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Effects of Using *Artemisia annua* Leaves, Probiotic Blend, and Organic Acids on Performance, Egg Quality, Blood Biochemistry, and Antioxidant Status of Laying Hens

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This study was performed to investigate and compare the effects of using *Artemisia annua* leaves, probiotic, and organic acid on the performance, egg quality, blood biochemistry, and antioxidant status of laying hens. In total, 288 Hy-Line W-36 commercial layers (32 weeks old) were divided into six groups with six replicates per group (eight birds per replicate) and were fed one of six experimental diets. The hens were fed either a corn-soybean meal basal diet (control) or the basal diet supplemented with 2.5% *A. annua* leaves (AA1), 5% *A. annua* leaves (AA2), 7.5% *A. annua* leaves (AA3), 0.1% probiotic (Pro), and 0.005% organic acid (Org), respectively. The experiment lasted 10 weeks. Results showed that there were differences in the feed conversion ratio (FCR) among experimental groups ($P<0.05$). The highest yolk color index and shell thickness were observed in hens fed AA3 and AA2 diets ($P<0.05$). Egg yolk cholesterol was decreased ($P<0.01$) by the diet containing AA3 and Pro compared to the other groups. The atherogenic index was lower ($P<0.01$) in the plasma of hens fed AA3 than those in other groups. The glutathione peroxidase activity (GSH-Px) and malondialdehyde (MDA) contents in layers fed AA3 were lower and higher ($P<0.05$), respectively, than in layers fed the other diets. Moreover, the concentration of plasma cholesterol was decreased ($P<0.05$) in layers fed AA3 and Pro. In conclusion, feeding laying hens with *A. annua* leaves positively influenced the plasma antioxidant status, and the dietary inclusion of *A. annua* leaves plus a probiotic significantly decreased the egg yolk cholesterol, with no adverse effect on the egg productive traits.

Key words: *Artemisia annua*, egg quality, laying hens, organic acids, probiotics

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Introduction

In the last years, numerous studies have focused on the physiological and biochemical structure and function of natural feed additives, such as probiotics, organic acids, and phytogenic additives (Tufarelli *et al*., 2017). In particular, phytogenic feed additives are plant-derived products used in animal feed in order to improve livestock performance (Dhama *et al*., 2015). This class of feed additives has recently gained interest, especially for use in swine and poultry, as can be observed by the significant increase in scientific literature since 2000 (Jayaparkasha *et al*., 2005). One of the best medicinal plants is Artemisia, belonging to the *Asteraceae* family, which contains artemisinin, an important bioactive compound (Kim *et al*., 2015; Mesa *et al*., 2015). Powdered leaves of *Artemisia absinthium*, *Artemisia biennis*, *Artemisia frigida*, and *Artemisia ludoviciana* have been applied by North American native people for treating sores and wounds and also to treat chest infections (Kershaw, 2000). Khalaji *et al*. (2011) reported that the inclusion of *Artemisia* leaves had a positive effect on broiler gut health and growth performance.

Probiotics have been defined as live microbial feed supplements that beneficially affect host animals by improving their intestinal microbial balance (Tufarelli *et al*., 2017). The

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addition of probiotics to diet has been found to improve growth performance and feed efficiency in broilers, as well as egg production and quality in laying hens (Kurtoglu *et al*., 2004; Rezaei *et al*., 2015). Khan *et al*. (2011) found that the supplementation of enzymes and probiotic blends in layer diets did not appear to cause any adverse effects on egg production and quality, immunity response, or HDL-cholesterol level.

Organic acids are promising alternative compounds to antibiotics (Paul *et al*., 2007). Dietary organic acids and their salts are able to inhibit the microorganism growth in feed by preserving the microbial balance in the gastrointestinal tract of poultry. In addition, by modifying the intestinal pH, organic acids also improve the solubility of feed ingredients, digestion, and absorption of nutrients (Rahman *et al*., 2008). Yesilbag and Colpan (2006) showed that the supplementation of dietary organic acids in laying hens resulted in an improved egg production and protein metabolism efficiency.

