JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0190076

Feeding to Produce n-3 Fatty Acid-enriched Table Eggs

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This study aimed to modify the feed mixtures of laying hens to enrich the consumer eggs with n-3 polyunsaturated fatty acids (PUFA): α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). One hundred and twenty Tetra-SL laying hens used in the study were divided into three groups of 40 laying hens arranged in five repetitions: C, control with 5% soybean oil; E1, 0.5% fish oil + 0.5% microalgae *Schizochytrium limacinum*; and E2, 0.75% fish oil + 0.75% microalgae. The composition of the mixtures was balanced at the level of 17.5% raw protein and 11.81 MJ/kg metabolic energy (ME). Feed and water were provided *ad libitum*, and the experiment lasted for 21 days. In this study, the different physical and chemical properties of eggs, the fatty acid profile and lipid oxidation of fat in egg yolks were analyzed. The results of the study showed that the weight of the egg yolk and that of the shell depended on the feeding treatments ($P=0.014$ and $P<0.001$), and the weight of eggs and basic parts, as well as the thickness of the shell depended on the storage duration ($P<0.001$). The storage time affected the pH of egg yolks and albumen and the reduction in Haugh units and albumen height ($P<0.001$). Significant differences were observed in the content of ALA, DHA, Σn-3 PUFA (mg/100 g) and the n-6/n-3 PUFA ratio between the C and E1/E2 egg groups ($P<0.001$). The results of the study indicate that it is sufficient to use a lower level of fish oil and the microalgae *Schizochytrium limacinum* in hens' feed to achieve a satisfactory increase in n-3 PUFA in eggs, while maintaining optimal values of egg quality and freshness indicators.

Key words: egg quality, fish oil, n-3 PUFA, *Schizochytrium limacinum*, table eggs

J. Poult. Sci., 57: 138-147, 2020

Introduction

Production of designer or functional eggs enriched with n-3 polyunsaturated fatty acids (n-3 PUFAs) has become popular in many countries. Nutrition in Western European countries is inadequate as meals contain higher levels of n-6 fatty acids (n-6 PUFAs) and lower levels of n-3 fatty acids, resulting in nutritional imbalances, which is associated with higher risks for certain diseases. Conventional eggs derived from laying hens fed standard feed mixture contain low levels of α-linolenic acid (ALA, C18:3n-3) and do not contain eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic acids (DHA, C22:6n-3). For balancing antioxidant activity in the body, it is recommended to consume antioxidant-rich food such as eggs enriched with n-3 PUFAs (Surai and Sparks, 2001). Studies have indicated that egg enrichment with nutricines depend on the laying hens' feed mixture composition, as n-3 PUFAs are incorporated into egg yolks (Fraeye *et al*., 2012; Kralik *et al*., 2012; Zotte *et al*., 2015). Poultry has limited storage capacity of n-3 PUFAs owing to their own metabolism (Kralik *et al*., 2015).

The definition of functional food varies, and the one used by experts from the European Commission (FuFoSE, Functional Food Science in Europe), in coordination with the International Life Science Institute (ILSI), is considered the most appropriate: "the product may be considered functional if, together with the basic nutritional effect, it has an additional effect on one or more functions of the human body, which improves the general and physical condition and/or

Received: June 14, 2019, Accepted: August 8, 2019
Released Online Advance Publication: August 25, 2019
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reduces the risk of developing the disease." Functional food should not be in the form of tablets or capsules, but in the normal natural form of food (Siro *et al*., 2008).

According to Commission Regulation (EU) No. 116/2010, food is a source of omega-3 fatty acids if it contains at least 0.3 g ALA per 100 g and 100 kcal of the product and/or at least 40 mg EPA + DHA per 100 g and 100 kcal of the product. Food is a high source of omega-3 fatty acids if it contains at least 0.6 g ALA per 100 g and 100 kcal of the product and/or at least 80 mg EPA + DHA per 100 g and 100 kcal of the product.

