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Effects of different levels of L-carnitine and fish oil supplementation in diets on performance, egg quality traits and egg yolk fatty acid profile in aged laying hens

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Abstract

Nutritional interventions play a pivotal role in sustaining egg production performance and improving egg quality parameters in aged laying hens. The present study was conducted to investigate the effect of L-carnitine (LC) supplementation in diets containing different levels of fish oil (FO) on performance, egg quality parameters and egg yolk fatty acids (FAs) profile in aged laying hens. In this study, 432 laying hens (Hy-line-W36, 65 weeks of age) were used and allocated in a 3 × 3 factorial design with six replications and eight birds *per* replication. The experimental treatments included diets containing three levels of FO (0.00, 1.50 and 3.00% of the diet) and three levels of LC (0.00, 300, and 600 mg kg^{-1} of the diet). The results showed that hens treated with 3.00% FO and 300 and 600 mg LC had the highest egg production rate, egg weight and egg mass which was significantly higher than the treatments without FO and LC. With increasing usage level of FO to 3.00% of diet, egg yolk pH was significantly decreased, however, yolk pH was increased when diet was supplemented with 600 mg LC. The percentage of polyunsaturated FAs, the ratio of polyunsaturated FA/saturated FAs and the percentage of omega-3 FAs were increased significantly with increasing FO usage level in the diet. Overall, these findings suggested that dietary supplementation with FO and LC could synergistically improve productive performance and enhance the nutritional value of eggs in aged laying hens.

Introduction

Oils have multiple effects, including improved palatability, increased feed intake, safety and performance.[1] Due to distinct physiological status and increased susceptibility to lipid metabolism disorders, laying hens require less oil added to their diet than broilers.[2] Because they lower the risk of inflammatory and cardiovascular disorders, omega-3 fatty acids (FAs) are crucial for human nutrition.[3] Omega-3 poly-unsaturated FAs (omega-3 PUFAs), particularly long-chain ones, are abundant in fish oil (FO).[4] Omega-3 PUFAs make up than 30.00% of this oils FA composition, according to its FA profile.[5] This oil omega-6 to omega-3 acid ratio is roughly one, and it has very low saturated FAs (SFAs). The most significant family of PUFAs, omega-3 FAs, are involved in a number of physiological processes including ovulation as well as lowering blood cholesterol, enhancing immuno-logical response, increasing fertility, lowering blood lipids and managing cardiovascular disease.[6] By generating prostaglandins, estrogens, and progesterone, changing the synthesis of eicosanoids or steroids, changing the fluidity of cell membranes, causing oxidative stress, and/or taking part in signal transduction, these omega-3 PUFAs can influence ovulation in a number of ways.[7]

Foods enhanced with omega-3 PUFAs are currently more popular with customers. People prefer to eat eggs with greater levels of omega-3 PUFAs, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), as they are the most economical source of protein.[8] However, it is undesirable that products with higher amounts of omega-3 FAs are more prone to lipid peroxidation.[9] The FO causes lipid peroxidation and reduced liver function in laying hens, according to an investigation that found a significant rise in blood malondialdehyde and aspartate transaminase activity in the hens.[10] Because PUFAs, particularly PUFAs contain more than one double bond, are more vulnerable to lipid peroxidation than SFA, the FA profile of the oil influences how susceptible oils are to oxidation.[11] As a result, it appears that including antioxidants in diets that contain FO is essential.

