied the promising effects exerted by ACP.

**Key words:** *Antrodia cinnamomea*, immune, antioxidant, chicken, feed additive

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## INTRODUCTION

Oxidative stress, an imbalance condition when the reactive oxygen species (ROS) formation exceeds cellular antioxidant capacity in the animal body (Lee et al., 2017), is confronting the modern poultry industries and mainly attributing to factors such as heat exposure, handling, rearing density, and pathological status. Worse yet, it is responsible for compromised

growth, productive performance, and economic loss (Sahin et al., 2010; Est´eve, 2015; Surai, 2016). For instance, Zheng et al. (2016) employed lipopolysaccharides (LPS) to induce oxidative stress in broiler chickens, and average daily weight and feed consumption were remarkably decreased. Similar detrimental effects also were found in chickens suffering high ambient temperatures (Sohail et al., 2012). Moreover, the annual economic loss caused by heat-induced stress was estimated at \$128 to \$165 million in the poultry industry (St-Pierre et al., 2003). Animal cells have developed delicate systems, including enzymatic, fat, and water soluble antioxidants, to balance the formation

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and elimination of these constant oxidative elements and prevent the oxidative stress induced either internally or externally (Surai, 2002). However, these adaptive systems have limited abilities that are not only prone to sudden oxidative damage, but also lead to an immunosuppressive condition (Surai, 2016). The birds' ability to cope with an environment composed of interacting stressors by which they are surrounded is therefore critical in maintaining or restoring a homeostatic state (Shini et al., 2010). Since the defense systems consist of numerous molecules involved in signal transduction pathways, and the interaction between animals and their diet is a complex physiological event (Mutch et al., 2005), the regulatory mechanisms can be elucidated more deeply through evaluating the expression of numerous genes. Nutrigenomics therefore sheds light on how the interactions between genes and nutrition may affect antioxidant and immune responses, and final performance traits.

Nuclear factor-2 erythroid related factor-2 (Nrf2) and nuclear factor kappa light chain enhancer of B cells (NF- $\kappa$ B) are the 2 main redox-sensitive and -regulated transcription factors that coordinate with one another (Ganesh Yerra et al., 2013; Lee et al., 2013). Transcription factors are protein complexes controlling gene expression by binding to specific DNA sequences, thereby turning the downstream signaling cascades on and off (Latchman, 1997). Nrf2 is a cytoprotective transcription factor that regulates the antioxidant response element (**ARE**); this in turn mediates the expression of phase II detoxifying antioxidant enzymes (Kim et al., 2010). NF- $\kappa$ B participates in immune and inflammatory reaction in response to various stimuli, including free radicals, thereby controlling cytokine production and some immunological functions (Gilmore, 2006). In addition, it is reported that in some situations, inflammation and oxidative stress have mutual influences, illuminating the pivotal roles that NF- $\kappa$ B and Nrf2 play in maintaining redox homeostasis, which may be associated with the potential interfaces and crosstalk between Nrf2 and NF- $\kappa$ B (Cheng et al., 2014; Yen et al., 2015).

*Antrodia cinnamomea* (Syn. *Antrodia camphorata* and *Taiwanofungus camphorata*), a precious and unique medical fungus existing exclusively in Taiwan, has been utilized by Taiwanese aborigines in folk remedies for centuries, thanks to its antioxidant, hepatoprotective, and immunomodulatory properties (Wang et al., 2013; Yang et al., 2013), mainly due to its active components, such as triterpenoids, polysaccharides, and polyphenols (Hseu et al., 2005). Among the characteristic metabolites of *A. cinnamomea*, triterpenoids are pivotal representatives of phytoconstituents in both its fruiting body and mycelia (Geethangili and Tzeng, 2011; Lee et al., 2012). The potent activities of triterpenoid against ROS generation were emphasized and reviewed by Owusu-Ansah et al. (2015), proving that it holds potentiality to activate the Nrf2-dependent antioxidant pathway. Polysaccharides were reported to possess the greatest structural variability, rendering them capable of

carrying diverse biological information among all of the macromolecules (Lu et al., 2013). For instance, antioxidant potentiality in purified polysaccharide from fungi were exemplified via in vitro assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and ferrous ions chelating effect, and it could even retard lipid peroxidation in rat liver (He et al., 2017); additionally, polysaccharide derived from mushrooms such as glucan was documented to boost the immune defense system of the host by stimulating the proliferation of monocytes and macrophages and inducing nitric oxide production by macrophages (Borchers et al., 2004; Ooi, 2008; Vannucci et al., 2013). Finally, natural phenolic compounds, a group of secondary metabolites also derived from fungi, possess strong antioxidant capabilities in terms of lipid peroxidation inhibition and DPPH scavenging activity (Palacios et al., 2011; Lu et al., 2013; Wang et al., 2013). Furthermore, there are positive correlations between phenolic compounds and the detected antioxidant potential (Kim et al., 2008). These positive actions are mainly derived from its hydroxyl groups (Zhang and Tsao, 2016). Studies showed that *A. cinnamomea* extract could effectively inhibit inflammation, oxidative stress, and the growth of various types of cancer cells (Arias-Salvatierra et al., 2011). Moreover, the anti-inflammatory and antioxidant potential of isolated compounds derived from this fungus demonstrated that, when exposed to LPS and 2,2'-azobis(2-amidinopropane) dihydrochloride (**AAPH**), NF- $\kappa$ B and Nrf2 were down- and up-regulated, respectively, in selected cells of mice (Gokila Vani et al., 2013; Shie et al., 2016). The activity of inducible nitric oxide synthase (**iNOS**), one of the target dominating inflammatory molecule nitric oxide (**NO**) productions via the NF- $\kappa$ B pathway, was inhibited, and oxidative stress was also concomitantly reduced through Nrf2 activation (Tsai et al., 2011). This further demonstrates the possible crosstalk between Nrf2 and NF- $\kappa$ B, the controllers of the antioxidant and inflammation signaling cascades (Contestabile, 2000; Brown, 2007; Shi et al., 2009). This supporting evidence, however, is largely based on the use of artificial stress models and cells rather than the animal itself, and the impact of the modern poultry industry upon cell-mediated immunity has not been addressed (El-Lethey et al., 2003).

Based on the above considerations, we examined the hypothesis that supplementation of *A. cinnamomea* product in diet could alleviate repressed antioxidant machinery and prevent abnormally amplified inflammatory signaling via the Nrf2 and NF- $\kappa$ B pathways in conventional rearing chickens.

## MATERIALS AND METHODS

### *Mycelium of Antrodia Cinnamomea*

Solid-state cultured *A. cinnamomea* mycelia powder (**ACP**), which has been certificated as a health

food (A00190) by the Department of Health, Taiwan, was obtained from the R&D Center of Taiwan Leader Biotechnology Corp. (Taichung, Taiwan).

### **Preparation of *Antrodia Cinnamomea* Water Extract**

The ACP was weighed to exactly 5.0 g in the 100 mL Erlenmeyer flask, and then about 45 mL of distilled water were added. The solution was extracted in a 95°C water bath and left to stand for 1 hour. After the extraction, the Erlenmeyer flask was taken out and then cooled under ambient temperature. The final concentration was made to 50 mg/mL with distilled water, and then the solution was filtered (Advantec No. 1, Tokyo, Japan) in order to obtain the near transparent sample. Finally, the finished sample was stored at -20°C for subsequent analysis.

### **Determination of Total Phenolic Content**

The total phenolic content of *A. cinnamomea* water extract (ACW) was measured based on the procedures described by Kujala et al. (2000) with minor modifications. Briefly, an aliquot of 50 µL extract was mixed with 0.5 mL Foline-Ciocalteau phenol reagent and 1 mL 7.5% sodium carbonate, and allowed to react for 30 min at room temperature (RT), comparative to a gallic acid standard. Absorbance was measured at 730 nm using an automated microplate reader. The results were expressed as mg gallic acid equivalence/g extracts.

### **Crude Polysaccharides**

The water-soluble polysaccharides were precipitated by adding 4 volumes of 95% ethanol. The precipitated polysaccharides were collected via centrifugation at 3,000 rpm for 10 min, and then subsequently dried at 60°C to remove residual ethanol. The total amount of polysaccharides in the culture medium was determined by phenol-sulfuric acid assay, as per Dubois et al. (1956).

