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Dietary Supplementation with Fermented *Radix astragalus-ginkgo* Leaves Improves Antioxidant Capacity and Meat Quality in Broilers

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Authors' Contribution
XZ and FC conceived and designed the study. ZS bred the broiler chicks and collected the samples. JC and GW analyzed the samples. ZZ analyzed the data and wrote the article. LZ assisted in manuscript preparation.

Key words
Fermentation, *Radix astragali-ginkgo* leaves, Antioxidant system, Growth, Meat quality.

ABSTRACT

The objective of this study was to compare the effect of non-fermented (NF) and fermented *Radix astragali-ginkgo* leaves (FR) products produced by fermentation using *Aspergillus niger* on growth, antioxidant capacity and meat quality of broilers. Three hundred day-old commercial Arbor Acres (AA) broiler chicks were randomly allocated into 5 dietary treatments including five variations: control group, fed with the basal diets; NF group, the addition of 3 g/kg NF products; FR1, FR2 and FR3 groups, addition of FR products 1 g/kg, 3 g/kg and 6 g/kg, respectively. There was no difference in body weight gain, feed intake and feed intake/gain ratio between treatments. Compared with the control group, the percentage of the abdominal fat, sarcous fat and lactic acid (LD) for FR groups were significantly decreased. Birds had significantly higher water holding capacity (WHC) when they were provided with the FR3 diet. Moreover, serum glutathione (GSH) and α-tocopherol (α-TOH) contents in the FR groups were increased, while, levels of total cholesterol (TC), malondialdehyde (MDA) in serum and hepatic reactive oxygen species (ROS) in FR groups were significantly decreased compared with the control or NF group at 42 d of age. Furthermore, serum total superoxide dismutase (T-SOD) activity of birds from group FR3 was significantly increased (42d) compared with NF group. Additionally, the expression of antioxidant enzyme genes including nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), SOD, and glutathione peroxidase (GPx) were improved by FR supplemented into the broiler diets. Based on these results, FR could be acted as a beneficial feed additive with antioxidant capacity and meat quality-improving effect in broiler diets.

INTRODUCTION

With the development of meat industry, considerable attention has been paid to the improvement of meat quality parameters. Meat producers consistently produce safe, healthy and tasty meat for consumers, accompanied by eliminating deteriorative phenomenon that negatively affects meat quality (Xia *et al*., 2017). As a major cause of meat deterioration (Asghar *et al*., 1988), lipid oxidation can produce toxic compounds, such as fatty acid peroxides, cholesterol hydroperoxide and reactive oxygen species (ROS) that adversely influence muscle oxidative stability (Grün *et al*., 2006). What is more, muscle oxidative stability is related to many aspects of meat quality that are represented by postmortem pH, flavour, color, water holding capacity (WHC), and nutritive value (Luciano *et al*., 2009; Karami *et al*., 2011). It decreases the shelf life of meat which leads to economic losses in the meat industry.

In order to improve meat quality and to maximize the oxidative stability of meat, antioxidants are added to feeds. Although synthetic antioxidants, for example, butylated hydroxytoluene and butyl hydroxy anisol have been widely used in the meat industry. The consumer concern over their safety and toxicity have initiated search for natural sources of antioxidants (Nuala *et al*., 2006). It has been found that plant polyphenolic flavonoids were one of the major groups of compounds acting as primary antioxidant free-radical terminators (Singh *et al*., 2005). Havsteen (2002)

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reported that, flavonoids in the cell membrane protect the unsaturated fatty acids against oxidants as ascorbate. These flavonoids work as a part of cellular antioxidant systems and in close cooperation with other cellular antioxidants, particularly the ascorbate and glutathione (GSH) systems.

Ginkgo biloba L. (Family: Ginkgoaceae) is a famous traditional herb drug in China, which has been used in many parts of the world showing high physiological activities in therapies for diseases (Pietri *et al*., 1997; Goh *et al*., 2003; Naik *et al*., 2006; Naik and Panda, 2007; Chen *et al*., 2011). China has had a large-scale production of Ginkgo leaves for years, about 40,000 t every year. In the last few years, large-scale cultivation of Ginkgo has been initiated (Zhang *et al*., 2012). Therefore, it is important to find a way to use this herbal resource as a feed ingredient and unveil its medicinal potential in the poultry feed industry. Chemically, the active constituents of *Ginkgo biloba* leaf are flavonoids (flavone glycosides, primarily composed of quercetin, kaempferol and isorhamnetin glycosides) and terpenoids fraction (ginkolides A, B, C, J and bilobalides) (Kleijnen and Knipschild, 1992; Abdel-Kader *et al*., 2007). The combination of flavonoids (such as quercetin), terpenoids (ginkgolides and bilobalide), and the organic acids contribute to the anti-oxidative properties and free radical scavenging activities of *Ginkgo biloba* (Le Bars *et al*., 1997). Up to the present, the assumption that the beneficial effects of *Ginkgo biloba* leaves are due to its free radical scavenging action has been shown in several studies *in vitro* (Maitra, *et al*., 1995; Smith and Luo, 2004) and *in vivo* (Smith and Luo, 2003; Sarikçioğlu *et al*., 2004; Shi *et al*., 2009). Moreover, extract of *Ginkgo biloba* leaf has been found to be more effective than water-soluble antioxidants (ascorbic acid, glutathione and uric acid), and as effective as lipid-soluble antioxidants (alpha-tocopherol and retinol acetate) in reducing H_2O_2-induced oxidative stress in human erythrocytes (Kőse and Doğan, 1995). However, there are also considerations with regard to the seasonal variations and storage conditions of the leaves (Ellnain-Wojtaszek *et al*., 2002).

Radix astragali (RA) is the dried root of *Astragalus membranaceus Bge. Var. mongholicus* (Huangqi), which is the most popular health-promoting herb in China (Yan *et al*., 2010). The main constituents of RA roots are polysaccharides, saponins, flavonoids, amino acids, and trace elements (Ma *et al*., 2002). Therefore, it is important to find out a way to utilize resource of *Radix astragali-ginkgo* leaves as feed ingredient and unveil its potential economic value in feed industry. Toward promoting processing of *ginkgo* leaves, in this study, a process for *Aspergillus niger* fermentation was developed wherein the functionality of *Radix astragali-ginkgo* leaves is preserved and enhanced, with the aim of investigating the effect of FR on the growth performance, antioxidant capacity, meat quality and plasma biochemical parameters of broiler chicks, and its comparison with the non-fermented (NF) products.