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The L-carnitine (LC) is synthesized from precursor amino acids (methionine and lysine) and many vitamins as cofactors such as vitamins C, B_3, B_6 and B_9 and iron.[12] The LC biosynthesis generally occurs in the liver and kidneys, with approximately 75.00% of it derived from dietary sources and 25.00% produced endogenously.[13] One of the important roles of LC is to help transport FAs into mitochondria for energy production which is critical for maintaining optimal physiological functions in various animals.[14] The LC supplementation may accelerate lipid metabolism in the yolk, thus, accelerating follicular growth. The LC may also increase metabolic rate in the gland magnum and shell, thereby, leading to albumin deposition and shell calcification followed by increased egg weight (EW).[15] Recently, the antioxidant effects of LC have been well reported.[3,16] While LC is known to have antioxidant properties,[16] a major metabolic role of LC is to reduce the availability of lipids for peroxidation by facilitating the transport of long-chain, short- and medium-chain FAs that tend to accumulate because of normal and abnormal metabolism.[17] Thus, the presence of LC in the diet can enhance beta-oxidation in these FAs to produce adenosine triphosphate as a source of energy to boost energy utilization.[18] An increase in LC in the diet caused an accumulation of LC in the egg yolk. Therefore, the presence of more LC in the egg yolk can prevent further oxidation of the FAs in the egg.[16] In recent years, although FO has been used to enrich eggs with omega-3 FAs and its positive effects on egg-laying physiology, the problem of its storage and oxidation has always been a matter of debate. Considering the different metabolic activities of LC and sources of long-chain omega-3 FAs in laying hens, it seems that adding LC to diets containing FO can not only increase egg-laying performance, but also increase the storage of these FAs in eggs. So, the present study was conducted to investigate effect of LC supplementation in diets containing different levels of FO on performance, egg quality parameters and egg yolk FA profile in older laying hens.

Materials and Methods

Birds and experimental treatments. A total number of 432 laying hens (Hy-line-W36, 65 weeks of age) were used in this study. Birds with similar body weight (1,530 ± 50.00 g) were selected and allocated in a 3 × 3 factorial design with six replications and eight birds *per* replication. To acclimatize the birds to the experimental conditions, they were first fed on the basal diet for 2 weeks and then on the experimental treatments for 8 weeks (56 days). The experimental treatments included diets containing three levels of FO (0.00, 1.50 and 3.00% of the diet) and three levels of LC (0.00, 300, and 600 mg kg^{-1} of the diet), which were adjusted according to the nutritional recommendations of the Hy-line-W36 strain in 2020 (Table 1).[19] The lighting schedule was set as 14 hr of lightning with a light intensity of 30.00 lux and 10 hr of darkness with a light intensity of 3.00 lux.[20] This study was approved by the Animal Care Committee and Animal Research Ethics Board of Urmia University, Urmia, Iran (Approval No. IR-UU-AEC-3/99).

Production performance. The egg production (EP) and mean EW were measured (using a 0.01 g electronic scale) daily, and the egg mass (EM), feed conversion ratio (FCR) and feed intake were calculated weekly throughout the experiment. Daily mean EW was multiplied to hen day EP of each replicate to calculate the EM. Also, weekly feed intake was divided into the EM to estimate the FCR.

Egg quality traits. At the end of the experimental period (8 week), 2 eggs (59.00 ± 18.00 g) were collected from each replication and the most important internal quality traits of eggs including albumin weight, yolk weight, pH of yolk and egg albumen, Haugh unit (HU), yolk color index, shell strength were measured. The egg albumen height was measured using a manual Haugh Micrometer (Analog Baxlo Haugh Micrometer; Baxlo Precisión S.L., Barcelona, Spain). So that after breaking the eggs, the whites were placed on a flat surface. Then, the values of the albumen height were recorded at every point where the tip of the height gauge touched the egg white (1.00 cm around the yolk). The following equation was used to calculate the HU:[21]

$$HU = \log_{10}(AH - 1.7\,EW^{0.37} + 7.57)$$

where, *AH* is egg albumen height (mm), and *EW* is egg weight (g).

To measure the pH of yolk and egg albumen, 2.00 g of egg albumen and egg yolk were mixed with distilled water at a ratio of 1:9 and stirred well until foam was formed (5 min). After subsiding the produced foam by inserting a pH meter sensor (MTT-65 pH meter; Metrohm AG, Herisau, Switzerland) the pH value was recorded.[22] A force reader machine (Ogawa Seiki, Tokyo, Japan) was provided to determine the shell strength of the eggs.