### **Determination of Total Triterpenoid Content**

The total triterpenoid content of ACW was determined according to Lu et al. (2011) with a slight modification. Briefly, after a 200-µL sample solution in a 10 mL volumetric flask was heated to evaporation in a water bath, 1 mL newly mixed 5% (W/V) vanillin-acetic solution and 1.8 mL sulfuric acid were added, mixed, and incubated at 70°C for 30 minutes. The solution was then cooled and diluted to 10 mL with acetic acid. The absorbance was measured at 573 nm against a blank, using a spectrophotometer. The blank consisted of all reagents and solvents without a sample solution. The content was determined using the standard ursolic acid (Sigma) calibration curve.

### **Ferrous Chelating Capacity Assay**

The chelating effect was determined according to the methods of Dinis et al. (1994). In brief, 0.25 mL ACW was mixed with 0.025 mL 2 mM ferrous chloride solution and 0.925 mL methanol. After 30 min at RT, 0.05 mL 5 mM ferrozine was added to initiate a reaction that lasted for 10 min at RT. The absorbance of the mixture was then determined at 562 nm. The inhibiting percentage of ferrozine-Fe<sup>2+</sup> complex formation was calculated as:

$$\text{Ferrous ion chelating (\%)} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100$$

Where: A0 and A1 represent the absorbance of the control and ACW, respectively. In this experiment, ethylenediaminetetraacetic acid (EDTA) was used as a positive control.

### **Scavenging Effect on 1,1-diphenyl-2-picrylhydrazyl Radicals**

Two mL of each ACW was mixed with 0.5 mL 1 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) ethanol solution. The mixtures were shaken vigorously and left to stand for 30 min in the dark; the final absorbance was then measured at 517 nm against a blank. The values were converted into the percentage scavenging activity using the following formula:

$$\text{Scavenging activity (\%)} = \frac{(\text{Abs sample} - \text{Abs blank}) \times 100}{\text{Abs control}}$$

Butylated hydroxytoluene (BHT) was used as the control in this assay.

### **Anti-commensal Bacteria Colonizing Chickens**

Samples of fresh chicken droppings were collected from 3 birds. One gram of the fresh sample was suspended in 24 mL LB broth (BD Difco) and vortexed until homogenized. The sediments were removed by filtration using 3 pieces of gauze. The suspension was incubated at 37°C in an anaerobic incubator for 24 hours. The ACW was then added to the fecal suspension at a 1:1 ratio before anaerobic incubation at 37°C for another 24 hours. At the same time, the counts of coliform and lactic acid bacteria were determined. After 24 h, the cultured mixture was taken out and spread onto freshly prepared MRS and McConkey agar plates before being cultured anaerobically for a further 24 h to calculate the final bacterial count. PBS was used as a negative control in the experiment. The antibacterial effect was expressed as compared to the PBS-treated results.

**Table 1.** Ingredients and chemical composition of the experimental diets of broilers.

Ingredient	g/kg	
	Starter diet	Finisher diet
Yellow corn	472.6	518.0
Soybean meal (CP 44.0%)	345.2	295.9
Full fat soybean meal	100.0	100.0
Soybean oil	35.0	45.0
Calcium carbonate	16.2	13.4
Monocalcium phosphate	18.6	16.6
DL-Methionine	2.0	1.3
L-Lysine-HCl	3.7	3.2
NaCl	3.9	3.8
Choline-Cl	0.8	0.8
Vitamin premix <sup>1</sup>	1.0	1.0
Mineral premix <sup>2</sup>	1.0	1.0
Total	1000	1000
Calculated nutrient value		
ME, kcal/kg	3050.1	3175.1
Crude protein, %	23.0	21.0
Calcium, %	1.05	0.90
Total Phosphorus, %	0.75	0.70
Available Phosphorus, %	0.50	0.45
Lysine, %	1.43	1.25
Methionine+Cystein, %	1.07	0.96
Analyzed nutrition value		
Crude protein, %	23.3	21.2
Dry matter, %	87.8	88.1

<sup>1</sup>Supplied per kg of diet: Vit. A 15,000 IU; Vit. D3 3000 IU; Vit. E 30 mg; Vit. K3 4 mg; Riboflavin 8 mg; Pyridoxine 5 mg; Vit. B12 25 µg; Ca-pantothenate 19 mg; Niacin 50 mg; Folic acid 1.5 mg; Biotin 60 µg.

<sup>2</sup>Supplied per kg of diet: Co (CoCO<sub>3</sub>) 0.255 mg; Cu (CuSO<sub>4</sub>•5H<sub>2</sub>O) 10.8 mg; Fe (FeSO<sub>4</sub>•H<sub>2</sub>O) 90 mg; Zn (ZnO) 68.4 mg; Mn (MnSO<sub>4</sub>•H<sub>2</sub>O) 90 mg; Se (Na<sub>2</sub>SeO<sub>3</sub>) 0.18 mg.

## Experimental Birds and Housing

Two-hundred-forty one-day-old male broiler chickens (Ross 308) were randomly allocated to one of 4 treatments. Each treatment group had 4 replicates per treatment, with 15 birds per pen (totaling 60 birds per treatment). The initial body weights of the birds were similar (approximately 38.0 to 38.5 g/bird). The temperature was maintained at 34 ± 1°C until the birds reached 7 d of age; it was then gradually decreased to 26 ± 1°C until the birds reached 21 d of age. After this point, the broilers were maintained at RT (approximately 27°C). The experiment was conducted at National Chung Hsing University, Taiwan, and the experimental protocol was approved by the Animal Care and Use Committee.

## Feeding Schedule and Dietary Treatment

Birds were fed a corn-soybean meal basal diet (Table 1) supplemented with 0% (control), 0.1, 0.2, or 0.4% ACP until 35 d of age. All birds received starter (1 to 21 d of age) and finisher (22 to 35 d of age) diets ad libitum and had free access to water. The proximate composition of the diets was analyzed according to the AOAC (2000). Crude protein, crude fat, ash, and acid detergent fiber levels were determined using methods 990.03 (Kjeldahl N × 6.25), 945.16, 967.05, and 973.187, respectively; the results showed no major deviations from the calculated values. During the entire

experimental period (35 d), the diets were formulated to meet the requirements suggested by the Ross Broiler Management Manual (2014) and the NRC (1994).

## Performance and Sample Collection

Body weights were recorded at 1, 21, and 35 d of age. Body weight gain and feed conversion ratio (FCR) were calculated on the basis of the above data. At the end of the experiment (35 d), 8 birds (2 birds per pen) were randomly selected for sampling. Blood samples were collected via wing-vein puncture into a tube containing 1% EDTA. The samples were then centrifuged at 3,000 × g for 10 min to obtain the serum, and the aliquots were transferred into microfuge tubes. Sera were kept on ice and protected from light to prevent any artifactual oxidation during sample collection. Samples were stored at -20°C until analysis or catalase (CAT) and superoxide dismutase (SOD) activities. The birds were euthanized by exsanguination, and the abdominal cavities were opened for liver, ileum, and ceca collection. The liver samples were stored at -80°C until analysis; the ileal and cecal contents were collected for the study below.

## Determination of Ileal and Cecal Microbial Population

Strains of lactic acid bacteria and coliform from ileal and cecal contents were cultured with MRS (de Man Rogosa and Sharpe agar, Difco 288,130) and McConkey agar medium, respectively. After aerobic and anaerobic incubation, respectively, at 37°C for 48 h, the microflora numbers were calculated. Bacterial populations were expressed as log<sub>10</sub> colony forming units (CFU) per gram of intestinal contents.

## Determination of Serum Antioxidant Enzyme Activities

Total SOD and CAT activities were assayed using kits purchased from Cayman Chemical Co., Ltd. (Ann Arbor, MI). Serum samples were measured in triplicate and at the appropriate dilutions to allow enzymatic activities to achieve the linear range of standard curves. Antioxidant enzyme activities were expressed as unit (U) per milliliter of serum.

