

Paper type: Original Research

## Synergistic effects of probiotic, vitamin C, and organic selenium on performance, eggshell quality, antioxidant capacity, and yolk fatty acid composition in laying hens

Mohamad Hossein Khalesi, Mohamad Salarmoini\*, Mohsen Afsharmanesh, Mohamad Khajeh Bami

Department of Animal Science, Faculty of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran

\*Corresponding author,  
E-mail address:  
salarmoini@uk.ac.ir

Received: 23 Nov 2025,  
Received in revised form: 05 Jan  
2026,  
Accepted: 13 Mar 2026,  
Published online: 14 Mar 2026,  
© The authors, 2027.

### ORCID

Mohamad Hossein Khalesi  
0009-0003-3720-439X  
Mohamad Salarmoini  
0000-0003-3240-8521  
Mohsen Afsharmanesh  
0000-0002-4782-8687  
Mohamad Khajeh Bami  
0000-0001-9671-0952

**Abstract** This study evaluated the effects of dietary supplementation with probiotic, vitamin C, and organic selenium (selenium-enriched yeast), individually or in combination, on productive performance, eggshell quality, antioxidant status, immune response, and yolk fatty acid profile in laying hens. A total of 180 Bovans hens aged 45 weeks were randomly assigned to five dietary treatments for 12 weeks: (1) Control diet (basal diet supplemented with 0.3 mg/kg inorganic selenium); (2) control diet supplemented with 0.3 g/kg probiotic; (3) control diet supplemented with 0.1 g/kg vitamin C; (4) basal diet supplemented with 0.3 mg/kg organic selenium; and (5) basal diet supplemented with probiotic + vitamin C + organic selenium. Productive traits, including egg production, egg weight, and feed intake, were recorded, and the feed conversion ratio (FCR) was calculated, while blood samples were analyzed for antibody titer against sheep red blood cells (SRBC), antioxidant enzyme activities, and total antioxidant capacity (TAC). Eggshell quality parameters and yolk fatty acid composition were also determined. The results indicated that hens receiving the combination of probiotic, vitamin C, and organic selenium exhibited the lowest FCR and highest egg mass and egg weight compared with most other treatments ( $P<0.05$ ). Organic selenium improved several aspects of eggshell quality, including shell strength and egg shape index ( $P<0.05$ ). Vitamin C supplementation increased total antibody titer relative to the probiotic group, although no differences were observed compared with the control group, and IgM and IgG levels remained unchanged. Activities of antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) were not affected by dietary treatments, but TAC was significantly elevated in the combination group ( $P<0.05$ ). Combined dietary inclusion of probiotic, vitamin C, and organic selenium enhances yolk polyunsaturated fatty acids. In conclusion, concurrent dietary inclusion of probiotic, vitamin C, and organic selenium improved feed efficiency, certain aspects of eggshell quality, total antioxidant capacity, and yolk fatty acid composition in laying hens. These findings suggest that the combined supplementation of these nutrients represents a promising nutritional strategy to enhance productive performance, oxidative stability, and yolk nutritional value, while single additives may produce more limited effects.

**Keywords:** laying hens; probiotic; vitamin C; selenium; eggshell quality

### Introduction

Maintaining optimal laying performance, eggshell quality, and egg nutritional value is critical in commercial egg production. However, oxidative stress, environmental

challenges, and age-related declines in physiological functions can adversely affect these traits. Nutritional interventions that enhance antioxidant defense, immune competence, and nutrient utilization have therefore

gained increasing attention. Among these, organic selenium, vitamin C, and probiotics are of particular interest due to their synergistic roles in improving the health and productivity of laying hens.

Selenium is an essential trace mineral required for the synthesis of selenoproteins such as glutathione peroxidase and thioredoxin reductase, which protect cells against oxidative damage and maintain redox homeostasis. Organic selenium, typically supplied as selenium-enriched yeast (Se-yeast), exhibits greater bioavailability than inorganic forms and is more effectively deposited into egg yolk and tissues. Supplementation with Se-yeast has been shown to improve egg selenium concentration, antioxidant enzyme activities, and egg quality, while reducing lipid peroxidation and oxidative stress in laying hens (Abbas et al., 2022; Liu et al., 2023). Moreover, organic selenium may enhance eggshell strength and specific gravity by supporting mineral metabolism and collagen crosslinking in the shell membrane.

Vitamin C (ascorbic acid) is a potent water-soluble antioxidant that interacts with vitamin E and selenium-dependent enzymes to regenerate oxidized antioxidants and protect biological membranes from oxidative damage. Although poultry can synthesize vitamin C endogenously, supplementation has been shown to be beneficial under stressful conditions, improving immune response, antioxidant capacity, and potentially improving shell quality (Skřivan et al., 2013). Vitamin C also contributes to collagen synthesis, which is vital for proper eggshell formation. Combined supplementation of vitamin C and selenium has demonstrated enhanced antioxidant enzyme activity and improved productive performance compared with either nutrient alone (Pečjak et al., 2022).

Probiotics, which are live microorganisms such as *Lactobacillus*, *Bacillus*, and *Pediococcus* species, are widely used to modulate gut microbiota, enhance nutrient absorption, and stimulate the immune system. In laying hens, probiotics have been associated with improved feed conversion ratio, egg production, eggshell quality, and changes in yolk fatty acid composition, including increased polyunsaturated fatty acids (Mikulski et al., 2012; Macit et al., 2021). Probiotics also reduce intestinal oxidative stress and promote systemic antioxidant status, indirectly supporting reproductive and metabolic performance.

The combination of selenium and probiotics has been reported to improve antioxidant capacity, selenium deposition, and gut morphology (Deng et al., 2022), whereas co-supplementation of selenium and vitamin C further enhances antioxidant status and immune responses (Skřivan et al., 2013; Pečjak et al., 2022). However, despite these promising outcomes, comprehensive evaluations of the combined use of selenium-enriched yeast, vitamin C, and probiotics in laying hens remain limited, particularly in relation to eggshell characteristics, antioxidant capacity, immune response, and yolk fatty acid composition.

Therefore, the present study was designed to investigate the individual and combined effects of dietary supplementation with probiotic, vitamin C, and organic selenium on productive performance, eggshell quality, antioxidant status, immune response, and yolk fatty acid profile in laying hens. It was hypothesized that the simultaneous inclusion of these supplements would exert synergistic effects, resulting in enhanced production efficiency and improved egg quality compared with individual supplementation in the diet.