Therefore, the aim of this study was to investigate the effect of dietary *Artemisia annua* leaves on the performance, egg quality, blood biochemistry, and antioxidant status of laying hens, and also to compare the effects of *A. annua* leaves with those of a probiotic blend and an organic acid supplement.

Materials and Methods

Animals and Diets

Procedures with animals were performed following good veterinary practice for animal welfare, according to the national laws in force and in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Islamic Azad University, Iran (no. 93/10-14).

A total of 288 Hy-Line W-36 White Leghorn (32 weeks old) layers were used in six treatments, with six replicates and eight hens per replicate. The nutrient profile of the *A. annua* leaves were analyzed (dry matter, crude protein, gross energy, ether extract, ash, and phenolic contents) by the Arya Analytical Laboratory (Table 1). The hens were allocated individual cages (41×23×43 cm), and four cages were considered as one replicate. Before starting the feeding trial, the egg production of hens was individually assessed, and hens with equal egg production were replaced in each replicate. Diets were formulated to meet the nutrient requirements of laying hens, according to the National Research Council guidelines (NRC, 1994).

Dietary treatments were as follows: a basal-diet as control, or the basal diet supplemented with 2.5% (AA1), 5% (AA2), and 7.5% *A. annua* leaves (AA3); a 0.1% probiotic blend (PrimaLac, Star Labs Inc., Clarksdale, MO, USA; a combination of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Enterococcus faecium*) (Pro), and 0.005% organic acids (Org) (Sunzen, Selangor, Malaysia; a combination of formic, lactic, malic, citric, tartaric, and orthophosphoric acids containing 38% organic acids and 62% silicates as carriers). The composition of the basal diet is shown in Table 2. Feed and water were offered *ad libitum* during the whole trial, and light was provided for 16 h per day. Before starting the feeding trial, hens were fed a balanced commercial diet for two weeks, covering their daily requirements. This allowed the birds to adapt and reach a standard level of egg production (data not shown). The feeding trial lasted 10 weeks.

Sample Preparation

Fresh *A. annua* leaves were provided from Darvash Giah Khazar Medicinal Herbs Complex Company (Ltd.) in Guilan Province (Iran), then ground and mixed with the diet. The Folin–Ciocalteu reagent method was used for the total phenol determination. Briefly, 1 g of dried plant sample was ex-

Table 1. **Nutrient profile of *Artemisia annua* leaves**

Item	%
Dry matter (%)	92.86
Crude protein (%)	22.64
Ether extract (%)	6.38
Crude fiber (%)	24.85
Ash (%)	11.57
Ca (%)	1.49
P (%)	0.47
Mg (%)	0.82
Gross energy (kcal kg^{-1})	4521
Total phenols (mg g^{-1})	65.00

Table 2. **The ingredients and chemical compositions of the basal diet**

Ingredients (%)	
Corn	61.87
Soybean meal (44% CP)	23.02
Vegetable oil	3.00
Oyster shell	5.00
Limestone	4.13
Dicalcium phosphate	2.18
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
Salt	0.30
Calculated composition (%)	
Crude protein	16.30
Crude fiber	3.67
Ca	4.00
Available P	0.50
Met	0.21
Met + Cys	0.75
Lys	0.86
AME_n (kcal kg^{-1})	2900

[1] Vitamin supplement provided per kg of diet: vitamin A, 8000 IU; vitamin E, 20 IU; menadione, 3.0 mg; vitamin D3, 2000 IU; riboflavin, 4.0 mg; pantothenate, 12 mg; nicotinic acid, 50 mg; choline 300 mg; vitamin B12, 15 mg; vitamin B6, 0.12 mg; thiamine, 1.5 mg; folic acid, 1.00 mg; d-biotin, 0.10 mg.

