Synergistic effects of quercetin and vitamin E on egg production, egg quality, and immunity in aging breeder hens

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ABSTRACT Laying hens experience a rapid decline in egg production, egg quality, and immunity, usually at the end of the peak laying period. Quercetin, a known flavonoid, exerts biological activities, including phytoestrogenic, immunity, antibiotic, antioxidant, and anti-inflammatory properties. Vitamin E also shows egg production and immunoregulatory potential in animals. This study evaluated the capacity of dietary quercetin, vitamin E, and the combination of both, to promote egg production and egg quality, and to improve the immunity of aging breeder hens. We also elucidated how quercetin and vitamin E combination could synergistically affect egg production, egg quality, and immunity in aging breeder hens. A total of 400 Tianfu broiler breeders at the age of 52 wk were randomly allotted to 4 treatments with 4 replicates, 100 hens per treatment and 25 hens per replicate. They were fed diets containing quercetin at 0.4 g/kg, Vitamin E (200 mg/kg), quercetin and vitamin E (0.4 g/kg and 200 mg/kg), and a basal diet (control) for a period 10 wk. Daily feed intake and egg production rate were recorded, and weekly records were recorded on egg quality tests. At the end of the 10-wk experimental period, blood samples and immune organ (spleen) were collected from 2 birds per replicate, totaling 32 birds. Feed intake, immune organ index, serum cytokines, and immunoglobulins were evaluated, and the mRNA expression of genes related to immunity was determined from the spleen tissue. Generally, the results showed that separately or as a combination, supplemental quercetin and vitamin E significantly improved performance and egg quality ($P < 0.05$), and significantly increased serum immunoglobulins (IgA, IgM, and IgG) and cytokines (IFN-γ and IL-2) concentrations, as well as promoted immune organ development and index, and promoted the expression of splenic immune-related genes (IL-2 and INF-γ) ($P < 0.05$), compared with the control. It was confirmed in this study that the combination of quercetin and vitamin E exert synergistic effects on egg production, egg quality, and immune function in aging hens.

Key words: quercetin, vitamin E, aging breeder hen, egg production, immunity

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INTRODUCTION

In breeder chickens, once peak production starts declining, the percentage of infertile eggs increases, and egg laying per day and egg quality also decline (Durape, 2007; Liu et al., 2018). This may be due to the decline in reproductive potentials including vitellogenesis, lipogenesis, and antioxidant and immune status of the breeder chickens, and of course also due to management, environment, nutrition, genetics, or the combined effect of these factors (Joyner et al., 1987; Durape, 2007; Liu et al., 2013; Liu et al., 2018). Recently, our study revealed that sexual maturity promotes yolk precursor synthesis and follicle development in breeder hens via the liver-blood-ovary signal axis, which regulates the transport and exchange of synthetic substances, including yolk precursors and reproductive hormones (e.g., estradiol) to ensure synchronous development and functional coordination between the liver and ovary to promote egg production in breeder hens (Cui et al., 2020).

With advancing age, the reproductive performance of broiler breeders begins to decline. For instance, in female breeders, egg weight, egg number, egg quality, go down, and hatchability decrease. In addition, cases of infertility, embryonic mortality, and an increase in the number of cull chick's increase (Durape, 2007; Liu et al., 2013; Liu et al., 2014). Therefore, pragmatic management

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practices must be employed to enhance the reproductive, health, and egg quality potential of aging breeder hens.

The importance of nutrition in breeder chicken reproductive and health processes is undisputable. In the past, researchers employed the supplementation of synthetic feed additives such as antibiotics, into the diet of breeder chickens to promote egg laying rate, egg quality, and immunity (Liu et al., 2014). However, due to the ban on synthetic antibiotic usage in animal production and the incidence of drug residues in animal products (Liu et al., 2013; Dalia et al., 2018), researchers have recently made use of naturally safe dietary supplements to maximize animal production. For instance, in chickens and other animal models, phytochemicals and various dietary supplements such as flavonoids (Liu et al., 2013; Zhao et al., 2017; Shu et al., 2020), vitamins (Sahin et al., 2003; Abedi et al., 2017; Dalia et al., 2018; Liu et al., 2019), and minerals (Sahin et al., 2003; Dalia et al., 2018) have been reported to exhibit reproductive, stress-fighting, anti-inflammatory, antioxidant, antiaging, and immunity related potential.