Yolk fatty acid (FA) profile. In the case of yolk FA profile, the yolk samples of 18 eggs *per* treatment (three eggs *per* replicate) were collected at the end of experiment and stored at – 20.00 ˚ C prior to FA profile determination. The yolk samples were extracted using the Soxhlet extraction method, homogenized by vortexing, and 100 mg of each sample was weighed. They were then saponified by adding 3.00 mL of 2.00 M potassium hydroxide (Merck, Darmstadt, Germany) and esterified by adding 5.00 mL of 12.00% methanolic sulfuric acid (Merck). The methyl ester of FAs was injected into gas chromatography machine (Agilent 6890N; Agilent Technologies, Santa Clara, USA) using 0.10 mL of extracted normal heptane and 1.00 µL of normal heptane phase. The

initial oven temperature was set at 75.00 ˚C for 1 min and gradually increased to 240 ˚C (25.00 ˚C *per* min) and maintained for 8 min. Nitrogen gas (99.99% purity) was used as a carrier at a flow rate of 45.00 mL *per* min. The injector temperature was set at 250 ˚C and the detector at 280 ˚C. A mixture of all FAs standards was also injected, and its retention time was used for comparison with egg yolk FAs. The results of the retention time comparison were analyzed using Chemstation Software (version A.10.02; Agilent Technologies) in the Windows environment. The matrix value (FA profile) of the diets used in the present experiment is shown in Table 2.

Statistical analysis. All data from this experiment were statistically analyzed using SAS Software (version 9.2; SAS Institute, Cary, USA) and the Mixed procedure.

$$y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$$

where, y_{ijk} is value of the trait of interest, μ is total mean, A_i is effect of the i-th level of FO, B_j is effect of the j-th level of LC, AB_{ij} is interaction effect of the level of FO and LC, and e_{ijk} is effect of experimental error or unknown factors in each observation. The significance of the differences between the data means was examined using the Tukey test at a probability level of 5.00%.

Table 1. The ingredient and chemical composition of experimental diets.

Ingredients (% of diet)	Diet 1 (Control)	Diet 2 (1.50% fish oil)	Diet 3 (3.00% fish oil)
Corn grain	60.17	56.73	53.01
Soybean meal (44.00%)	25.66	25.54	26.43
Wheat bran	0.00	1.00	2.00
Soybean oil	0.30	0.30	0.30
Fish oil	0.00	1.50	3.00
Dicalcium phosphate	2.16	2.27	2.48
Calcium carbonate	10.86	11.31	11.89
Common salt	0.32	0.32	0.32
Sodium bicarbonate	0.10	0.10	0.10
DL-Methionine	0.09	0.09	0.10
Vitamin premix[1]	0.30	0.30	0.30
Mineral premix[2]	0.30	0.30	0.30
Chemical composition			
Metabolizable energy (kcal kg^{-1})	2,685.00	2,685.00	2,685.00
Energy intake (kcal *per* bird *per* day)	278.97	278.70	278.97
Crude protein (%)	15.70	15.70	15.70
Calcium (%)	4.65	4.65	4.65
Available phosphorus (%)	0.39	0.39	0.39
Sodium (%)	0.17	0.17	0.17
Methionine (%)	0.37	0.37	0.37
Methionine + Cysteine (%)	0.65	0.65	0.65
Lysine (%)	0.79	0.79	0.79
Fatty acid composition			
C14:0	0.09	0.31	0.53
C16:0	27.09	30.51	31.98
C16:1	0.07	0.05	0.04
C18:0	3.91	4.17	6.14
C18:1 n-9	31.66	29.02	26.69
C18:1 n-7	0.67	0.61	0.60
C18:2 n-6	35.01	31.47	27.92
C18:3 n-3	0.31	0.33	0.59
C20:4 n-6	0.33	0.38	0.53
C20:5 n-3	0.01	1.11	2.39
C22:6 n-3	0.01	0.92	1.61
SFA	31.01	34.99	38.65
PUFA	35.66	33.73	32.84
PUFA: SFA	1.15	0.96	0.85
Omega-3	0.33	2.35	4.39
Omega-6	35.33	31.38	28.45
Omega-6 : Omega-3	107.10	13.35	6.48
LA : ALA	112.90	93.93	47.32

[1] Supplied *per* kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,500IU; vitamin E, 10.00 IU; vitamin B_1, 22.00 mg; vitamin B_2, 4.00 mg; vitamin B_3, 8.00 mg; vitamin B_4, 2.00 mg; vitamin B_9, 0.56 mg; vitamin B_{12}, 0.015 mg; choline, 200 mg.
[2] Supplied *per* kilogram of diet: manganese, 80.00 mg; iron, 50.00 mg; zinc, 60.00 mg; copper, 12.00 mg; sodium selenite, 0.30 mg.
PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, LA: linoleic acid, ALA: α-linolenic acid.