## Peripheral Blood Mononuclear Cell Isolation

Whole blood was collected via wing-vein using a hypodermic syringe and inserted into tubes containing EDTA. The blood was gently layered on to Ficoll-Paque Plus (GE Healthcare Uppsala, Sweden) and centrifuged at 200 g for 10 minutes. Peripheral blood mononuclear cells (PBMC) were collected from the gradient interface; the plasma suspension was combined and washed 3 times with PBS and then centrifuged at 200 × g for

**Table 2.** Characteristics and performance data of the primers used for q-PCR analysis.

Gene	Forward primer (from 5' to 3') Reverse primer (from 5' to 3')	PCR product size (bp)	NCBI GenBank
$\beta$ -actin	CTGGCACCTAGCACAATGAA ACATCTGCTGGAAGGTGGAC	109	X00182.1
HO-1 <sup>1</sup>	AGCTTCGCACAAGGAGTGT GGAGAGGTGGTCAGCATGTC	106	X56201.1
NOX1 <sup>2</sup>	CAATGCAGCACTCCACTTTG GACAAGATCTCCGCAAGACC	185	NM_0,011,01830.1
GST <sup>3</sup>	AGTCGAAGCCTGATGCACTT TCTAGGCGTGGTTTCCCTTTG	121	L15386.1
Nrf2 <sup>4</sup>	GGAAGAAGGTGCTTTTTCGGAGC GGGCAAGGCAGATCTTCCAA	171	NM_205,117.1
ROMO1 <sup>5</sup>	AGCCCAGCTGCTTCGACAGAGT CGTCCTCTCATGCCGATCCTGA	115	NM_0,011,98821.1
NF- $\kappa$ B <sup>6</sup>	GAAGGAATCGTACCGGGAACA CTCAGAGGGCCTTGTGACAGTAA	131	NM_205,134
IL-1 $\beta$ <sup>7</sup>	GCTCTACATGTCTGTGTGATGAG TGTCGATGTCCCGCATGA	80	NM_204,524
IL-6 <sup>8</sup>	AGGACGAGATGTGCAAGAAGTTC TTGGGCAGGTTGAGGTTGTT	78	NM_204,628
COX-2 <sup>9</sup>	TGTCTTTTCACTGCTTTCCAT TTCCATTGCTGTGTTTGAGGT	84	NM_0,011,67718.1

<sup>1</sup>HO-1: Heme oxygenase -1.<sup>2</sup>NOX1: NADPH oxygenase 1.<sup>3</sup>GST: Glutathione S-transferase.<sup>4</sup>Nrf2: Nuclear factor (erythroid-derived 2)-like 2.<sup>5</sup>ROMO1: Reactive oxygen species modulator protein 1.<sup>6</sup>NF-Kb: Nuclear factor of kappa light polypeptide gene enhancer in B-cells p50.<sup>7</sup>IL-1 $\beta$ : Interleukin 1, beta.<sup>8</sup>IL-6: Interleukin 6.<sup>9</sup>Cyclooxygenase 2.

10 minutes. After the suspension was removed, 1 mL Trizol reagent (Invitrogen, Carlsbad, California, USA) was added and the mixture was stored at  $-80^{\circ}\text{C}$ .

### PBMC Viability Assay

Chicken PBMC were isolated from whole blood by the density gradient centrifugation as described above. The mitogen, including AAPH and LPS, were mixed with PBMC cell line ( $1 \times 10^7$  cell/well) and incubated on air incubator at  $37^{\circ}\text{C}$ . After 48 h, to each well were added  $20\mu\text{l}$  of MTT (3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution, which incubated at  $37^{\circ}\text{C}$  for 4 hours. Then, the medium was removed and Dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. The absorbance of the solution was measured by a microplate reader at 517 nm.

### RNA Isolation and Quantitative Reverse Transcription-polymerase Chain Reaction

The PBMC treated with Trizol mentioned above were further extracted with chloroform and then precipitated with isopropanol. The RNA pellet was briefly washed with 75% ethanol and then re-suspended in RNase-free water. RNA concentration was determined by spectrophotometry and diluted to  $50\text{ ng}/\mu\text{L}$ . Total RNA concentration and purity, cDNA synthesis, and qPCR analysis were determined as per the methods of Lin et al. (2014). Gene-specific primers were designed based

on the genes of *Gallus gallus* (chickens); Table 2 lists the features of the primer pairs. After the normalization of gene expression data using the calculated GeNorm normalization factor, the means and standard deviations (SD) were calculated for samples from the same treatment groups.

### Western Blot Analysis

For Western blot analysis, the chicken liver was extracted, homogenized, and immersed overnight in T-PER, Tissue Protein Extraction Reagent (78,510, Thermo), containing  $5\mu\text{M}$  of a protease inhibitor cocktail (78,430, Thermo). The tissue homogenate was centrifuged at  $15,000 \times g$  at  $4^{\circ}\text{C}$  for 30 min, and the supernatant was transferred into fresh tubes. The Protein Assay kit (Bio-Rad, California, Hercules, USA) was used to measure the concentration of protein in the supernatant. Equal amounts of protein ( $20\mu\text{g}$ ) were electrophoresed and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Science). Upon protein transfer, the blots were washed 5 times for 5 min in PBS and blocked with blocking buffer (37,515, Thermo) for 1 h prior to the application of the primary antibody. Chicken antibodies against NF- $\kappa$ B (#ab16502) and Nrf2 (#ab31163) were purchased from Abcam (Cambridge, UK). The primary antibody was diluted (1:1000) in the same buffer containing 0.05% Tween-20. The PVDF membrane was incubated overnight at  $4^{\circ}\text{C}$  with the

**Table 3.** Content of bioactive compounds of *Antrodia cinnamomea* powder.<sup>1</sup>

Item	Crude triterpenoid (mg of UAE <sup>2</sup> /g DW <sup>3</sup> )	Crude polysaccharide (mg of GCE <sup>4</sup> /g DW)	Total phenolics (mg of GAE <sup>5</sup> /g DW)
	20.1 ± 0.2	29.3 ± 0.2	12.1 ± 0.2

<sup>1</sup>Value is expressed as the mean ± standard deviation (n = 4).

<sup>2</sup>UAE: Ursolic acid equivalent.

<sup>3</sup>DW: Dry weight.

<sup>4</sup>GCE: Glucose equivalent.

<sup>5</sup>GAE: Gallic acid equivalent.

primary antibody. The membrane was then washed 5 times with 0.05% phosphate buffer saline and Tween 20 (PBST) for 5 min before being incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (#ab205718, Abcam, Cambridge, UK). Specific binding was detected using hydrogen peroxide as substrates. Protein loading was controlled using a monoclonal-mouse antibody against  $\beta$ -actin antibody (Biorbyt or b40714). Band intensities of the proteins were quantified by densitometric analysis using an image analysis system (Image J; National Institute of Health, Bethesda, Maryland, USA). Samples were analyzed in triplicate; a representative blot is shown in the respective figures. Results were normalized to the  $\beta$ -actin expression in each group (mean ± SEM) as a percentage of control.

### Statistical Analysis

The data were analyzed by performing ANOVA for completely randomized designs and executing the general linear model procedure implemented in SAS software (SAS, 1999). Additionally, the data were conducted using contrasts to measure the linear and quadric effects of increasing concentration of ACP.

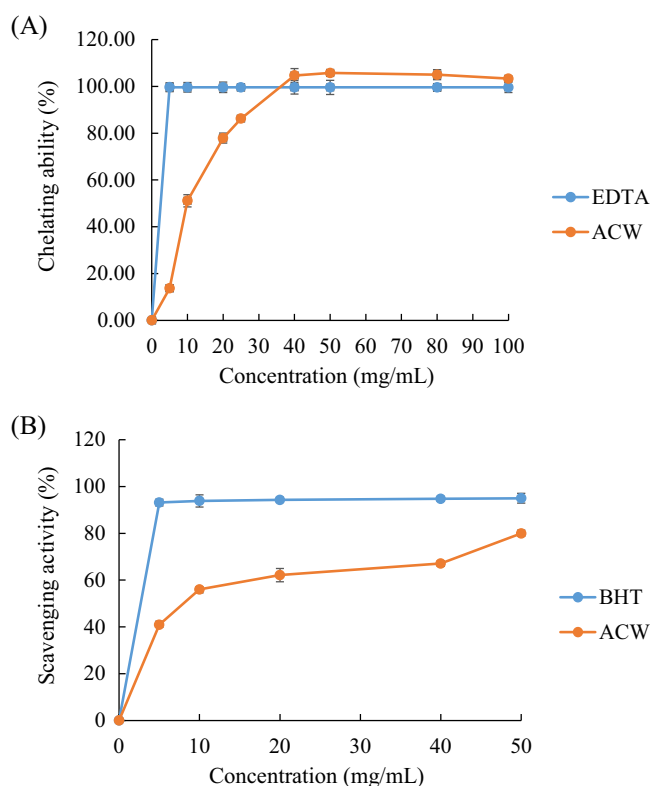
## RESULTS

### Bioactive Compounds in *Antrodia Cinnamomea* Mycelial Powder

Table 3 shows the concentration of the 3 main bioactive compounds in ACP; the crude triterpenoid content was  $20.08 \pm 0.17$  mg of UAE/g DW, the crude polysaccharide content was  $29.34 \pm 0.22$  mg of GCE/g DW, and the total phenolic content was  $12.14 \pm 0.19$  mg of GAE/g DW.