## Materials and methods

### *Experimental design and birds*

This experiment was conducted using 180 Bovans laying hens with an initial average body weight of 1,542 g. The study followed a completely randomized design (CRD), consisting of five dietary treatments, each with six replicates and six hens per replicate housed in individual cages. The trial lasted 14 weeks, including a 2-week adaptation period and a 12-week production phase (from 45 to 57 weeks of age), which was divided into three 4-week intervals.

The experimental groups were designed as follows: (1) Control diet (basal diet supplemented with 0.3 mg/kg inorganic selenium in the form of sodium selenite); (2) control diet supplemented with 0.3 g/kg probiotic; (3) control diet supplemented with 0.1 g/kg vitamin C; (4) basal diet supplemented with 0.3 mg/kg organic selenium; and (5) basal diet supplemented with probiotic + vitamin C + organic selenium.

All hens were provided *ad libitum* access to feed (in mash form) and water throughout the experiment. Diets were formulated according to the nutritional requirements for Bovans layers (Table 1). Before the initiation of the experiment, the birds were evaluated for body weight, egg production rate, and flock uniformity to ensure no significant variation among groups.

The inorganic selenium source ( $\geq 99\%$  purity) was procured from Sigma (St. Louis, MO, USA). The probiotic supplement, containing *Bacillus licheniformis* and *Bacillus subtilis* ( $1.6 \times 10^{11}$  CFU/kg), was supplied by Takgene Co. (Tehran, Iran) at a recommended inclusion rate of 0.3 g/kg of feed (provided approximately  $4.8 \times 10^8$  CFU per kilogram of diet). Vitamin C was obtained from Zagros Pharmed Pars Co. (Tehran, Iran) and used at 0.1 g/kg of feed. The selenium-enriched yeast (organic selenium) was provided by Radin Dam Co. (Tehran, Iran).

### *Productive performance*

Weekly feed intake was determined throughout the experimental period. Eggs were collected daily at 12:00 AM, and both egg number and average egg weight were recorded for each replicate. Total egg mass per replicate was calculated using the following formula:

$$\text{Egg mass (g)} = (\text{egg production} \times \text{average egg weight}) / 100$$

Feed conversion ratio (FCR) was calculated on a weekly basis as the ratio of feed intake (kg) to egg mass

(kg). Mortality was also monitored and recorded daily.

**Table 1.** Ingredients and calculated chemical composition of the basal diet

Ingredients	Percentage (as-fed basis)
Corn	50.90
Soybean meal (41%)	27.80
Wheat	10.00
Calcium carbonate	9.10
Dicalcium phosphate	1.10
Salt	0.40
DL-Methionine	0.20
Vitamin supplement <sup>1</sup>	0.25
Mineral supplement <sup>2</sup>	0.25
Chemical composition	
Metabolizable energy (kcal/kg)	2,700
Crude protein (%)	17
Methionine (%)	0.45
Lysine (%)	0.86
Methionine + Cystine (%)	0.72
Available phosphorus (%)	0.34
Calcium (%)	3.80
Sodium (%)	0.17
Selenium, analyzed (mg/kg)	0.05

<sup>1</sup>Per kg of diet: 120 mg manganese, 40 mg iron, 16 mg copper, 1 mg iodine, 100 mg zinc.

<sup>2</sup>Per kg of diet: 12,000 IU vitamin A (retinol), 5,000 IU vitamin D<sub>3</sub> (cholecalciferol), 3 mg vitamin B<sub>1</sub> (thiamine), 7.5 mg vitamin B<sub>2</sub> (riboflavin), 51 mg vitamin B<sub>3</sub> (niacin), 4.5 mg vitamin B<sub>6</sub> (pyridoxine), 0.02 mg vitamin B<sub>12</sub> (cyanocobalamin), 2.55 mg vitamin K<sub>3</sub>, 1.5 mg vitamin B<sub>9</sub> (folic acid), 13.5 mg vitamin B<sub>5</sub>.

### Eggshell quality characteristics of fresh and stored eggs

At the end of each experimental period (the last two days of every 28-day cycle), two eggs were randomly collected from each replicate to evaluate eggshell traits, including shell percentage, specific gravity, shell thickness, shell strength, and shell ash content.

To assess the effects of dietary treatments on these parameters under storage conditions, two eggs per replicate were also stored at 4°C for 28 days (Kralik et al., 2021). To determine the shell thickness and shell ash, the eggshell was separated from the albumen and yolk, and air-dried at room temperature for 24h.

Egg length and width were measured using 0.01mm digital caliper, and the egg shape index was calculated by dividing egg width (mm) by egg length (mm) and multiplying by 100 (Nemati et al., 2020). The specific gravity of eggs was determined using the salt solution flotation method (VanEmous, 2023).

Eggshell breaking strength was measured using a shell strength tester. The eggs were placed horizontally in the device, and a 5 mm diameter probe was used. The texture analyzer operated at a speed of 100 mm/min, and the maximum force exerted on the shell (measured in Newtons) was recorded as shell strength, providing an indication of how much force the eggshells could withstand before breaking (Ahmadi et al., 2022).

Shell thickness was measured using a digital micrometer at three points of each egg without the eggshell membrane (top, bottom, and middle). The average of the three measurements was recorded as the eggshell thickness (Aydin et al., 2008).

To calculate the eggshell ash content, the eggshells were dried for 48h at room temperature, weighed and then ground. Approximately 2 g was weighed into a crucible, and ashed in a furnace at 650°C for 12 hours (Aydin et al., 2008).

### Immune response evaluation

To evaluate the primary and secondary humoral immune responses, 2 hens per replicate (12 hens/treatment) were injected with 1 mL of a 5% sheep red blood cell (SRBC) suspension into the breast muscle. The second injection was administered after 7 days to ensure a stable immune response during the second sampling. Seven days after the injections, blood samples (2 mL/hen) were collected from the brachial vein. The blood samples were centrifuged to separate the serum, which was then stored at -20°C until further analysis. The antibody titers, including total anti- bodies and IgG anti-SRBC antibodies (mercaptoethanol- resistant antibodies against SRBC), were measured using hemagglutination assays. In brief, for the assessment of total antibodies, 50 mL of PBS was placed in the first row of wells in a fresh 96-well V-bottom microtitration plate. Subsequently, 50 mL of serum (after inactivation at 56°C for 30 min) were added to the same wells. The plates were then sealed and incubated at 37°C for 30 min. Following incubation, 50 mL of PBS were added to the 11 remaining wells in each row. A 2-fold serial dilution of the samples was carried out on successive rows. Furthermore, 50 mL of a 2.5% SRBC suspension were added to each well, and the plates were sealed and incubated for an additional 30 min (Wegmann and Smithies, 1966).