Different feed mixtures of plant and animal origin are used in the egg enrichment process with n-3 PUFA. Flax and canola products have been successfully used to increase the ALA content (Ceylan *et al*., 2011; Zotte *et al*., 2015). Oils derived from marine organisms such as algae and fish or their combinations are used to increase the EPA and DHA content (Ao *et al*., 2015; Kaewsutas *et al*., 2016). The purpose of our research was to enrich table eggs with n-3 PUFA using fish oil and the sea algae *Schizochytrium limacinum* (SL). We also investigated the physicochemical properties and oxidative stability of n-3 PUFA-enriched eggs. The aim of this study was to determine which combination of fish oil and the microalgae *Schizochytrium limacinum* should be added to the hens' feed in order to increase the n-3 PUFA content in eggs, so that they could be marketed as an enriched product. Furthermore, the aim of the study was to determine how feeding treatments affect the quality and freshness indicators of table eggs.

Materials and Methods

Laying Hens and Feed

The study was performed on 120 Tetra-SL laying hens, divided into three groups of 40 hens (five repetitions; eight hens per repetition) that were housed in an enriched cage. Feed and water were provided *ad libitum* and the experiment lasted for 21 days. The composition of the feed is shown in Table 1. Three nutrition treatments used were: C, control with 5% soybean oil; E1, 0.5% fish oil + 0.5% microalgae *Schizochytrium limacinum*; and E2, 0.75% fish oil + 0.75% microalgae. The laying hens were in the middle of the laying cycle and were in the 47th week of their life at the beginning of the experiment.

The nutrient content in the mixtures was determined using the following reference methods: HRN ISO 6496: 200; HRN EN ISO 5983-2: 2010; HRN EN ISO 6865: 2001, changed according to the instructions from manual FOSS Fiber Cap; HRN ISO 5984: 2004; HRN ISO 6492: 2001, and modified according to the instructions of the extraction system ANKOM XT15; RU-5.4.2-11 (internal method).

All three laying hen groups were in the same poultry house under controlled microclimatic conditions with a light length of 16 h per day. The eggs were collected manually, and analyses were conducted on fresh eggs. The eggs were stored for 28 days at 4℃ in the refrigerator to monitor changes in the physico-chemical properties and lipid peroxidation (thiobarbituric acid reactive substances or TBARS values).

Fatty Acid Analysis in Feed Components, Feed, and Eggs

The fatty acid profile in feed components, feed, and egg yolk was analyzed as follows: the fat of the homogenized samples was extracted using the method of Folch *et al*. (1957). All solvents used were of ultrapure-grade from Sigma-Aldrich (Schnelldorf, Germany). Butylated hydroxytoluene (100 mg/L) was added to the extraction mixture (chloroform: methanol, 2:1 v/v) as an antioxidant. Subsequently, fatty acid-containing lipids were transmethylated per the base-catalyzed sodium methoxide method of Christie (1982). Gas liquid chromatography was performed on a Bruker 430-GC apparatus (Bruker, Billerica, MA, USA), equipped with a FAMEWAX (RESTEK, Bellefonte, PA, USA) type capillary column (30 m×0.32 mm internal diameter, 0.25 μm film) and flame ionization detector. The characteristic operating conditions were as follows: injector temperature, 220℃; detector temperature, 230℃; helium flow, 25 ml/min. The oven temperature was graded from 50 to 225℃ at 6℃/min and held for 21 min at 225℃. To identify the individual fatty acids in the chromatogram, a fatty acid standard mixture (Supelco 37 Component FAME Mix, SUPELCO® Analytical, Bellefonte, PA, USA) was used. Portions of individual and total fatty acids were shown as percentage of total fatty acids in the lipids of feed components and feed, and in eggs in mg/100 g edible part. Fatty acid analysis was performed for two samples of feed components (oils and microalgae), two samples of feed, and five samples of eggs per group.

Table 2 shows the average fatty acid content in the feed mixtures for laying hens (% of total fatty acids). Table 3 shows the average fatty acid content in soybean and fish oil and in microalgae *Schizochytrium limacinum* (% of total fatty acids).

Physical and Chemical Properties of Eggs

The physical and chemical properties of eggs were determined on L grade eggs. According to the current Croatian Rulebook on Egg Quality (Narodne Novine, 115/06), L grade indicates large eggs weighing 63 to 73 g. For calculating the shape index (SI), the length and width of the eggs were measured using a sliding scale, and the shape index was calculated from these measurements according to the following formula: SI=[(egg width (mm)/egg length (mm)]×100. The weight of the eggs and their basic parts were measured using an electronic scale BBK 422-6 DXS, (Mettler Toledo, Greifensee, Switzerland). The shell strength was measured using the automatic device Eggshell Force Gauge Model-II (Robotmation Co., Tokyo, Japan), and the values were expressed in kg/cm^2. The shell thickness was measured in the middle of the egg using an electronic micrometer and the average value was used. The pH values of albumen and yolk were measured using a digital pH meter model Seven Easy (Mettler Toledo, Greifensee, Switzerland). The albumen height and Haugh unit (HU) values, as well as the yolk color, were measured using the device Egg Multi-Tester EMT-5200 (Robotmation Co.). All measurements were made on fresh eggs and eggs stored for 28 days at 4℃. Sample size was 25 eggs per group in each term of measurement,