MATERIALS AND METHODS

Culturing of Aspergillus niger

The *Aspergillus niger* used in this study was a laboratory strain obtained from the College of Chemical Engineering, Nanjing Forest University, Nanjing, Jiangsu, China. It was cultured by an agar plating technique using Sabouraud dextrose agar (Oxoid Ltd., Basingstoke, UK) and incubated at 24°C for 7 d. *Aspergillus niger* spores were harvested by tapping the top of the plate when turned upside down. Spore counts were determined using a hematocytometer according to the Fuchs-Rosenthal technique to be approximately 4.0×10^6 spores, which were equivalent to 0.25 g.

Preparation of fermented ginkgo leaves sample

Comminuted (2.0 -mm sieve) dried *ginkgo* leaves picked in September (*ginkgo* garden for leaf use, Nanjing Forestry University, Jiangsu Province, P.R. China) and *Radix astragali* (purchased from Bozhou Company of Traditional Chinese Medicine, Anhui Province) were used for this study. Samples were divided into 2 lots after autoclave sterilization. One lot was untreated (no fermentation), and the other lot was fermented using *Aspergillus niger*. *Radix astragali-ginkgo* leaves were mixed in the mass based ratio of 1:4 (1 part *Radix astragali* to 4 parts *ginkgo* leaves). The fermentation medium contained 10 g solid medium (*Radix astragali-ginkgo* leaves : wheat bran : corncob = 8 : 1.5 : 0.5, mass : mass) and 16 mL nutritive salt (glucose : urea: $(NH_4)_2SO_4$: peptone : KH_2PO_4: $MgSO_4 \cdot 7H_2O$ = 4 : 2 : 6 : 1 : 4 : 1, mass : mass) and was inoculated with 1 g/kg of the spores of *Aspergillus niger*. The mixture was packed in a plastic container, gently firmed, and sealed with adhesive film before being kept in a room at ambient temperature (24°C). The sample was fermented for 48 h. Although *Aspergillus niger* is an aerobic organism, there would be a production phase under micro-aerobic conditions that existed in the closed container (David *et al*., 2003). The fermented sample was spread on a polythene sheet in a room at 30 to 40°C, dried for 6 d up to about 90% of the dry matter, and ground to pass through a 0.15-mm sieve. The changes of the ingredients before and after the fermentation are shown in Table I. Repetitious examination showed that the proportion of components in polysavone was constant within a minute range.

Table I.- Nutrient and amino acids composition of *Radix astragalis-ginkgo* leaves before and after fermentation[1].

***Radix astragali-ginkgo* leaves**	**Total flavonoids/mg of quercetin equivalents/g**	**Polysaccharides (%)**	**Protein (%)**	**Total amino acid (%)**	**Total ginkgolic acid (g/kg)**
Before	9.3	0.97	10.63	8.60	1.465
After	8.8	2.05	18.29	15.88	0.033
Increment	-0.5	1.08	7.66	7.28	-1.432
Concentration of amino acid (%)					
Indispensable	Before	After	Dispensable	Before	After
Lysine	0.422	0.515	Aspartate	0.882	1.134
Threonine	0.327	0.419	Serine	0.396	0.476
Leucine	0.499	0.652	Glutamic acid	1.260	1.505
Isoleucine	0.296	0.386	Alanine	0.436	0.564
Methionine	0.061	0.049	Cystine	0.034	0.036
Tryptophan	0.019	0.085	Valine	0.428	0.547
Phenylalanine	0.387	0.483	Proline	0.592	0.565
Tyrosine	0.202	0.265	Total	4.028	4.827
Histidine	0.366	0.353		Increment	0.799%
Glycine	0.585	0.665			
Arginine	0.407	0.489			
Total	3.571	4.361			
Increment	0.790%				

[1]Mean values. All samples were made in triplicate. The variation coefficient <2% batch to batch.

Experimental animal and feeding

A total of 300 healthy, day-old commercial Arbor Acres (AA) broiler chicks, obtained from a local commercial hatchery (Haian, Jiangsu, China), were randomly allocated to 5 treatment groups consisting of 6 replicates of 10 birds each (male and female were equal). The average initial body weight was 52 g ± 3.8 g. The birds were fed a basal diet consisting of maize-soybean meal in which the respective experimental treatments, 3 g/kg NF products, 1 g/kg, 3 g/kg and 6 g/kg products, were included (control, NF, FR1, FR2 and FR3, respectively). All the diets (mash form) of the five treatments were formulated for starter (1 to 21 d) and grower (22 to 42 d) broiler growth periods (Table II) according to Nutrient Requirements of Poultry (NRC, 1994). Birds in the experimental group received a diet supplemented with different NF or FR levels at the priority expense of wheat bran and posteriorly of maize, based on the control diet; other factors were the same as those in the control group as shown in Table II. Dietary total flavonoids and polysaccharides contents are shown in Table III.

All birds were placed in wire cages in a 3-level battery, each replicate was assigned to a cage (150 cm×100 cm×60 cm) of 10 chickens (0.15 m^2 per chick) and housed in an environmentally controlled room maintained at 34°C to 35°C for 5 d, and then gradually decreased to 24°C, after which it was maintained at room temperature and then kept constant. The light regimen was a 12 h light–dark cycle (06:00 to 18:00 h light) throughout the trial. Both feed and water were provided for *ad libitum* consumption. Fresh diets were prepared once a week and were stored in sealed bags at 4°C. The rearing period was 6 weeks.

Broiler growth performance responses such as body weight (BW), body weight gain (BWG), feed intake (FI) and feed intake/gain ratio (F/G) were determined on a weekly basis during the 6 experimental weeks. Mortality was recorded on a daily basis. Weekly FI per broiler was calculated on a pen basis by dividing the amount of weekly feed consumption (corrected for the feed consumed by the birds that died during the week) by the number of birds alive at the end of the week. For practical reasons performance data were presented on a growth period (*i.e.* starter and grower period) basis. In addition, overall BWG, FI and F/G were calculated and presented for the entire duration of the experiment.

The experimental design and procedures were approved by the Animal Care and Use Committee of Nanjing Forestry University following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals of China.

Table II.- Ingredients and nutrient composition of broiler diets on as-fed basis.