Table 2. Effects of using L-carnitine in diets containing different levels of fish oil on the performance of laying hens.

Treatments	Egg production (%)	Egg weight (g)	Egg mass (g day^{-1} *per* bird)	FI (g day^{-1} *per* bird)	FCR
Fish oil (%)					
0.00	87.79^{b}	58.57^{c}	51.41^{c}	103.90	2.02^{a}
1.50	88.52^{ab}	59.14^{b}	52.37^{b}	103.80	1.98^{b}
3.00	89.15^{a}	59.68^{a}	53.22^{a}	103.90	1.95^{c}
SEM	0.260	0.159	0.254	0.142	0.008
***p*-value**	0.023	0.013	0.011	0.929	0.019
L-carnitine (mg kg^{-1})					
0.00	86.98^{b}	57.11^{b}	49.69^{b}	103.80	2.09^{a}
300	88.66^{a}	59.65^{a}	52.91^{a}	103.90	1.96^{b}
600	89.79^{a}	60.64^{a}	54.45^{a}	104.00	1.91^{c}
SEM	0.282	0.159	0.254	0.142	0.008
***p*-value**	0.039	0.029	0.007	0.789	0.022
Fish oil (%) × L-carnitine (mg kg^{-1})					
0.00 × 0.00	87.30^{b}	56.83^{c}	49.60^{b}	103.80	2.09^{a}
0.00 × 300	87.74^{b}	58.96^{b}	51.72^{b}	104.00	2.01^{b}
0.00 × 600	88.33^{ab}	59.93^{ab}	52.92^{ab}	103.90	1.96^{b}
1.50 × 0.00	86.69^{b}	56.91^{c}	49.32^{b}	103.80	2.10^{a}
1.50 × 300	88.32^{ab}	59.74^{ab}	52.76^{ab}	104.30	1.97^{b}
1.50 × 600	90.55^{a}	60.78^{a}	55.05^{a}	103.80	1.88^{c}
3.00 × 0.00	87.05^{b}	57.61^{bc}	51.17^{b}	104.00	2.07^{a}
3.00 × 300	89.91^{a}	60.20^{a}	54.13^{a}	103.60	1.91^{c}
3.00 × 600	90.51^{a}	61.22^{a}	55.39^{a}	104.30	1.88^{c}
SEM	0.450	0.376	0.570	0.646	0.014
***p*-value**	0.033	0.047	0.016	0.171	0.021

FI: Feed intake, FCR: Feed conversion ratio, and SEM: standard error of means.
^{abc} Means in the same column with different superscripts are significantly different ($p < 0.05$).

Results

Production performance. Table 2 shows the effect of using LC in diets containing different levels of FO on the performance of laying hens. According to the results obtained, the percentage of production, EW, EM and FCR were affected by the main effects of FO and LC and the interaction effect of FO × LC, and the hens treated with 3.00% FO and 300 and 600 mg of LC had the highest percentage of production, EW and mass, which was significantly higher than the treatment without FO and LC ($p < 0.05$). The FCR was also increased in the treatments without LC supplementation compared to the other experimental treatments ($p < 0.05$).

Egg quality traits. The qualitative traits of eggs produced by laying hens fed on experimental treatments containing different levels of FO and LC supplementation are reported in Table 3. The results showed that by increasing the level of FO to 3.00%, the pH of egg yolks was decreased significantly, however, the pH of the yolk of the group fed on a diet containing 600 mg kg^{-1} LC was significantly increased ($p < 0.05$). Yolk weight, HU and yolk color index were significantly increased with the use of 600 mg kg^{-1} LC in the diet ($p < 0.05$).