Table 1. **Composition of the diet**

Ingredients (g/kg)	Experimental groups		
	C	E1	E2
Maize	491.9	491.9	491.9
Alfalfa	15.0	15.0	15.0
Roasted soybean	33.3	33.3	33.3
Soybean meal	210.0	210.0	210.0
Sunflower meal	50.0	50.0	50.0
Yeast	5.0	5.0	5.0
Salt	3.3	3.3	3.3
Limestone	106.8	106.8	106.8
Mono-calcium phosphate	13.3	13.3	13.3
Methionine	1.5	1.5	1.5
[1]Sal-CURB™	3.3	3.3	3.3
[2]Nanofeed-zeolite	3.3	3.3	3.3
[3]Premix	13.3	13.3	13.3
Soybean oil (SO)	50.0	40.0	35.0
Fish oil (FO)	—	5.0	7.5
Microalgae (SL)	—	5.0	7.5
	1000.00	1000.00	1000.00
[4]Chemical analysis of mixtures for laying hens (g/kg)			
Moisture	82	82	86
Ash	193	144	134
Crude protein	176.1	176.7	170.1
Fat	77	84	78
Crude fibers	34	31	31
Energy value of mixtures			
ME MJ/kg	11.81	11.81	11.77

[1] Sal-CURB™ feed additive used to control contamination of the feed mixture with *Salmonella* (Kemin Industries. Des Moines. IA USA)

[2] Nanofeed-zeolite is a natural mineral functional additive for animal feed, which is added to 0.2−0.4% concentration.

[3] Premix: calcium 33%, vit. A 833,340 I.U., vit. D_3 208,340 I.U., vit. E 8,350 mg, vit. K_3 170 mg, vit. B_1 150 mg, vit. B_2 375 mg, pantothenic acid 590 mg, niacin 2,100 mg, choline chloride 33,340 mg. vit. B_6 200 mg, vit. B_{12} 960 mcg, biotin 7,100 mcg, folic acid 70.5 mg, vit. C 1,900 mg, iron 2,500 mg, copper 415 mg, zinc 5,200 mg, manganese 5,835 mg, iodine 75 mg, selenium-yeast 35 mg, antioxidant (Apo-ester 85 mg, canthaxanthin 250 mg).

[4] Reference methods applied for chemical analysis of feed: HRN ISO 6496: 200; HRN EN ISO 5983-2: 2010; HRN EN ISO 6865: 2001. Changed according to the instructions from manual FOSS Fiber Cap; HRN ISO 5984: 2004; HRN ISO 6492: 2001. Modified according to the instructions of the extraction system ANKOM XT15; RU-5.4.2-11 (internal method)

totaling to 150 analyzed eggs.

Oxidation of lipids in the yolks of fresh and stored eggs (28 days at 4℃) was determined using the TBARS value (μg malondialdehyde (MDA)/g egg yolk). Samples were prepared as follows: 10% trichloroacetic acid was added to the weighed egg yolk, and the mixture was homogenized and centrifuged at 5,500 rpm, 4℃. After centrifugation, a solution of thiobarbituric acid (pH 2.5) was added to the supernatant, following which the tubes were closed and immersed in water bath at 95℃ for 30 min. After cooling, distilled water was added and the mixture was centrifuged at 5,500 rpm, 4℃. The content of the colored product formed by the reaction of lipid peroxidation products with thiobarbituric acid was measured spectrophotometrically at 534 nm. The obtained values were compared with the standard curve prepared using standard malondialdehyde tetrabutylammonium salt (Sigma-Aldrich, Switzerland) and expressed in μg MDA/g of egg yolk. In total, 36 eggs were used for determining oxidation (6 per group in each term of measurement (fresh and stored eggs)).