Ingredients (%)	Dietary treatments (1 to 21 d)[3]					Dietary treatments (22 to 42 d)[3]				
	Cont.	NF	FR1	FR2	FR3	Cont.	NF	FR1	FR2	FR3
Maize	60.34	60.34	60.34	60.34	60.24	64.68	64.68	64.68	64.68	64.48
Soybean meal (43%)	30.22	30.22	30.22	30.22	30.22	24.23	24.23	24.23	24.23	24.23
Maize gluten meal (60%)	2.9	2.9	2.9	2.9	2.9	3.8	3.8	3.8	3.8	3.8
Wheat bran	0.5	0.2	0.4	0.2	0	0.4	0.1	0.3	0.1	0
Non-fermented *Radix astragali-ginkgo* leaves	0	0.3	0	0	0	0	0.3	0	0	0
Fermented *Radix astragali-ginkgo* leaves	0	0	0.1	0.3	0.6	0	0	0.1	0.3	0.6
Lard	2.03	2.03	2.03	2.03	2.03	2.83	2.83	2.83	2.83	2.83
Limestone	1.1	1.1	1.1	1.1	1.1	1.27	1.27	1.27	1.27	1.27
Dicalcium phosphate	1.49	1.49	1.49	1.49	1.49	1.27	1.27	1.27	1.27	1.27
Sodium chloride	0.2	0.2	0.2	0.2	0.2	0.25	0.25	0.25	0.25	0.25
L-Lysine	0.07	0.07	0.07	0.07	0.07	0.16	0.16	0.16	0.16	0.16
DL-Methionine	0.15	0.15	0.15	0.15	0.15	0.11	0.11	0.11	0.11	0.11
Premix[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	100	100	100	100	100	100	100	100	100	100
Chemical composition[2]										
Metabolizable energy (MJ/kg)	12.3	12.3	12.3	12.3	12.3	12.6	12.6	12.6	12.6	12.6
Crude protein (%)	20.07	20.07	20.07	20.07	20.04	20.3	20.3	20.3	20.3	20.3
Calcium (%)	1.0	1.0	1.0	1.0	1.0	0.9	0.9	0.9	0.9	0.9
Available phosphorus (%)	0.45	0.45	0.45	0.45	0.45	0.4	0.4	0.4	0.4	0.4
Lysine (%)	1.13	1.13	1.13	1.13	1.13	1.0	1.0	1.0	1.0	1.0
Methionine (%)	0.5	0.5	0.5	0.5	0.5	0.44	0.44	0.44	0.44	0.44
Methionine + cystine (%)	0.82	0.82	0.82	0.82	0.82	0.74	0.74	0.74	0.74	0.74
Threonine	0.77	0.77	0.77	0.77	0.77	0.71	0.71	0.71	0.71	0.71

[1]Premix provided per kilogram of diet: transretinyl acetate, 30 mg; α-tocopherol acetate, 30 mg; cholecalciferol, 6 mg; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyidoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamin), 0.013 mg; Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 7.5 mg; Mn (from manganese sulfate), 110 mg; Zn (from zinc oxide), 65 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg; Bacitracin Zinc, 30 mg.
[2]Calculated based on analyzed values of individual feed ingredient except for energy which was based on published ME values.
[3]Cont. = Basal diet; NF = Basal diet with 3 g/kg non-fermented *Radix* astragali-*ginkgo* leaves; FR1, FR2 and FR3 = Basal diet with 1 g/kg, 3 g/kg and 6 g/kg, respectively, fermented *Radix* astragali-*ginkgo* leaves.

Table III.- Total flavonoids and polysaccharides contents in the diets[1] of broilers.

Dietary treatments	Total flavonoids/mg of quercetin equivalent/kg		Total polysaccharides/ mg/kg	
	1 to 21 d	22 to 42 d	1 to 21 d	22 to 42 d
Basal diet	2.85[d]	3.34[d]	1.51[d]	1.48[d]
Non-fermented *Radix* astragali-*ginkgo* leaves (3 g/kg)	19.82[b]	19.75[b]	20.34[c]	19.80[c]
Fermented *Radix astragali-ginkgo* leaves (1 g/kg)	9.11[c]	8.75[c]	21.75[c]	20.34[c]
Fermented *Radix astragali-ginkgo* leaves (3 g/kg)	17.46[b]	17.03[b]	44.14[b]	43.87[b]
Fermented *Radix astragali-ginkgo* leaves (6 g/kg)	29.65[a]	28.63[a]	63.71[a]	61.97[a]
Standard error of mean	1.91	1.83	4.40	4.32
***P*-values**				
ANOVA	0.001	0.001	0.001	0.001
Regression				
Linear	0.001	0.001	0.001	0.001
Quadratic	0.001	0.001	0.001	0.001

[a, b, c] Means having a common superscript in a sub-column do not vary significantly ($P > 0.05$).
[1]Six different samples from each diet were randomly selected and determined.

Sampling procedures

At 21 and 42 days of age, one bird was randomly selected from each replicate (6 birds per treatment, 3 males and 3 females) and weighed after feed deprivation for 12 h. Individual blood samples were taken via brachial vein and serum were separated by centrifugation at 350 *g* for 15 min and at 4°C. Serum samples were frozen at -20°C for further analyzing. At 42 day, after collection of blood samples, all birds were euthanized by exsanguination immediately. After that, samples of liver and muscle were rapidly excised, frozen in liquid nitrogen and stored at -80°C until body composition analysis. The carcasses were then necropsied and the abdominal fat pad were removed and weighed. Relative weight of abdominal fat was calculated as [weight (g)/BW (100g)].

Serum analysis

Plasma α-tocopherol (α-TOH) was analyzed as described by Kayden *et al*. (1973). The samples were saponified by mixing 1 mL serum with a 2% pyrogallol solution (5 mL) and heated for 2 min in a 70°C shaking water bath. The tubes were removed, and 0.25 mL of 11N KOH was added. The tubes were heated again in (shaking) 70°C water bath for 30 min, and then placed in an ice bath. Two milliliters of hexane (used to extract the VE) and 0.5 mL of water were added to the saponified samples and shaken vigorously for 2 min. One milliliter of the hexane layer was transferred to a 4 mL glass test tube for analysis. Standards of 1, 2, 4, 6, 8, and 10 μg/mL of α-TOH were prepared at the same time. A 0.2% bathophenanthroline solution (200 μL) was added to all the samples and standards and thoroughly mixed. Two hundred microliters of 1 mM $FeCl_3$ was added and samples were vortexed. After 1 min, 200 μL of an H_3PO_4 solution was added and vortexed again. The tubes were read on a spectrophotometer at 534 nm. The standard curve was used to calculate the concentration of α-TOH in each sample. The concentrations of α-TOH were expressed as μg/mL.

