The effect of adding L-carnitine to omega-3 fatty acid diets on productive performance, oxidative stability, cholesterol content, and yolk fatty acid profiles in laying hens

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ABSTRACT In this study, different levels of Omega-3 fatty acids and L-carnitine (**LC**) were used in diets for laying hens. The effects of these supplements were examined on productive performance, antioxidant properties, cholesterol content, and yolk fatty acid profiles in the laying hens. A population of 120 Lohmann LSL-Lite laying hens (34-wk-old) were used in 2 × 3 factorial arrangements with 2 diets (control = 0.031 and 0.48% omega-3 fatty acids) and 3 levels of L-carnitine (0, 100, 200 mg/kg of diet) in a completely randomized design with 6 treatments. While having 5 replicates and 4 birds per replicate, the total period of the experiment lasted for 10 wk. The eggs were weighed daily, parallel to measurements of egg production, daily feed intake, feed conversion ratio, and egg mass. When the hens reached 44 wk of age, the measurements were aimed at fatty acid profiles, malondialdehyde (**MDA**), and cholesterol concentration in egg yolk. Feeding the hens on diets enriched by omega-3 fatty acids led to higher levels of egg production than those fed on control diets, but their daily feed intake was generally lower ($P < 0.05$). Egg weight decreased in birds that were fed on diets enriched with omega-3 fatty acids without L-carnitine, or with diets which contained 100 mg/kg L-carnitine, compared to control diets which contained 0 mg/kg L-carnitine ($P < 0.05$). Egg mass increased in birds that were fed on diets enriched with omega-3 fatty acids and which contained 200 mg/kg L-carnitine, compared to the control diet with 0 or 100 mg/kg L-carnitine ($P < 0.05$). The analysis of fatty acid profiles showed that L-carnitine and omega-3 fatty acids caused a significant increase in the percentage of eicosapentaenoic acid (**EPA**), docosahexaenoic acid (**DHA**), C18: 1 (n-9), arachidonic acid (**ARA**) C20: 4 (n-6), and Σ n-3 in the eggs of birds ($P < 0.05$). Based on the results, adding L-carnitine (200 mg/kg) to diets that were already enriched with omega-3 fatty acids increased the level of production and led to a longer maintenance of fatty acids in the eggs. Also, oxidative stability was enhanced in the yolk of eggs.

Key words: omega-3 fatty acids, L-carnitine, MDA, fatty acid profile, laying hen

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INTRODUCTION

The egg production industry has always been a unique manufacturing sector, because eggs are nutritious functional foods that contain high-quality proteins. There are currently 2 different general and specific approaches to the egg production industry. In the general approach, eggs are considered as a natural, rich food package of proteins, vitamins, and minerals that have always been easily available to consumers. In the specific approach, eggs are considered as a specially structured food (Miranda et al., 2015) with over 30% egg yolk weight which primarily comprises cholesterol and fatty acids. Accordingly, the research sector has partly focused on providing the market with enriched eggs, so that the content of minerals, vitamins, and omega fatty acids can be enhanced.

Polyunsaturated fatty acids (**PUFAs**) are essential for the body's metabolism, growth, and development, since they play an important role in regulating reproduction (Chien et al., 2000). Omega-3 fatty acids are the most important family of polyunsaturated fatty acids and play important roles in many physiological activities such as ovulation, while lowering blood cholesterol levels, improving the immune response, enhancing the dynamics of fertility, lowering blood lipids, and controlling cardiovascular diseases (Zolfaghari et al., 2014). These n-3 PUFAs can assist in ovulation through a

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variety of ways, such as producing prostaglandins, estrogen, and progesterone, altering the production of eicosanoids or steroids, changing cell membrane fluidity, causing oxidative stress, and/or participating in signal transduction, among other functions (Wakefield et al., 2008; Yi et al., 2012). The alteration of eicosanoids, namely, prostaglandins (**PG**), by PUFAs can affect ovulatory functions (Abayasekara and Wathes, 1999; Sirois et al., 2004). At present, n-3 PUFA-enriched foods have gained popularity among consumers. Eggs are a complete part of people's daily diets. The nutritional value of an egg can be increased by incorporating more omega-3 fatty acids in the diets of the birds (Bruneel et al., 2013). The flaxseed, canola seed, millet seed, soybean seed, or edible oil are rich in linolenic acid, while marine-sourced additives usually contain long-chain omega-3 fatty acids such as docosahexaenoic acid (**DHA**) and eicosapentaenoic acid (**EPA**). Since salmon fish oil is a strong source of omega-3 fatty acids, it can have a wide range of effects as an additive and, thus, can improve yield and other related traits in laying hens. Nonetheless, fish odors and flavors have been reported in eggs when chickens were fed with fish (Baucells et al., 2000) or rapeseed meal, except for the '00′ variety (Clandinin and Robblee, 1981).

Carnitine (β-hydroxy-γ-trimethylaminobutyrate) is a vitamin-like amino acid and contains a quaternary ammonium mixture with poly-functional roles in reproduction. L-carnitine is important in the catabolism of fats, especially high-chain fatty acids that tend to accumulate in adipose tissue, as they usually reduce the accumulation rate of fats (Adabi et al., 2011). The continuous addition of L-carnitine to experimental diets for laying hens have reportedly resulted in a decrease of total proteins, cholesterol, calcium, and phosphorus in blood plasma (Thiemel and Jelinek, 2004). Since L-carnitine plays a pivotal role in transporting long-chain fatty acids across the inner mitochondrial membrane, its function is partly focused on energy production through β-oxidation (Durán et al., 2005). L-carnitine supplementation may accelerate fat metabolism in the yolk and, thus, expedite follicular development. L-carnitine may also increase metabolic rates in the magnum and shell gland, thereby resulting in albumen deposition and shell calcification, followed by an increase in egg weight (Peebles et al., 2007).