Statistical Analysis

The results were processed using Statistica for Windows v.13.3 (Stat Soft Inc., 2017). The significance of the differences between and within the groups was determined using the GLM procedure of the analysis of variance (ANOVA). The following statistical plan was applied: 3×2

Table 2. **Content of fatty acids in mixtures (% of total fatty acids, $n=2$ per group*)**

Fatty acids	Experimental groups		
	C	E1	E2
Caproic (C6:0)	0.04	0.03	0.20
Caprylic (C8:0)	0.03	0.02	0.18
Myristic (C14:0)	0.10	0.47	0.45
Pentadecanoic (C15:0)	0.03	0.11	0.12
Palmitic (C16:0)	12.39	11.92	11.72
Heptadecanoic (C17:0)	0.10	0.11	0.12
Stearic (C18:0)	6.10	4.07	4.13
Arachidic (C20:0)	0.58	0.47	0.54
Heneicosanoic (C21:0)	0.49	0.34	0.37
Behenic (C22:0)	0.51	0.31	0.36
Lignoceric (C23:0)	—	0.27	0.32
ΣSFA	20.37	18.12	18.51
Myristoleic (C14:1)	0.02	0.01	0.03
Palmitoleic (C16:1)	0.13	0.40	0.26
cis-10-heptadecenoic (C17:1)	0.05	0.08	—
Oleic (C18:1 cis 9)	25.81	31.16	28.00
Elaidic (C18:1 trans 9)	0.92	0.91	1.44
Eicosenoic (C20:1)	0.24	0.87	0.41
ΣMUFA	27.17	33.43	30.14
Linoleic (C18:2 n-6)	47.93	34.85	36.79
Σn-6 PUFA	47.93	34.85	36.79
α-linolenic (C18:3 n-3)	4.15	12.36	13.75
EPA (C20:5 n-3)	0.38	0.43	0.46
DHA (C22:6 n-3)	—	0.81	0.35
Σn-3 PUFA	4.53	13.60	14.56
n-6 PUFA /n-3 PUFA	10.58	2.56	2.53

SFA＝saturated fatty acids; MUFA＝monounsaturated fatty acids; PUFA＝polyunsaturated fatty acids; *the average value of the results of two sample analyses is shown.

treatments; feeding treatments: C, E1, and E2; storage, 1 and 28 days. The calculated F value was compared with the critical theoretical F value at the significance levels $P<0.05$, $P<0.01$, and $P<0.001$. The significance of differences between mean values was determined using the Fisher's least significant difference (LSD) test.

Results

The fatty acid content in the feed mixtures for laying hens is shown in Table 2. As the aim of the study was to increase the content of ALA, EPA, and DHA or Σn-3 PUFA in eggs, E1 and E2 were modified with respect to the control group using fish oil and microalgae. Analysis of the n-3 PUFA profile showed that the experimental mixtures E1 and E2 contained higher levels of ALA (12.36% and 13.75%, respectively), EPA (0.43% and 0.46%, respectively) in relation to control mixture (4.15% ALA and 0.38% EPA), and also DHA (0.31% and 0.35%, respectively), which was not present in the control feed mixture. The n-6/n-3 PUFA ratio in the experimental mixtures E1 (2.56) and E2 (2.53) was significantly lower than that in the control mixture (10.57).

The fatty acid profile of oils and microalgae is shown in Table 3. Soybean oil has a higher content of ΣSFA (saturated fatty acids) and Σn-6 PUFA, and lower content of ΣMUFA (monounsaturated fatty acids) and Σn-3 PUFA than fish oil. The microalgae *S. limacinum* has considerably high proportion of DHA (21.33 %).

Table 4 shows the quality of L class eggs, in terms of relative and absolute indicators such as SI (%), egg weight (g), albumen weight (g), yolk weight (g), and shell weight (g). Feeding treatments and storage time affected the yolk weight and weight of the shell ($P=0.014$ and $P<0.001$), although their interaction was not statistically significant ($P>0.05$). Diffusion between the egg yolk and albumen affects the changes in the weight of the base parts. During the 28 days of egg storage in the refrigerator, the egg weight decreased in the E1 and E2 group compared to that in the control group ($P<0.001$). The storage duration reduced egg shell strength ($P=0.032$), as well as shell thickness ($P<0.001$).