Flavonoids are dietary polyphenolic compounds including quercetin, rutin, resveratrol, and catechin which are ubiquitously present in plants and play major roles in improving the performance of chickens (Vazquez Prieto et al., 2015; Zhao et al., 2018; Shu et al., 2020) Reports indicate that flavonoids, minerals, and vitamins promote egg production and quality in breeder hens (Kirunda et al., 2001; Sahin et al., 2003; Liu et al., 2013; Abedi et al., 2017; Dalia et al., 2018; Liu et al., 2019). Shu et al. (2020) revealed that bamboo leaf flavones enhance cytokine secretion in the bloodstream and splenic cytokine immune genes to exert immunoregulatory effects in chickens.

Quercetin is a major dietary polyphenolic flavonoid found in a variety of beverages, fruits, and vegetables with diverse biological activities including phytoestrogenic, anti-inflammatory, and immunoregulatory potential (Goliomytis et al., 2014; Yang and Chaudhry, 2018). Dietary quercetin supplementation in chicken diets has been reported to improve egg production and immunity via its estrogenic immunoglobulin and cytokine regulation properties (Muir et al., 2001; Liu et al., 2013; Liu et al., 2014; Yang and Chaudhry, 2018).

The National Research Council (1994) stated that chickens required vitamin E and hence recommended 4 to 6 IU VE/kg supplementation to the diets of healthy laying hens. However, vitamin E concentrations above physiological requirements do not have any side effects on laying hens (Sunder and Flachowsky, 2001). Vitamin E supplementation significantly increased egg production and egg quality in laying hens by facilitating the release of yolk precursor (vitellogenin) from the liver, and by acting as an anti-stressor (Bollengier-Lee et al., 1998; Puthpongsiriporn et al., 2001; Çiftci et al., 2005). It also promotes serum immunoglobulin and cytokine concentrations (El-Shenawy et al., 2015; Dalia et al., 2018). Several reports indicate that vitamin E and other vitamins or minerals promote egg laying performance and egg quality parameters of hens (Sahin et al., 2003; Çiftci et al., 2005; Scheideler et al., 2010; Abd El-Hack et al., 2019).

Becker et al. (2007) conducted a multiphasic systems analysis between quercetin and other chain-breaking antioxidants including rutin and α-tocopherol in lipid systems based on their increasing structural organization and concluded that quercetin may have a strong synergistic effect with α-tocopherol and rutin. This suggests that quercetin and α-tocopherol (vitamin E), and quercetin and rutin can be combined for in vivo experiments without any antagonism or negative interactions between them (Becker et al., 2007). Other reports also indicated that dietary quercetin exhibited strong synergistic effects by interacting with other dietary polyphenol extracts including catechin (Vazquez Prieto et al., 2015) and resveratrol (Zhao et al., 2017), to enhance biological processes, including immune and reproductive functions in animal models.

Currently, scientific data on the effects of quercetin and vitamin E in aging broiler breeders are limited, and to the best of our knowledge, there is a lack of information on the effects of combining quercetin and vitamin E on egg production, egg quality, and immunity on aging hens. Therefore, the aim of this study was to evaluate the effects of dietary quercetin and vitamin E supplementation alone and together on egg production, egg quality, serum immunoglobulins, and serum and splenic cytokine concentrations in aging Tianfu broiler breeders.

MATERIALS AND METHODS

Birds, Experimental Design, and Managements

A total of 400 Tianfu broiler breeders (52-wk-old) obtained from Sichuan Agricultural University, Chicken Breeding Experimental Unit, were used in this study, and the experiment was conducted at the same facility. The birds were randomly assigned to four (4) treatment groups (100 birds each) consisting of 4 replicates of 25 birds each. The birds were kept in an individual wire cages (width: 48.8 cm, depth: 38.1 cm, height: 38.1 cm) and the lighting system was controlled (16 h light per day) and optimal ventilation was maintained throughout the experimental period.

The birds were fed either a basal diet (Control); a basal diet supplemented with 0.4 g quercetin/kg of diet; a basal diet supplemented with 200 mg Vitamin E/kg of diet, and a basal diet supplemented with the combination of 0.4 g quercetin and 200 mg Vitamin E /kg of diet. Quercetin (95%, HPLC) and vitamin E were supplied by Shanxi Huike Plant Development Co., Ltd (Xi'an, Shaanxi, China). The purity of quercetin was determined by HPLC. Each hen was given 120 g per diet daily, and water was provided ad libitum. The diets were formulated to meet or exceed the National Research Council (1994) nutrient

recommendations (National Research Council, 1994). The composition and nutritional values of the basal diet for the experiment have been reported (Amevor et al., 2021). The experiment lasted for 10 wk, and throughout the experiment, daily observations and measurements were taken on egg laying rate, feed intake, and weekly determination of egg quality.

This study was approved by the Animal Care and Use Committee of Sichuan Agricultural University. Animals used in this experiment were cared for under the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching of Sichuan Province, China (No. 2019502005).