The IgG antibody titers were assessed using a similar procedure as for total titers, with the exception that 50 mL of 2-mercaptoethanol-resistant were added to the first row of wells. The antibody titers were determined by observing the wells showing agglutination while holding plates over a lighted mirror. The antibody titers were reported as log<sub>2</sub> of the reciprocal of the last dilution in which agglutination was observed. The IgM titer was determined by calculating the difference between the total antibody titer and the IgG titer (Khajeh Bami et al., 2022).

### Antioxidant enzyme activity

On the final day of the experiment, blood samples were collected from 2 hens per replicate (12 hens/treatment) through the wing vein and transferred into sterilized tubes. The tubes were then centrifuged at 3,000 g for 10 min at 4°C to separate the serum. The activity of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) was assessed using a Randox Assay Kit (London, UK) according to the manufacturer's instructions. The activity of catalase (CAT) was determined using the Sinha (1972) method, which involves the use of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as a substrate. In this method, a mixture containing phosphate buffer, distilled water, and the necessary

enzyme was prepared, to which 1 mL of H<sub>2</sub>O<sub>2</sub> was added to initiate the reaction. The solution was then incubated at 37°C for 2 min. To terminate the reaction, 2.5 mL of the DDA reagent (potassium dichromate solution 5% and glacial acetic acid) were added (at a ratio of 1:3 v/v). Finally, the absorbance of the reaction mixture was measured at 570 nm (Rafeenia et al., 2022).

**Yolk fatty acid profile**

At the end of the experiment, one egg per replicate was collected and the yolks were stored at 20°C for further analysis. The fatty acid concentration in the yolks was determined using the Folch et al. (1957) extraction method. Gas chromatography (Agilent 7890A, Santa Clara, CA) was employed to separate the different fatty acids, with specific temperature settings and an internal standard used. The peaks corresponding to the fatty acids were identified and quantified based on their retention times and peak areas. The results were expressed as a percentage of the total fatty acids in the yolk (Ghasemi et al., 2022).

**Statistical analysis**

The data were analyzed as a Completely Randomized Design (CRD) using the SAS (2005) software; and

treatment means were compared using the Tukey's multiple range test at a 5% probability level.

**Results**

**Feed intake, feed conversion ratio, and egg production traits**

The effects of dietary treatments on feed intake and feed conversion ratio (FCR) are presented in Table 2. Hens fed diets supplemented with either probiotic or organic selenium exhibited significantly lower daily feed intake compared to those fed the control diet (P<0.05).

The lowest FCR was observed in hens fed the combination diet (probiotic + vitamin C + organic selenium), which differed significantly from both the control and vitamin C-supplemented groups (P<0.05).

Dietary treatments significantly influenced egg production percentage, egg weight, and egg mass (Table 2). Hens fed the combination diet achieved the highest egg production, which was only significantly greater than that of the vitamin C group (P<0.05).

Average egg weight was highest in the combination group and differed significantly from all other treatments except the vitamin C group (P<0.05). Egg mass followed a similar trend, with the combination diet yielding significantly greater values than all other dietary treatments (P<0.05).

**Table 2.** Effects of experimental diets on feed intake, feed conversion ratio, and body weight gain of laying hens

Treatments	Feed intake (g/hen/d)	FCR	Egg production (%)	Egg weight (g)	Egg mass (g/hen/d)
Control	117 <sup>a</sup>	2.42 <sup>ab</sup>	79.37 <sup>ab</sup>	61.31 <sup>b</sup>	48.66 <sup>b</sup>
Probiotic	114 <sup>b</sup>	2.32 <sup>bc</sup>	81.21 <sup>a</sup>	60.73 <sup>b</sup>	49.31 <sup>b</sup>
Vitamin C	117 <sup>a</sup>	2.44 <sup>a</sup>	77.54 <sup>b</sup>	62.13 <sup>ab</sup>	48.17 <sup>b</sup>
Organic Se	114 <sup>b</sup>	2.33 <sup>abc</sup>	80.97 <sup>ab</sup>	60.66 <sup>b</sup>	49.10 <sup>b</sup>
Probiotic + vitamin C + organic Se	117 <sup>a</sup>	2.26 <sup>c</sup>	81.40 <sup>a</sup>	64.03 <sup>a</sup>	52.11 <sup>a</sup>
SEM	0.502	0.027	0.877	0.501	0.559
P-value	0.001	0.001	0.021	0.001	0.001

<sup>a,b</sup>Means in a column with common superscript(s) do not differ (P>0.05)  
SEM: Standard error of the mean

**Egg shape index and eggshell quality**

As shown in Table 3, dietary treatments had different effects on the egg shape index of fresh and stored eggs during the experimental periods. In the first production period, organic selenium supplementation significantly increased the shape index of stored eggs compared with the probiotic treatment (P<0.05). During the third period,

the combined supplementation of vitamin C, probiotic, and organic selenium resulted in a significantly higher egg shape index than the organic selenium group (P<0.05). However, no significant differences were observed among treatments during the second period or across the overall experimental period for either fresh or stored eggs.

**Table 3.** Shape index of fresh and stored eggs of laying hens fed experimental diets (%)

Treatments	Weeks 46-49		Weeks 50-53		Weeks 54-57		Whole period	
	Fresh	Stored	Fresh	Stored	Fresh	Stored	Fresh	Stored
Control	77.07	76.77 <sup>ab</sup>	77.52	77.33	77.20 <sup>ab</sup>	75.83	77.26	76.64
Probiotic	78.04	77.75 <sup>b</sup>	78.05	76.92	76.50 <sup>ab</sup>	77.73	77.53	76.80
Vitamin C	78.24	77.14 <sup>ab</sup>	76.90	77.57	76.54 <sup>ab</sup>	77.58	77.23	76.80
Organic Se	77.79	78.86 <sup>a</sup>	77.21	77.40	75.82 <sup>b</sup>	77.74	76.94	78.00
Probiotic + vitamin C + organic Se	78.08	77.70 <sup>ab</sup>	78.32	77.83	78.66 <sup>a</sup>	77.62	78.35	77.72
SEM	0.746	0.615	0.603	0.668	0.594	0.691	0.415	0.393
P value	0.817	0.013	0.440	0.481	0.018	0.239	0.165	0.056

<sup>a,b</sup>Means in a column with common superscript(s) do not differ (P>0.05)  
SEM: Standard error of the mean

**Additive effects of probiotic, vitamin C, and Se in hens**

Table 4 presents the effects of experimental diets on the specific gravity of fresh and stored eggs. In the first period, hens receiving probiotic supplementation produced fresh eggs with a higher specific gravity than those fed organic selenium ( $P < 0.05$ ). Stored eggs from hens fed the combined supplement diet showed significantly greater specific gravity than those from other treatments ( $P < 0.05$ ). In contrast, during the second

period, the combined supplementation significantly reduced the specific gravity of stored eggs compared with other treatments ( $P < 0.05$ ). Throughout the entire experimental period, specific gravity of fresh eggs was not influenced by dietary treatments, whereas organic selenium and the combined supplement diet significantly increased the specific gravity of stored eggs compared with the control group ( $P < 0.05$ ).