Determination of serum total cholesterol (TC), glutathione (GSH), malondialdehyde (MDA) concentrations, total superoxide dismutase (T-SOD) activities, and sarcous lactic acid (LD) were done using the corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China) according to the instructions of the manufacturer.

Hepatic mitochondria isolation and ROS determination

Hepatic mitochondria were prepared according to the method described by Bai *et al*. (2017). Namely, liver tissue was homogenized in ice-chilled Dounce homogenizers (1:10, w/v) using isolation buffer containing 10 mM MOPS pH 7.4, 250 mM sucrose, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, and 0.1% fatty acid-free BSA, and centrifuged at 1,000 × g for 5 min at 4°C. The supernatants were removed and the mitochondria-enriched pellets gently resuspended and washed with the isolation buffer, then the pellets obtained by centrifugation at 12,000 × *g* for 5 min. Mitochondria were lysed and the protein was measured using the Micro BCA protein assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturers' instructions.

The ROS concentrations in the liver mitochondria of broiler chickens were detected using a ROS assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturers' instructions. Briefly, the mitochondria were incubated with DCFH-DA (10 μM) and DNA stain Hoechst 33342 (10 mmol/L) at 37°C for 30 min. Then the DCFH fluorescence of the mitochondria was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm with a microplate fluorescence reader (BioTek Instruments Inc., Synergy™2). The results were expressed as the mean DCFH-DA fluorescence intensity over that of the control.

Quantitative real-time PCR analyses

Total RNA was obtained from the liver using Trizol Reagent (TaKaRa, Dalian, China) and then reverse-transcribed using a commercial kit (Perfect Real Time, SYBR® PrimeScript™, TaKaRa) following the instructions of the manufacturer. The mRNA expression levels of specific genes were quantified via real-time PCR, using SYBR®Premix Ex Taq™ II (Tli RNaseH Plus) and an ABI StepOnePlus™ 167 Real-Time PCR system (Applied Biosystems, Grand island, NY, USA).

Table IV.- Primer sequences used for Real-time PCR assay.

Name[1]	Sequence (5′→3′)[2]	Genbank Accession No.
β-Actin	TGCTGTGTTCCCATCTATCG	NM_205518.1
	TTGGTGACAATACCGTGTTCA	
Nrf2	GATGTCACCCTGCCCTTAG	NM_205117.1
	CTGCCACCATGTTATTCC	
HO-1	GGTCCCGAATGAATGCCCTTG	HM237181.1
	ACCGTTCTCCTGGCTCTTGG	
SOD	CCGGCTTGTCTGATGGAGAT	NM_205064.1
	TGCATCTTTTGGTCCACCGT	
GPx	GACCAACCCGCAGTACATCA	NM_001277853.1
	GAGGTGCGGGCTTTCCTTTA	

[1]Nuclear factor erythroid 2-related factor 2 (Nrf2); Heme oxygenase 1 (HO-1); Superoxide dismutase (SOD); Glutathione peroxidase (GPx). [2]Shown as forward primer followed by reverse primer.

The SYBR Green PCR reaction mixture consisted of 10 µL SYBR®Premix Ex Taq (2X), 0.4 µl of the forward and reverse primers, 0.4 µL of ROX reference dye (50X), 6.8 µL of ddH_2O and 2 µL of cDNA template. Each sample was amplified in triplicate. The fold-expression of each gene was calculated according to the $2^{-\Delta\Delta Ct}$ method (Bai *et al*., 2017), in which the β-Actin gene was used as an internal standard. The primer sequences used are given in Table IV.

Carcass and meat quality analysis

At 42 d, after all birds were killed by manual exsanguinations and manually eviscerated, the carcass and eviscerated carcass yield, abdominal fat (including fat around the gizzard), breast meat (including pectoralis major and pectoralis minor), and leg meat (including thigh and drumstick) were equally measured. Carcass yield and eviscerated carcass percentage was calculated as the ratio between the carcass or eviscerated carcass and live BW after fasting. The weight percentages of breast meat, leg meat, and abdominal fat were calculated as a percentage of eviscerated carcass weight.

Muscle samples were collected from the left side of the pectoralis major muscle for the determination of physico-chemical characteristics (water, protein, fat, water-holding capacity and pH) of the breast muscle.

The ultimate pH values (measurements done in triplicate) of the pectoralis muscles were measured 45 min postmortem, using a calibrated portable pH meter (HI9023, Hanna Instruments, Padova, Italy) equipped with an insertion glass electrode (FC 230B, Hanna Instruments). The meat samples were always measured at the same place for pH.

Water holding capacity was estimated by determining expressible juice using modification of the filter paper press method described by Wang *et al*. (2009). A raw meat sample weighing 1,000 mg was placed between 18 pieces of 11-cm-diameter filter paper and pressed at 35 kg for 5 min. Expressed juice was defined as the loss in weight after pressing and presented as a percentage of the initial weight of the original sample.

Proximate analyses of water, protein (nitrogen), and fat contents of the breast muscle were determined by AOAC (2000) methods. Nitrogen was determined using the Kjeltec Analyzer Unit (2300, Foss, Höganäs, Sweden) and crude lipid was determined by ether extraction using the Soxtec Auto Extraction Unit (2050, Foss, Tecator, Sweden). The amino acid composition of the NF and FR products was determined using an automatic amino acid analyzer L-8500 (Hitachi, Tokyo, Japan) after the samples were hydrolyzed in 6N HCl for 22 h at 110°C, as described in Gui *et al*. (2010). Gross energy content of diets was determined on a Parr 1281 adiabatic calorimeter (Moline, Illinois, USA). Apparent metabolisable energy (AME) content was corrected for nitrogen retention by assuming that weight gain consisted of 200 g protein/kg, that protein consisted of 160 g nitrogen/kg, and that the energy equivalent was 34.36 kJ/g nitrogen gained (Bourdillon *et al*., 1990).