Yolk fatty acid (FA) profile. Regarding the content of FA ratios in egg yolks (Table 4), the results showed that the percentage of PUFA, the ratio of PUFA/SFA and the percentage of omega-3 FAs were increased and total SFAs were decreased in egg yolk with increasing levels of FO in the diet ($p < 0.05$). The interaction effect of FO and LC levels showed that the content of omega-3 FAs in egg yolk was significantly increased with LC supplementation in FO diets ($p < 0.05$). The content of omega-6 FAs and omega-6: omega-3 ratio, as expected, was decreased in egg yolk significantly with increasing levels of dietary FO ($p < 0.05$). The linoleic acid (LA)/α-LA (ALA) ratio was also affected by the interaction between FO and LC levels, in a way with increasing levels of LC in FO diets, this ratio was significantly decreased ($p < 0.05$). Increasing the amount of FO in the diet increased the content of EPA FAs (omega-3) in egg yolk compared to the control group ($p < 0.05$). The use of 300 and 600 mg kg^{-1} LC supplementation in diets containing FO had synergistic effects on the percentage of DHA omega-3 FAs in egg yolk and significantly increased them.

Discussion

In agreement with the findings of the present study (Table 2), recent studies using 1.20 and 1.50% FO in the diet of laying hens reported improved EP and weight.[23] Previous reports indicated positive effects of omega-3 FAs on physiological activities of birds, including egg laying, growth rate, immunity, skeletal system and reproduction.[24] Although in this study all diets were tried to be energy-efficient, it seems that increasing the level of omega-3 FAs in the diet provided more available energy at the cellular level for the birds reproductive system, which

Table 3. Effects of using L-carnitine in diets containing different levels of fish oil on egg quality traits of laying hens.

Treatments	Albumen weight (%)	Yolk weight (%)	Albumen pH	Yolk pH	Haugh unit	Yolk color index	Shell strength (kg *per* m²)
Fish oil (%)							
0.00	62.67	27.50	8.18	7.13[a]	93.24	6.56	4.45
1.50	63.35	28.09	8.31	6.99[ab]	92.11	6.65	4.42
3.00	62.90	28.46	8.27	6.27[b]	92.79	6.93	4.41
SEM	0.428	0.372	0.049	0.058	0.589	0.144	0.152
***p*-value**	0.157	0.067	0.154	0.003	0.397	0.185	0.979
L-carnitine (mg kg⁻¹)							
0.00	62.74	27.61[b]	8.24	6.45[b]	91.17[b]	6.32[b]	4.46
300	62.98	27.45[b]	8.26	6.89[ab]	92.72[ab]	6.41[b]	4.37
600	63.21	29.25[a]	8.27	7.05[a]	93.24[a]	7.42[a]	4.45
SEM	0.428	0.372	0.049	0.058	0.589	0.144	0.152
***p*-value**	0.433	0.008	0.963	0.021	0.046	0.010	0.881
Fish oil (%) × L-carnitine (mg kg⁻¹)							
0.00 × 0.00	61.10	27.32	8.19	6.54[b]	91.87	6.42	4.62
0.00 × 300	64.46	25.97	8.11	7.52[a]	93.65	6.20	4.40
0.00 × 600	63.44	29.96	8.23	7.34[a]	93.21	7.08	4.34
1.50 × 0.00	63.58	26.95	8.36	6.60[b]	90.36	6.14	4.48
1.50 × 300	62.10	28.71	8.28	6.85[ab]	91.68	6.28	4.20
1.50× 600	61.38	28.60	8.30	7.52[a]	93.27	7.54	4.58
3.00 × 0.00	61.53	28.55	8.18	6.21[b]	91.29	6.40	4.30
3.00 × 300	62.36	27.67	8.37	6.32[b]	92.83	6.76	4.50
3.00 × 600	63.80	29.21	8.27	6.70[ab]	93.26	7.64	4.44
SEM	0.739	0.113	0.086	0.101	1.021	0.249	0.263
***p*-value**	0.268	0.645	0.427	0.001	0.891	0.556	0.744

SEM: standard error of means.
[abc] Means in the same column with different superscripts are significantly different ($p < 0.05$).