**Table 4.** Specific gravity (g/cm<sup>3</sup>) of fresh and stored eggs from hens fed with the experimental diets

Dietary treatments	Weeks 46-49		Weeks 50-53		Weeks 54-57		Whole period	
	Fresh	Stored	Fresh	Stored	Fresh	Stored	Fresh	Stored
Control	1.100 <sup>ab</sup>	1.085 <sup>c</sup>	1.100	1.091 <sup>a</sup>	1.101	1.085	1.100	1.087 <sup>b</sup>
Probiotic	1.102 <sup>a</sup>	1.085 <sup>c</sup>	1.099	1.091 <sup>a</sup>	1.101	1.087	1.101	1.087 <sup>ab</sup>
Vitamin C	1.100 <sup>ab</sup>	1.086 <sup>bc</sup>	1.099	1.090 <sup>a</sup>	1.101	1.087	1.100	1.088 <sup>ab</sup>
Organic Se	1.097 <sup>b</sup>	1.089 <sup>b</sup>	1.100	1.092 <sup>a</sup>	1.101	1.087	1.099	1.089 <sup>a</sup>
Probiotic + vitamin C + organic Se	1.100 <sup>ab</sup>	1.095 <sup>a</sup>	1.101	1.085 <sup>b</sup>	1.101	1.088	1.101	1.089 <sup>a</sup>
SEM	0.001	0.001	0.001	0.100	0.100	0.001	0.001	0.001
P value	0.005	0.001	0.275	0.001	0.852	0.299	0.125	0.007

<sup>a,b</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ )

SEM: Standard error of the mean

As shown in Table 5, dietary treatments did not significantly affect eggshell percentage in fresh or stored eggs during the first and third periods. In the second period, dietary vitamin C supplementation significantly reduced the eggshell percentage of fresh eggs compared with the control ( $P < 0.05$ ). Moreover, in both

the second period and the overall experimental period, the combination of all three supplements significantly increased the eggshell percentage of stored eggs compared with diets containing vitamin C or probiotic alone ( $P < 0.05$ ).

**Table 5.** Eggshell percentage in laying hens fed with the experimental diets

Dietary treatments	Weeks 46-49		Weeks 50-53		Weeks 54-57		Whole period	
	Fresh	Stored	Fresh	Stored	Fresh	Stored	Fresh	Stored
Control	9.447	10.48	10.300 <sup>a</sup>	10.423 <sup>ab</sup>	9.924	10.86	9.893	10.59 <sup>ab</sup>
Probiotic	10.039	10.36	9.835 <sup>ab</sup>	9.887 <sup>b</sup>	10.179	11.11	10.017	10.45 <sup>b</sup>
Vitamin C	9.851	10.04	9.569 <sup>b</sup>	9.977 <sup>b</sup>	9.941	11.28	9.787	10.43 <sup>b</sup>
Organic Se	10.140	10.01	9.977 <sup>ab</sup>	10.846 <sup>a</sup>	10.042	11.35	10.053	10.73 <sup>ab</sup>
Probiotic + vitamin C + organic Se	10.063	10.44	10.11 <sup>ab</sup>	10.929 <sup>a</sup>	10.279	11.68	10.152	11.02 <sup>a</sup>
SEM	0.200	0.198	0.166	0.207	0.188	0.232	0.105	0.134
P value	0.119	0.289	0.034	0.001	0.620	0.162	0.139	0.018

<sup>a,b</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ )

SEM: Standard error of the mean

The effects of the experimental diets on the eggshell thickness of fresh and stored eggs of laying hens during different experimental periods are presented in Table 6. In the first period, the control group exhibited a significantly greater eggshell thickness compared with the other dietary treatments, except for the group receiving the combined supplementation ( $P < 0.05$ ).

During the second period and over the entire experimental period, no significant differences were observed in the thickness of fresh or stored eggshells among the dietary treatments. However, in the third period, hens fed the organic selenium diet showed a significantly higher thickness of stored eggshells compared with the other experimental groups ( $P < 0.05$ ).

**Table 6.** Eggshell thickness (mm) of fresh and stored eggs in laying hens fed with the experimental diets

Dietary treatments	Weeks 46-49		Weeks 50-53		Weeks 54-57		Whole period	
	Fresh	Stored	Fresh	Stored	Fresh	Stored	Fresh	Stored
Control	0.271 <sup>a</sup>	0.250	0.276	0.252	0.232	0.233 <sup>b</sup>	0.260	0.245
Probiotic	0.230 <sup>b</sup>	0.243	0.235	0.233	0.256	0.245 <sup>b</sup>	0.240	0.240
Vitamin C	0.233 <sup>b</sup>	0.245	0.268	0.234	0.243	0.248 <sup>b</sup>	0.248	0.242
Organic Se	0.233 <sup>b</sup>	0.239	0.271	0.235	0.233	0.322 <sup>a</sup>	0.246	0.265
Probiotic + vitamin C + organic Se	0.254 <sup>ab</sup>	0.226	0.265	0.245	0.262	0.238 <sup>b</sup>	0.260	0.236
SEM	0.010	0.011	0.014	0.009	0.010	0.010	0.006	0.007
P value	0.031	0.657	0.305	0.599	0.184	0.001	0.166	0.074

<sup>a,b</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ )

SEM: Standard error of the mean

The effects of the experimental diets on eggshell strength during different periods are shown in Table 7. In the first period, eggshell strength was significantly higher in hens fed the control diet and in those receiving the diet supplemented with all three additives, compared with the other treatments ( $P < 0.05$ ). During the second period, hens fed the diet containing the combination of the three supplements exhibited significantly higher eggshell strength

than the other treatments, except for the group supplemented with vitamin C ( $P < 0.05$ ). In the third period, hens receiving each supplement individually or in combination showed greater eggshell strength compared with the control group ( $P < 0.05$ ). Overall, combined supplementation with probiotic, organic selenium, and vitamin C resulted in a significant improvement in eggshell strength compared with the other experimental groups ( $P < 0.05$ ).