Total flavonoids, polysaccharides and ginkgolic acids analysis

The contents of total flavonoids, polysaccharides and ginkgolic acids were analyzed according to our previous study (Yu *et al*., 2015; Zhang *et al*., 2015). The aluminumchloride colorimetric method described by Chang *et al*. (2002) and Verzelloni *et al*. (2007) was used to determine the total contents of flavonoids with some modifications. The concentration of total polysaccharides was determined by colorimetric method of phenol-suluric acid assay (Dubois *et al*., 1956) taking D-glucose as standard control. The content of total ginkgolic acids in *G. biloba* leaves was determined by HPLC. A Alltima C18 (4.6 mm × 250 mm, 5 microm) and the mobile phase of methanol and 1% acetic acid (90 : 10) were used, the flow rate was 1.0 mL × min ($^{-1}$), and the wavelength was 310 nm. The content was calculated with external standard method.

Statistical analysis

The effects of dietary treatment were determined by one-way analysis of variance (ANOVA, SAS 9.0) followed, where appropriate, by Tukey's comparison test. Percentage data and data which were identified as nonhomogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. The effect of supplementing graded concentrations of FR on various dependent parameters was analysed by regression analysis. Differences among treatments were separated using polynomial orthogonal contrasts to determine linear and quadratic responses. Values in the tables were means and pooled SEM. Statistical significance was determined at $P \leq 0.05$.

RESULTS

Growth performance

Table V showed that no differences ($P > 0.05$) occurred in BWG, FI and F/G among the treatments in the period of 1 to 21 d, 22 to 42 d and the overall period of 1 to 42 d. No differences were observed on the mortality during the whole period of 1 to 42 d ($P > 0.05$).

Table V.- Growth performance of broilers fed maize-basal diets supplemented with NF or FR products.

Items	Dietary treatments[1]					SEM[3]	n[4]	P-values		
	Cont.	NF	FR1	FR2	FR3			ANOVA	Regression	
									Linear	Quadratic
1 to 21 d										
BWG[2], kg	0.618	0.617	0.638	0.644	0.647	0.005	6	0.168	0.190	0.471
FI[2], g/bird/d	46.74	45.39	46.05	47.42	46.80	0.443	6	0.332	0.568	0.758
F/G[2]	1.588	1.545	1.516	1.546	1.519	0.093	6	0.099	0.494	0.738
22 to 42 d										
BWG[2], kg	1.470	1.456	1.517	1.521	1.531	0.015	6	0.422	0.094	0.188
FI[2], g/bird/d	135.88	133.21	136.61	138.52	137.97	0.904	6	0.234	0.469	0.750
F/G[2]	1.941	1.921	1.891	1.913	1.892	0.013	6	0.715	0.255	0.448
1 to 42 d										
BWG[2], kg	2.088	2.073	2.155	2.165	2.178	0.016	6	0.258	0.054	0.114
FI[2], g/bird/d	91.31	89.30	91.33	92.97	92.39	0.654	6	0.477	0.437	0.728
F/G[2]	1.837	1.809	1.780	1.804	1.782	0.012	6	0.629	0.304	0.439
Mortality rate (%)	2.69	2.56	2.42	2.05	2.03	0.08	6	0.565	0.648	0.865

[1]Cont. = Basal diet; NF = Basal diet with 3 g/kg non-fermented *Radix* astragali-*ginkgo* leaves; FR1, FR2 and FR3 = Basal diet with 1 g/kg, 3 g/kg and 6 g/kg, respectively, fermented *Radix* astragali-*ginkgo* leaves.
[2]BWG = body weight gain; FI = feed intake; F/G = feed intake/gain ratio.
[3]Standard error of mean.
[4]*n*= number of replicates per treatment.

Table VI.- Meat qualities of broilers fed maize-basal diets supplemented with NF or FR products.

Items	Dietary treatments[1]					SEM[4]	n[5]	P-values		
	Cont.	NF	FR1	FR2	FR3			ANOVA	Regression	
									Linear	Quadratic
Water (g/kg)	754.7	758.9	753.3	758.9	761.4	0.03	6	0.104	0.124	0.369
Protein (g/kg)	228.3	223.8	227.3	233.8	231.5	1.88	6	0.554	0.389	0.696
Fat (g/kg)	14.8^{a}	13.5^{ab}	12.2^{b}	12.5^{b}	12.7^{b}	0.30	6	0.010	0.064	0.007
WHC[2]	54.69^{b}	56.93^{a}	57.21^{a}	57.34^{a}	58.21^{a}	2.93	6	0.001	0.004	0.011
pH_{45min}	5.72^{c}	5.74^{c}	5.75^{c}	5.86^{b}	5.91^{a}	0.021	6	0.001	0.003	0.001
LD[2] (mmol/g of protein)	3.64^{a}	3.41^{ab}	3.19^{b}	3.17^{b}	3.12^{b}	0.016	6	0.046	0.075	0.058
Percentage of abdominal fat[3]	1.53^{a}	1.49^{ab}	1.40^{bc}	1.43^{bc}	1.37^{c}	0.019	6	0.017	0.095	0.006

[a, b, c] Means having a common superscript in a sub-column do not vary significantly (P >0.05).
[1]Cont. = Basal diet; NF = Basal diet with 3 g/kg non-fermented *Radix* astragali-*ginkgo* leaves; FR1, FR2 and FR3 = Basal diet with 1 g/kg, 3 g/kg and 6 g/kg, respectively, fermented *Radix* astragali-*ginkgo* leaves.
[2]WHC = water-holding capacity. LD = Lactic acid.
[3]Calculated as a percentage of eviscerated carcass weight.
[4]Standard error of mean.
[5]*n*= number of animals per treatment.

Meat quality

As seen in Table VI, there was no (P >0.05) difference between dietary treatments in terms of the contents of water and protein of the muscle. The contents of sarcous fat from chicks fed the diets FR1, FR2 and FR3 was lower (P <0.05) than those from birds fed the control diet. The WHC in group NF, FR1, FR2 and FR3 exerted a 4.10%, 4.61%, 4.85% and 6.44% increase (P <0.05) respectively, compared with the control group. While group FR2 (P <0.05) and FR3 (P <0.01) exhibited higher pH compared with the control, NF and FR1 group. In addition, group FR3 had higher (P <0.05) muscle pH than that of group FR2. While the contents of sarcous LD were decreased (P =0.046) by the inclusion of FR. Percentage of the abdominal fat of broilers for group FR1 and FR2 were decreased (P <0.05) by 8.47% and 6.51%, respectively, in comparison with the control.