Table 4. Effects of using L-carnitine in diets containing different levels of fish oil on fatty acid ratios of egg yolk at the end of the period

Treatments	SFA	PUFA	PUFA/SFA	∑ omega-3	∑ omega-6	omega-6 : omega-3	LA /ALA	EPA	DHA
Fish oil (%)									
0.00	37.37[a]	12.34[c]	0.331[c]	1.33[c]	11.09[a]	8.25[a]	12.08[a]	0.148[c]	0.288[c]
1.50	35.49[b]	13.42[b]	0.378[b]	2.77[b]	10.64[b]	3.93[b]	10.15[b]	0.581[b]	1.219[b]
3.00	33.54[c]	13.97[a]	0.416[a]	3.60[a]	10.37[c]	2.88[c]	9.08[c]	1.006[a]	1.540[a]
SEM	0.380	0.088	0.004	0.016	0.083	0.038	0.127	0.014	0.006
***p*-value**	0.026	0.034	0.011	0.016	0.019	0.011	0.006	0.001	0.010
L-carnitine (mg kg⁻¹)									
0.00	35.94	13.06[b]	0.364[b]	2.47[c]	10.59	5.11[a]	10.62[a]	0.538	0.981[c]
300	35.36	13.26[ab]	0.379[a]	2.56[b]	10.69	4.98[b]	10.60[a]	0.586	1.015[b]
600	35.10	13.41[a]	0.384[a]	2.67[a]	10.74	4.88[b]	10.10[b]	0.605	1.042[a]
SEM	0.357	0.071	0.003	0.024	0.094	0.032	0.138	0.014	0.006
***p*-value**	0.147	0.048	0.002	0.016	0.444	0.007	0.028	0.055	0.033
Fish oil (%) × L-carnitine (mg kg⁻¹)									
0.00 × 0.00	37.31	12.25	0.328	1.30[c]	10.95	8.43	12.30[a]	0.140[c]	0.282[c]
0.00 × 300	37.57	12.26	0.326	1.33[c]	10.93	8.19	11.95[a]	0.154[c]	0.278[c]
0.00 × 600	37.22	12.51	0.336	1.37[c]	11.14	8.13	12.01[a]	0.152[c]	0.304[c]
1.50 × 0.00	36.35	13.33	0.368	2.73[b]	10.59	3.88	10.16[b]	0.560[b]	1.188[b]
1.50 × 300	35.15	13.48	0.382	2.79[b]	10.68	3.82	10.20[b]	0.590[b]	1.216[b]
1.50 × 600	34.97	13.46	0.386	2.81[b]	10.65	3.79	10.10[b]	0.592[b]	1.224[b]
3.00 × 0.00	34.15	13.61	0.396	3.38[a]	10.22	3.02	9.40[c]	0.914[a]	1.470[a]
3.00 × 300	33.37	14.04	0.422	3.57[a]	10.47	2.93	9.66[c]	1.016[a]	1.552[a]
3.00 × 600	33.10	14.26	0.432	3.84[a]	10.42	2.71	8.18[d]	1.072[a]	1.598[a]
SEM	0.312	0.153	0.006	0.028	0.145	0.066	0.219	0.024	0.010
***p*-value**	0.162	0.194	0.194	0.018	0.832	0.274	0.008	0.049	0.014

PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, LA: linoleic acid, ALA: α-linolenic acid EPA: Eicosapentaenoic DHA: docosaheaenoic, SEM: standard error of means.
[abc] Means in the same column with different superscripts are significantly different ($p < 0.05$).

was associated with higher egg laying and more efficient FCR. Therefore, the increased level of EP could be attributed to the role of omega-3 FAs in the physiological activities of reproduction. In this regard, the positive effect of omega-3 FAs in birds has been reported on many physiological activities such as EP and fertility.[25] On the other hand, Shahryari *et al*.[26] measured the apparent digestibility of FO and soybean oil, confirmed the findings of previous researchers on the effect of increasing metabolizable energy on improving animal performance, and evaluated the intestinal histology and stated that the improvement in body weight gain of broilers fed diets containing FO and soybean oil could be due to the effect of these fat sources on the development of intestinal tissue and increased persistence of consumed food in the intestinal environment, because these conditions increased the digestion and absorption of nutrients. Therefore, in the present experiment, FO at a level of 3.00% probably improved the performance of laying hens due to increasing the level of omega-3 FAs in the diet and providing more energy generation at the cellular level for the liver or ovary as well as reducing the speed of passage of materials in the small intestine.