Determination of Feed Intake and Laying Performance

Feed consumption was recorded weekly by calculating the average weekly feed consumption of the hens for each replicate/treatment. Eggs were collected daily and the laying rates were recorded. Egg weight (g) was measured with an electronic weighing scale using eggs produced every day of the week. Moreover, feed-egg ratio was calculated as: feed-egg ratio = daily feed consumption/average egg weight. Subsequently, egg mass was calculated as the laying rate × egg weight. The performance was calculated and presented for the 10-wk experimental period.

Determination of Egg Quality Parameters

Two eggs were randomly collected from each replicate (totaling 32 eggs) per week and used for egg quality determination. Egg quality parameters such as egg weight (g), albumen height (mm), yolk weight (g), yolk diameter (cm), yolk height (mm), yolk color, Haugh unit, and eggshell thickness (mm) were determined. All egg quality parameters were evaluated for eggs collected at the end of each week. The egg weight (g), albumen diameter (cm), height (mm), yolk color, and Haugh units were determined using an Egg Multi-Tester machine following the manufacturer's instructions (Robotmotion Co. Ltd, Takanawa Minato-ku, Tokyo, Japan). Eggshell thickness (mm) was determined by measuring the average thickness taken at 3 locations on the egg shell (sharp end, blunt end, and middle section of the egg shell), and the yolk diameter (cm) and height (mm) were measured using Vernier calipers. An electronic weighing scale was used to measure the yolk weight (g).

Sample Collection and Procedure

At the end of the 10th wk of the experiment, 2 birds per replicate (8 birds per treatment, totaling 32) were randomly selected for blood sample collection. Blood samples (5 mL) were obtained from birds via the wing vein and were subsequently centrifuged at 3,000 rpm for 10 min at 4°C to separate the serum, and then stored at −80°C for further analyses. Subsequently, the chickens whose blood samples were collected were euthanized and their spleen samples were collected and weighed. Parts of the spleen samples were collected, snap-frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction and qRT-PCR analysis.

Immune Organs Index and Serum Immunological Assays

The immune organ index was calculated as the spleen organ weight divided by body weight. Serum immunoglobulin M (**IgM**), IgA, and immunoglobulin G (**IgG**) levels were determined using commercially specific enzyme-linked immunosorbent assay (**ELISA**) kits following the protocols provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, China).

Determination of Serum Cytokines

Serum concentrations of inter-leukins-2 (**IL-2**) and interferon-γ (**IFN- γ**) were determined using commercially specific ELISA kits following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Gene Expression Abundances of IL-2 and INF-γ in the Spleen

Total RNA was isolated from the spleen tissues using Trizol reagent (Takara, Japan) following the protocols provided by the manufacturer, and the concentration and purity were determined using a Nanodrop 2000C (Thermo Fisher Scientific, Waltham, MA) through the A260/280 absorbance ratio. First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. qRT-PCR was conducted using a CFX96 real-time system (Bio-Rad, Hercules, CA). Each qRT-PCR reaction was performed with volumes of 15 μL containing 6.25 μL TB Green Premix (Takara), 0.3 μL forward and reverse primers, 1.5 μL cDNA, and 6.65 μL DNase/RNase-Free Deionized Water (Tiangen, Beijing, China). GAPDH was used as an endogenous control to normalize the gene expression. The fold change in gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔCt = Ct target gene − Ct housekeeping gene, and $\Delta\Delta Ct = \Delta Ct - \Delta Ct$ reference. Primer 5 software was used to design the gene-specific primers used for qRT-PCR analysis according to the coding sequences of the target genes (Table 1).

Statistical Analysis

All data were analyzed by one-way analysis of variance (**ANOVA**) using GraphPad Prism version 6.01 statistical package for Windows (GraphPad Software Inc., San Diego, CA). All experimental data are

Table 1. Primers used for qRT-PCR.

Target gene	Primer sequences (5′-3′)	Product size (bp)	Gene bank no.
INF-γ	Forward: GCTCCCGATGAACGACTTGA Reverse: TGTAAGATGCTGAAGAGTTCATTCG	150	NM_205149
IL-2	Forward: GCTTATGGAGCATCTCTATCATCA Reverse: ACTCCTGGGTCTCAGTTGGT	130	NM_204153.1

Table 2. Effects of Quercetin, vitamin E, and Q + VE on feed intake, body weight, egg laying rate, egg weight, feed-egg ratio, and egg mass of aging hens.