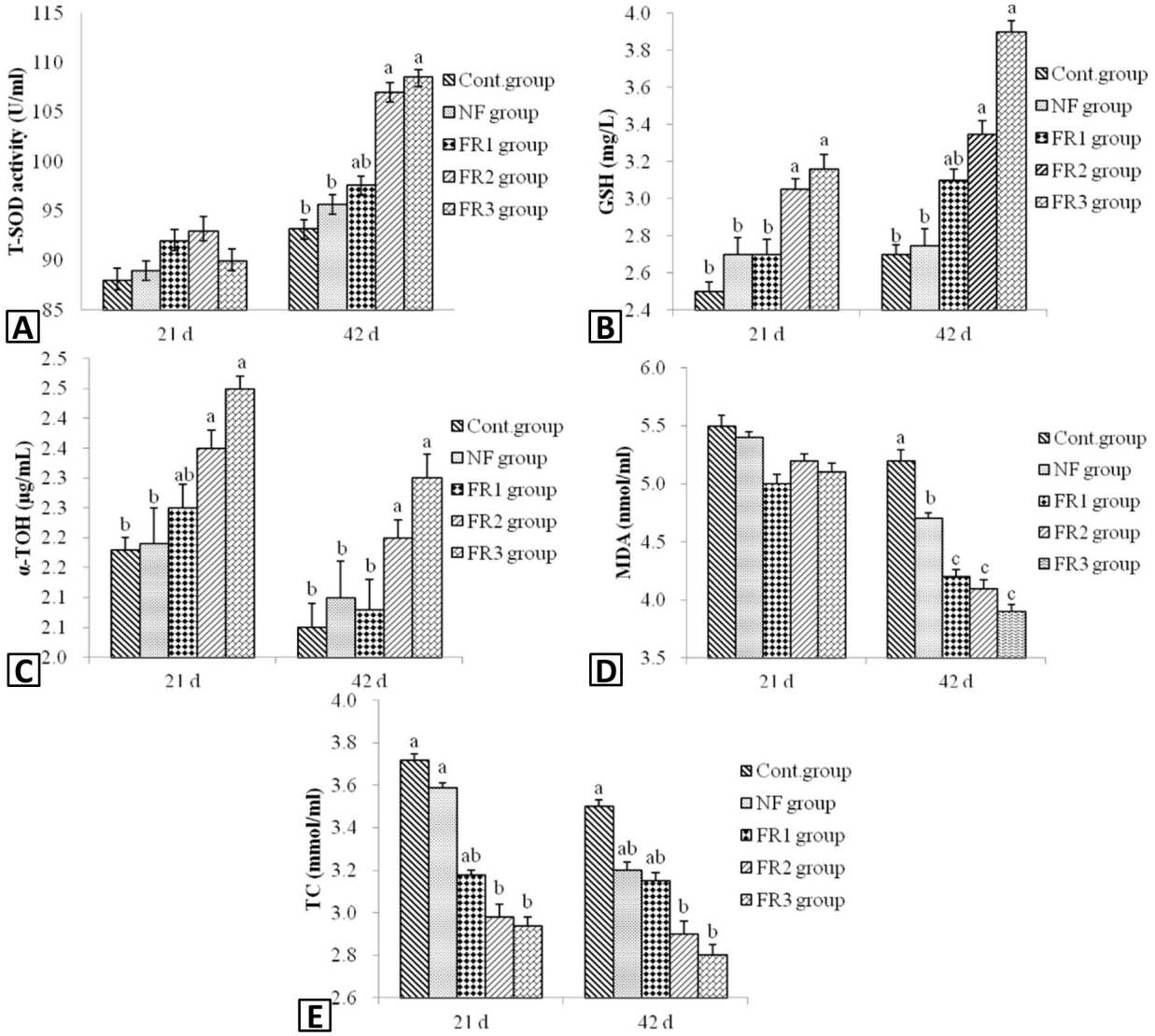

Fig. 1. Serum T-SOD activities (A), GSH (B), MDA (C) α-TOH (D) and TC concentrations (E) of broilers fed maize-basal diets supplemented with NF or FR products. [a, b, c] means with different letters differ significantly (P<0.05 or P<0.01), compared with the control group within the same time period.
[1]Cont. = Basal diet; NF=Basal diet with 3 g/kg non-fermented *Radix astragali-ginko* leaves; FR1, FR2 and FR3=Basal diet with 1 g/kg, 3 g/kg and 6 g/kg, respectively, fermented *Radix astragali-ginko* leaves.
[2]T-SOD, total superoxide dismutase; GSH, glutathione; MDA, malondialdehyde; TC, total cholesterol; α-TOH, α-tocopherol.
[3]n=6 (number of animals per treatment).

Also, group FR3 exerted the greatest effect, as it led to a decrease of 10.46% and 8.05% as compared to the control (P <0.05) or NF group (P <0.05), respectively.

Indicators in serum

The results of the T-SOD activities, concentrations of GSH, α-TOH, MDA, and TC in the serum are summarized in Figure 1. The T-SOD activities were significantly increased in FR2 and FR3 groups compared with that in the NF or control group at 42 days of age (P <0.05), and no difference (P >0.05) was observed for the T-SOD activities at 21 days of age, although there was an increasing tendency in the FR groups (Fig. 1A). Concentrations of GSH and α-TOH were significantly improved (P < 0.05) in groups FR2 and FR3 compared with that in NF and FR1 group, at 21 and 42 day of age (Fig. 1B, C). In addition, a significant increase (P <0.05) of serum GSH (21 d) and α-TOH (42 d) contents were observed in group FR2 and

FR3 compared with that in FR1 group.

Serum MDA levels did not differ among the treatments (P <0.05), showing a tendency to be decreased in the FR groups at 21 d (Fig. 1D), while MDA levels for group FR1, FR2 and FR3 at 42 d were decreased compared with the control (P <0.01) and NF group (P <0.05). A significant reduction (P <0.05) of serum TC concentration was observed in FR2 and FR3 groups (Fig. 1E) compared with that in control (21 d and 42 d) and NF group (42 d).