### In Vitro Antioxidant Activities

Fig. 1 shows the ferrous chelating capacity and DPPH free radical scavenging ability of ACW. The ferrous chelating capacity was elevated in the concentration range of 5 to 40 mg/mL, and saturated after 40 mg/mL (Fig. 1 A). While the concentration was above 40 mg/mL, the chelating ability of ACW was

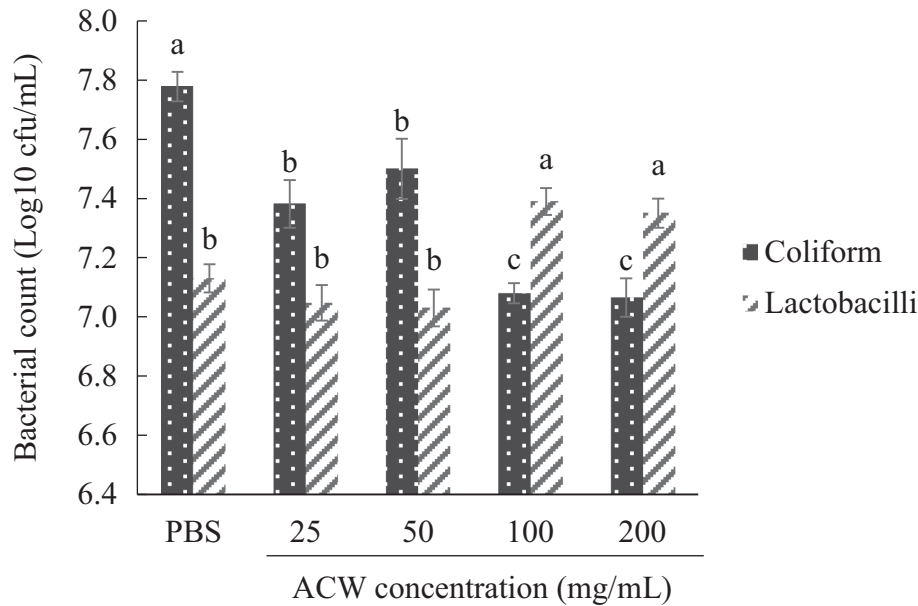


**Figure 1.** (A) Ferrous ion chelating and (B) DPPH (1,1-Diphenyl-2-picrylhydrazyl) free radical scavenging ability of hot water extracted *Antrodia cinnamomea* (ACW).

higher than that of EDTA. On the other hand, the scavenging effects of ACW on DPPH free radicals increased as the concentration rose from 0 to 20 mg/mL; from 20 to 80 mg/mL, there was a slight increase until the value peaked at 80 mg/mL.

### In Vitro Antimicrobial Activity

We tested the possible antimicrobial activity of ACW toward coliform and lactobacilli in fresh chicken excreta. As seen in Fig. 2, chicken excreta incubated with ACW for 24 h exhibited better antibacterial activity in terms of reduced coliform count than those with the blank of PBS liquid. It is interesting that in comparison with PBS treatment, the lactobacilli count was diminished by incubation with either 25 or 50 mg/mL ACW. Conversely, 100 and 200 mg/mL



**Figure 2.** In vitro antimicrobial activity of hot water extracted *Antrodia cinnamomea* (ACW) by chicken intestinal bacteria (Coliform and *Lactobacilli*). The value is provided as the means of 3 samples (n = 5).

**Table 4.** Effect of *Antrodia cinnamomea* powder supplemented in diet on growth performance of one- to 35-day-old broilers.

Experimental period (d) and parameter	Experimental diets				SEM <sup>3</sup>	P-value	
	Control	0.1% ACP	0.2% ACP	0.4% ACP		Linear	Quadratic
<b>1 to 21 d</b>							
Body weight (g/bird) <sup>2</sup>	647	730	705	725	17.24	0.011	0.149
Feed consumption (g/bird) <sup>1</sup>	805	908	855	911	45.55	0.235	0.575
Weight gain (g/bird) <sup>2</sup>	603	686	661	681	17.17	0.012	0.152
FCR	1.335	1.324	1.309	1.338	0.08	0.871	0.942
<b>22 to 35 d</b>							
Body weight (g/bird)	1821	2024	1930	1995	62.05	0.074	0.533
Feed consumption (g/bird)	1817	1897	1967	1937	71.64	0.400	0.659
Weight gain (g/bird)	1174	1294	1225	1270	24.97	0.038	0.795
FCR	1.625	1.531	1.565	1.510	0.05	0.687	0.687
<b>1 to 35 d</b>							
Feed consumption (g/bird)	2622	2806	2764	2781	103.37	0.270	0.581
Weight gain (g/bird)	1777	1980	1959	1951	51.96	0.018	0.133
FCR	1.521	1.418	1.473	1.424	0.03	0.086	0.075

<sup>1</sup>Results are provided as the means of 4 replicates (12 birds/replicate) in each control and treatment group (n = 4).

<sup>2</sup>Results are provided as the means of 48 birds in each control and treatment group (n = 48).

<sup>3</sup>SEM: standard error of the mean.

ACP: *Antrodia cinnamomea* powder.

ACW increased the lactobacilli count, which matches the results of the PBS group.

slight increment of feed intake among the experimental groups.

## Growth Performance

The effects of ACP supplementation on the growth performance of broiler chickens are shown in Table 4. From days 1 to 21, there was a significant linear effect in the body weight and weight gain of birds that received the ACP-supplemented diet compared to those fed the control diet. Moreover, the significant linear effect of ACP on weight gain continued to be observed during 22 to 35 d, denoting an increased weight gain for the entire experimental period. While no significant changes were found among any of the groups in the FCR, there was a

## Microbial Population in Ileum and Ceca

The effects of dietary supplementation with ACP on microbial populations in broiler ileum and ceca after 35 d are shown in Table 5. There were no significant differences in either lactic acid bacteria or coliform counts among all groups after 21 days. However, it is noteworthy that, compared to the control group, the coliform count in the ileum was decreased in both linear and quadratic manner by ACP supplementation, while only a linear effect was observed in the cecum coliform count.

**Table 5.** Effect of *Antrodia cinnamomea* powder supplemented in diet on lactic acid bacteria and coliform count in intestinal content of 21- and 35-day-old broilers.<sup>1</sup>

Parameter	Experimental diets				SEM <sup>2</sup>	P-value		
	Control	0.1% ACP	0.2% ACP	0.4% ACP		Linear	Quadratic	
D21								
Lactic acid bacteria		log cfu/g						
Ileum	8.32	9.06	9.06	8.55	0.16	0.08	0.74	
Cecum	11.00	11.38	11.27	11.48	0.12	0.47	0.56	
			Coliform					
Ileum	7.16	6.66	6.91	6.34	0.14	0.61	0.57	
Cecum	10.48	10.15	10.25	10.49	0.12	0.05	0.79	
D35								
Lactic acid bacteria		log cfu/g						
Ileum	9.26	9.34	9.60	9.43	0.08	0.56	0.21	
Cecum	13.67	13.27	13.77	13.67	0.13	0.36	0.35	
			Coliform					
Ileum	9.67	8.81	8.84	8.49	0.10	0.04	0.02	
Cecum	11.57	10.37	10.57	10.67	0.15	0.02	0.27	

<sup>1</sup>Results are provided as the means of 4 samples corresponding to 3 birds for the control group (corn-soybean meal diet) and 0.1, 0.2, and 0.4% ACP group (n = 4).

<sup>2</sup>SEM: standard error of the mean.

ACP: *Antrodia cinnamomea* powder

**Table 6.** Effects of *Antrodia cinnamomea* powder supplementation on the levels of superoxide dismutase (SOD) and catalase (CAT) in the serum in broilers aged 21 and 35 days.<sup>1</sup>

Items	Experimental diets				SEM <sup>2</sup>	P-value	
	Control	0.1% ACP	0.2% ACP	0.4% ACP		Linear	Quadratic
D21							
CAT (U/mL)	80.0	147.9	248.9	170.7	10.02	0.005	<.0001
SOD (U/mL)	58.3	97.7	111.1	109.0	2.84	<.0001	<.0001
D35							
CAT (U/mL)	151.8	210.7	258.4	201.1	10.23	0.009	0.012
SOD (U/mL)	82.8	113.5	108.4	111.3	1.46	<.0001	0.001

<sup>1</sup>Value is provided as the means of 3 samples (n = 5).