A review of functions performed by L-carnitine can reveal the main aspects of this valuable supplement in the nutritional management of poultry. In recent years, Omega-3 fatty acids have been considered as a suitable feed additive, as they are capable of increasing egg production and nutritional value. Regarding the different metabolic activities of L-carnitine and omega-3 fatty acids in laying hens, it seems that adding L-carnitine to diets that are already enriched with omega-3 fatty acids can not only increase the storability of these fatty acids in egg yolk but also improve laying performance and antioxidant properties. The present study aimed to evaluate the effects of different levels of omega-3 fatty acids and L-carnitine on productive performance, antioxidant properties, cholesterol content, and fatty acid profiles in the yolk of eggs.

MATERIALS AND METHODS

Animal Ethics

All experimental procedures were confirmed by the Animal Welfare Committee of the Department of Animal Science, University of Tehran.

Bird Management and Treatments

A population of 120 Lohmann LSL-Lite laying hens (34-wk-old) was used in 2 × 3 factorial arrangements and was fed with 2 diets, that is, the control and the omega-3 fatty acid diet, which contained 0.031 and 0.48% omega-3, respectively. A number of laying hens at 32 wk of age were fed a standard diet. The adaptation period lasted for 14 d. During this period, laying hens were examined for production. The laying hens were selected based on body weight approximately the same and with similar production and were randomly distributed among the experimental units. In addition, there were 3 levels of L-carnitine (0, 100, 200 mg/kg of diet) in a completely randomized design with 6 treatments, 5 replicates, and 4 birds per replicate. The experimental duration lasted for 10 wk. Salomega (Agritech, Ireland) was used as the source of omega-3 fatty acids with graded levels (0 and 3%) in the diet to provide the fatty acids. Salomega was prepared using salmon oil, while corn cob was used as a carrier in this product. Salomega contained 52% fat and about 17% total omega-3 fatty acids. Measurements determined the fat content and fatty acid profiles (Table 1). All laying hens were fed

Table 1. Total fat content and fatty acids composition (%) of Salomega.

Fatty acids	Reported[1] (%)	Measured (%)
Dry mater	95.6	95.1
Total fat	52.0	51.3
Myristic acid (C14:0)	3.2	2.1
Palmitic acid (C16:0)	10.9	11.4
Stearic acid (C18:0)	2.9	2.9
Arachidic acid (C20:0)	0.3	0.4
Palmitoleic acid (16:1n7)	3.6	3.8
Oleic acid (C18:1 n9)	34.3	35.4
Eicosenoic acid (20:1n9)	3.8	3.5
Erucic acid (22:1n9)	0.6	0.2
Tetracosenoic acid (24:1n9)	0.4	0.2
Linoleic acid (C18:2 n6)	13.1	11.9
γ-linolenic acid(18:3n6)	0.1	ND
Eicosadienoic acid(20:2n6)	0.8	0.6
Eicosatrienoic acid(20:3n6)	0.2	0.9
Arachidonic acid (C20:4n6)	0.4	0.8
Alpha linolenic acid (C18:3 n3)	4.3	4.9
Eicosapentaenoic acid (C20:5 n3)	3.7	3.6
Docosapentaenoic acid (C22:5n3)	1.6	1.6
Docosahexaenoic acid (C22:6 n3)	4.6	5.4
Other fatty acids	10.8	10.4
Total of saturated fatty acids	17.9	16.8
Total of monounsaturated fatty acids	50.1	43.0
Total of polyunsaturated fatty acids	32.0	29.9
Total of omega-3 fatty acids	16.6	15.6
Total of omega-6 fatty acids	14.6	14.2

ND, not detected.

[1]Fatty Acid Analysis of Salomega reported by Irish Agritrch company (Compiled by: Nutrition Analytical Service, Institute of Aquaculture, University of Stirling).

Table 2. Ingredient and nutrient composition of basal diets.

Ingredient (%)	Total omega-3 fatty acids (% of diet)	
	Control	Enriched
Yellow corn	52.20	49.00
Soybean meal[1]	30.80	31.50
Bran wheat	2.50	2.50
Salomega	0.00	3.00
Canola oil	1.83	1.37
Dicalcium phosphate	1.53	1.52
Limestone	6.50	6.50
Oyster shell	3.66	3.66
Vitamin-mineral premix[2]	0.50	0.50
DL-Methionine	0.18	0.16
Sodium chloride	0.15	0.15
Sodium bicarbonate	0.15	0.15
Total	100	100
Calculated nutrients		
AMEn (kcal/kg)	2,620	2,620
Crude protein (%)	18.50	18.50
Crude fiber (%)	2.64	2.71
Calcium (%)	4.25	4.25
Available phosphorus (%)	0.43	0.43
Lysine (%)	1.03	1.02
Methionine (%)	0.48	0.44
Methionine + cysteine (%)	0.78	0.74
Measured nutrients		
Crude protein (%)	17.33	18.09
Crude fat (%)	4.58	5.81
Total omega-3 fatty acid (%)	0.031	0.507

[1]Non-Dehulled Soybean meal (44% crude protein).
[2]Vitamin and mineral Premix supplied per kilogram of diet: Vitamin A, 9,000 IU. Vitamin D3, 2,000 IU. Vitamin E, 18 IU. Vitamin K3, 2 mg. thiamin, 1.8 mg. riboflavin, 6.6 mg. Niacin, 30 mg. Calcium pantothenate, 10 mg. Vitamin B6, 3 mg. Folic acid 1 mg. Vitamin B12, 0.015 mg., Biotin 0.1 mg., Choline 500 mg., manganese oxide 100 mg., ferrous sulfate 50 mg., zinc oxide 100 mg., copper sulphate 10 mg., calcium iodate 1 mg., sodium selenite, 0.2 mg.