In other experiment, researchers investigated the effects of different levels of LC on the production performance of laying hens. Their results showed that LC supplementation (100 and 150 mg kg^{-1}) significantly increased EP and EM.[27] In addition, Pignatelli *et al*.[28] reported that LC mediated the nicotinamide adenine dinucleotide phosphate oxidase system by interfering with the arachidonic acid linkage between phospholipids and protein kinase-c. On the other hand, omega-3 FAs and LC can help maintain a healthy digestive tract by regulating the intestinal microbial balance and increasing the secretion of digestive enzymes from related organs such as the pancreas.[29] The LC supplementation has been shown to increase FA beta-oxidation and adenosine triphosphate production.[30] The increased FA oxidation induced by LC may reduce the availability of long-chain FAs for esterification into triglycerides while increasing acetyl-coenzyme A levels in mitochondria.[3] The activity of pyruvate carboxylase, an acetyl-coenzyme A-dependent enzyme that may provide carbon chains for amino acid biosynthesis, is likely to be affected in this condition.[31] It has been shown that additional LC may improve production performance in part due to its amino acid storage function as well as its involvement in FA metabolism. LC may stimulate the biosynthesis of estrogen and progesterone by increasing FA oxidation. Thus, by increasing the regeneration of reductants required for the cholesterol side chain scission reaction, it causes more production of sex hormones and also affects the growth and maturation of small ovarian follicles and accelerates the ovulation process.[32] Also, the use of LC supplementation may theoretically reduce the demand for LC production from methionine and, by saving methionine, it could be used for other biological purposes.[33]

The improvement in egg quality with 300 and 600 mg kg^{-1} LC could be due to the fact that LC probably performed its antioxidant role to modulate the function of the ovary and oviduct and to stimulate the efficient absorption of nutrients transported to the egg (Table 3). Carnitine has been suggested to metabolize energy after recycling to produce adenosine triphosphate via creatine phosphokinase in muscle tissues. Also, it may protect against oxidative, apoptotic and toxic reactions in most vital tissues such as the ovary.[34] In agreement with our results, Al-Shammari *et al*.[12] reported that the use of LC in the diet of laying hens increased yolk weight, HU and yolk color index. The optimal metabolic rate in the magnum is due to the synthesis and secretion of β-omucin, which is responsible for the gelatinous structure of the egg albumen and can be controlled by dietary LC,[35] and was observed by optimizing the HU in the present study. The increase in yolk weight and color may be attributed to the stimulatory effect of LC on the formation of yolk precursors in the liver tissue and the subsequent activation of their transport to developing follicles and mature oocytes from the sites of synthesis in the liver.[36] The present study showed that the decrease in yolk pH was likely due to lipid peroxidation in the 3.00% FO group. The FO was rich in PUFAs that were susceptible to peroxidation. Consequently, the decrease in yolk pH might be due to lipid peroxidation. Previous studies have shown that when albumin pH increases, the binding between lysosomes and eosin weakens, which in turn causes a decrease in HU.[37] However, in this study, albumin pH was not decreased and HU remained constant. Limited studies have investigated the effects of FO or even omega-3 PUFA on yolk pH and albumin and HU. Therefore, further experiments are needed to clarify the reasons for the changes. Findings of the present study suggested that antioxidants in the LC content prevented the deterioration of yolk and albumin quality by inhibiting pH changes and weakening albumin protein bonds. Additionally, the increase in yolk color index may indicate that the albumin protein binding remained intact and water did not penetrate into the egg yolk. We hypothesized that LC supplementation might have increased the deposition of pigments, especially β-carotene, in the yolk, leading to an increase in yolk color index. This hypothesis was based on the solubility of pigments such as astaxanthin and β-carotene in lipids.[2]