Hepatic mitochondria ROS and mRNA expression levels

Supplemental effects of dietary FR on hepatic mitochondria ROS and mRNA expression levels of antioxidant genes of broilers at 42 d are shown in Figures 2 and 3. The ROS contents in liver mitochondria (Fig. 2) were significantly decreased (P <0.05) in group FR2 and FR3 compared with that in the control, NF and FR1 groups.

The expression levels of antioxidant gene, including *Nrf2*, *HO-1* and *GPx* in broiler liver were significantly improved (P <0.05) in both FR2 and FR3 groups compared with that in control and NF group. Higher expression level of *HO-1* gene was noted in both FR2 and FR3 groups compared with that in group FR1. The expression level of SOD was significantly enhanced (P <0.05) in FR3 group compared with that in control and NF group.

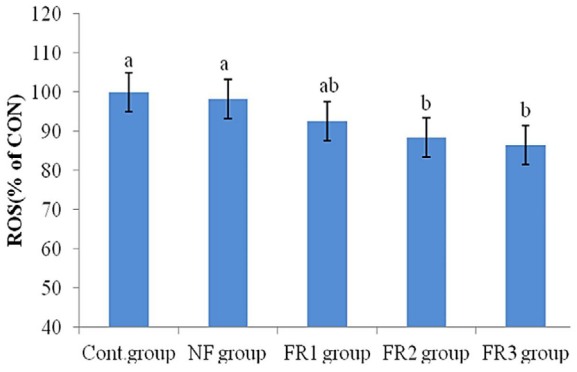

Fig. 2. Contents of hepatic mitochondria ROS of broilers fed maize-basal diets supplemented with NF and FR products.
[2]ROS, reactive oxygen species. For other statistical details, see Figure 1.

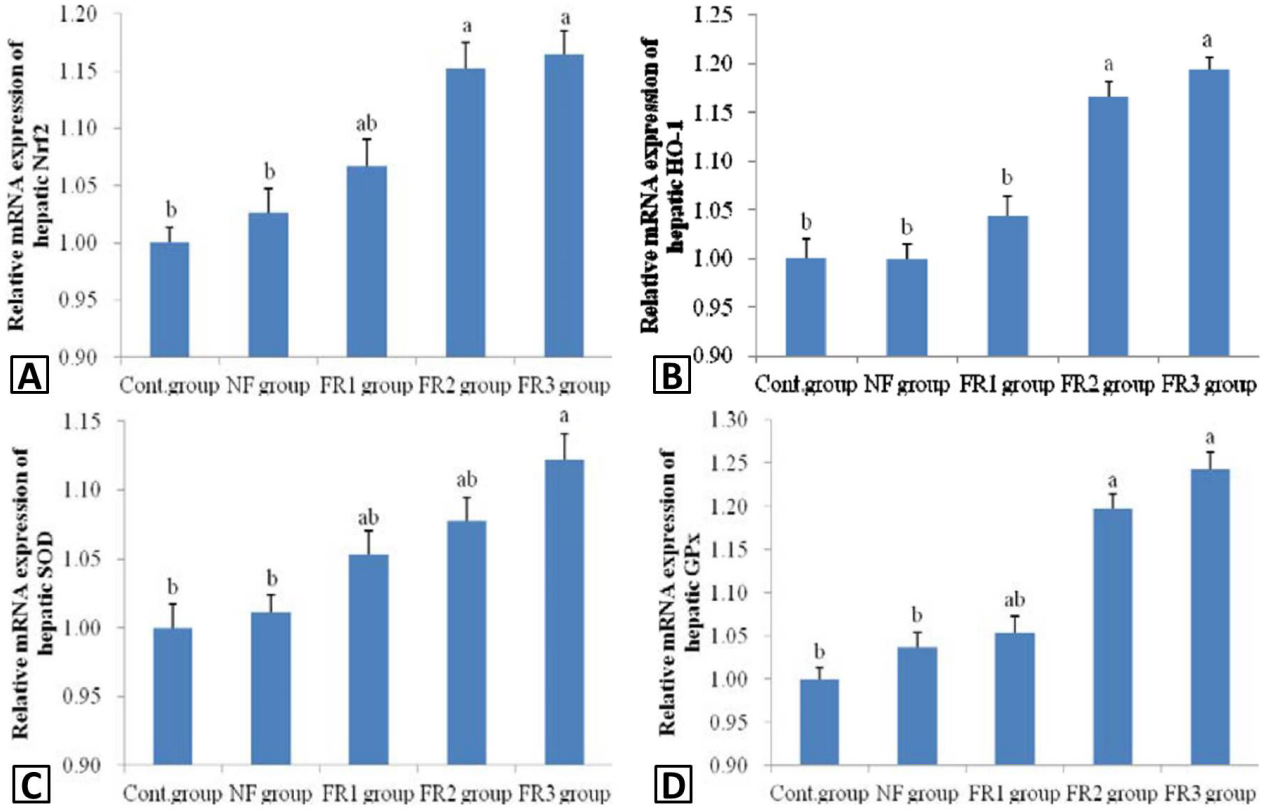

Fig. 3. Expression levels of antioxidant gene in liver of broilers fed maize-basal diets supplemented with NF or FR products.
[2]Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, Heme oxygenase 1; SOD, superoxide dismutase; GPx, glutathione peroxidase. For other statistical details, see Figure 1.

DISCUSSION

Microbial fermentation has been identified as a fruitful process for improving the health-promoting properties of medicinal plants. The fermentation process can alter the original bioactivities and nutritional composition of Chinese herbs (Yamamoto *et al*., 2007; Ng *et al*., 2011; Ahmed *et al*., 2016), reduce the anti-nutrient effects (Deacon, 2005), resulting in new treatment effects, and enhance the original treatment efficacy of active ingredients (Miyake *et al*., 2005; Dei *et al*., 2008) by the action of enzymes produced by bacteria, yeast and molds. The fermentation process does not require the use of chemicals and is easy to manage under farm conditions or on an industrial scale. Fermentation processes using *aspergilli* have been used to improve the nutritive value of some feedstuffs (Mathivanan *et al*., 2006; Yamamoto *et al*., 2007) for poultry. Our previous researches have confirmed that the use of up to 0.5% (in the starter phase) and 1.0% (in the grower phase) *Aspergillus niger*-fermented-*Ginkgo biloba* leaves (FR) products had a positive influence on growth performance, lipid metabolism, antioxidant capacity, breast meat quality, and fatty acid composition (Cao *et al*., 2012; Zhang *et al*., 2012).