with a standard iso-caloric (2,620 kcal/kg) and an iso-nitrogenous diet (18.5% protein; Table 2, Lohmann's manual for laying hens). The experimental diets were produced, first, by mixing all ingredients, except the L-carnitine. The required L-carnitine was measured using a sensitive digital scale with an accuracy of 0.01 g. Separately, the L-carnitine was mixed with a portion of other ingredients using a mixer and was added to the diets. The diets were divided into 3 parts. Each part was then supplemented with L-carnitine at levels of 0 (LC-0), 100 (LC-100), and 200 (LC-200) mg/kg. The fatty acid profiles of the experimental diets are presented in Table 3. The laying hens were maintained under similar conditions with a controlled photoperiod regimen (16 h light: 8 h dark, and at a temperature of 24 ± 2°C, water temperature at about 20 to 24°C and RH = 60%). The laying hens were kept in individual cages (29 × 43 × 51 cm) as 3-tier battery, environmentally controlled poultry houses (4 × 11 m). Eggs were counted, weighed by a scale with an accuracy of 0.01 g and collected by cage between 14:30 and 15:30 daily. All cages and their compartments, including coils, feeders, waste trays, etc., were made of hot-dip galvanized steel Q235. During the experiment, the light sources were white light-emitting diodes (**LEDs** = 6-Watt, 365 lm, 6200 Kelvin, 100% light, 220−240 Volts, 50−60 Hz). All light sources were equalized to a light intensity of 20 lx, as recorded by a lux meter (TES 1335, Digital Light Meter). The diets and fresh water were available for the hens ad libitum

Table 3. Fatty acids profiles (%) of the experimental diets.

Fatty acids (%)	Diets	
	Control	Enriched
Myristic acid (C14:0)	-	3.03
Palmitic acid (C16:0)	15.87	14.77
Palmitoleic acid (C16:1)	-	1.23
Stearic acid (C18:0)	5.49	4.66
Oleic acid (C18:1 n9)	29.45	31.19
Linoleic acid (C18:2 n6)	46.92	36.39
Alpha linolenic acid (C18:3 n3)	2.23	3.26
Arachidic acid (C20:0)	-	1.85
Arachidonic acid (C20:4)	-	1.42
Eicosapentenoic acid (C20:5 n3)	-	0.78
Docosapentaenoic acid (C22:5)	-	0.34
Docosahexaenoic acid (C22:6 n3)	-	1.09
Total of saturated fatty acids	21.36	24.30
Total of mono unsaturated fatty acids	29.45	32.41
Total of polyunsaturated fatty acids	49.19	43.29
Total of omega-6 fatty acids	46.92	37.81
Total of omega-3 fatty acids	2.28	5.48
Total of omega-6/total omega-3 fatty acids ratio	20.79	6.90

throughout the study. Feed allocation for the 2-wk period was weighed into individual labeled feed buckets for each replicate cage and daily allocation was provided at approximately 8 a.m. from each individual feeding bucket. At the end of the 2-wk period, any remaining feed in the feed tray was returned to the corresponding feed bucket and weighed to determine feed intake.

Productive Performance

The feed intake, egg count, and mean egg weight were recorded on a daily basis at first, and then weekly, followed by calculations of egg production, egg mass, daily feed intake, egg shape index, and feed conversion ratio (FCR as "kg feed/1 kg eggs").

Fatty Acids Profile in the Diet and Egg Yolk

To determine the fatty acid profile in the diet, 500 g samples of the diets were poured into zippered plastic bags and then stored at −20°C until further analyses. To determine fatty acid profiles in egg yolk, 10 eggs per treatment were collected and broken at the end of the experiment. After breaking the eggs, the yolks were separated from the albumen. Corresponding to each replicate, the fresh yolks were mixed, homogenized, and stored in 50 mL falcons at −20°C until further analyses. The fatty acids profile in the diet was determined by gas chromatography (GC; Agilent 6890 N, Agilent Technologies, Paris, France) which was interfaced with mass spectroscopy (MS; Agilent 5973 N, Agilent Technologies) according to a method used by Metcalf et al. (1966). Briefly, 0.5 g of the sample (diet or egg yolk) was mixed with 5 mL of methanol solution (2%) and was placed in a water bath for 10 min. Then, 175 mL of the solution, along with boron tri-fluoride methanol (BF3 20%), were added before placing the tubes in a boiling water bath for 3 min. This was followed by adding n-hexane (1 mL) and NaCl solution (1 mL). Finally, the

upper layer was separated with a micro-pipette and poured into a 1.5 mL falcon. Then, 2.0 μL of the extract was injected by gas chromatography and the fatty acid methyl esters were separated by GC (Metcalfe et al., 1966). FA methyl esters were reconstituted in 1 mL of hexane (Loor et al., 2005) and the concentration of FA was specified by gas chromatography (Model YL 6100, Make: Young Lin, Korea) with a 60 m capillary column (0.0.25 mm I.D.; Dikmacap-2330). The carrier gas was hydrogen. The initial and final temperatures were set at 170 and 230°C, while the detector and injector temperatures were set at 300 and 260°C.