In this study, evaluation of egg yolk FAs in laying hens fed on diets supplemented with FO showed increased PUFA, especially omega-3 FAs, including EPA, DHA and ALA. In contrast, LA levels, omega-6: omega-3 ratio and LA:ALA ratio was significantly reduced compared to the control group (Table 4). These results were consistent with previous studies that reported changes in FA profiles

in laying hens fed on diets supplemented with FO. In a meta-analysis by Irawan *et al*.[38] increasing dietary ALA levels linearly increased EPA, DHA, and total omega-3 PUFAs while simultaneously decreased LA concentrations in eggs. The results could be interpreted as suggesting that feeding 3.00% FO resulted in eggs with a high percentage of DHA. The total omega-3 PUFA content in the diet could also predict DHA formation because ALA was the predominant omega-3 PUFA in many FA sources. Our findings were consistent with the existing theory that ALA serves as the main precursor for the synthesis of EPA and DHA. Consumption of ALA sources by laying hens causes Δ-6-desaturase enzymes to desaturate ALA by removing hydrogen atoms and then elongating it by adding carbons to form EPA. In this perspective, EPA is mostly converted to DHA by elongation and saturation processes.[39] The efficiency of ALA to DHA conversion varies among experimental settings, which is mainly due to the LA ratio.[40] This is because Δ-6-desaturase can also use LA as a substrate, although its activity is lower than that of ALA. Therefore, increasing the LA/ALA ratio may impair DHA formation in eggs due to substrate competition.[37] Several recent studies support the argument that decreasing the LA/ALA ratio from different FA sources linearly increased DHA content in eggs.[41,42] In addition to the LA/ALA ratio, other factors that explain differences in DHA deposition are the age of the hens and the source of FAs. Older hens have a more efficient metabolism, especially in the formation of DHA from ALA sources, due to their larger livers.[39]

The ALA sources have different efficiencies for the formation of DHA and total omega-3 PUFA in eggs. FO has been reported to have the highest conversion efficiency for the formation of DHA.[38] This is plausible because FO is inherently rich in DHA content. Comparative studies have shown that egg yolk DHA content was significantly higher with a diet containing FO compared to flaxseed oil and microalgae.[43] A recent study showed that the use of 1.50% FO produced 142 mg DHA *per* 60.00 g of eggs,[23] which was higher than the 106 mg reported by Lee *et al*.[42] with flaxseed oil and 89.00 mg with microalgae.[44] Most importantly, it should be kept in mind that one of the major problems for including omega-3 PUFA in the diet is its susceptibility to lipid oxidation.[45] which directly affects egg quality and flavor. Previous studies using FO and flaxseed reported increased lipid oxidation in eggs.[23] Therefore, the addition of antioxidants along with omega-3 PUFA sources has been proposed as an effective strategy to inhibit oxidation, which can spoil eggs and shorten egg shelf life.

In a recent study, LC at levels of 300 and 600 mg kg^{-1} improved the FA profile of egg yolk. Some birds, including chickens, have a limited ability to synthesize long-chain omega-3 FAs from LA. They also have a poor ability to transfer them to eggs. However, chickens have a high ability to synthesize SFA and can compensate for the deficiency even if their diet is reduced in quantity.[46] In one study, the addition of 500 mg kg^{-1} LC to the diet increased the beta-oxidation of mitochondrial long-chain FAs by facilitating their transport across the inner mitochondrial membrane.[47] Therefore, LC can improve the body use of energy and FAs.[48] The enzyme Δ-9-desaturase is responsible for the conversion of stearic acid (C18:0) to oleic acid (C18:1n-9), while dietary LC may stimulate the synthesis of Δ-9-desaturase and, therefore, increase the total amount of unsaturated FAs.[49] This explains how LC plays an important role in the mitochondrial oxidation of FAs for energy production.[48] The level of DHA in egg yolk can be related to direct deposition of DHA from the diet or the end result of a new synthesis that produces its precursors ALA and EPA.[50]

In conclusion, the results of this study indicated that use of LC in a diet containing FO not only increased production performance and egg quality (HU and yolk color index), but also increased the level of omega-3 FAs in the yolk. Also, where 3.00% FO is used, the use of LC as an antioxidant is recommended to prevent lipid peroxidation and maintain yolk pH.

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Conflicts of interest

There is not conflict of interest with any person or institute/organization regarding this manuscript.

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