[2] Mineral supplement provided per kg of diet (mg kg^{-1}): Mn, 100; Zn, 70; Fe, 50; Cu, 10; Iodine, 1; Se, 0.30; antioxidant, 50.

tracted with 10 mL of 80% methanol. For this purpose, 0.50 mL of the extract, gallic acid (as the standard, Sigma-Aldrich, Steinheim, Germany), Folin (Sigma; diluted with water at a 1:10 ratio), and 4.00 mL of sodium carbonate (Sigma; 1 M) solution were mixed and kept at 18℃ for 15 min. Standards with concentrations of 0, 25, 50, 100, 250, and 500 mg mL^{-1} were prepared, and, following incubation, the mixture was submitted to conventional spectrophotometry (model UV-160A; Shimadzu, Tokyo, Japan) in the range of 450 to 765 nm. Finally, total phenols were expressed as mg g^{-1} dry matter (Baghban-Kanani *et al*., 2017).

Sample Collection

During the trial, the daily feed intake, egg weight and mass, egg production, feed conversion ratio (FCR), and mortality rate were measured. The body weights of hens were recorded at the beginning and the end of the experiment. The feed intake was measured weekly by subtracting the leftover feed from the amount supplied to animals. Eggs from individual hens were collected daily and weighed. The egg production and feed efficiency were calculated as the rate of production per hen per day, and feed intake/egg mass, respectively. The egg quality parameters (shell thickness, shell strength, yolk index, Haugh unit, and yolk color) were assessed 24 h after egg collection. The shell strength was measured using a specific instrument (Digital Egg Shell Force Gauge; Wagner Instruments, USA). The shell thickness was measured at three locations (air cell, equator, and sharp end) using a digital micrometer (0.01 mm; Mitutoyo, Japan). The yolk height (HY) was measured using a tripod micrometer (0.01 mm, Mitutoyo, Kawasaki, Japan), and the yolk diameter (D) by compass (0.02 mm; Swordfish, China). The yolk index was calculated by the following formula: yolk index＝(HY/D)×100. The Haugh unit was calculated using the following formula: Haugh unit＝100 logHA＋7.57 －1.7 $WE^{0.37}$), where HA is the albumen height, and WE is the egg weight. The yolk color was evaluated with a DSM Yolk Color Fan, which included 15 colorimetric blades according to the yellow intensity.

At the end of the trial, 30 eggs from each treatment were collected for the determination of yolk cholesterol, as previously described by Baghban-Kanani *et al*. (2018). Four hens from each replicate group were randomly selected, and blood from the brachial wing vein was drawn into ethylenediaminetetraacetic acid (EDTA) -containing tubes and centrifuged for 10 min at 3000 rpm for plasma collection; these samples were used to determine the GSH-Px activity, and concentrations of MDA, triglycerides, total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol in low-density lipoprotein (LDL-C), and cholesterol in high-density lipoprotein (HDL-C), using commercial diagnostic kits. The obtained samples were stored at －80℃ until further analyses. The atherogenic index was calculated as the ratio of LDL to HDL cholesterol (LDL/HDL).

Antioxidant Status Parameters

The GSH-Px activity was determined in plasma samples using RANDOX kits (Randox, Crumulin, UK) according to the manufacturer's instruction. Plasma lipid peroxidation (LP) was determined following the method proposed by Kei (1978) and Yagi (1984), but using 1,1,3,3-tetraethoxypropane as the standard. This method is based on the reaction between MDA (an aldehyde lipid peroxidation product) and thiobarbituric acid (TBA). The MDA forms a pink-colored complex with TBA. The absorbance of the solution containing the complex was measured at 532 nm using a spectrophotometer (UV-1201, Shimadzu, Japan). The plasma LP values were expressed, in terms of MDA, as nmol mL^{-1} plasma.

Statistical Analysis

Data were analyzed in a completely randomized design. The obtained data were submitted to ANOVA, using the general linear model (GLM) procedure of SAS software (2001). Means were compared using Tukey's test at the 5% probability. All values were expressed as means±standard error of the means (SEM).