Traits	Control	Quercetin	Vitamin E	Q + VE
Feed intake (g/day/laying hen)	91.75 ± 4.16^{c}	$104.79 \pm .2.61^{b}$	113.37 ± 4.09^{a}	108.91 ± 4.61^{ab}
Live body weight (g)	2602.81 ± 311.96	2655.94 ± 310.09	2637.50 ± 223.17	2675.00 ± 214.41
Laying rate %	56.53 ± 6.89^{c}	82.13 ± 4.66^{ab}	72.65 ± 4.36^{b}	84.55 ± 4.77^{a}
Egg weight (g)	43.55 ± 2.74^{b}	51.32 ± 1.16^{a}	51.66 ± 2.90^{a}	52.00 ± 1.90^{a}
Egg mass (g/day)	53.70 ± 17.76^{b}	103.17 ± 16.10^{a}	92.43 ± 17.52^{ab}	107.79 ± 21.80^{a}
Feed-egg ratio[1]	2.40 ± 0.17^{a}	2.16 ± 0.08^{b}	2.23 ± 0.05^{ab}	2.09 ± 0.02^{b}

The values are presented as mean ± standard deviation.
[a,b,c]Means in the same row without a common superscript letter differed statistically ($P < 0.05$).
[1]Feed-egg ratio = daily feed consumption/average egg weight.

indicated as the mean ± standard deviation (**SD**) and differences among treatments were examined using Tukey's test. Calculated Δ Ct (corrected sample) = mean value of target gene − mean value of internal reference gene, ΔΔ Ct = Δ Ct-mean value of control group. The values were significantly different at $P < 0.05$.

RESULTS

Effects of Quercetin, Vitamin E, and Their Combination on Performance of Aging Broiler Breeder Hens

The effects of dietary supplementation with quercetin (**Q**), vitamin E (**VE**), and the combination of Q + VE on feed intake, laying rate, egg weight, feed-egg ratio, and egg mass are shown in Table 2. The results showed that the relative amount of feed intake was increased by the addition of Q, VE, or their combination ($P < 0.05$), compared with the control. However, between individual Q and VE, there was a significant difference ($P < 0.05$) in which VE recorded the highest feed intake, while VE and Q + VE were similar ($P > 0.05$), but QVE differed from Q ($P < 0.05$). In addition, the laying rate of the aging broiler breeders was decreased in the control by 56.53% ($P > 0.05$), whereas, compared to the control, the Q, VE, and QVE significantly increased ($P < 0.05$) the laying rate by 82.13, 72.65, and 84.55%, respectively. The highest laying rate was recorded in the combination group which also differed from the VE group ($P < 0.05$) but similar to the Q group ($P > 0.05$). Again, we observed that the egg weight of the breeder chickens fed the basalt diet was significantly reduced by 15.43 g ($P > 0.05$) compared with the Q, VE, and the combination groups ($P < 0.05$) which were not different ($P > 0.05$) from each other. Moreover, Q and Q + VE differed significantly ($P < 0.05$) from the control for egg mass but were similar to the VE ($P > 0.05$), while the egg masses of the VE and the control were similar ($P > 0.05$). In addition, the group that received the combination of quercetin and vitamin E, showed a significant decrease in the feed-egg ratio during the experimental period compared to the control group ($P < 0.05$; Table 2).

Effects of Quercetin, VE, and Their Combination on Egg Quality of Aging Broiler Breeders

The results shown in Table 3 indicate that quercetin, vitamin E, and the combination of Q + VE significantly improved ($P < 0.05$) the yolk weight, yolk height, yolk diameter, and Haugh unit compared to the control

Table 3. Effects of Quercetin, vitamin E, and Q + VE on egg quality of aging hens.

Traits	Control	Quercetin	Vitamin E	Q + VE
Yolk weight (g)	15.43 ± 2.06^{b}	20.00 ± 2.07^{a}	19.17 ± 1.49^{a}	19.75 ± 2.17^{a}
Yolk height (mm)	15.35 ± 1.37^{b}	18.50 ± 0.93^{a}	17.91 ± 0.72^{a}	19.37 ± 2.03^{a}
Yolk diameter (mm)	38.08 ± 2.07^{b}	44.13 ± 2.77^{a}	42.16 ± 2.52^{a}	44.86 ± 4.32^{a}
Haugh unit	66.16 ± 11.23^{c}	89.00 ± 9.95^{ab}	80.73 ± 2.92^{b}	93.96 ± 12.51^{a}
Albumen height (mm)	6.29 ± 0.33^{b}	7.36 ± 0.76^{a}	7.11 ± 0.46^{ab}	8.03 ± 1.26^{a}
Yolk color	5.49 ± 0.73^{b}	7.96 ± 1.72^{ab}	6.61 ± 0.72^{ab}	9.38 ± 5.21^{a}
Eggshell thickness (mm)	0.28 ± 0.04^{b}	0.36 ± 0.03^{a}	0.30 ± 0.06^{b}	0.36 ± 0.04^{a}

The values are presented as mean ± standard deviation.
[a,b]Means in the same row without a common superscript letter differed statistically ($P < 0.05$).