**Table 7.** Eggshell breaking strength (N/cm<sup>2</sup>) of laying hens fed with the experimental diets

Dietary treatments	Weeks 46-49	Weeks 50-53	Weeks 54-57	Whole period
Control	43.56 <sup>a</sup>	41.58 <sup>b</sup>	37.16 <sup>b</sup>	40.77 <sup>b</sup>
Probiotic	38.45 <sup>b</sup>	39.50 <sup>c</sup>	45.08 <sup>a</sup>	41.01 <sup>b</sup>
Vitamin C	37.79 <sup>b</sup>	43.41 <sup>ab</sup>	44.09 <sup>a</sup>	41.76 <sup>b</sup>
Organic Se	39.90 <sup>b</sup>	37.89 <sup>c</sup>	43.06 <sup>a</sup>	40.29 <sup>b</sup>
Probiotic + vitamin C + organic Se	43.33 <sup>a</sup>	45.36 <sup>a</sup>	43.35 <sup>a</sup>	44.02 <sup>a</sup>
SEM	0.709	0.497	0.923	0.434
P value	0.001	0.001	0.001	0.001

<sup>a,c</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ )  
SEM: Standard error of the mean

The effects of the experimental diets on eggshell ash content of laying hens during different periods are presented in Table 8. Comparison of the means indicated that, in the first period, eggshell ash content was significantly higher in hens fed diets supplemented with organic selenium or vitamin C compared with the control and probiotic groups ( $P < 0.05$ ). During the second period and over the entire experimental period, eggshell

ash content was significantly increased in hens receiving any of the experimental supplements, either individually or in combination, compared with those fed the control diet ( $P < 0.05$ ). In the third period, hens supplemented with probiotics exhibited significantly higher eggshell ash content compared with the control and organic selenium groups ( $P < 0.05$ ).

**Table 8.** Eggshell ash percentage (%) of laying hens fed with the experimental diets

Dietary treatments	Weeks 46-49	Weeks 50-53	Weeks 54-57	Whole period
Control	90.52 <sup>c</sup>	90.68 <sup>b</sup>	89.22 <sup>b</sup>	90.14 <sup>b</sup>
Probiotic	89.95 <sup>c</sup>	95.24 <sup>a</sup>	92.49 <sup>a</sup>	92.56 <sup>a</sup>
Vitamin C	93.93 <sup>a</sup>	92.49 <sup>ab</sup>	91.60 <sup>ab</sup>	92.67 <sup>a</sup>
Organic Se	92.84 <sup>ab</sup>	95.27 <sup>a</sup>	90.00 <sup>b</sup>	92.70 <sup>a</sup>
Probiotic + vitamin C + organic Se	90.88 <sup>bc</sup>	92.90 <sup>ab</sup>	91.21 <sup>ab</sup>	91.66 <sup>a</sup>
SEM	0.514	0.825	0.615	0.366
P value	0.001	0.001	0.003	0.001

<sup>a,b</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ )  
SEM: Standard error of the mean

**Immune response of laying hens**

The effects of the experimental diets on the immune response of laying hens are presented in Table 9.

Vitamin C supplementation significantly enhanced total antibody titer against SRBC compared to the probiotic group ( $P < 0.05$ ), though no treatment effects were observed on IgM or IgG individually.

**Table 9.** Immune response (log<sub>2</sub> antibody titer) of laying hens fed with the experimental diets

Dietary Treatments	IgM		IgG		Total Antibody	
	primary	secondary	primary	secondary	primary	secondary
Control	1.50	2.33	2.33	3.00	3.83	5.33 <sup>ab</sup>
Probiotic	2.33	1.66	2.00	2.50	4.33	4.16 <sup>b</sup>
Vitamin C	1.66	3.00	2.66	2.66	4.33	5.66 <sup>a</sup>
Organic Se	2.83	2.50	1.83	2.83	4.66	5.33 <sup>ab</sup>
Probiotic + vitamin C + organic Se	2.66	1.83	3.00	3.00	4.66	4.83 <sup>ab</sup>
SEM	0.518	0.408	0.428	0.413	0.448	0.301
P value	0.293	0.178	0.319	0.891	0.085	0.016

<sup>a,b</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ ).  
SEM: Standard error of the mean.

**Antioxidant enzyme concentrations**

The effects of the experimental diets on the concentrations of antioxidant enzymes in the blood of

laying hens are presented in Table 10. Serum antioxidant enzyme activities (SOD, CAT, GSH-Px) were unaffected by dietary treatments; however, total

antioxidant capacity (TAC) was significantly elevated in the combination group ( $P < 0.05$ ).

**Table 10.** Concentration of blood antioxidant enzymes in laying hens fed with the experimental diets.

Dietary Treatments	Catalase (KU/L)	Glutathione Peroxidase (U/L)	Superoxide Dismutase (U/ml)	Total Antioxidant Capacity (mmol/L)
Control	42.61	19.53	73.91	910.16 <sup>b</sup>
Probiotic	50.86	18.60	58.04	997.50 <sup>ab</sup>
Vitamin C	47.41	23.97	62.33	976.16 <sup>ab</sup>
Organic se	50.90	19.84	48.86	1072.66 <sup>ab</sup>
Probiotic + vitamin C + organic Se	48.03	35.18	52.69	1299.9 <sup>a</sup>
SEM	2.281	19.53	6.992	85.05
P value	0.137	0.470	0.194	0.032

<sup>a,b</sup>Means in a column with common superscript(s) do not differ ( $P > 0.05$ ).  
SEM: Standard error of the mean.

### Yolk fatty acid composition

The effects of the experimental diets on yolk fatty acid profile are presented in Table 11. The concentration of myristic acid was not affected by any of the experimental treatments. Organic selenium supplementation resulted in significantly higher oleic acid concentration compared to the control and vitamin C groups. Supplementation with probiotics, or the combination of all three additives

(probiotic, organic selenium, and vitamin C) significantly increased the concentrations of linoleic and linolenic acids in yolks compared with the control and vitamin C groups ( $P < 0.05$ ). Additionally, the use of probiotics, organic selenium, or all three supplements simultaneously significantly increased yolk arachidonic acid concentration compared with the control group ( $P < 0.05$ ).

