Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Oct - Dec 2016 / v.18 / n.4 / 663-668
http://dx.doi.org/10.1590/1806-9061-2016-0356

The Effects of Dietary Flavonoid Supplementation on the Antioxidant Status of Laying Hens

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■Keywords

Antioxidant, Flavonoid, Hesperidin, Naringin, Quercetin.

ABSTRACT

Ninety-six 28-week-old Lohmann White laying hens were utilized to test the antioxidant effects of flavonoids (hesperidin, naringin, and quercetin at 0.5 g/kg diet) during an 8-wk experimental period. At the end of the experiment blood samples were collected to determine total protein, cholesterol, and malondialdehyde (MDA) serum levels as well as activities of glutathione reductase (GR), glutathione peroxidase (GSH-Px), glutathione-*S*-transferase (GST), and superoxide dismutase (SOD) and level of glutathione (GSH) in erythrocyte lysates. Data were analyzed using one-way ANOVA. Naringin supplementation did not alter serum cholesterol concentration, whereas hesperidin and quercetin supplementations decreased serum cholesterol concentration. Naringin and quercetin supplementations did not affect serum protein concentration. All flavonoids decreased MDA concentration as well as increased GSH-Px, GR, GST, and SOD activities and GSH level, being quercetion superior to hesperidin and naringin. In conclusion, flavonoids, especially quercetin, exert antioxidant activity, which may help improve wellbeing when laying hens are exposed to stressors.

INTRODUCTION

Poultry meat and eggs are cheap and valuable foods for human nutrition. The demand for these foods is likely to increase as the global population increases (Florou-Paneri *et al.*, 2005). At a farm level, bird health is critical to maximize production efficiency (reducing mortality, eliminating diseases and treatment costs, and improving feed efficiency). Due to public health concerns regarding antibiotic resistance and residue issues, the use of antimicrobial agents to improve productivity has been banned (Ertaş *et al.*, 2005). Various alternative feed additives have been tested to improve the feed efficiency and to enhance the health status of poultry, and are expected to exert antimicrobial, antioxidant, and immune-enhancing activities and be retained in the poultry products (Abdel-Rahman *et al.*, 2013; Kamboh *et al.*, 2015).

Defense systems in the body that prevent damages caused by reactive oxygen species (ROS) during exposure to infections, inflammation, and stressors are called antioxidant defense systems. Antioxidants prevent lipid peroxidation by blocking peroxidation chain reactions and acting as ROS scavengers (Valko *et al.*, 2007; Melhem *et al.*, 2005). Antioxidant systems, which may be classified as endogenous/exogenous or enzymatic/non-enzymatic, are in equilibrium the production of free radicals under normal conditions. Enzymatic antioxidants include the enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione-*S*-transferase (GST), and glutathione reductase (GR) (Mates *et al.*, 2000; Pham-Huy *et al.*, 2008). Glutathione (GSH), a non-enzymatic antioxidant, is one of the most fundamental means for the protection of the body against oxidative stress (Mates *et al.*, 2000).

Submitted: February/2016
Approved: August/2016

The enzymes located of the body's antioxidant defense system provide a strong defense against the destructive effects of free radicals. Although free radicals affect many compounds and molecules present on the cell membranes, their most obvious effect is to initiate lipid peroxidation. Malondialdehyde (MDA) is one of the best known aldehydes occurring as a result of lipid peroxidation. It reflects the degree of cellular damage, changes in the polymerization of cell membrane components, in enzymatic activity, in the aggregation state of the cell surface determinants, and intrinsic membrane features (Kamboh *et al.*, 2015).

Flavonoids are polyphenols that are abundantly and widely found in phytonutrients and give plants yellow, orange, and red colors (Pikulski *et al.*, 2003). These compounds are not produced by the body and need to be ingested (Abdel-Rahman *et al.*, 2013; Kamboh *et al.*, 2015). They have antioxidant properties (Urso *et al.*, 2003). Herbal flavonoids activates appetite mechanisms in poultry and contribute to balance the gastrointestinal microflora system, and also have anti-inflammatory and antioxidant effects (Goliomytis *et al.*, 2014). Hesperidin is one of the most abundant flavonoids found in citrus species. It is an antioxidant, inhibiting lipid peroxidation and reducing oxidative stress (Jain *et al.*, 2015; El-Shafey *et al.*, 2014). Quercetin is a flavonoid compound that belongs to flavonol class and has strong antioxidant activity (Goliomytis *et al.*, 2014; Sternberg *et al.*, 2008; Boots *et al.*, 2011). Naringin is another flavonoid found mainly in grapefruit and other citrus fruits. Naringin is also a strong antioxidant that has protective effects against metal chelation and lipid peroxidation, acting as a free radical scavenger. In addition, due to their blood cholesterol-lowering effect, flavonoids can improve the health status of laboratory animals and prolong the shelf life of poultry products (Ali *et al.*, 2014; Jeon *et al.*, 2002; Lee *et al.*, 2001). This experiment was conducted to compare the antioxidant and cholesterol-reducing effects of hesperidin, quercetin, and naringin in laying chickens.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