Table 4. Effects of Quercetin, vitamin E, and Q + VE supplementation on the immune organ weight and index of the aging hens.

Parameters	Control	Quercetin	Vitamin E	Q + VE
Spleen weight (g)	2.56 ± 0.77^{b}	4.62 ± 1.39^{a}	4.81 ± 1.42^{a}	4.45 ± 1.70^{a}
Immune organ index[1]	0.11 ± 0.03^{b}	0.17 ± 0.05^{a}	0.17 ± 0.07^{a}	0.21 ± 0.08^{a}

The values are presented as mean ± standard deviation.
[a,b]Means in the same row without a common superscript letter differed statistically ($P < 0.05$).
[1]Immune organ index (Splenic index) = Organ weight/body weight.

group. For the yolk height, yolk diameter, and Haugh unit, the birds fed Q in combination with VE obtained the highest values compared to the control, Q, and VE groups. Moreover, the albumen height was significantly improved by the addition of Q and QVE ($P < 0.05$), compared with the control, but they (Q and Q + VE) were not statistically different from the VE group ($P > 0.05$). The combination group showed the highest albumen height. Furthermore, the results in Table 3 showed that the eggs obtained from the birds fed the combinatory diet produced a higher yolk coloration (deep yellow coloration) in comparative to the control ($P > 0.05$), meanwhile, between the control, Q, and VE, there were no significant differences observed for the yolk coloration in this study ($P > 0.05$). Again, Q, VE, and Q + VE showed no differences in yolk color ($P > 0.05$). The eggshell thickness obtained from quercetin alone or in combination with vitamin E group was significantly improved ($P < 0.05$) compared with the control and VE groups, whereas the control and VE groups did not differ ($P > 0.05$; Table 3).

Effects of Q, VE, and QVE on Immunity of Aging Broiler Breeders

Immune Organ Weight and Index (Splenic Index)

The positive effects of quercetin, VE, and Q + VE on aging broiler breeder's immune organ (spleen) wet weight and splenic organ index (since the thymus and bursa of fabricius disappeared in aging chickens) are presented in Table 4. Separately or in combination, supplemental quercetin and vitamin E increased spleen wet weight and the splenic index compared with the control ($P < 0.05$; Table 4).

Serum Immunoglobulin Concentration The effects of quercetin, vitamin E, and their combination on serum immunoglobulin concentrations in aging hens are shown in Figure 1. Supplementation with Q, VE, and Q + VE increased IgM and IgA concentrations ($P < 0.05$), compared to the control group. Except for IgM, the combinatory group had the highest IgA and IgG concentrations (Figures 1A and 1B). However, for serum IgG concentration, there were no differences observed between the control and VE groups ($P > 0.05$; Figure 1C). In contrast, Q and Q + VE significantly increased the concentration of IgG compared to the control and VE groups ($P < 0.05$; Figure 1C).

Serum Cytokines (INF-γ and IL-2) Levels The effects of Q, VE, and Q + VE on serum cytokine (IL-2 and INF-γ) concentrations in aging broiler breeders are shown in Figure 2. Compared to the control group, IFN-γ concentrations were significantly increased in the Q, VE, and Q + VE groups ($P < 0.05$; Figure 2A). Similarly, IL-2 concentration was increased by the addition of Q, VE, and Q + VE compared to the control group ($P < 0.05$; Figure 2B).

Splenic Cytokines Gene Expression (INF-γ and IL-2) The gene expression of INF-γ and IL-2 was examined in the spleen as shown in Table 5. In comparison to the control group, the INF-γ gene was significantly expressed in the spleen of the birds fed quercetin, vitamin E, and the combination of quercetin and vitamin E ($P < 0.05$). However, in the expression of IL-2 gene in the spleen, there were no significant differences ($P >$