**Table 11.** Comparison of the effects of experimental diets on yolk fatty acid composition

Fatty acids	Control	Probiotic	Vitamin C	Organic selenium	Vitamin C + probiotic + organic selenium	SEM	P value
Myristic (C14:0)	56.04	57.74	49.26	60.28	54.16	5.16	0.60
Palmitic (C16:0)	2027.06 <sup>b</sup>	2436.69 <sup>ab</sup>	2004.17 <sup>b</sup>	2683.19 <sup>a</sup>	2224.26 <sup>ab</sup>	195.89	0.040
Palmitoleic (C16:1)	275.26 <sup>b</sup>	359.82 <sup>a</sup>	276.41 <sup>b</sup>	371.54 <sup>a</sup>	341.52 <sup>ab</sup>	31.77	0.040
Stearic (C18:0)	614.42 <sup>b</sup>	718.61 <sup>ab</sup>	617.22 <sup>b</sup>	831.78 <sup>a</sup>	646.28 <sup>ab</sup>	70.37	0.020
Oleic (C18:1)	2911.17 <sup>b</sup>	3471.63 <sup>ab</sup>	2771.75 <sup>b</sup>	3897.18 <sup>a</sup>	3067.90 <sup>b</sup>	297.89	0.008
Linoleic (C18:2)	398.31 <sup>c</sup>	851.36 <sup>a</sup>	364.17 <sup>c</sup>	629.97 <sup>ab</sup>	740.52 <sup>ab</sup>	63.85	0.006
Linolenic (C18:3)	0.74 <sup>b</sup>	11.67 <sup>a</sup>	1.14 <sup>b</sup>	6.30 <sup>ab</sup>	11.75 <sup>a</sup>	3.22	0.020
Arachidonic (C20:4)	32.22 <sup>b</sup>	134.29 <sup>a</sup>	45.61 <sup>ab</sup>	95.56 <sup>a</sup>	111.42 <sup>a</sup>	18.70	0.030

<sup>a,c</sup>Means in a row with common superscript(s) do not differ ( $P > 0.05$ ).  
SEM: Standard error of the mean.

## Discussion

The present study demonstrated a pronounced synergistic effect of combined dietary supplementation with probiotic, vitamin C, and organic selenium on key performance and egg quality traits in laying hens. Hens receiving the combination diet showed the lowest feed conversion ratio (FCR), highest egg mass and egg weight, markedly increased total antioxidant capacity (TAC), and elevated yolk polyunsaturated fatty acids (PUFAs) compared with individual supplements or control. These results highlight that multi-nutrient strategies can achieve benefits beyond single-nutrient supplementation, simultaneously enhancing productivity, oxidative balance, and egg nutritional value.

### Productive performance

Hens supplemented with probiotic or organic selenium exhibited reduced feed intake without compromising egg production, suggesting enhanced nutrient digestibility and metabolic efficiency. Similar findings have been

reported where organic selenium improved thyroid hormone metabolism and basal metabolic rate (Yang et al., 2012), while Bacillus-based probiotics enhanced gut enzyme activity and nutrient absorption (Rajput et al., 2013). The combination of probiotic, vitamin C, and organic selenium further improved feed conversion ratio (FCR), egg weight, and egg mass, likely due to complementary mechanisms: probiotics optimize intestinal health, selenium supports selenoprotein-dependent antioxidant defense, and vitamin C regenerates oxidized antioxidants, stabilizing cell membranes (Abd El-Hack et al., 2017). In contrast, vitamin C alone did not markedly enhance performance under thermoneutral conditions, in agreement with earlier reports indicating its benefits are primarily observed under stress. The improved FCR in the combined group suggests that multi-nutrient strategies targeting oxidative and digestive efficiency can yield greater productivity gains than single-nutrient approaches (Carvalho et al., 2022).

### Egg shape index and eggshell quality

Findings from the present study indicate that dietary supplementation with probiotic, vitamin C, and organic selenium either individually or in combination exerts meaningful effects on multiple indicators of eggshell quality in laying hens. Consistent with earlier reports, improvements in specific gravity and shell mineralization parameters appear particularly responsive to organic selenium and to the combined supplementation strategy.

Organic selenium has been shown to enhance shell gland function through its role in antioxidant defense and regulation of mineral metabolism, thereby improving calcium utilization and stabilizing shell deposition processes (Surai et al., 2018; Ahmadiyan et al., 2020). This aligns with the elevated specific gravity and shell thickness observed in the selenium-supplemented groups, particularly in stored eggs, where shell quality tends to deteriorate more rapidly.

Probiotic supplementation, especially strains such as *Bacillus subtilis*, has been associated with enhanced calcium absorption through modulation of gut microbiota, increased production of short-chain fatty acids, and upregulation of calcium-binding protein and carbonic anhydrase activity (Mazanko et al., 2019; Raveschot et al., 2020). These mechanisms can collectively improve shell matrix formation and mechanical resistance, supporting the increased breaking strength detected in probiotic-treated hens during certain periods. However, inconsistencies reported in other studies (Upadhaya et al., 2019; Wang et al., 2020) emphasize the importance of factors such as probiotic strain specificity, hen age, and environmental conditions.

Vitamin C, known for its involvement in collagen synthesis and antioxidative processes, likely contributed to improved eggshell strength when used in combination with organic selenium and probiotics. Its role in supporting the organic matrix of the eggshell may explain the higher shell percentage and breaking strength observed in the combination group.

The most pronounced and consistent enhancements across traits including specific gravity, shell percentage, breaking strength, and shell ash content were associated with the combined supplementation of probiotic, vitamin C, and organic selenium. This suggests a synergistic interaction wherein improved antioxidant capacity, enhanced mineral metabolism, and optimized gut function collectively support more efficient shell formation. The elevated total antioxidant capacity (TAC) observed in the combination group further supports this integrative mechanism.

Overall, the synergistic effect of improved calcium absorption (probiotic), enhanced collagen matrix formation (vitamin C), and optimized antioxidant protection (organic Se) in the shell gland created a more favorable physiological environment for consistent, high-quality calcification.

#### *Immune response and antioxidant capacity*

In the present study, vitamin C supplementation resulted in a higher total antibody titer against sheep red blood

cells (SRBC) compared to the probiotic group, although no significant difference was observed relative to the control group. This suggests that vitamin C may modulate humoral immune responsiveness under specific conditions, but its effect relative to a standard diet requires further investigation. No significant differences were observed in IgM or IgG levels individually. Although total antibody titer was higher in the vitamin C group compared to the probiotic group, the lack of significant change in IgM or IgG suggests that vitamin C's effect on specific immunoglobulin classes is limited under the conditions tested.

Regarding antioxidant status, activities of key enzymatic antioxidants, including SOD, CAT, and GSH-Px, were not significantly affected by any of the individual or combined dietary treatments. However, total antioxidant capacity (TAC) was significantly elevated in the group receiving the combination of probiotic, vitamin C, and organic selenium. This finding indicates that while single additives did not alter enzymatic activities, their combination can improve the overall redox status of the hens. The observed increase in TAC may result from synergistic interactions among probiotics, selenium, and vitamin C: Probiotics can produce metabolites (e.g., butyrate) that boost cellular antioxidant defenses like glutathione (Zheng et al., 2019). Selenium serves as an essential cofactor for selenoenzymes involved in peroxide detoxification (Dalia et al., 2017), and vitamin C contributes to the regeneration of other antioxidants like vitamin E, collectively enhancing systemic antioxidant capacity.