Malondialdehyde Assay and Cholesterol Values in the Yolk

To measure the rate of lipid peroxidation, the last 2 d of the 44-wk period involved collecting and cracking 10 eggs per treatment. The yolks were separated from the albumen and the fresh yolks were stored at −20°C until further analyses. In this research, Malondialdehyde (**MDA**) was measured by the TBA method (Botsoglou et al., 1994) but with slight modifications (Galobart et al., 2001). Cholesterol concentration in the yolk was determined based on a method used by Pasin et al. (1998). Briefly, 1 g of egg yolk was mixed with 9 mL NaCl solution (2%). The sample was then gently shaken for 2 h. A solubilized yolk sample (1 mL) was further diluted (10-fold) with 9 mL NaCl solution (2%) and was used as a working sample. A cholesterol reagent kit (Zist shimi, Tehran, Iran) and a spectrophotometer (UV-Visible S2100, Scinco, Korea) were used for measuring the amount of cholesterol. The samples were prepared by adding an additional amount of enzyme reagent (1 mL) to the working sample and the standard solution (10 μL). A blank was prepared by substituting 10 μL of deionized water with the working sample or with the standard solution. They were vortexed for 30 s and left for 15 min in a water bath at 37°C. Absorbance values were read at 505 nm using a spectrophotometer (Perkin Elmer Lambda25). To examine the lipid oxidation, the yolks were first stored in the refrigerator for 3 d and, then, the lipid peroxidation was estimated by determining the MDA via the spectrophotometer (Botsoglou et al., 1994). In brief, 1 g of each sample was transferred into a 15-mL centrifuge tube, followed by adding 4 mL aqueous trichloro acetic acid (**TCA**, 5%), and 2.5 mL butylated hydroxy toluene (**BHT**) (0.8%) in hexane, respectively. The content of the tube was Ultra-Turraxed for 30 s at high speed and centrifuged for 3 min at 3,000 *g*, and the top hexane layer was discarded. The bottom aqueous layer was filtered (Whatman filter paper), and then relocated to a volume of 5 mL using TCA. Then, aqueous thiobarbituric acid (3 mL, 0.8%) (**TBA**) was added to the content. This mixture was heated in a water bath at 70°C for 30 min and, after cooling, the samples were measured for absorbance at 521 nm.

Statistical Analysis

Prior to the analysis of variance, Shapiro-Wilk and Levene tests were used for normality in the residues and homogeneity of variances, respectively. Data were analyzed in a 2 × 3 factorial arrangement using the GLM procedure of SAS (version 9.4) to determine the main effects of L-carnitine, omega-3 fatty acids, and all two-way interactions. Each cage was an experimental unit. Four replicates were used per treatment. Productive performance was measured at different times by repeated data methods, with mixed procedures which involved Tukey's test and a comparison of mean values. In case of no interaction, the factors were analyzed separately. Tukey's test was used when the main effects or interactions were significant ($P < 0.05$).

RESULTS

Productive Performance

The results on productive performance of the laying hens are shown in Table 4. The effects of interaction between omega-3 fatty acids and L-carnitine were not significant on egg production, daily feed intake, and FCR ($P > 0.05$). A higher level of egg production was observed in hens fed on diets that were enriched with omega-3 fatty acids, compared to those fed on the control diet, but their daily feed intake was lower ($P < 0.05$). Average egg weight decreased in response to diets with omega-3 fatty acids, without L-carnitine, or with 100 mg/kg L-carnitine, compared to control diets with 0 mg/kg L-carnitine ($P < 0.05$). Egg mass increased in response to omega-3 fatty acids and 200 mg/kg L-carnitine, compared to the control diet with 0 or 100 mg/kg L-carnitine ($P < 0.05$). Egg shape index increased in birds that were fed with an enriched diet of omega-3 fatty acids and 200 mg/kg L-carnitine, compared to diets enriched with a combination of omega-3 fatty acids and with 0 mg/kg L-carnitine or 100 mg/kg L-carnitine ($P < 0.05$).

MDA and Cholesterol Values in the Yolk

The results demonstrated positive interactions between L-carnitine and omega-3 fatty acids on lipid oxidation and cholesterol content in the yolk of eggs that were stored for 3 d (Table 5). Lower levels of MDA were observed in the egg yolk of birds that received diets with omega-3 fatty acids and 200 mg/kg L-carnitine, and also in those that received the control diet, with 0 mg/kg L-carnitine, compared to diets that were enriched with omega-3 fatty acids and 100 mg/kg L-carnitine ($P < 0.05$). There were no effects of interaction between L-carnitine and omega-3 fatty acids on the cholesterol content of the yolk (mg/g of yolk and mg/yolk; $P \geq 0.05$).

Table 4. Effect of different levels of L-carnitine and Omega-3 fatty acids supplementation on production performance in laying hens.

Item		Egg production (%)[1]	Mean egg weight (g)	Egg mass (g/hen/d)[2]	Daily feed intake[3] (g/hen/day)	Feed conversion ratio[4]	Egg shape index[5]
Omega-3 fatty acids (%)	L-carnitine (mg/kg)						
Control	0	94.2	62.8[a]	59.3[a]	110.9	1.76	75.2[ab]
Control	100	95.3	61.9[ab]	59.0[a]	110.2	1.78	75.7[ab]
Control	200	95.1	61.5[ab]	58.5[b]	110.4	1.79	75.7[ab]
Enriched	0	96.2	60.5[b]	58.2[b]	108.8	1.80	74.8[b]
Enriched	100	96.5	61.0[b]	58.9[b]	109.9	1.80	74.8[b]
Enriched	200	96.4	61.5[ab]	59.3[a]	109.5	1.78	77.3[a]
SEM[6]		0.36	0.22	0.29	0.57	0.02	0.40
Main effect							
Omega-3 fatty acids (%)							
Control		94.9[b]	62.1[a]	58.9	110.5[a]	1.78	75.5
Enriched		96.4[a]	61.1[b]	58.8	109.4[b]	1.79	75.6
SEM		0.21	0.13	0.17	0.33	0.01	0.23
L-carnitine levels (mg/kg)							
0		95.2	61.7	58.7	109.9	1.78	74.9[b]
100		95.9	61.4	58.9	110.0	1.79	75.2[ab]
200		95.7	61.5	58.9	109.9	1.79	76.5[a]
SEM		0.25	0.18	0.21	0.40	0.01	0.28
P-value							
Omega-3 fatty acids (%)		<0.0001	<0.0001	0.72	0.01	0.44	0.68
L-carnitine levels (mg/kg)		0.11	0.53	0.74	0.94	0.89	0.0004
Omega-3 fatty acids × L-carnitine		0.47	<0.0001	0.006	0.22	0.42	0.003
Time		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a–b]Means with different superscripts within a column are different at $P < 0.05$.
[1]Egg production = (100 × number of eggs laid) / (number of hens × days).
[2]Egg mass = (egg production × mean egg weight) / 100.
[3]DFI=The amount of feed given at the beginning of the period (g) − The amount of feed left at the end of the period (g) / number of hen days.
[4]FCR = (kg feed / 1 kg eggs).
[5]Egg Shape index = [Egg width (mm) / Egg height (mm)] × 100.
[6]SEM = Standard error of means.