In the present experiment, the increase of polysaccharides may be due in part to the decreased carbohydrate content after fermentation (Hong *et al*., 2004). In detail, *Aspergillus niger* is a fungus that has the capacity to produce enzymes such as cellulose, hemicellulases, β-glucosidase, hydrolases, pectinases, lipases, and tannases (Mathivanan *et al*., 2006; Dei *et al*., 2008). These enzymatic components act synergistically in the hydrolysis of 1, 4-β bonds within cellulose molecules, cellobiose and low-molecular-weight cellodextrins, thereby yielding glucose (Leschine, 1995). Total flavonoids contents of FR were found to be decreased slightly after fermentation, suggesting that β-glucosidase from *Aspergillus niger* might have converted the flavonoids glycones into aglycones, which has been proved to have greater activity than flavonoid glycones (Hsu and Chiang, 2009). In addition, ginkgolic acids have been recognized as hazardous compounds with suspected cytotoxic, allergenic, mutagenic and carcinogenic properties, and they were found to be decreased sharply after fermentation in the current study (Liu and Zeng, 2009; Yu *et al*., 2015).

There are several published works on the lipolytic activity of flavonoids in *vitro* and in mammals (Nakagawa *et al*., 2004; Hsu and Yen, 2007; Zarrouki *et al*., 2010). Previous studies suggested (Honda *et al*., 2009; Aoki *et al*., 2009) that flavonoid exerts potent antilipogenic effect by decreasing adipose tissue deposition and ameliorating diet-induced obesity in mice. Moreover, it was well documented that some flavonoids form insoluble complexes with cholesterol in the digesta and inhibit the intestinal absorption of endogenous and exogenous cholesterol (Rao and Gurfinkel, 2000). Jang *et al*. (2007) demonstrated that TC content was decreased by fermented ginseng culture in laying hens, and this effect was confirmed in the present study that serum TC contents was decreased by FR supplementation, as well as abdominal fat deposition and sarcous fat.

Lipid peroxidation is a natural phenomenon that has a significant effect on meat quality (Wood *et al*., 2004). It was reported that lipid peroxidation led to disruption of muscle cell membrane integrity, which may badly induce exudative loss from meat (Buckley *et al*., 1995). The lipid content is relatively high in broiler chickens, which tends to damage the body through producing ROS. The MDA content is the main end-products of lipid peroxides that generally used as an indicator of an increased oxidative stress in the body (Gaweł *et al*., 2004), which can endanger the safety of food (Fernandez *et al*., 1997). Numerous studies have documented that dietary natural antioxidant supplementation can improve meat quality by decreasing lipid peroxidation and improving antioxidative status (Zhao *et al*., 2018; Salami *et al*., 2016). It is well known that flavonoids act as antioxidants and free radical scavengers (Procházková *et al*., 2011). It also has been reported that administration of RA polysaccharides possess effective antioxidant potential, can significantly promote serum and hepatic antioxidant enzyme activities in chicks and rats and decrease lipid peroxidative levels (Xu *et al*., 2008; Yang *et al*., 2009; Sun and Wang, 2010; Yan *et al*., 2010; Deng and Hu, 2011). In our study, the increased GSH and decreased serum MDA and hepatic ROS may contribute to the potent antioxidative effect of increased dietary total polysaccharides and flavonoids by the modulation of the cellular free radical/antioxidant balance. In addition, elevated T-SOD activities and α-TOH contents were observed in the present study. This could be a sparing effect of dietary total polysaccharides and flavonoids, which also might be explained by a more general mechanism of reducing GSH and α-TOH consumption by reducing the burden of the enzymatic and non-enzymatic antioxidative system.

The Nrf2 binds to the antioxidant response element in the promoter regions of a number of genes encoding for antioxidative enzymes (Zhu *et al*., 2005). GSH related enzymes, HO-1, SOD and GPx play an important role in antioxidant system. HO-1 is the rate-limiting enzyme of heme decomposition reaction, and the reaction could produce endogenous carbon monoxide, biliverdin, and ferrous ion Fe^{2+} (Morse *et al*., 2009). Biliverdin can be converted to bilirubin under the influence of biliverdin

reductase. Biliverdin and bilirubin are the two most potential of endogenous antioxidants, which are involved in the composition of the defense system against ROS in the body (Chiu *et al*., 2002). As expected, our results indicated that supplemental of FR in broiler diets can enhance the antioxidant gene expression of Nrf2, HO-1, SOD, and GPx. We only measured the activities of T-SOD in this study, which was in accordance with the gene expression of SOD. So we can not judge the relationship between the expression of antioxidant enzymes-related genes and antioxidant enzymes activities. An in-depth study of the dietary FR effects on broiler antioxidant system is needed in the future.

In the present study, the results of improved pH and WHC of birds from FR groups may be attributed to a decreased LD production in muscles postmortem (Raj *et al*., 1990, 1992). Jiang *et al*. (2007) reported that the inability of muscle cells to rid themselves of metabolic by-products such as LD causes a decrease in pH, and this decrease in pH can affect WHC. Lower WHC indicated losses in the nutritional value through exudates that were released and this resulted in drier and tougher meat (Dabes, 2001). The present results showed that FR addition reduced the concentration of LD in breast muscles, which suggested that improved antioxidative status of broilers by FR supplementation may protect skeletal muscle cells against metabolic by-products such as LD. A previous study showed that improvements in WHC and pH development postmortem were due to increased antioxidative status in the chickens (Young *et al*., 2003). In the present study, the data showed that WHC is consistent with that of pH values. The improvement of pH and WHC must be related to the anti-oxidative status, enhanced antioxidative status and elevated α-TOH contents, and the sparing effect of dietary total polysaccharides and flavonoids may play a beneficial role. Therefore, the presence of supplemental FR would be expected to reduce meat oxidation and improve meat quality.

CONCLUSION

Considering the results obtained herein, it can be included that, the use of up to 6 g/kg FR products had a positive influence on meat quality and antioxidant capacity. Total flavonoids and polysaccharides were possibly the key compounds responsible for the health-improving effect of the fermentation products.

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Statement of conflict of interest

The authors declare no conflict of interest.

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