Results

As indicated in Table 3, the feed intake, egg production,

Table 3. **Effect of the experimental diets on the performance and egg parameters of laying hens**

	Feed consumption (g d^{-1} $bird^{-1}$)	Egg production (%)	Egg weight (g)	Egg mass (g d^{-1} $bird^{-1}$)	FCR (kg feed: kg egg)
Basal diet	103.81	80.64	60.54	48.82	2.12[a]
AA1	100.13	81.77	60.75	49.68	2.01[b]
AA2	99.13	81.68	60.77	49.64	1.99[b]
AA3	97.68	82.78	60.75	50.29	1.94[b]
Pro	99.05	82.69	60.72	50.21	1.97[b]
Org	98.89	81.84	60.75	49.72	1.98[b]
SEM	0.57	0.20	0.11	0.17	0.01
P-value	0.27	0.10	0.49	0.10	0.03

[a–b] Values in the same column not sharing a common superscript differ significantly ($P<0.05$). AA1, 2.5% *Artemisia annua* leaves; AA2, 5% *Artemisia annua* leaves; AA3, 7.5% *Artemisia annua* leaves; Pro, 0.1% probiotic; Org, 0.005% organic acid.

Table 4. **Effect of the experimental diets on egg quality parameters of laying hens**

	Shell thickness (mm)	Eggshell strength (kg cm^{-2})	Yolk index (%)	Haugh unit	Yolk color
Basal diet	0.31^{b}	3.51	42.15	80.94	8.47^{c}
AA1	0.31^{b}	3.50	42.59	81.28	9.21^{bc}
AA2	0.33^{a}	3.52	42.35	81.40	9.74^{b}
AA3	0.33^{a}	3.51	43.11	81.70	10.65^{a}
Pro	0.32a^{b}	3.51	42.78	81.61	8.99^{bc}
Org	0.31^{b}	3.51	42.48	81.59	8.86^{bc}
SEM	0.006	0.006	0.21	0.10	0.16
P-value	0.03	0.78	0.97	0.37	0.002

$^{a-b}$ Values in the same column not sharing a common superscript differ significantly ($P<0.05$). AA1, 2.5% *Artemisia annua* leaves; AA2, 5% *Artemisia annua* leaves; AA3, 7.5% *Artemisia annua* leaves: Pro, 0.1% probiotic; Org, 0.005% organic acid.

Table 5. **Effects of the experimental diets on egg weight, yolk weight, and cholesterol**

	Egg weight (g)	Yolk weight (g)	Yolk cholesterol (mg egg yolk^{-1})	Yolk cholesterol (mg g^{-1} yolk)
Basal diet	60.54	13.28	198.68^{a}	14.96^{a}
AA1	60.75	13.29	194.57^{ab}	14.63^{ab}
AA2	60.77	13.34	192.71^{ab}	14.46^{ab}
AA3	60.75	13.28	184.67^{b}	13.90^{b}
Pro	60.72	13.32	185.89^{b}	13.96^{b}
Org	60.75	13.32	191.89^{ab}	14.40^{ab}
SEM	0.11	0.07	2.19	0.17
P-value	0.49	0.78	0.0001	0.0001

$^{a-b}$ Values in the same column not sharing a common superscript differ significantly ($P<0.05$). AA1, 2.5% *Artemisia annua* leaves; AA2, 5% *Artemisia annua* leaves; AA3, 7.5% *Artemisia annua* leaves; Pro, 0.1% probiotic; Org, 0.005% organic acid.