Yolk Fatty Acids Profiles

L-carnitine and dietary omega-3 fatty acids had variable effects on the fatty acid profiles of egg yolk (Tables 6 and 7). The interaction between L-carnitine and omega-3 fatty acids had no significant effect on the amount of C18:0, C18:1, C18:2, C20:1, C20:4, C22:5, total unsaturated fatty acids, the ratio of UFA/SFA, total omega-3 fatty acids, total omega-6 fatty acids, other fatty acids, and the ratio of total omega-6: total omega-3 fatty acids ($P \geq 0.05$). Adding 100 mg/kg L-carnitine to the control diets significantly increased the C16:0, compared to the control and to the diet enriched with omega-3 fatty acids and 200 mg/kg L-carnitine (P

Table 5. Effect of different levels of L-carnitine and Omega-3 fatty acids supplementation on MDA and cholesterol in laying hens.

Item		Yolk cholesterol (mg/g of yolk)	Yolk cholesterol (mg/yolk)	Malondialdehyde (μg/yolk)
Omega-3 fatty acids (%)	L-carnitine (mg/kg)			
Control	0	16.7	156	0.48[c]
Control	100	13.3	187	0.85[b]
Control	200	14.3	178	0.52[b,c]
Enriched	0	13.2	241	1.25[ab]
Enriched	100	13.1	155	1.31[a]
Enriched	200	13.4	163	0.41[c]
SEM[d]		2.32	24.55	0.14
Main effect				
Omega-3 fatty acids (%)				
Control		15.8	187	0.59[b]
Enriched		13.4	173	1.02[a]
SEM		1.34	14.17	0.08
L-carnitine (mg/kg)				
0		14.9	198	0.83[ab]
100		13.8	172	1.08[a]
200		15.4	170	0.50[b]
SEM		1.64	17.36	0.09
P-value				
Omega-3 fatty acids (%)		0.28	0.51	0.001
L-carnitine (mg/kg)		0.79	0.47	0.002
Omega-3 fatty acids × L-carnitine		0.49	0.09	0.01

[a–c]Means with different superscripts within a column are different at $P < 0.05$.
[d]SEM = Standard error of means.

Table 6. Effect of different levels of L-carnitine and Omega-3 fatty acids supplementation on fatty acids[2] content in yolk of laying hens.

Item		C16:0	C18:0	C18:1 (n-9)	C18:2 (n-6)	C18:3 (n-3)	C20:1	C20:4 (n-6) ARA	C20:5 (n-3) EPA	C22:4 (n-6) *DTA*	C22:5 (n-3) DPA	C22:6 (n-3) DHA
Omega-3 fatty acids (%)	L-carnitine (mg/kg)											
Control	0	28.2^{bc}	9.4	41.9	13.5	0.33^{c}	0.14	1.8	0.00^{c}	0.31^{ab}	0.00	0.64^{c}
Control	100	37.3^{a}	9.2	43.1	12.8	0.34^{c}	0.15	1.7	0.00^{c}	0.29^{b}	0.00	0.63^{c}
Control	200	30.3^{b}	9.5	44.5	14.8	0.36^{b}	0.13	1.9	0.00^{c}	0.34^{a}	0.00	0.70^{bc}
Enriched	0	27.8^{bc}	8.8	39.3	15.5	0.69^{a}	0.23	1.3	0.03^{bc}	0.05^{c}	0.10	1.57^{b}
Enriched	100	28.4^{bc}	9.2	39.9	14.4	0.50^{ab}	0.22	1.3	0.05^{b}	0.06^{bc}	0.08	1.78^{a}
Enriched	200	26.7^{c}	8.7	40.1	15.9	0.59^{ab}	0.24	1.4	0.09^{a}	0.04^{c}	0.09	1.86^{a}
SEM[1]		0.44	0.18	0.46	0.68	0.03	0.02	0.05	0.007	0.007	0.008	0.04
Main effect												
Omega-3 fatty acids (%)												
Control		28.6^{a}	9.3^{a}	43.2^{a}	13.7^{b}	0.34^{b}	0.14^{b}	1.8^{a}	0.00^{b}	0.31^{a}	0.00^{b}	0.65^{b}
Enriched		27.6^{b}	8.9^{b}	39.8^{b}	15.3^{a}	0.59^{a}	0.23^{a}	1.3^{b}	0.05^{a}	0.05^{b}	0.09^{a}	1.73^{a}
SEM		0.26	0.10	0.26	0.39	0.02	0.01	0.03	0.004	0.004	0.004	0.02
L-carnitine (mg/kg)												
0		28.0	9.0	40.6^{b}	14.5	0.51^{a}	0.19	1.5^{b}	0.01^{b}	0.18^{ab}	0.05	1.10^{b}
100		27.9	9.2	41.5^{ab}	13.6	0.42^{b}	0.18	1.5^{b}	0.03^{ab}	0.17^{b}	0.04	1.20^{ab}
200		28.5	9.1	42.3^{a}	15.4	0.48^{ab}	0.19	1.7^{a}	0.04^{a}	0.19^{a}	0.05	1.28^{a}
SEM		0.31	0.13	0.32	0.48	0.02	0.02	0.04	0.005	0.005	0.005	0.03
P-value												
Omega-3 fatty acids (%)		0.02	0.02	<0.0001	0.02	<0.0001	0.0006	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
L-carnitine (mg/kg)		0.40	0.51	0.01	0.07	0.03	0.95	0.02	0.005	0.02	0.47	0.002
Omega-3 fatty acids × L-carnitine		0.0007	0.15	0.19	0.84	0.02	0.72	0.48	0.005	0.003	0.47	0.02