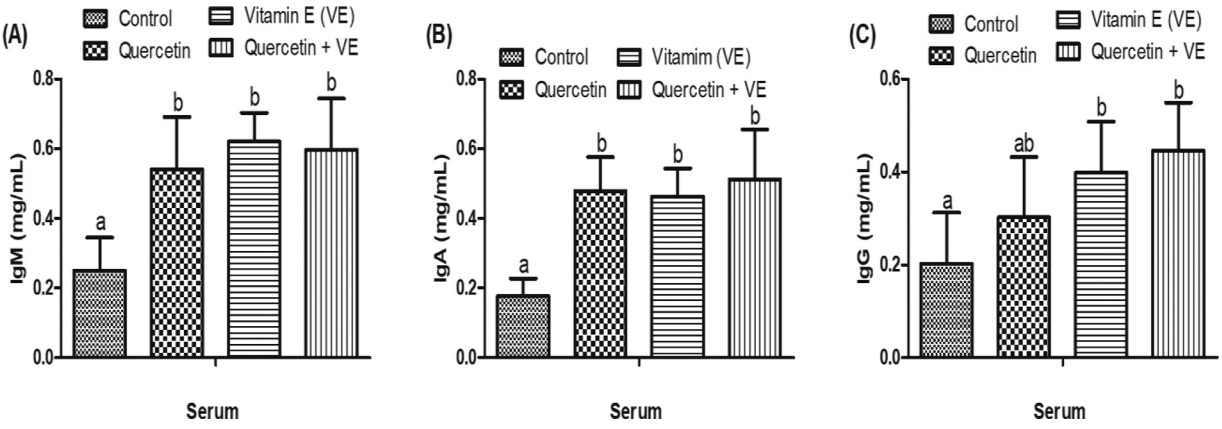

Figure 1. Effect of dietary Quercetin (Q), Vitamin E (VE), and Q + VE on serum immunoglobulins of aging hens. The values are presented a mean ± standard deviation. Bars without the same letter differed significantly ($P < 0.05$). A−C represents serum IgA, IgM, and IgG, respectively. Abbreviations: IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

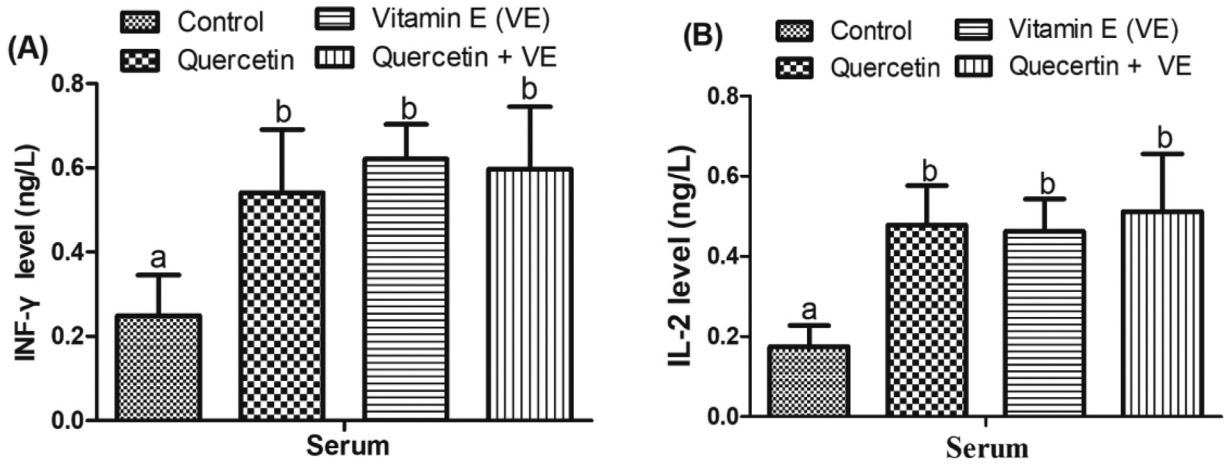

Figure 2. Effects of Quercetin, Vitamin E, and Q + VE on serum cytokines levels (INF-γ and IL-2) of aging hens. The values are presented as mean ± standard deviation. Bars without the same letter differed significantly ($P < 0.05$). A and B represent serum INF-γ and IL-2, respectively.

Table 5. Effects of Quercetin, vitamin E, and Q + VE on gene expression abundances of immune cytokines in the spleen of aging hens.

Genes	Control	Quercetin	Vitamin E	Q + VE
IFN-γ	1.33 ± 0.89^{b}	4.43 ± 3.38^{a}	5.70 ± 2.37^{a}	5.79 ± 3.02^{a}
IL-2	0.56 ± 0.46^{b}	1.69 ± 1.14^{a}	1.19 ± 0.44^{ab}	1.50 ± 1.09^{a}

The values are presented as mean ± standard deviation.
[a,b]Means in the same row without a common superscript letter differed statistically ($P < 0.05$).

0.05) between the control and the vitamin E group, whereas, compared to the control, the expression of spleen IL-2 gene was significantly increased in both quercetin and combination (Q + VE) groups ($P < 0.05$; Table 5).

DISCUSSION

In the present study, it was shown that quercetin, vitamin E, and their mixture improved feed intake, egg production, egg mass, egg weight, egg quality, and immune related factors in the aging breeder hens. To date, there have been no reports on the combined synergistic effects of quercetin and vitamin E on the performance of aging broiler breeders.