<sup>2</sup>SEM: standard error of the mean.

ACP: *Antrodia cinnamomea* powder.

## Serum Antioxidant Enzyme Assessment

Table 6 shows supplementation of ACP in broiler diets on levels of SOD and CAT activities at 21 and 35 d of age. There were linear and quadratic effects in SOD activity of birds fed diets containing either supplementation dose, opposing to the corresponding control group at both 21 and 35 days. In addition, the CAT activity of groups having ACP addition showed the same manner of elevation at 21 d ( $P < 0.001$ ). As for the CAT activity of birds at 35 d, chickens in the 0.2% ACP group showed the highest activity, followed by 0.1 and 0.4% ACP ( $P < 0.05$ ). Overall, both SOD and CAT activity in chicken serum were significantly increased as diets were supplemented with ACP, as compared to birds fed the control diet.

## Cell Viability Assay Against 2,2' Azobis (2-amidinopropane) Dihydrochloride and Lipopolysaccharide in Vivo

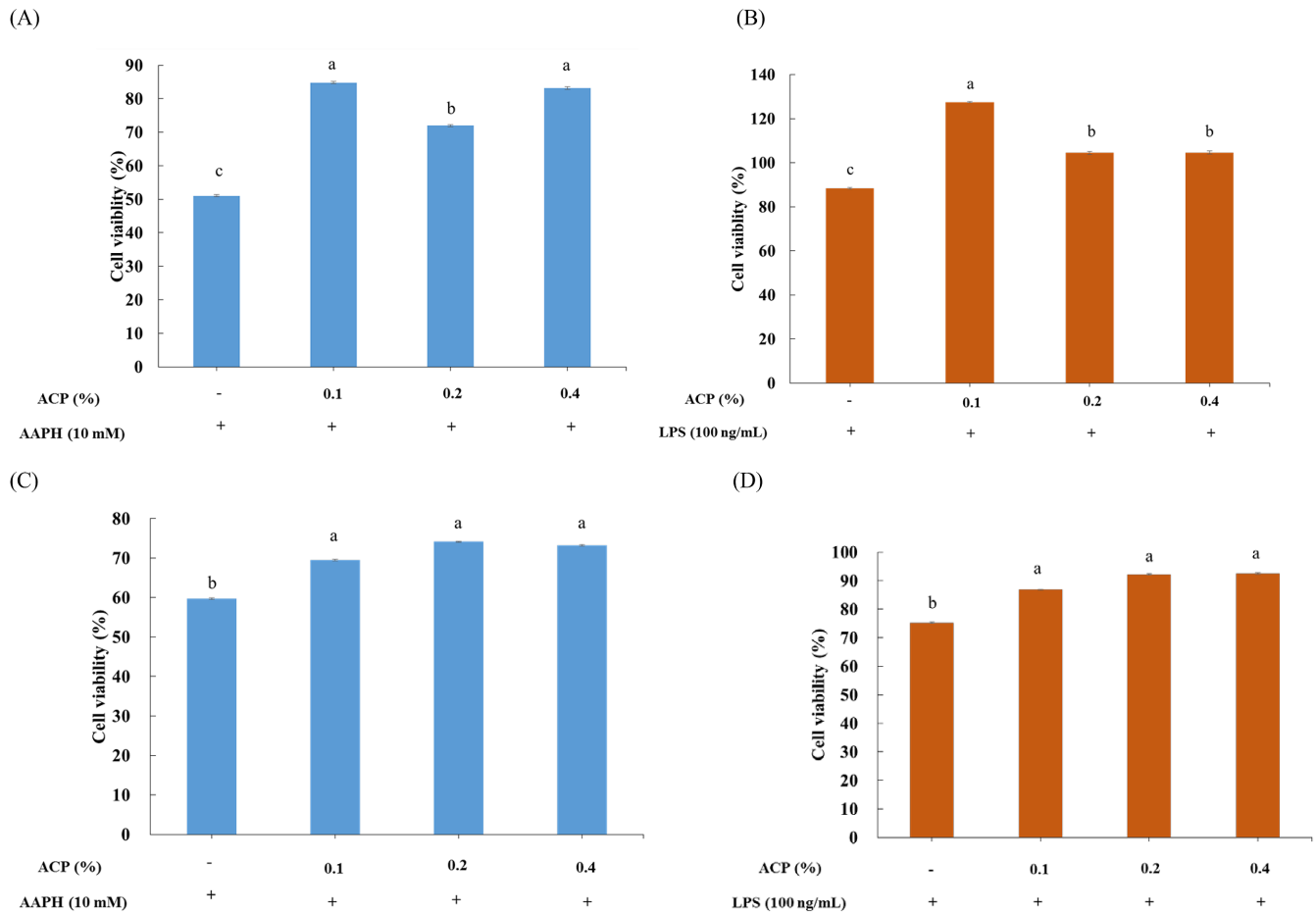
As shown in Fig. 3 (A), AAPH-induced reduction in cell viability (50%) was significantly inhibited in the PBMC of birds that received 0.1, 0.2, or 0.4% ACP supplementation. Cell viability of nearly 85% was

observed in both the 0.1 and 0.4% ACP treatment groups after 21 days. Moreover, the viability ratios of cells challenged by 100 ng/mL LPS were improved in the ACP-supplemented groups; chicken PBMC exposed to LPS at a dosage of 100 ng/mL exhibited above 100% and a nearly 90% viability ratio [Fig. 3 (B)]. The results in chickens after 35 d of age showed similar patterns in both AAPH and LPS treatments [Fig. 3 (C) and (D)]. Despite suffering either challenge, PBMC in birds fed diets containing ACP at all concentrations exhibited higher cell viability than those that received the conventional diet ( $P < 0.05$ ). These results indicate that treatment with ACP confers a significant protective effect against AAPH- or LPS-induced cell death in chickens.

## Antioxidant and Inflammatory Gene Expression in Chicken PBMC

The mRNA expressions of antioxidant (Fig. 4 A-F) and inflammatory genes (Fig. 4. G-K) in chickens were examined at 21 and 35 d of age; the results are shown in Fig. 4. In general, the antioxidant genes were up-regulated in the PBMC of chickens that received APC-supplemented diets. The expression of Nrf2 gene, which





**Figure 3.** Effects of dietary supplementation of *Antrodia cinnamomea* powder (ACP) on the viability of AAPH and LPS induced peripheral blood mononuclear cells (PBMC) in 21- (A-B) and 35-day-old chickens (C-D). The value is provided as the means of 6 samples ( $n = 6$ ). ACP: *Antrodia cinnamomea* powder. <sup>a-c</sup> Means among groups without the same superscript letter are significantly different ( $P < 0.05$ ).

controls the antioxidant signaling pathway, was significantly elevated at d 35 (Fig. 4 A), similar to the pattern of heme oxygenase 1 (**HO-1**) (Fig. 4 B). Glutamate-cysteine ligase catalytic subunit (**GCLC**) (Fig. 4 C), one of the downstream targets of the Nrf2 antioxidant pathway, was significantly higher at d 21 in the 0.2% APC treatment group. A similar increase was found after 35 d in the 0.4% APC group, though no significant differences were observed in either the 0.1% ACP group or the control. Similar phenomena were found in the SOD mRNA (Fig. 4 D). ROS modulator 1 (**Romo1**) and NADPH oxidase 1 (**Nox1**) are 2 modulators that promote ROS production; the expression of Nox1 was significantly lower in the 0.2% ACP group on both sampling d (d 21 and 35). The other ACP-supplemented groups also demonstrated lower Nox1 levels than the control group. Similarly, both 0.2 and 0.4% ACP supplemented groups exhibited decreased Romo1 levels compared to birds in the control group.