[a-c]Means with different superscripts within a column are different at $P < 0.05$.
[1]SEM = Standard error of means.
[2]C16:0 (palmic acid), C18:0 (Stearic acid), C18:1n9 (oleic acid), C18:2n6 (linoleic acid), C18:3n3 (linolenic acid), C20:4n6 (arachidonic acid), C20:5n3 (eicosapentaenoic acid), C22: n-6 (docosatetraenoic acid), C22:5n3 (docosapentaenoic acid) and C22:6n3 (docosahexaenoic acid);

< 0.05). The level of linolenic acid (C18: 3n3) increased in the egg yolk in response to omega-3 fatty acids with 0 mg/kg L-carnitine, compared to the control diet with 0, 100, and 200 mg/kg L-carnitine ($P < 0.05$). EPA increased in birds which received diets of omega-3 fatty acids along with 200 mg/kg L-carnitine, compared to diets enriched with omega-3 fatty acids and 100 mg/kg L-carnitine, and also compared to the control diet with 0, 100, and 200 mg/kg L-carnitine ($P < 0.05$). On the other hand, docosatetraenoic acid (DTA) was affected significantly by the control diet containing 200 mg/kg L-carnitine, compared to birds fed on the control, with

Table 7. Effect of different levels of L-carnitine and Omega-3 fatty acids supplementation on fatty acids content in yolk of laying hens.

Item		Σ SFA	Σ UFA	UFA/SFA	Σ n-3	Σ n-6	Other FA	Σ n-6/Σ n-3
Omega-3 fatty acids (%)	L-carnitine (mg/kg)							
Control	0	37.7^{b}	63.9	1.6	0.9	15.6	7.6	16.2
Control	100	37.7^{b}	62.3	1.6	0.9	14.8	8.6	15.4
Control	200	40.1^{a}	66.2	1.6	1.1	17.1	1.4	16.2
Enriched	0	37.8^{b}	62.2	1.6	2.4	16.8	9.2	7.0
Enriched	100	38.3^{ab}	61.7	1.6	2.4	15.8	8.2	6.6
Enriched	200	36.3^{b}	61.7	1.7	2.6	17.4	8.4	6.6
SEM[1]		0.45	1.22	0.03	0.07	0.73	1.50	0.39
Main effect								
Omega-3 fatty acids (%)								
Control		38.5^{a}	63.5	1.6	0.9^{b}	15.8	5.9^{b}	15.9^{a}
Enriched		37.5^{b}	62.5	1.6	2.5^{a}	16.7	8.6^{a}	6.7^{b}
SEM		0.26	0.66	0.02	0.04	0.42	0.87	0.23
L-carnitine (mg/kg)								
0		37.8	62.1	1.6	1.8^{b}	16.2	8.4	11.6
100		37.9	62.0	1.6	1.9^{ab}	15.3	8.4	10.9
200		38.2	64.9	1.1	1.8^{a}	17.2	4.9	11.4
SEM		0.32	0.85	0.02	0.05	0.51	1.06	0.28
P-value								
Omega-3 fatty acids (%)		0.02	0.36	0.34	<0.0001	0.17	0.05	<0.0001
L-carnitine (mg/kg)		0.63	0.05	0.07	0.05	0.06	0.06	0.31
Omega-3 fatty acids × L-carnitine		0.0008	0.52	0.07	0.54	0.83	0.07	0.64

Abbreviations: SFA, saturated fatty acid; UFA, unsaturated fatty acid.
[a–b]Means with different superscripts within a column are different at $P < 0.05$. Σ SFA= The sum of C14:0 (myristic acid), C16:0 (palmic acid), C18:0 (Stearic acid), C20:0 (Arachidic acid), and C22:0 (behenic acid); Σ UFA= The sum of C16:1 (palmitoleic acid), C18:1n9 (oleic acid), C18:2n6 (linoleic acid), C18:3n3 (linolenic), C20:4n6 (arachidonic acid), C20:5n3 (eicosapentaenoic acid), C22: n-6 (docosatetraenoic acid), C22:5n3 (docosapentaenoic acid) and C22:6n3 (docosahexaenoic acid); Σ n-3 = The sum of C18:3n3, C20:5n3, C22:5n3 and C22:6n3 fatty acids; Σ n-6 = The sum of C18:2n6, C20:4n6 and C22: n-6 fatty acids; Other fatty acids = 100- (total fatty acids).
[1]Standard error of means

100 mg/kg L-carnitine, and also compared to the effect of diets with omega-3 fatty acids with 0 or 200 mg/kg L-carnitine ($P < 0.05$). A higher level of DHA was observed in the egg yolk of birds that received diets enriched with omega-3 fatty acids and 100 or 200 mg/kg L-carnitine, compared to diets enriched with omega-3 fatty acids and 0 mg/kg L-carnitine, or compared to control diets containing 0 or 100 mg/kg L-carnitine ($P < 0.05$). The total saturated fatty acids increased in response to the control diet containing 200 mg/kg L-carnitine, compared to the other diets, except those that were enriched with omega-3 fatty acids and 100 mg/kg L-carnitine ($P < 0.05$). Regarding the main effects on C18:2 (n-6), C20:1, DPA C22:5 (n-3), Σ n-3, other FAs increased in response to omega-3 fatty acids, whereas a decrease occurred in the amounts of C18:0, C18:1 (n-9), C20:4 (n-6), total n-6/total n-3, compared to the values caused by the control diet ($P < 0.05$). The values of C18:1 (n-9), C20:4 (n-6), and ARA, Σ n-3 increased in response to 200 mg/kg L-carnitine, compared to the effect of the control diet ($P < 0.05$).