Ninety-six 28-week-old Lohmann White laying hens with 1.5 kg average body weight were housed in tier cages (50 × 46 × 46 cm, with 4 chickens per cage). After a 1-wk adaptation period, birds were randomly distributed into four treatment groups, with six replicates each. The experimental unit was a cage housing four birds.

Treatments consisted of a basal diet or the basal diet with the inclusion of hesperidin (80%, HPLC), naringin (90% from citrus fruit), or quercetin (97%, HPLC) at a rate of 0.5 g/kg (Sigma-Aldrich, St. Louis, MO). The basal diet was formulated to meet nutrient requirements proposed by NRC (1994) (Table 1). During the experimental period (8 wks), layers were exposed to a 17-h daily photoperiod and were offered feed and water *ad libitum*.

Table 1 – Ingredient composition and chemical analysis of the basal diet.

Ingredient	g/kg	Chemical composition	
Corn	596.3	Dry matter (%)	88.36
Soybean meal (46% CP)	195.0	Crude protein (%)	17.58
Sunflower meal (36% CP)	74.0	Crude fiber (%)	3.19
Soybean oil	14.9	Fat (%)	3.75
Meat and bone meal	15.0	Crude Ash (%)	13.77
Monacalcium phosphate	0.7	Calcium (%)	3.80
Marble	95.0	Phosphorus (%)	0.47
Vitamin and mineral premix[1]	3.0	Metabolizable energy (MJ/kg) (kkal/kg)	11.40
Salt (NaCl)	2.0		
Sodium bicarbonate	1.5		
Toxin Binder[2]	1.0		
Organic acid mixture[3]	1.0		
Methionine[4]	0.6		

[1]The premix provided per 1 kg of diet: 1.200 mg Vitamin A retinol; 20 mg cholecalciferol (Vit D_3); 10.000 mg α-tocopheryl acetate (Vit E); 1.333 mg menadione sodium (Vit K_3); 1.000 mg thiamine mononitrate (Vit B_1); 1.667 mg riboflavin (Vit B_2); 8.333 mg niacin (Vit B_3); 3.333 mg Ca-D- panthotenic acid (Vit B_5); 1.667 mg pyridoxine (Vit B_6); 333 mg folic acid (Vit B_9); 5 mg cyanocobalamin (Vit B_{12}); 15 mg D-biotin (Vit H); 16.667 mg (Vit ascorbic acid (C); 100.000 mg Choline chloride; 200 mg Lutein; 12.5 mg zeaxanthin; 26.667 mg manganese oxide; 20.000 mg zinc oxide; 1.667 mg copper sulfate; 67 mg cobalt carbonate; 333 mg calcium iodate; 50 mg sodium selenite; 300 mg hydroxy methionine.

[2]Contained sepiolite, 1000.000 mg/kg (Mycobond, Optivite, Nottinghamshire, United Kingdom).

[3]Contained formic acid 35.000 mg/kg; ammonium propionate, 85.000 mg/kg; ammonium formate 160.000 mg/kg; propionic acid, 20.000 mg/kg; sodium aluminosilicate as carrier, 700.000 mg/kg (Salgard Powder, Optivite, Nottinghamshire, United Kingdom).

[4]DL-methionine.

Sample Collections and Laboratory Analyses

At the end of the experiment, blood samples (5.0 mL) were collected from the axillary vein from all hens into non-heparinized tubes using sterilized needles. Blood samples were incubated at 37°C for 2 h and then centrifuged at 1,500 *g* at 4°C for 10 min. The serum samples were stored in 1.5 mL Eppendorf tubes at –80°C for the determination of total protein, total cholesterol, and MDA levels.

Additional blood samples were collected from axillary vein by venous puncture into heparinized tubes to determine the activities of GR, GSH-Px, GST, and SOD and the level of GSH in erythrocyte lysates. After centrifugation at 1,000 *g* for 15 min, the supernatant was removed and erythrocytes were washed three times with saline solution. A known volume of the erythrocytes was lysed with hypotonic phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2,500 *g* for 15 min at 2°C.