NF- $\kappa$ B (Fig. 4 G), the counterpart to Nrf2 that dominates the inflammatory pathway, was significantly down-regulated by the addition of 0.2% ACP at 21 and 35 d compared to the control group; birds that received 0.1% ACP showed a similar decrease at 35 d as well. Interleukin 1 beta (**IL-1 $\beta$** ) (Fig. 4 H) and

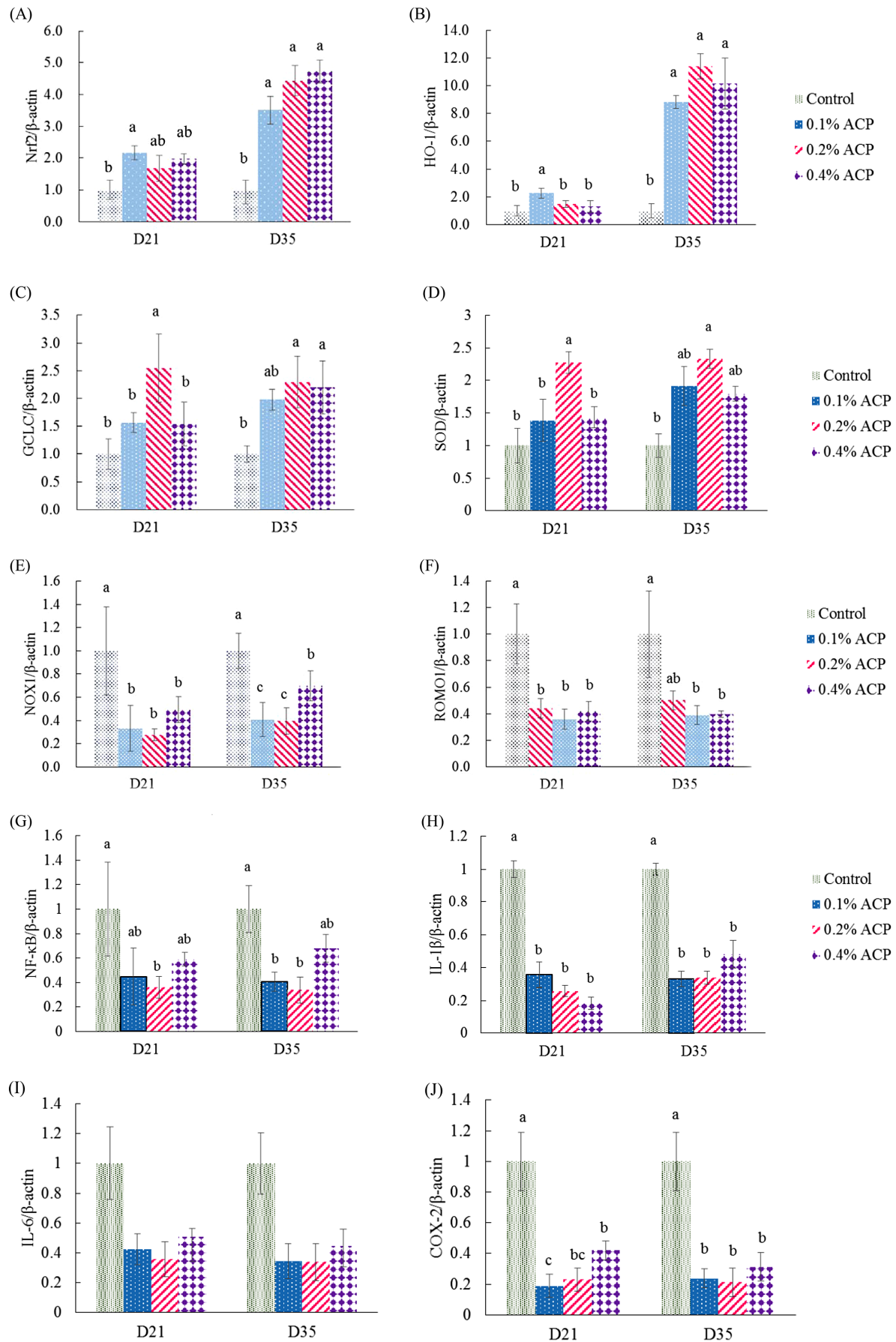
Interleukin 6 (**IL-6**) (Fig. 4 I) were selected to assess the downstream chain effects of the NF- $\kappa$ B pathway. Both were significantly decreased in the groups that received ACP supplementation. Moreover, cyclooxygenase 2 (**COX-2**) levels (Fig. 4 J) also were decreased, suggesting that ACP inhibited the expression of inflammatory-related genes.

### Transcription Factor-Nrf2 and NF- $\kappa$ B Protein Expression

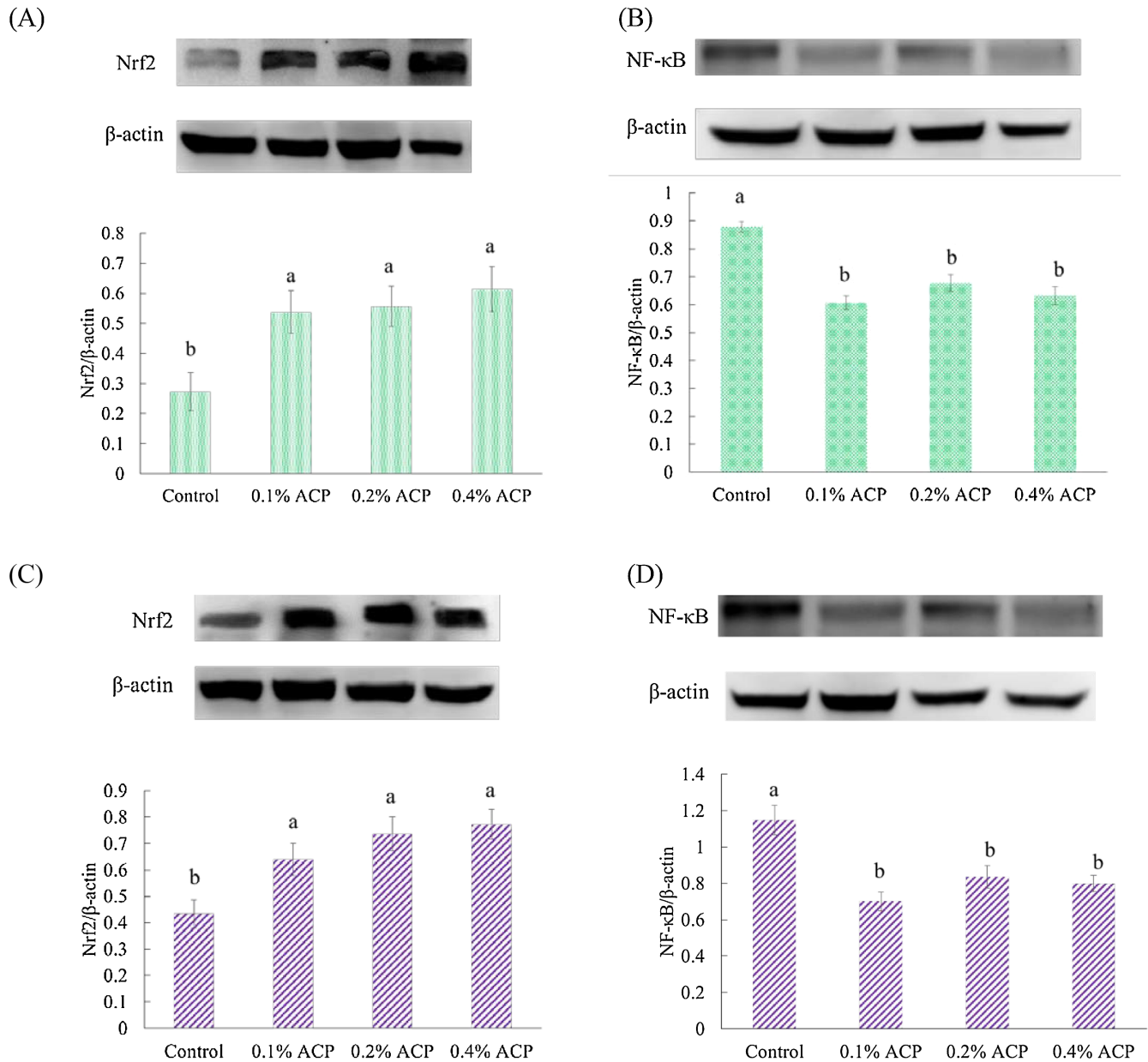
Fig. 5 illustrates the levels of the transcription factors Nrf2 (A and C) and NF- $\kappa$ B (B and D) in chicken liver. All of the ACP-supplemented groups showed significantly higher Nrf2 expression, whereas NF- $\kappa$ B was inhibited by ACP supplementation.

## DISCUSSION

*A. cinnamomea* has been extensively studied for its potential benefits, especially its immunomodulatory and antioxidant effects. Its toxicity in mammals also has been examined in order to evaluate its functional application to human food and health. This



**Figure 4.** Effects of dietary supplementation of *Antrodia cinnamomea* powder (ACP) on the mRNA expression levels of selected genes in the chicken PBMCs at 21 and 35 days. ACP: *Antrodia cinnamomea* powder. <sup>a-c</sup> Means among groups without the same letter within the same sampling d are significantly different ( $P < 0.05$ ). The value is provided as the means of 6 samples ( $n = 6$ ).