In the present study, feed intake, egg weight, feed-egg ratio, and egg mass were significantly influenced by quercetin, vitamin E, and a combination of both. This shows that the dietary supplements were palatable for easy consumption by the hens. Previous reports in support of the findings in this study indicated that the addition of synthetic soybean isoflavone from 10 to 80 mg/kg feed significantly increased feed intake in chickens (Jiang et al., 2007), while Maini et al. (2007) also reported an increased feed intake in birds fed 200 mg/kg of vitamin E. However, the current results were inconsistent with those reported by Yang et al. (2020), who reported no differences in feed intake between birds fed dietary quercetin and those fed basal diet, but we speculate that the differences in the results of the present and previous studies may be caused by age differences in the chickens used, breed, doses, and structure of various dietary flavonoids and phytonutrients used (Espín et al., 2017; Yang et al., 2020). Moreover, dietary supplements such as vitamin E can be directly stored in tissues as a fat-soluble vitamin in the form of α-tocopherol and is also involved in facilitating the transportation and deposition of yolk precursors into the oocyte (Jiang et al., 2013). In addition, the vitellogenesis functions of quercetin and the synergetic roles played by the mixture of quercetin and vitamin E may have contributed to the improved egg weight, egg mass, and yolk weights observed in the present study. Vitamin E has also been shown to promote egg production and quality in laying hens (Bollengier-Lee et al., 1998; Puthpongsiriporn et al., 2001; Çiftci et al., 2005; Yardibi and Gülhan Türkay, 2008; Liu et al., 2014; Abedi et al., 2017; Liu et al., 2019).

It has been well documented that flavonoids, including quercetin (a phytoestrogenic compound) exert agonistic and antagonistic effects depending on the intrinsic estrogen concentration (Cassidy, 2003; Yang and Chaudhry, 2018; Amevor et al., 2021). This is because estradiol ($\mathbf{E_2}$) actively facilitates the production and function of other reproductive hormones, including follicle stimulating hormone (**FSH**) in sexually mature hens (Liu et al., 2018; Cui et al., 2020). The results of this study first showed the potential of the combination of quercetin and vitamin E to improve the laying rate and decrease the feed-egg ratio in aging laying hens. Therefore, the combination of quercetin and vitamin E may synergistically exhibit estrogenic and vitellogenic attributes to enhance egg production.

During the late laying period, egg quality decreases with increasing age (Liu et al., 2014). Moreover, the results obtained in the present study showed that dietary supplementation with quercetin, vitamin E, and the combination of both improved the egg quality parameters including yolk weight, yolk height, yolk diameter, yolk color, albumen height, Haugh unit, and

eggshell thickness. Similar results were reported by Liu et al. (2013) and Liu et al. (2014), who reported that dietary quercetin supplementation in layer diets improves the quality of chicken eggs, and others also reported significant increases in egg mass, egg yolk, and Haugh unit of layer hens fed a diet supplemented with vitamin E (58.5 mg) (Puthpongsiriporn et al., 2001). Jiang et al. (1994) reported evidence indicating a direct relationship between dietary α-tocopherol acetate levels and egg yolk concentration, but the magnitude of response and the potential interaction with other dietary constituents have not been clearly established. The egg Haugh unit is an indicator of protein content in egg albumen (Jiang et al., 2013; Liu et al., 2013; Liu et al., 2014). These results showed that quercetin and vitamin synergistically improved egg protein content. The mixture of quercetin and vitamin E significantly improved the egg yolk coloration; this indicated that at aging laying periods the yolk coloration may be improved by synergistic effects of quercetin and vitamin E through their pigment compounds (xanthophyll and carotenoid). Reports have indicated that flavonoids improve egg shell thickness via calcium metabolism regulated by their estrogen-like effects (Liu et al., 2013). In this study, quercetin and a mixture of quercetin and vitamin E significantly improved eggshell thickness. These results are consistent with those reported by Liu et al. (2014) who stated that quercetin promotes egg shell quality and thickness. This finding shows that the synergistic effects of quercetin and vitamin E could positively influence calcium metabolism through hormonal pathways during the aging period to improve eggshell thickness. Therefore, the results obtained in this study show that the mixture of vitamin E and quercetin exerted synergetic activities on modulating processes involved in egg production and egg quality (vitellogenesis and hormone production) in the aging hens; however, further studies are required.

The immune system is responsible for fighting contaminants that enter the body (Work et al., 2015; Yasuma et al., 2016; Yang et al., 2020). Hence, homeostatic immune systems should be maintained (Work et al., 2015; Dalia et al., 2018). Immunoglobulins, such as IgM, IgA, and IgG, act as antibodies by regulating immune homeostasis. Usually, IgM is the first called for action to fight against foreign harmful substances and is mostly produced in abundance compared to IgG. In most cases, IgA is regarded as the main Ig in external secretions; hence, it is referred to as secretory IgA (**sIgA**) (Work et al., 2015; Yang et al., 2020).