Total Protein Concentration. Four hundred µL of 2x Lowry concentrate [3 volumes of copper reagent (20 g sodium carbonate, 0.4 g cupric sulfate (hydrated 5x), and 0.2 g sodium potassium tartrate in 300 mL water) + 1 volume of 1% sodium dodecyl sulfate + 1 volume of 1 M NaOH] was added to 10 µL serum samples, mixed thoroughly, and incubated at 25°C for 10 min. Then, 200 µL of 0.2 N folin reagent was added and immediately vortexed. The solution was incubated at 25°C for 30 min and the absorbance was read at 750 nm. Results were calculated by using a standard albumin solution (5 g/dL) (Lowry *et al.*, 1951).

Total Cholesterol Concentration. A volume of 1000 µL of total cholesterol assay reagent [horseradish peroxidase 166.7 µkat/L, phenol 5.2 mmol/L, cholesterol oxidase 3.3 µkat/L, cholesterol esterase 3.3 µkat/L, 4-aminoantipyrine 0.31 mmol/L, in 103 mmol/L phosphate buffer (pH 6.5)] was added to 10 µL serum samples. Tubes were incubated at 37°C for 15 min. Absorbance was measured at 500 nm against the blank reagent. Results were calculated by using a standard cholesterol solution (50 mg/dL) (Allain *et al.*, 1974).

Antioxidant Status. Serum MDA levels (nmol/mL) were measured at 532 nm wavelength using the spectrophotometric method (Ohkawa *et al.*, 1979). Erythrocyte SOD activity is based on the spectrophotometric measurement of the color intensity, resulting from the release of superoxide anion ($O^{2\bullet-}$) by the action of xanthine-xanthine oxidase and then reaction of the anion with nitroblue tetrazolium (NBT). The reaction is inhibited according to CuZn-SOD activity (U/g hemoglobin) in the medium, and the inhibition % is then calculated (Sun *et al.*, 1988). Erythrocyte GSH levels (µmol g/hemoglobin) were measured at 412 nm wavelength using the spectrophotometric method (Beutler *et al.*, 1975). Erythrocyte GST activity (U/g hemoglobin) was measured by monitoring the increase in absorbance at 340 nm, using 1-chloro-2,4-dinitro benzene as substrate (Habig *et al.*, 1974). In the GSH-Px activity assay, glutathione reduced in the presence of H_2O_2 is oxidized to oxidized glutathione (GSSG) by GSH-Px. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the medium was used during the transformation to GSH through the GR enzyme of the oxidized GSSG. The NADPH consumption was measured at 340 nm wavelength in the form of decrease in absorbance. GSH-Px activity was expressed as U/g hemoglobin (Beutler, 1984). GR catalyzes the reduction of GSSG to GSH by NADPH. The GR activity (U/g hemoglobin) was determined by measuring the absorbance difference of NADPH oxidized during the reaction at 37 °C and then reading at 340 nm wavelength (Andersen *et al.*, 1997).

Statistical Analysis

One-way analysis of variance was used to evaluate the effect of treatments. Means were compared by the LSD test (SPSS 10.01, SPSS Inc., Chiago, IL). Statistical significance was declared at $P < 0.05$.

RESULTS

Changes in serum metabolites in response to the dietary supplementation with flavonoids are shown in Table 2. In comparison with the control group, serum protein concentration was the lowest in the hesperidin group, whereas it remained unchanged in the naringin and quercetin groups. Dietary naringin supplementation did not affect serum cholesterol

Table 2 – Effect of dietary hesperidin, naringin and quercetin supplementation (0.5 g/kg) on serum total protein (g/dL) and cholesterol (mg/dL) level of laying hens*

Parameter	Control	Hesperidin	Naringin	Quercetin
Total protein	4.90±0.03^{a}	4.80±0.03^{b}	4.89±0.03^{a}	4.83±0.02^{ab}
Cholesterol	199±2^{a}	179±2^{a}	200±2^{a}	184±+2^{b}

*Results are expressed as mean ± SE (n=6 per group). Different superscripts within the same columns differ ($p< 0.05$).

concentration, whereas dietary hesperidin and quercetin supplementations reduced serum cholesterol concentration compared with the control diet.

Table 3 shows the effects of dietary hesperidin, naringin, and quercetin supplementation on the antioxidant status and antioxidant enzymes. All flavonoids reduced serum MDA concentration compared with the control group, with stronger effects of naringin and quercetin relative to hesperidin. All flavonoids increased the activities of GSH, GSH-Px, GR, and GST. Dietary hesperidin and naringin supplementation did not affect SOD activity, whereas dietary quercetin supplementation increased SOD activity. Quercetin promoted stronger activation of antioxidant enzymes than hesperidin and naringin.