egg weight, and egg mass were not different among treatments ($P>0.05$), whereas feeding with experimental treatments decreased the FCR of hens compared to that of hens fed the basal diet ($P<0.05$). The effect of the experimental diets on the egg quality of laying hens is summarized in Table 4. The dietary treatments had no effect on the shell strength, yolk index and Haugh unit throughout the trial< however, an increase in yolk color intensity was observed in the AA3 group ($P<0.01$). In addition, there were significant increases in the shell thickness and yolk color intensity in laying hens fed AA3 and AA2 diets ($P<0.05$). The egg weight, yolk weight, and yolk cholesterol of laying hens are reported in Table 5, and it was found that dietary treatments had no effect ($P>0.05$) on the egg and yolk weight; conversely, the egg yolk cholesterol was decreased ($P<0.01$) by the diet containing AA3 and Pro, respectively. In Table 6, the effect of experimental diets on plasma biochemical parameters of laying hens is shown. In this study, triglycerides and HDL levels were not affected by diet ($P>0.05$), whereas plasma LDL was influenced by the experimental diet ($P<$ 0.01). The atherogenic index was lower ($P<0.01$) in the plasma of hens fed AA3 compared to that of hens in the other groups. The effect of the dietary treatments on plasma GSH-Px, MDA, ALT, AST, and total cholesterol is shown in Table 7. There were no significant differences among diets in terms of plasma ALT and AST compared to that of the control. On the other hand, the GSH-Px and MDA levels in hens fed AA3 were significantly lower and higher than those in other treatments, respectively. Furthermore, the concentration of plasma cholesterol decreased ($P<0.05$) in laying hens fed AA3 and Pro diets.

Discussion

The findings of the present study indicated that the dietary treatments decreased the FCR of laying hens. A decrease in feed efficiency when fed *A. annua* could be due to its antibacterial and antifungal effects, which can lead to a decrease in the number of harmful microbes in the digestive system, improving the immunity and performance of the chicken (Mansoub, 2011). The lower FCR indicated that *A. annua* leaves can be successfully used as a natural feed additive for laying hens without affecting their production performance. This is not surprising, because *A. annua* leaves contain over >4271 kcal kg^{-1} of gross energy, 27% crude protein, and >50% essential fatty acids (Cherian *et al*., 2013). To the best of our knowledge, the effects of *A. annua* dietary supplementation on laying hens have not been investigated. Therefore, we not found any literature for comparing our

Table 6. **Effect of the experimental diets on plasma biochemical parameters of laying hens**

	LDL cholesterol (mg dL^{-1})	HDL cholesterol (mg dL^{-1})	Atherogenic index	Triglycerides (mg dL^{-1})
Basal diet	98.25[a]	43.07	2.28[a]	1335.8
AA1	96.34[b]	44.51	2.16[b]	1335.0
AA2	96.31[b]	45.47	2.12[bc]	1335.0
AA3	95.25[b]	46.63	2.04[c]	1334.8
Pro	96.07[b]	45.61	2.10[bc]	1334.9
Org	96.34[b]	44.51	2.15[bc]	1335.1
SEM	0.28	0.35	0.02	1.23
P-value	0.0002	0.22	0.005	0.95

[a–b] Values in the same column not sharing a common superscript differ significantly ($P<0.05$). AA1, 2.5% *Artemisia annua* leaves; AA2, 5% *Artemisia annua* leaves; AA3, 7.5% *Artemisia annua* leaves; Pro, 0.1% probiotic; Org, 0.005% organic acid.

Table 7. **Effect of the experimental diets on laying hens' plasma glutathione peroxidase (GSH-Px) activity, malondialdehyde (MDA), ALT, AST, and total cholesterol**

	GSH-Px (U mL^{-1})	MDA (nmol mL^{-1})	ALT (U L^{-1})	AST (U L^{-1})	Total cholesterol (mg dL^{-1})
Basal diet	811.27[b]	5.92[a]	166.65	6.87	100.03[a]
AA1	813.22[b]	5.55[a]	165.24	6.75	99.85[a]
AA2	813.24[b]	5.34[a]	163.66	6.45	98.31[ab]
AA3	822.38[a]	3.91[b]	163.66	6.16	96.14[b]
Pro	813.98[b]	5.33[a]	163.84	6.37	96.20[b]
Org	813.14[b]	5.47[a]	164.30	6.66	98.19[ab]
SEM	0.91	0.16	0.69	0.13	0.40
P-value	0.02	0.04	0.10	0.69	0.02

[a–b] Values in the same column not sharing a common superscript differ significantly ($P<0.05$). AA1, 2.5% *Artemisia annua* leaves; AA2, 5% *Artemisia annua* leaves; AA3, 7.5% *Artemisia annua* leaves, Pro, 0.1% probiotic; Org, 0.005% organic acid.

results regarding performance and egg quality traits.