**Figure 5.** Effects of dietary supplementation of *Antrodia cinnamomea* powder (ACP) on the expression of Nrf2 and NF- $\kappa$ B proteins in the liver of 21- (A and B) and 35-day-old (C and D) chickens. The graph represents the mean  $\pm$  standard error from 3 independent experiments. ACP: *Antrodia cinnamomea* powder. <sup>a-b</sup> Means among groups without the same superscript letter are significantly different ( $P < 0.05$ ).

led to the hypothesis that chickens would respond in a similar way as mammals to *A. cinnamomea*, and those well-established pathways could help in detecting and clarifying the molecular changes in the anti-inflammatory and the antioxidant pathways in chickens along with their effects on performance and related indices. As our results show in Table 2, the levels of crude triterpenoids, crude polysaccharides, and total phenols were detected in the present study. Song and Yen (2002) evaluated these 3 functional components in both culture medium (dry matter) and fermented *A. cinnamomea* broth. None of the 3 targets was detected in the culture medium; in contrast, the yields of crude triterpenoid, polysaccharides, and total phenols were 67 mg/g, 23.2%, and 47 mg/g in the

fermented broth, respectively, suggesting that during its growth period, *A. cinnamomea* metabolizes the culture medium and further releases active components. Moreover, they tested the antioxidant activity of these components, demonstrating a correlation between the antioxidant activity of *A. cinnamomea* and its polyphenol, triterpenoid, and polysaccharide contents. In the current study, the antioxidant properties of ACP in terms of ferrous ion chelating (Fig. 1 A) and DPPH free-radical scavenging abilities (Fig. 1 B) were analyzed. The scavenging effects of ACP on DPPH free radicals reached the maximum at 80 mg/mL, while its chelating ability transcended EDTA. Most of the previous literature employed organic solvents for extracting the lipophilic constituents of *A. cinnamomea* to

evaluate its antioxidant effects. Mau et al. (2003) reported that at 0.5 mg/mL, the scavenging effects of methanolic extracts from *A. cinnamomea* mycelia were comparable to those of butylated hydroxyanisole (BHA) and  $\alpha$ -tocopherol (about 93%), while extracts from white mycelia were 97.1% at 5.0 mg/mL (Huang et al., 1999). In addition, methanolic extracts in Mau's study showed a high percentage (92.3%) of chelating effects on ferrous ions at a relatively low concentration (10 mg/mL) (Mau et al., 2003). That said, water would be a preferable solvent from the point of view of both safety and accuracy while investigating its potential action inside the animal body. Our findings suggest that the antioxidant actions, in terms of ferrous ion chelating and DPPH free-radical scavenging abilities, demonstrated by the bioactive components, triterpenoids, polysaccharides, and polyphenols, were potentially exerted in vitro.

Few studies have evaluated the promising effects of *A. cinnamomea* on chickens. In our study, while no significant was found among groups in FCR, the approximate 7% increment in the corresponding control group as compared to other treatment groups during d 22 to 35 and 1 to 35 would be significant if more replicates had been used. Additionally, all of the ACP-supplemented groups exhibited higher weight gain than did those in the control group, suggesting that minimal supplementation with ACP (0.1%) could significantly improve weight gain in chickens. It is noticeable that the feed consumption showed an increased tendency, even though not significantly different from each other. Liu et al. (2017a) employed a mouse model to investigate the potentially ameliorating effects of *A. cinnamomea* on alcohol-challenged mice. During the two-week experimental period, the body weight of the AC-treated group increased up to 10%, compared to the alcohol-only control group. This effect was even more significant in mice treated with silymarin, a putative hepatoprotective pharmaceutical agent. In addition, Liu et al. (2017b) reported the anti-fatigue effect of *A. cinnamomea* cultured mycelium by its antioxidant effect through preventing ROS to damage body tissues. In our case, this theory may be supposed by the slightly increased feed intake of the broilers supplemented with ACP, since broilers performed actively to consume feed rather than resting. FCR was slightly improved but not significantly affected by ACP supplementation because of the simultaneous increment of feed consumption and body weight gain. It is noteworthy that the previous literature regarding AC proposed that diseases induced in the study are partially attributed to oxidative stress, implying promising mitigating effects of AC on oxidation and inflammation.

Oxidative stress, associated with various factors in the commercial poultry industry, has become a critical issue for research in recent yr given its potential to suppress livestock production (Sahin et al., 2010; Surai, 2016). Moreover, inflammation is thought to be pertinent, since its byproducts contribute to oxidative stress

(Zhang et al., 2012). Accordingly, biomarkers of oxidative stress and immunomodulation can be exploited to follow the concurrent progression of oxidation and inflammation (Zhang et al., 2012). For instance, it has been demonstrated that a compromised immune response due to stress jeopardizes the antimicrobial resistance of animals (El-Lethey et al., 2003). An in vitro antimicrobial assay using fresh chicken feces as a substrate was conducted. The decreased coliform counts in all ACP-supplemented groups, along with increased lactobacilli counts in the 100 and 200 mg/mL treatments, imply the possible utilization and elimination actions of components in ACP. Several polysaccharides from mushrooms have been used as immune enhancers, exhibiting antibacterial, antiviral, and even antiparasitic activities in chickens (Guo et al., 2003). Guo et al. (2003) used chicken cecal content as inoculum to investigate the in vitro fermentation characteristics of polysaccharides from different mushrooms and herbs. In their subsequent research in 2004, they confirmed the correlation between the fermentation of polysaccharides and a shift in the outcome of the cecal microbial community of chickens. Combined with previous studies, these findings demonstrate that certain bacteria in the large intestine hydrolyze large molecular carbohydrates, namely, polysaccharides, releasing small molecular weight carbohydrates and further fermenting them, resulting in greater bacterial numbers. Furthermore, the fermentation end products, such as short-chain fatty acids (SCFA), can eliminate harmful bacteria thereby stimulating beneficial bacteria (Guo et al., 2004). Interestingly, previous literature had proposed the potential toxic action of SCFA on bacteria. As small and uncharged nonionized acids freely enter the bacterial membrane, they dissociate and hence accumulate protons and SCFA anions, leading to the dissipation of a proton motive force that compromises metabolic reactions (Roe et al., 2002) and alters osmotic balance, impacting cellular physiology (Roe et al., 1998). Moreover, SCFA, like butyrate, had been reported to inhibit NF- $\kappa$ B activation and its downstream proinflammatory molecules in a LPS-treated macrophage cell line (Meijer et al., 2010). This could potentially explain the decreased coliform count observed in the ACP-supplemented groups (0.1, 0.2, and 0.4%) at 35 d of age, as seen in Table 5.

Cha et al. (2009) used methanol and hot water to extract *A. cinnamomea* and study its effects on the antioxidant capacity, in terms of SOD and CAT activities, of rat liver. Their results showed improvement in both SOD (from 7.1 to 18.9 U/mg protein) and CAT (from 110.5 to 125.3 KU/mg protein) activity, with the SOD activity even higher than silymarin (18.9 vs. 16.8 U/mg protein), the agent for hepatoprotection being used as a positive control in this instance. Wen et al. (2011) evaluated the in vivo anti-inflammatory activity of the methanol-extracted liquid cultured mycelia of *A. cinnamomea* (MEMAC) by measuring SOD and CAT activity in the livers of mice with carrageenan-induced

hind paw edema, a model of acute inflammation. Similar to other studies, the SOD and CAT activities that were suppressed after carrageenan injection improved significantly following administration with MEMAC in a dose-response manner.

Since oxidative stress is associated not only with the increased production of free radicals, but also with fluctuations in the scavenging capacity of antioxidant systems (Akbarian et al., 2015), the vital role of the antioxidant enzymes are key to maintaining the redox balance (Aluwong et al., 2013; Akbarian et al., 2015). The first line of antioxidant enzymatic defense includes SOD and CAT; SOD is in charge of catalyzing the dismutation of superoxide radicals to hydrogen-peroxide and oxygen, while CAT is regarded as the main ROS enzymatic scavenger that breaks down the hydrogen-peroxide into water and oxygen (Aluwong et al., 2013). In comparison with the control group, dietary supplementation with ACP elevates the activities of both enzymes. Nutrition plays a crucial role in maintaining the redox balance (Aluwong et al., 2013), suggesting the presence of antioxidants in the diet would determine the activity of these antioxidant enzymes. The antioxidant properties detected in our own study, as mentioned above, may support this hypothesis.