DISCUSSION

By common knowledge, Omega-3 fatty acids play a crucial role in reducing cardiovascular disease. A previous report indicated the positive effects of omega-3 fatty acids on the physiological activities of birds, for example, egg laying, growth rate, immunity, skeletal system, and reproduction (Alagawany et al., 2019). L-Carnitine has a variety of effects, including the increase in growth, improvement in feed efficiency, more hatching and the occurrence of fewer metabolic diseases (Chen et al., 2020). In this experiment, the diets that contained 100 and 200 mg/kg L-Carnitine improved the overall performance. The increase in egg production can be explained by the fact that L-carnitine and omega-3 fatty acids in the diet caused the chickens to show their highest genetic potential. Increasing the level of omega-3 fatty acids in the diets is likely to provide more energy and, thus, less feed intake. This was associated with more egg laying (%) and an efficient conversion factor, although it had a suppressive effect on egg weight or egg mass. An enhanced level of egg production can be attributed to the role of omega-3 fatty acids in reproductive activities. In this regard, the positive effect of omega-3 fatty acids in birds was reported previously, regarding many physiological activities such as egg production and fertility (Bozkurt et al., 2008). Further comparisons can be made with reports of common sources such as those that include the use of fish oil or flax. While PGF2α interacts with growth factors, egg weight and function of reproductive organs, the values of these factors tend to decrease by the action of omega-3 fatty acids, parallel to the depletion of estrogen (Gonzalez-Esquerra and Leeson, 2000). In a relevant research, a higher level of egg production was reported in chickens that were fed on fish oil, compared to the control (Basmacioglu et al., 2003). In another research, different fat supplements (1.5%) were incorporated into a diet of corn and soybean, which improved egg production, fertility, egg weight, and chick weight, while having no adverse effect on the bird's body weight or egg characteristics (Bozkurt et al., 2008).

The presence of L-carnitine in diets has reportedly resulted in larger egg yolk and more energy for the fetus to grow (Thiemel and Jelinek, 2004). In an experiment, researchers investigated the effects of different levels of L-carnitine on productive performance when W-36 breeder-laying hens were 90 days old. Their results showed that L-carnitine supplementation (100 and 150 mg/kg) increased egg production and egg mass significantly, while also improving egg shape index (Kazemi-Fard et al., 2015). Furthermore, Pignatelli et al. (2003) reported that L-carnitine mediated the NADPH oxidase system by interfering with the arachidonic acid linkage between phospholipids and protein kinase-c. In fact, Omega-3 fatty acids and L-carnitine are known to have compounds that can help maintain digestive health by regulating gut microbial balance and by increasing the secretion of digestive enzymes from relevant organs like the pancreas (Suresh and Srinivasan, 2007). In improving intestinal health and increasing the secretion of digestive enzymes, the uptake and accumulation of nutrients can be improved, more proteins and amino acids can enter egg tissues, especially the egg white, to improve egg quality (Aydin and Dogan, 2010). In addition, the occurrence of oviduct metabolic activity in the magnum and shell gland can increase albumen storage and mineralization of the shell, thereby increasing productive performance (Rouhanipour et al., 2021). Therefore, it seems that the combination of Omega-3 fatty acids and L-carnitine can improve egg laying performance.

In the current study, the diets that contained 100 and 200 mg/kg L-carnitine improved the MDA and cholesterol contents in the yolk. Since unsaturated fatty acids are susceptible to oxidation, there was an increase in MDA content or a decrease in the oxidative stability of egg yolk. This was especially expected in birds that received high levels of Omega-3 fatty acids because of the higher amount of unsaturated fatty acids, especially PUFAs. This increase in MDA can be due to enhanced levels of unsaturated fatty acids in the liver, as well as in the egg yolk, leading to a decrease in their oxidative stability. Eggs that have high levels of omega-3 fatty acids were reportedly more susceptible to oxidation (Kassis et al., 2012). Therefore, increasing the level of unsaturated fatty acids in eggs occurred by having more unsaturated fatty acids in the diet of hens, thereby leading to a greater susceptibility of the eggs to oxidation, as well as an increase in the MDA level of the egg yolk. It is likely that the antioxidant effects of L-carnitine may be attributed to a decrease in oxidative susceptibility of the egg yolk of birds that received L-carnitine. While L-carnitine is known to have antioxidant properties (Adabi et al., 2006), a major metabolic role of L-carnitine is to reduce the availability of lipids for peroxidation by

facilitating the transport of long-chain, short- and medium-chain fatty acids that tend to accumulate because of normal and abnormal metabolism (Kalaiselvi and Panneerselvam, 1998). Thus, the presence of L-carnitine in the diet can enhance beta-oxidation in these fatty acids to produce adenosine triphosphate (**ATP**) as a source of energy to boost energy utilization (Chatzifotis et al., 1995). An increase in L-carnitine in the diet caused an accumulation of L-carnitine in the egg yolk. Therefore, the presence of more L-carnitine in the egg yolk can prevent further oxidation of the fatty acids in the egg (Adabi et al., 2011).

It is likely that a decrease in cholesterol levels may reflect how omega-3 fatty acids tend to affect the inhibition of cholesterol-efficient enzymes in the liver (Shapiro et al., 2011). On the other hand, this decrease may result from a decline in cholesterol levels of the blood, due to the presence of omega-3 fatty acids in the diet, thereby reducing cholesterol transmission to the yolk. Also, since the liver synthesizes cholesterol, a decrease in cholesterol levels could be due to the diminishing effect of L-carnitine and Omega-3 fatty acids on liver hepatocytes, thereby resulting in a decrease of cholesterol levels in the blood (Hargis, 1988). In one experiment, it was reported that the cholesterol content decreased in eggs because of diets that contained omega-3 fatty acids (Lewis et al., 2000). Another study reported that omega-3 fatty acids in the diet had no effect on cholesterol levels in the egg yolk (Schreiner et al., 2004). One study reported that L-carnitine supplementation (100 and 150 mg/kg) reduced the amount of cholesterol in the yolk (Kazemi-Fard et al., 2015). The supplementary presence of L-carnitine and, possibly, Omega-3 fatty acids in the diets of laying hens can stimulate superoxide dismutase activity and increase the activity of this enzyme. This usually leads to fewer free radicals from lipid peroxidation, resulting in a decrease of MDA levels in the egg yolk.