Another possible alternative to antibiotics to enhance feed efficiency in poultry species is feeding with living microbial cultures (Nahashon *et al*., 1994, 1996). The mechanism by which probiotics affect poultry performance is well established. Supplementing probiotics do not improve growth by affecting feed intake per se, suggesting that the reduction of FCR by probiotics could be related to its promoting effects on the metabolic processes of digestion, utilization of nutrients, and enhancement of health status (Khan *et al*., 2011, 2012). Previous studies on poultry have revealed that dietary supplementation with probiotics and prebiotics improved growth performance and feed efficiency by improving the intestinal environment, reducing the intestinal pH, and increasing digestive enzyme activity in the gastrointestinal tract (Ghasemi *et al*., 2014; Jahromi *et al*., 2016). In the present study, FCR was significantly improved in laying hens fed organic acids compared to in those fed the basal diet. The improved FCR may be the result of the recovery of damaged digestive wall cells and the preservation of microbial balance and improved the nutrient utilization of hens in the supplemented groups (Rahman *et al*., 2008). These results were highly correlated with the findings of previous studies (Langhout and Sus, 2005; Soltan, 2008), where higher feed efficiencies were observed after organic acid supplementation in broiler diets. In our study, feeding with AA3 and AA2 diets significantly increased the eggshell thickness and yolk color intensity of hens. As in Table 1, *A. annua* leaves contained high concentrations of Ca, Mg, and P, and in agreement with our findings, Brisibe *et al*. (2009) reported that *A. annua* leaves have high concentrations of Ca, Mg, P, and S, which are necessary for shell formation. *A. annua* leaves in laying hens diet significantly increased the yolk color intensity compared to that of hens in other experimental groups, which could be due to the presence of pigments, such as carotenoids and xanthophylls, which are absorbed with high efficiency into the gastrointestinal tract, then transferred to egg yolk, making it more colorful.

In the present study, egg yolk cholesterol was decreased significantly ($P<0.01$) by the AA3 and Pro diets. Accordingly, Lutgen (2013) reported that several *Artemisia* species can enhance lipid metabolism and reduce blood cholesterol concentration. Kurtoglu *et al*. (2004) stated that in most animal species, cholesterol is eliminated by catabolism and