Flavonoids have been reported to improve immunity in animals (Shu et al., 2020; Yang et al., 2020). The immune organ weight and index are indicators of the capability of lymphoid cells to produce immune factors in response to stimulations (Jeurissen, 1991; Shu et al., 2020); our results in the current study showed that the combination of quercetin and vitamin E supplementation exerted synergistic effects on the splenic weight and splenic organ index by stimulating the development of the spleen, thereby improving the immune performance in the aging breeder hens. Similar findings reported by Shu et al. (2020) showed a significant increase in the weights and index of the spleen, bursa fabricius, and thymus of Arbor Acres broilers fed bamboo leaf flavone, and Yang et al. (2020), who reported that quercetin supplementation in the diet significantly increased the development of spleen and thymus organs (Yang et al., 2020). Reports indicate that immune organ weights are directly correlated with immune enhancement; thus, an increased immune organ weight signifies immunity enhancement, whereas the reverse indicates immunosuppression in chickens (Iftikhar et al., 2012). The findings of the present study indicate that combining quercetin and vitamin E may exert synergistic effects to enhance immune organ development and function in aging breeder hens. In addition, the combination of quercetin and vitamin E significantly increased the serum immunoglobulin (IgA, IgM, and IgG) concentrations. These results are consistent with those of previous studies. For instance, Liu et al. (2019) reported that supplementation with vitamin E significantly increased the concentrations of IgM, IgA, and IgG in chickens (Singh et al., 2006; Liu et al., 2019). Similarly, Yang et al. (2020) reported that dietary quercetin enhanced immunoglobulin response in a dose-dependent manner, and Liang et al. (2011) reported that flavonoids extracted from *Scutellaria baicalensis* significantly increased serum immunoglobulins (IgA and IgG) in broiler chickens.

Generally, the results obtained in this study showed synergistic effects exerted by the combination of quercetin and vitamin E on serum immunoglobulins. However, in this study, no difference was observed between the control and quercetin groups in serum IgG concentrations. This result is contrary to that reported by Yang et al. (2020), but this difference may be related to the age, origin, or structure of flavonoids and different breeds of chickens used. It has also been previously shown that the structure of flavonoids affects immune signaling pathways in chickens (Shin et al., 2011). However, there is the need for further studies to clarity this occurrence (Dinarello, 2000; Dalia et al., 2018). The cytokine profile is an important marker of the immune status in animals, because cytokines are protein messengers released by the host immune response to infection, inflammation, and trauma (Dinarello, 2000; Dalia et al., 2018). For instance, TNF-α, IFN-γ, and IL-2 are proinflammatory cytokines produced by activated monocytes, macrophages, T cells, and natural killer cells to enhance the host defense system (Dinarello, 2000; Fisher, 2010; Dalia et al., 2018; Liu et al., 2019; Shu et al., 2020). The results of this study showed that quercetin, vitamin E, and their combination significantly increased serum cytokine (IFN-γ and IL-2) levels. In addition, the expression of splenic immune related genes (INF-γ and IL-2) was also enhanced in the quercetin and the combinatory groups compared with the control and vitamin E groups. This indicates that the mixture of quercetin and vitamin E exerted synergetic effects and promoted the secretion of IFN-γ and IL-2 through the bloodstream

and subsequently improved the immunoregulatory functions of the aging hens.

Generally, the present study showed that, separately or in combination, supplemental quercetin and vitamin E improved performance and immunity in the aging hens compared with those fed a basal diet. Thus, when a significant effect was found for a parameter, the magnitude of the responses to quercetin and vitamin E supplements was greatest with the combination of quercetin and vitamin E, rather than that observed with each supplement separately. Consequently, the synergistic effects of quercetin and vitamin E combination increases plasma immunoglobulin (IgM, IgA, and IgG) levels, serum cytokine and splenic cytokine gene expression, egg production, and egg quality parameters. Hence, supplementation with a combination of quercetin and vitamin E should offer better results than supplementation with quercetin and vitamin E separately. It is therefore, recommended that the supplementation of quercetin and vitamin E combination may exert synergistic effects without any form of antagonism to improve egg production and immunity in aging breeder hens. We also recommend further studies to explore the molecular mechanisms through which the combination of quercetin and vitamin E synergistically regulate vitellogenesis, lipogenesis, and antioxidant capacity of aging hens.

DISCLOSURES

The authors declare that they have no competing interests.

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