In general, diets that contained 100 and 200 mg/kg L-Carnitine improved the fatty acids profiles of the egg yolk. Some birds, chicken included, have a limited ability to synthesize long-chain omega-3 fatty acids from linolenic acid. Also, they have a weak ability to transfer them to eggs. However, hens are highly able to synthesize saturated fatty acids and, even if their diets are reduced in quantity, they can compensate for the deficiency in this respect (Hargis et al., 1991). Meanwhile, Omega-3 fatty acids are abundant in unsaturated fatty acids and, thus, they cannot affect saturated fatty acids. Palmitoleic acid is an important fatty acid, so much so that supplementing diets with Salomega can change the amount of this fatty acid in the yolk, as observed in the current study, which perhaps is a result of the low variability in Palmitoleic acid in laying hens. Previous studies have reported that adding fish oil and sunflower oil to the diets of laying hens had no effect on the oleic acid content of egg yolk (Baucells et al., 2000; Garcia-Rebollar et al., 2008). In accordance with the results of the current experiment, using fish oil in the diet reduced the amount of arachidonic acid (**ARA**) in egg yolk, compared to the control group (Hargis et al., 1991; Zotte et al., 2015). It is possible that the difference in the amounts of linolenic acid among the different sources could be a reason for this unique observation. Other researchers observed an increase in DHA and EPA levels in egg yolk by adding fish oil to the diets, thereby confirming the results of the current experiment (Gonzalez-Esquerra and Leeson, 2000; Omidi et al., 2015). In one experiment, it was reported that high levels of fish oil in the diet actually reduced the total omega-6 fatty acids in the egg yolk (Zotte et al., 2015). The decrease in total omega-3 fatty acids usually results from a competition in the metabolic pathways that regulate the synthesis of omega-6 and omega-3 fatty acids. Parallel to an increase in the amount of precursors of a family of fatty acids in the diet, there have to be more enzymes for the synthesis of the same family of fatty acids, so much so that a disturbance could occur to the synthesis of fatty acids in another family. As a matter of research, the importance of the ratio of omega-6 to omega-3 fatty acids is more important than their percentage in edible sources (Milinsk et al., 2003). The lower the omega-6/omega-3 ratio, the better it benefits blood vessels and the more it tends to reduce the risk of cardiovascular strokes and sudden death. Consistent with the results of the present experiment, researchers reported that supplementation of fish oil in the diet significantly reduced the ratio of omega-6 to omega-3 fatty acids (Garcia-Rebollar et al., 2008; Omidi et al., 2015). In one study, adding 500 mg/kg L-carnitine to the diet enhanced the beta-oxidation of mitochondrial long-chain fatty acids by facilitating their transmission across the inner mitochondrial membrane (Corduk et al., 2008). Thus, L-carnitine can improve the body's utilization of energy and fatty acids (Mast et al., 2000). The Δ-9-desaturase enzyme is responsible for the conversion of stearic acid (C18: 0) to oleic acid (C18: 1n-9), while L-carnitine in the diet may stimulate the synthesis of Δ-9-desaturase and, thus, make an increase in total unsaturated fatty acid content (Cherian et al., 2007). This explains how L-carnitine has an important role in the mitochondrial oxidation of fatty acids for the purpose of producing energy (Mast et al., 2000). DHA levels in egg yolk can correlate with the direct deposition of DHA from the diet or from the end-result of a de novo synthesis that generate its α-linolenic and EPA precursors (Pappas et al., 2005). Hens are capable of converting some of these C18 precursors into C20−22 multiple unsaturated fatty acids (Surai, 1999). The enrichment of fatty acids can be explained by the fact that DHA can be made directly by the DHA diet and by the synthesis of its precursors (i.e., linolenic acid and eicosapentaenoic acid) which are present in the diet (Leskanich and Noble, 1997). In addition, high levels of linolenic acid (C18: 3n-3) tend to limit the synthesis of arachidonic acid (C20: 4n-6) from C18: 2n-6 because linolenic acid competes with linoleic acid by the Δ-6-desaturase enzyme (Mazalli et al., 2004). Similarly, an increase in omega-6 and omega-3 fatty acids in the diet can reduce MUFA in the egg by inhibiting the activity of Δ-9-desquoase in oleic acid production. Also, increasing the amount of saturated fatty acids may prevent the liver

from causing elongations of C16:0 and C18:0, thereby terminating the accumulation of saturated fatty acids and, thus, preventing the occurrence of changes in the composition of fatty acids in the egg yolk (Watkins et al., 2003). In one study, the inclusion of fish oil in the diets of laying hens ultimately reduced the ratio of omega-6 fatty acids to omega-3 fatty acids in the egg yolk (Cachaldora et al., 2006). Accordingly, the optimal dietary ratio of omega-6 to omega-3 fatty acids in the diet ranged from 3.5 to 10 (Pacetti et al., 2005). It could be probable that L-carnitine reduced the hepatic biosynthesis of precursors in the yolk or altered the translocation of these precursors from the liver to ovarian and oocyte follicles (Ringseis et al., 2018). L-carnitine facilitated the entry of fatty acids into the mitochondria for energy production through beta-oxidation (Durán et al., 2005). It is likely that carnitine can increase the rate of fatty acid transmission by enhancing the activity of carnitine palmitoyl transferase, an enzyme for fatty-acid beta-oxidation in the liver. In turn, this may cause nonesterification in fatty acids and in serum triacylglycerol concentrations (Lien and Horng, 2001). These effects can reduce the rate at which fatty acids enter the egg yolk.

CONCLUSIONS

The results of this study showed that adding L-carnitine to omega-3 fatty acid diets for laying hens not only increased production performance but also boosted the amount of omega-3 fatty acids in the yolk and improved the oxidative stability of lipids in the yolk during storage.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

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