DISCUSSION

Flavonoids are effectively used as supplemental agents to enhance the antioxidant status and boost the immune system (Kim *et al.*, 2004; Wilmsen *et al.*, 2005; Goliomytis *et al.*, 2014). Free radicals increase as oxygen consumption increases, and are neutralized by a defense system including enzymatic and non-enzymatic antioxidants (Göktepe M *et al.*, 2014). The enzymes SOD, GSH-Px, GST, and GR are the primary antioxidants of the enzymatic system and act at cellular level. Considering non-enzymatic antioxidants, GSH is the main intracellular redox buffer, and functions as a direct free radical scavenger, a co-substrate for GPx activity and a co-factor of many enzymes. It is also involved in the maintenance of exogenous antioxidants. The notable decline in the key cellular non-enzymatic antioxidant defense system considerably increases the susceptibility to oxidative stress (Rajadurai *et al.*, 2009; Pradeep *et al.*, 2008; Amália *et al.*, 2007).

A number of researchers have evaluated the antioxidant activity and radical scavenging properties of flavonoids, including hesperidin (Tirkey *et al.*, 2005; Jovanovic *et al.*, 1994; Suarez *et al.*, 1998), naringin (Mahmoud *et al.*, 2012), and quercetin (Rajadurai *et al.*, 2009; Pradeep *et al.*, 2008), using a variety of assay systems. Sahu *et al.* (2013) indicated that oral hesperidin administration significantly increased serum levels of GSH, GST, GR and GSH-Px as well as decreased the expression of lipid peroxidation in rats. It was reported that serum SOD levels were relatively high after hesperidin supplementation, resulting in a reduced superoxide anion concentration in laying hens (Lien *et al.*, 2008). In another study, the incorporation of hesperidin in the diet increased SOD activity in laying hens (Ting *et al.*, 2011). In our work, GSH levels increased along with SOD, GR, GPx, and GST activities, especially in the quercetin group (Table 3). In response to supplemental flavonoids, increased enzyme activities may suggest that birds had greater capacity to clear oxygen free radicals, resulting in lower MDA level (Wilmsen *et al.*, 2005; Rahman, 2007; Goliomytis *et al.*, 2014). As a by-product of lipid peroxidation, MDA reflects the degree of oxidation in the body. The administration of hesperidin, naringin, and quercetin may boost the immune response and improve the wellbeing (Tang *et al.*, 2012), by promoting cellular defense activity against oxidative stress (Karasu *et al.*, 2014). Moreover, in agreement with the literature (Selvakumar *et al.*, 2013), the dietary supplementation of hesperidin and quercetin decreased serum cholesterol and protein concentrations (Table 2).

In summary, all evaluated flavonoids (hesperidin, naringin, and quercetin) exerted antioxidant activity, as shown by the increased levels enzymatic and non-enzymatic defense system parameters and reduced lipid peroxidation level in laying chickens. Among these flavonoids, quercetin promoted the best results. Therefore, its utilization as a dietary additive for laying hens, especially under stress conditions, may help maintain their productivity and health status.

ACKNOWLEDGMENTS

This work was supported by the Department of Science Research Project at Artvin Coruh University.

Table 3 – Effect of dietary hesperidin, naringin, and quercetin supplementation (0.5 g/kg) on the antioxidant status of laying hens*

Parameter	Control	Hesperidin	Naringin	Quercetin
MDA (nmol/mL)	119±3^{a}	96.9±2.7^{b}	80.9±2.1^{c}	75.1±1.7^{c}
GSH (µg/g Hb)	1.39±0.04^{d}	2.51±0.13^{a}	2.03±0.07^{b}	1.66±0.09^{c}
GSH Px (U/g Hb)	29.6±2.1^{c}	122±4^{b}	136±7^{b}	157±7^{a}
GR (U/g Hb)	5.99±0.38^{c}	14.1±0.6^{b}	15.7±0.6^{a}	13.6±0.6^{b}
GST (U/g Hb)	2.22±0.14^{b}	4.63±0.16^{a}	4.54±0.24^{a}	4.24±0.22^{a}
SOD (U/g protein)	247±7^{b}	263±8^{b}	254±6^{b}	295±6^{a}

* Results are expressed as mean ± SE (n=6 per group). Different superscripts within the same columns differ ($p<0.05$). MDA, malondialdehyde; GSH, glutathione; GSH Px, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S transferase; SOD, superoxide dismutase. Hb, hemoglobin.

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