The cell's integrity and its tolerance toward oxidative stress have been addressed in previous literature. The improved antioxidant enzymatic activities in serum also could have been checked by the tolerance of the cell isolated from blood, under specific adverse conditions (Nunes et al., 2005). AAPH, an extensively studied generator of free radicals, causes various types of pathological changes through cellular oxidative damage (He et al., 2013), while LPS has been used as an effective model for studying cellular inflammation (Nunes et al., 2005). We therefore applied these agents to investigate the tolerance of chicken PBMC. Previous studies reported that AAPH treatment decreased the viability of various cells (Kusumoto et al., 2010). In the current study, the viability of PBMC exposed to AAPH increased in chickens whose diet included ACP at either 21 or 35 d of age. Gokila Vani et al. (2013) also showed that AAPH-induced reduction in cell numbers (62%) was ameliorated by antcin C (95%), a compound isolated from *A. cinnamomea*. These results demonstrate that the cytoprotective effects of *A. cinnamomea* are partially attributed to the inhibition of intracellular ROS generation. The cell viability of LPS-treated PBMC was also higher in the ACP-supplemented groups at 21 and 35 d of age. Shie et al. (2016) used 4,7-dimethoxy-5-methyl-1,3-benzodioxole (DMB), a benzenoid compound isolated from the mycelia of *A. cinnamomea*, to investigate its anti-inflammatory potential. Although no differences were found among LPS-challenged cells and cells treated with various concentrations of DMB in the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, the decreased production of pro-inflammatory molecules (such as NO, IL-1 $\beta$ , and tumor necrosis

factor- $\alpha$ ) in LPS-treated mouse macrophages demonstrated the anti-inflammatory effects of *A. cinnamomea*. Most previous studies used specific cell lines to identify the in vitro effects of selected materials on the cells (Gokila Vani et al., 2013; Liaw et al., 2013; Shie et al., 2016); others isolated cells and then tested the capacity of selected samples by stimulating the cells in vitro and comparing them to those challenged solely by an immunogen (Liu et al., 2010; Lin et al., 2015). In our study, in order to understand the protective effects of ACP, we isolated cells from the chickens after they had digested and absorbed the supplemented feed, and then the increase of viability of these cells was tested with a LPS challenge. The resulting improvement in cell tolerance, in terms of better cell viability when challenged by LPS, suggests that ACP may confer anti-inflammatory properties to chickens that consume it.

The above results provide a clear picture on ACP's potential protective effects against oxidation and immune-challenged conditions; however, these consequences may be the sum of several distinct mechanisms. Thanks to previous literature that examined the molecular mechanisms demonstrated by some cytoprotective materials, we can consider the components involved in these signal transduction pathways as potential targets for illuminating the sophisticated mechanisms underlying the final productive or physiological performance (Calabrese et al., 2008). The selected genes presented in Fig. 4 (A)-(D) are those representative of effective free-radical scavenging or antioxidant action. GCLC is the catalytic form of the rate-limiting enzyme in the GCL family, the first and most critical step in glutathione (GSH) synthesis. Given that GSH is the main non-protein thiol in animal cells responsible for antioxidant defense, a high expression level of GCLC would provide a powerful antioxidant weapon to battle oxidative factors. Accordingly, the higher GCLC expression in our results, as shown in Fig. 4 (C), implies an improved defense capacity compared to the control group. Among the targeted antioxidant genes, heme oxygenase (HO) is crucial in attenuating the overall production of ROS by degrading heme, producing carbon monoxide (CO), biliverdin/bilirubin, and releasing free iron with ferritin induction. These products all have their own actions related to oxidative species. It is widely accepted that the activation of HO-1 in response to the Nrf2/ARE pathway provides cells with a powerful means to counter the attack of ROS, thereby conferring an advantage to animals under various stress conditions (Sahin et al., 2012). Consequently, increased HO-1 expression (Fig. 4 B), especially at 35 d of age, not only confirms the elevation in Nrf2 activity, but also implies the potential contribution to improved body weight gain in chickens (Table 3). In contrast, Romo1 and Nox1 are regarded as positive regulators in intracellular ROS production (Shin et al., 2013; Zanetti et al., 2014). It has been proven that increased Romo1 expression, in response to external stress, enhances cellular ROS levels; its expression is also essential for cancer cell proliferation

(Lee et al., 2015; Swarnabala et al., 2015). Nox1 is one of the vital components of the enzymes superoxide and hydrogen peroxide, both of which are members of ROS. Therefore, the amelioration of these 2 gene expressions in the current study (Fig. 4 D and E) was in line with the activation of Nrf2 and its downstream effects.

The NF- $\kappa$ B pathway has long been deemed a prototypical pro-inflammatory signaling pathway considering its role in the expression of pro-inflammatory genes such as cytokines (Fujioka et al., 2004). By activating and regulating other cells and tissues, cytokines play a key role in the immune and inflammatory response (Wigley and Kaiser, 2003). It is reported that cytokines have great potential for controlling infectious disease in poultry. For instance, IL-1 $\beta$  is highly inflammatory, owing to its main function in activating the immune system in an acute phase response. The marked increase in IL-1 $\beta$  levels after LPS treatment were suppressed by treatment with DMB in a study conducted by Shie et al. (2016). Similarly, despite no extra stimuli to challenge the cells, ACP treatment may ameliorate the inflammatory response in the normal rearing field, which can be supported by IL-18, another cytokine responsible for the activation of interferon gamma (Wigley and Kaiser, 2003). COX is an enzyme responsible for converting arachidonic acid from cell membranes into prostaglandin. COX1 is a constitutively expressed isoform of COX. While COX2 is normally expressed only at low levels under basal conditions, it is markedly induced by pro-inflammatory stimuli under stressful conditions. COX2 therefore may be regarded as one of the important indices for the stress level of an organism (Crofford et al., 1997). Based on the above, it is assumed that decreased NF- $\kappa$ B activity resulted in lower levels of pro-inflammatory cytokines, which further led to a relatively low expression of COX2, representing a more steady immune condition for chickens supplemented with ACP.

It is noteworthy that Nrf2 activation and NF- $\kappa$ B amelioration were found in chickens fed an ACP-supplemented diet. Several studies have addressed the possible crosstalk between these 2 transcription factors. As a counterpart of NF- $\kappa$ B, Nrf2 is involved in cytoprotection against inflammation (Cardozo et al., 2013). More specifically, it has been demonstrated that the cycle of oxidative stress and overproduction of pro-inflammatory cytokines can be disrupted by the activation of the Nrf2/ARE signaling pathway. Once the NF- $\kappa$ B pathway is activated by oxidative stress, cytokines are produced in excess, inducing even more oxidative stress in the cells. Furthermore, the ROS generated would further prompt the activation of NF- $\kappa$ B, resulting in an inflammatory activation loop (Gessner et al., 2013). Compounds that have the ability to activate the Nrf2 pathway are therefore capable of down-regulating the overproduction of pro-inflammatory cytokines, which corresponds to our own results. Moreover, COX2 suppression is attributed mainly to the induced HO-1 expression that results

from Nrf2 activation, namely, blocking NF- $\kappa$ B activation (Kim et al., 2010). Shie et al. (2016) measured several known inflammatory-related targets to elucidate the mechanism underlying the anti-inflammatory effects of DMB. Notably, the increased production of pro-inflammatory molecules, such as IL1 $\beta$ , NO, and TNF- $\alpha$ , and the protein levels of COX2, via NF- $\kappa$ B activation after LPS-exposure, were suppressed by DMB treatment, which was attributed to the induction of HO-1 expression.

In conclusion, the functional metabolites of ACP, such as triterpenoids, polysaccharides, and phenols, may contribute not only to potential antioxidant activity but also an improved immunomodulatory capacity in terms of an ideal microbial balance. Moreover, ACP is a potential activator of the Nrf2/ARE-dependent pathway, which possibly through promoting HO-1 expression, antagonizes the NF- $\kappa$ B-dominated inflammatory signaling pathway. This process may therefore contribute to optimal body weight and enhanced antioxidant capacity and immunity of broiler chickens. A more comprehensive study is worth furthering not only for the immunological action in the corresponding tissue, but the exact interaction between immune and antioxidant pathways in chickens.

## ACKNOWLEDGMENTS

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