The enhancement of TAC in the combination group supports previous findings that concurrent supplementation of selenium and vitamin C can have additive or synergistic effects on antioxidant status compared with single nutrients (Skřivan et al., 2013; Pečjak et al., 2022). These results suggest that a multi-nutrient approach may be more effective in maintaining oxidative balance in laying hens than individual supplementation, potentially contributing to improved physiological resilience.

In conclusion, vitamin C supplementation resulted in a higher total antibody titer compared to the probiotic group, although no significant difference was observed compared with the control group. The combination of probiotic, vitamin C, and organic selenium significantly improved total antioxidant capacity, while enzymatic antioxidant activities remained unchanged. These findings suggest that strategic combinations of dietary supplements may support both humoral immune responses and systemic antioxidant status in laying hens, but further research is needed to confirm their effects relative to a standard diet.

#### *Yolk fatty acid composition*

Inclusion of probiotic, organic selenium, or their combination significantly increased yolk concentrations of linoleic (C18:2 n-6) and linolenic (C18:3 n-3) acids,

indicating improved incorporation of PUFAs into yolk lipids. These results agree with Mikulski et al. (2012), who reported higher levels of PUFA in yolks of hens supplemented with *Pediococcus acidilactici*. The mechanism may involve enhanced lipid metabolism and microbial synthesis of bioactive intermediates affecting hepatic lipid transfer. Organic selenium also altered yolk fatty acid distribution, increasing oleic and stearic acids, consistent with reports that selenium modulates desaturase activity and reduces lipid peroxidation (Buckiuniene et al., 2018). Furthermore, selenoprotein-mediated regulation of prostaglandin synthesis from arachidonic acid could explain variations in long-chain PUFA ratios (Pappas et al., 2005). Collectively, the present findings suggest that the combined dietary inclusion of probiotic, vitamin C, and organic selenium enhances yolk PUFA enrichment and overall egg nutritional value-attributes beneficial for human health and the functional egg industry.

## Conclusions

Synergistic improvements found in the present study can be attributed to the supplements' collective action: probiotics enhance gut health and nutrient absorption, vitamin C bolsters the non-enzymatic antioxidant pool and collagen synthesis, and organic selenium supports selenoprotein-dependent redox homeostasis.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Acknowledgments

The authors gratefully acknowledge the Vice Chancellor of Research and Technology, Shahid Bahonar University of Kerman, Iran.

## References

Abbas, A.O., Alaqil, A.A., Mehaisen, G.M.K., El-Sabry, M.I., 2022. Effect of organic selenium-enriched yeast on relieving the deterioration of layer performance, immune function, and physiological indicators induced by heat stress. *Frontiers in Veterinary Science* 9, 880790. <https://doi.org/10.3389/fvets.2022.880790>

Abd El-Hack, M.E., Mahrose, K., Askar, A.A., Alagawany, M., Arif, M., Saeed, M., Chaudhry, M.T., 2017. Single and combined impacts of vitamin A and selenium in diet on productive performance, egg quality, and some blood parameters of laying hens during hot season. *Biological Trace Element Research* 177, 169-179. <https://doi.org/10.1007/s12011-016-0862-5>

Ahmadi, F., Afsharmanesh, M., Salarmoini, M., Khajeh Bami, M., 2022. Investigating the effects of using biochar as a replacement for minerals premix in layers diet on physical and mechanical properties of their eggshells. *Biomechanism and Bioenergy Research* 1(1), 12-15.

Ahmadiyan, A., Moghadam, H., Khosravi, M., 2020. Effects of selenium sources on eggshell quality and calcium metabolism in laying hens. *Iranian Journal of Veterinary Research* 21, 211-217.

Aydin, R., Şahan, U., Ipek, A., 2008. Effects of dietary conjugated linoleic acid on eggshell quality of laying hens. *Turkish Journal of Veterinary and Animal Sciences* 32(5), 385-389.

Buckiuniene, V., Alencikiene, G., Miezeliene, A., Raceviciute-Stupeliene, A., Bliznikas, S., Gruzauskas, R., 2018. Effect of sunflower and rapeseed oil, organic and inorganic selenium and vitamin E in the diet on yolk fatty acids profile, malondialdehydes concentration and sensory quality of laying hens' eggs. *Veterinarija ir Zootechnika* 76(98).

Carvalho, C.L., Andretta, I., Galli, G.M., Stefanello, T.B., Camargo, N.D.O.T., Marchiori, M., Kipper, M., 2022. Effects of dietary probiotic supplementation on egg quality during storage. *Journal of Poultry Science* 1(3), 180-192.

Dalia, A.M., Loh, T.C., Sazili, A.Q., Jahromi, M.F., Samsudin, A.A., 2017. The effect of dietary bacterial organic selenium on growth performance, antioxidant capacity, and selenoproteins gene expression in broiler chickens. *BMC Veterinary Research* 13, 1-11. <https://doi.org/10.1186/s12917-017-1159-4>

Deng, S., Hu, S., Xue, J., Yang, K., Zhuo, R., Xiao, Y., Fang, R., 2022. Productive performance, serum antioxidant status, tissue selenium deposition, and gut health analysis of broiler chickens supplemented with selenium and probiotics-A pilot study. *Animals* 12(9), 1086. <https://doi.org/10.3390/ani12091086>

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* 226(1), 497-509. [https://doi.org/10.1016/S0021-9258\(18\)64849-5](https://doi.org/10.1016/S0021-9258(18)64849-5)

Ghasemi, H.A., Hajkhodadadi, I., Hafizi, M., Fakhrazadeh, S., Abbasi, M., Kalanaky, S., Nazaran, M.H., 2022. Effect of advanced chelate compounds-based mineral supplement in laying hen diet on the performance, egg quality, yolk mineral content, fatty acid composition, and oxidative status. *Food Chemistry* 366, 130636. <https://doi.org/10.1016/j.foodchem.2021.130636>

Hendrix Genetics, 2020. Nutrition Management Guide. Version L7121-2. Hendrix Genetics BV, The Netherlands.