Table 5 shows the egg yolk color and egg freshness indicators. Yolk color depended on feeding treatment, storage

Table 3. **Content of fatty acids in soybean and fish oil and microalgae *Schizochytrium limacinum* (% of total fatty acids; $n=2$ per sample*)**

Fatty acids	Soybean oil	Fish oil	Microalgae *Schizochytrium limacinum*
Lauric (C12:0)	0.00	0.00	0.19
Myristic (C14:0)	0.00	2.15	5.62
Pentadecanoic (C15:0)	0.00	0.00	1.90
Palmitic (C16:0)	10.31	9.40	57.18
Heptadecanoic (C17:0)	0.00	0.00	0.58
Stearic (C18:0)	6.12	2.87	2.24
Heneicosanoic (C21:0)	0.00	1.13	0.00
Arachidic (C20:0)	0.00	0.00	0.37
Behenic (C22:0)	0.00	0.00	0.20
Tricosanoic (C23:0)	0.00	0.00	0.16
Lignoceric (C24:0)	0.00	0.00	0.14
ΣSFA	16.44	15.54	68.58
Palmitoleic (C16:1)	0.00	2.78	0.31
cis-10-heptadecenoic (C17:1)	0.00	0.00	0.05
Oleic (C18:1 cis 9)	26.89	40.25	3.45
Elaidic (C18:1 trans 9)	1.56	3.15	0.34
Eicosenoic (C20:1)	0.00	4.82	0.00
Erucic (C22:1)	0.00	3.19	0.00
Nervonic (C24:1)	0.00	0.00	0.16
ΣMUFA	28.45	54.18	4.31
Linoleic (C18:2 n-6)	49.42	14.41	2.97
Eicosadienoic (C20:2 n-6)	0.00	0.00	0.13
Arachidonic (C20:4 n-6)	0.00	0.00	0.79
Σn-6 PUFA	49.42	14.41	3.89
α-linolenic (C18:3 n-3)	5.69	6.33	1.47
Eicosatrienoic (C:20:3 n-3)	0.00	0.51	0.00
EPA (C20:5 n-3)	0.00	3.81	0.43
DHA (C22:6 n-3)	0.00	5.23	21.33
Σn-3 PUFA	5.69	15.88	23.23
n-6 PUFA /n-3 PUFA	8.69	0.91	0.17

SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids; *the average value of the results of two sample analyses is shown.

duration, and their interactions ($P<0.001$). Yolk color is an important indicator of industrial egg processing. HU also depended on storage duration as well as albumen height ($P<0.001$). The albumen pH was affected by feeding treatments, storage duration, and their interaction ($P<0.001$); the pH of the egg yolk was affected by feeding treatments and storage time ($P<0.001$). Degradation of the yolk and albumen affected the pH, which was affected by feeding treatments ($P<0.001$) and egg storage time in the refrigerator ($P<0.01$). Statistical analysis showed that HU depended on the time of egg storage in the refrigerator ($P<0.001$).

Table 6 shows the levels of SFA, MUFA, n-6 PUFA, n-3 PUFA (mg/100 g of egg), and n-6/n-3 PUFA in the eggs of control and experimental groups. Statistically significant differences ($P<0.05$) were observed in the content of saturated fatty acids such as pentadecanoic (C15:0, $P=0.008$), heneicosanoic (C21:0, $P=0.002$), and ΣSFA ($P=0.006$). High statistically significant differences ($P<0.001$) were observed in the content of stearic (C18:0) fatty acid, oleic acid (C18:1 cis 9) and ΣMUFA content between the control and experimental groups. Among the n-6 PUFA, statistically significant differences ($P<0.01$) were observed for linoleic fatty acid and Σn-6 PUFA, and highly significant differences ($P<0.001$) were observed for γ-linolenic (C18:3 n-6) and arachidonic fatty acids. Statistically significant differences ($P<0.001$) were observed in the contents of α-linolenic acid (C18:3 n-3), docosahexaenoic acid (C22:6 n-3), and Σn-3 PUFA. The n-6/n-3 PUFA ratio was significantly lower in E1 and E2 than in the control group (4.23 and 4.72 for E1 and E2, respectively, versus 10.65 for the control; $P<0.001$).

Σn-3 PUFA was higher in the experimental groups E1 and E2 than in the control group by 2.03- and 2.0-fold. ALA was 2.5 and 2.24 times higher, and DHA was 1.69 and 1.55 times higher in the experimental groups than in the control group.