excretion in the feces as biliary acids, but hens eliminate considerable amounts of cholesterol in the egg. Furthermore, there is a positive correlation between egg cholesterol and serum cholesterol. The fiber content of *A. annua* leaves (Table 1) may stimulate the binding of cholesterol with bile acids, and the inhibition of micelle formation combined with the effect of fermentation on short-chain fatty acid production are mechanisms that have been proposed to explain the potential cholesterol-lowering effects (Baghban-Kanani *et al*., 2018). In addition, probiotic supplementation may decrease cholesterol concentrations in blood and egg yolks (Khan *et al*., 2011). Cholesterol metabolism in laying hens was studied by determining the effect of dietary factors on the levels of blood and egg yolk cholesterol. Probiotics are known, as a dietary factor, to affect the egg yolk cholesterol level of laying hens (Kurtoglu *et al*., 2004). This reduction could be attributable to the reduced absorption and synthesis of cholesterol in the gastrointestinal tract (Mohan *et al*., 1995). It is also possible that some microorganisms present in the probiotic blend could assimilate cholesterol in the gastrointestinal tract for their own cellular metabolism or precipitate the cholesterol with deconjugated bile salts (Kurtoglu *et al*., 2004). In the current experiment, dietary treatments decreased the plasma LDL cholesterol, and also the atherogenic index was significantly lower in hens fed experimental dietary treatments compared to those fed the control basal diet. As mentioned above, medicinal plants such as *A. annua* and probiotics have cholesterol-lowering effect, and a lower *de novo* cholesterol synthesis has been reported to increase the expression of LDL cholesterol receptors on hepatocytes, resulting in a higher LDL uptake by the liver hepatocytes, and ultimately lowering blood LDL concentrations (Ghasemi *et al*., 2014). The findings of our study on plasma LDL cholesterol are in agreement with those of Kamal and Ragaa (2014), who reported that serum LDL was significantly decreased by dietary acidifiers. The beneficial role of organic acids in reducing the blood lipid profile may be interpreted through their influence in decreasing the microbial intracellular pH; thus, inhibiting the action of important microbial enzymes and forcing the bacterial cell to use energy to release the acid protons, leading to an intracellular accumulation of acid anions (Kamal and Ragaa, 2014). Moreover, the hyperthyroidism associated with dietary organic acids could explain the reduction in the serum lipid profile. The results of the present study depicted no significant differences in ALT and AST concentrations among dietary treatments compared to those of the control. The GSH-Px is one of the main antioxidant enzymes of the antioxidant system, and the MDA concentration is usually measured to evaluate the level of lipid peroxidation. The GSH-Px concentration was increased and the MDA was decreased in the plasma of layers fed the AA3 diet. Previous studies have shown that extracts of *Artemisia* species increased GSH-Px activity, as well as decreased MDA production in the liver of rats (Ryu *et al*., 2013). Recently, Wan *et al*. (2016) reported that *A. annua* leaves enhanced GSH-Px activity and reduced MDA level in the serum and liver, indicating that *A. annua* can successfully improve the antioxidant status of poultry.

It was assessed that *A. annua* contains phenolic compounds and flavonoids with excellent antioxidant activity (Brisibe *et al*., 2009; Gouveia and Castilho, 2013), and this would enhance the antioxidant status of hens fed a diet supplemented with *A. annua* leaves. Aside from the nutritional values of *A. annua* leaves, the advantages associated with other antioxidant compounds in the diet, such as vitamin E and other phenolic compounds, make this plant a natural phytogenic feed additive with antioxidant potential that could be incorporated into poultry rations (Cherian *et al*., 2013). Feeding with AA3 and probiotic diets significantly decreased the plasma total cholesterol concentrations. Only few studies have investigated the effect of *A. annua* leaves on blood parameters in laying hens. One mechanism through which *A. annua* leaves may exert their hypocholesterolemic action is via bile acids. The cholic and deoxycholic bile acids are produced from cholesterol by hepatocytes and are conjugated with glycine and taurine, respectively. These acids enter the small intestine, where they are absorbed and directed to the liver, and a decrease in bile acid recycling would ultimately result in a lowering of the serum cholesterol concentration, because this cholesterol is used for bile acid synthesis (Baghban-Kanani *et al*., 2018). However, we did not measure the amount of bile acid synthesis to support this speculation. Thus, the hypocholesterolemic effect of *A. annua* leaves demonstrated in the present study was not clearly understood. However, fiber in *A. annua* leaves produces short-chain fatty acids when fermented by intestinal microorganisms, which mainly contained acetate, propionate, and butyrate. In agreement with the present study, Khan *et al*. (2011) reported that the supplementation of probiotics in laying hen's diet significantly decreased the plasma cholesterol concentration. Some of the microorganisms present in the probiotic may also modify the blood cholesterol by deconjugating bile acids to produce free bile acids or directly binding dietary cholesterol to the small intestine during fat digestion before cholesterol can be absorbed into the blood (Ghasemi *et al*., 2014).

In conclusion, supplementing *A. annua* leaves at 75 g/kg to the diet of laying hens improved the feed efficiency and some egg quality parameters. In overall, feeding laying hens a diet with *A. annua* leaves positively influenced the plasma antioxidant status. Moreover, *A. annua* leaves and probiotic blend significantly decreased the concentration of egg yolk cholesterol, with no adverse effect on the egg weight and production of laying hens.

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