Khajeh Bami, M., Afsharmanesh, M., Espahbodi, M., Esmaeilzadeh, E., 2022. Effects of dietary nano-selenium supplementation on broiler chicken performance, meat selenium content, intestinal microflora, intestinal morphology, and immune response. *Journal of Trace Elements in Medicine and Biology* 69, 126897. <https://doi.org/10.1016/j.jtemb.2021.126897>

- Kralik, G., Kralik, Z., Grčević, M., Galović, O., Hanžek, D., Biazik, E., 2021. Fatty acid profile of eggs produced by laying hens fed diets containing different shares of fish oil. *Poultry Science* 100(10), 101379. <https://doi.org/10.1016/j.psj.2021.101379>
- Liu, Z., Cao, Y., Ai, Y., Lin, G., Yin, X., Wang, L., et al., 2023. Effects of selenium yeast on egg quality, plasma antioxidants, selenium deposition and eggshell formation in aged laying hens. *Animals* 13(5), 902. <https://doi.org/10.3390/ani13050902>
- Macit, M., Karaoglu, M., Celebi, S., Esenbuga, N., Yoruk, M.A., Kaya, A., 2021. Effects of supplementation of dietary humate, probiotic, and their combination on performance, egg quality, and yolk fatty acid composition of laying hens. *Tropical Animal Health and Production* 53, 63. <https://doi.org/10.1007/s11250-020-02546-6>
- Mazanko, M.S., Makarenko, M.S., Chistyakov, V.A., Usatov, A.V., Prazdnova, E.V., Bren, A.B., Chikindas, M.L., 2019. Probiotic intake increases the expression of vitellogenin genes in laying hens. *Probiotics and Antimicrobial Proteins* 11, 1324-1329. <https://doi.org/10.1007/s12602-019-9519-y>
- Mikulski, D., Jankowski, J., Naczmanski, J., Mikulska, M., Demey, V., 2012. Effects of dietary probiotic (*Pediococcus acidilactici*) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poultry Science* 91(10), 2691-2700. <https://doi.org/10.3382/ps.2012-02370>
- Nemati, Z., Ahmadian, H., Besharati, M., Lesson, S., Alirezalu, K., Domínguez, R., Lorenzo, J.M., 2020. Assessment of dietary selenium and vitamin E on laying performance and quality parameters of fresh and stored eggs in Japanese quails. *Food Science* 9(9), 1324. <https://doi.org/10.3390/foods9091324>
- Pappas, A.C., Acamovic, T., Sparks, N.H.C., Surai, P.F., McDevitt, R.M., 2005. Effects of supplementing broiler breeder diets with organic selenium and polyunsaturated fatty acids on egg quality during storage. *Poultry Science* 84(6), 865-874. <https://doi.org/10.1093/ps/84.6.865>
- Pečjak, M., Leskovec, J., Levart, A., Salobir, J., Rezar, V., 2022. Effects of dietary vitamin E, vitamin C, selenium, and their combinations on meat oxidative stability under heat stress conditions. *Animals* 12(14), 1789. <https://doi.org/10.3390/ani12141789>
- Rafeeina, A., Asadikaram, G., Karimi-Darabi, M., Abolhassani, M., Abbasi-Jorjandi, M., Moazed, V., 2022. Organochlorine pesticides, oxidative stress biomarkers, and leukemia: a case-control study. *Journal of Investigative Medicine* 70(8), 1736-1745.
- Rajput, I.R., Li, L.Y., Xin, X., Wu, B.B., Juan, Z.L., Cui, Z.W., Li, W.F., 2013. Effect of *Saccharomyces boulardii* and *Bacillus subtilis* B10 on intestinal ultrastructure modulation and mucosal immunity development mechanism in broiler chickens. *Poultry Science* 92(4), 956-965. <https://doi.org/10.3382/ps.2012-02845>
- Raveschot, C., Coutte, F., Frémont, M., Vaeremans, M., Dugersuren, J., Demberel, S., Cudennec, B., 2020. Probiotic *Lactobacillus* strains from Mongolia improve calcium transport and uptake by intestinal cells *in vitro*. *Food Research International* 133, 109201. <https://doi.org/10.1016/j.foodres.2020.109201>
- SAS. 2005. User's Guide: Statistics. Version 9.1. SAS Institute Inc., Cary, North Carolina, USA.
- Sinha, A.K., 1972. Colorimetric assay of catalase. *Analytical Biochemistry* 47(2), 389-394.
- Skřivan, M., Marounek, M., Englmaierová, M., Skřivanová, E., 2013. Influence of dietary vitamin C and selenium, alone and in combination, on the performance of laying hens and quality of eggs. *Czech Journal of Animal Science* 58(2), 91-97. <https://doi.org/10.17221/6619-CJAS>
- Surai, P.F., Kochish, I.I., Fisinin, V.I., Velichko, O.A., 2018. Selenium in poultry nutrition: From sodium selenite to organic selenium sources. *The Journal of Poultry Science* 55(2), 79-93. <https://doi.org/10.2141/jpsa.0170135>
- Upadhaya, S.D., Rudeaux, F., Kim, I.H., 2019. Efficacy of dietary *Bacillus subtilis* and *Bacillus licheniformis* supplementation continuously in pullet and lay period on egg production, excreta microflora, and egg quality of Hyline-Brown birds. *Poultry Science* 98(10), 4722-4728. <https://doi.org/10.3382/ps/pez184>
- VanEmous, R.A., 2023. Effects of feeding strategies during lay on broiler breeder production performance, eggshell quality, incubation traits, and behavior. *Poultry Science* 102, 102630. <https://doi.org/10.1016/j.psj.2023.102630>
- Wang, W.W., Wang, J., Zhang, H.J., Wu, S.G., Qi, G.H., 2020. Effects of *Clostridium butyricum* on production performance and intestinal absorption function of laying hens in the late phase of production. *Animal Feed Science and Technology* 264, 114476. <https://doi.org/10.1016/j.anifeedsci.2020.114476>
- Wegmann, T. G., Smithies, O., 1966. A simple hemagglutination system requiring small amounts of red cells and antibodies. *Transfusion* 6(1), 67-73.
- Yang, Y.R., Meng, F.C., Wang, P., Jiang, Y.B., Yin, Q.Q., Chang, J., Zuo, R.Y., Zheng, Q.H., Liu, J.X., 2012. Effect of organic and inorganic selenium supplementation on growth performance, meat quality and antioxidant property of broilers. *African Journal of Biotechnology* 11(12), 3031-3036.
- Zheng, W., Dawei, Z., Wenjun, Y., Changhai, Q., Jiang, Y., 2019. Probiotic cocktails alleviate heat-induced intestinal barrier dysfunction and oxidative stress in broiler chickens. *Animal Husbandry and Feed Science* 11(3/4), 107-114.