Table 4. **Quality of eggs and egg shell (n=25 per group)**

Factors	Groups	Shape index (%)	Egg weight (g)	Albumen weight (g)	Yolk weight (g)	Egg shell weight (g)	Egg shell strength (kg/cm^2)	Egg shell thickness (mm)
Feeding treatment (FT)	C	76.54	68.85	41.12	16.71^{b}	9.01^{a}	3.09	0.442
	E1	75.83	66.90	40.96	16.88^{b}	9.00^{a}	2.90	0.414
	E2	76.17	66.85	41.14	17.40^{a}	8.34^{b}	2.85	0.401
Storage time (ST)	Fresh	76.17	68.03^{a}	42.48^{a}	16.63^{b}	8.96^{a}	3.04^{a}	0.440^{a}
	Storage	76.19	65.66^{b}	39.67^{b}	17.36^{a}	8.62^{b}	2.86^{b}	0.385^{b}
Interaction	C×Fresh	76.31	68.49	43.28^{a}	16.15	9.05	3.12	0.431^{ab}
	E1×Fresh	75.94	67.69	41.82^{b}	16.68	9.18	2.98	0.447^{a}
	E2×Fresh	76.33	68.06	42.34^{ab}	17.08	8.64	3.02	0.442^{a}
	C×Storage	76.78	65.22	38.96^{c}	17.28	8.98	3.06	0.413^{b}
	E1×Storage	76.41	66.02	40.10^{c}	17.09	8.83	2.82	0.381^{c}
	E2×Storage	75.32	65.74	39.95^{c}	17.73	8.05	2.69	0.360^{d}
P value								
FT		0.662	0.994	0.922	*0.014*	*<0.001*	0.054	0.083
ST		0.969	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.032*	*<0.001*
Interaction		0.558	0.296	*0.027*	0.329	0.259	0.427	*<0.001*

C, control, 5% soybean oil; E1, 0.5% fish oil + 0.5% microalgae; E2, 0.75% fish oil + 0.75% microalgae

Table 5. **Egg yolk color and indicators of egg freshness (n=25 per group)**

Factors	Groups	Yolk color	HU	Albumen height (mm)	Albumen pH	Yolk pH
Feeding treatment (FT)	C	13.20^{a}	78.92	6.51	8.33^{b}	6.14^{b}
	E1	12.10^{b}	76.63	6.44	8.62^{a}	6.23^{a}
	E2	13.22^{a}	75.21	6.13	8.63^{a}	6.23^{a}
Storage time (ST)	Fresh	12.45^{b}	79.79^{a}	6.80^{a}	8.36^{b}	6.09^{b}
	Storage	13.26^{a}	74.05^{b}	5.92^{b}	8.68^{a}	6.31^{a}
Interaction	C×Fresh	13.20^{bc}	82.21	7.00	8.26^{c}	6.05
	E1×Fresh	13.00^{c}	78.85	8.84	8.42^{b}	6.09
	E2×Fresh	11.16^{d}	78.32	6.56	8.41^{b}	6.12
	C×Storage	13.32^{ab}	75.64	6.01	8.40^{b}	6.24
	E1×Storage	13.44^{a}	74.40	6.05	8.84^{a}	6.36
	E2×Storage	13.04^{c}	72.11	5.70	8.83^{a}	6.34
P value						
FT		*<0.001*	0.071	0.176	*<0.001*	*<0.001*
ST		*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Interaction		*<0.001*	0.782	0.892	*<0.001*	0.067

C, control, 5% soybean oil; E1, 0.5% fish oil + 0.5% microalgae; E2, 0.75% fish oil + 0.75% microalgae

The n-6/n-3 PUFA ratio was reduced from 10.65 in control group to 4.23 and 4.72 in the experimental groups E1 and E2, respectively ($P<0.001$).

Table 7 shows the differences in TBARS values between and within the examined groups of fresh and stored eggs. TBARS values are expressed as μg MDA/g of egg yolk. The TBARS values did not vary significantly between the groups in the analysis of fresh eggs (C=1.453 μg MDA/g; E1=1.455 μg MDA/g, and E2=1.508 μg MDA/g; $P=0.929$). However, a statistically significant difference ($P=0.020$) was observed between the control (1.397 μg MDA/g) and E1 (1.745 μg MDA/g) and E2 (1.829 μg MDA/g) groups of stored eggs. Within the same group, a statistically significant difference in TBARS values was observed in the yolk analysis of the E1 group, where oxidation was more intense after storage (fresh eggs=1.455 μg MDA/g versus stored eggs=1.745 μg MDA/g; $P=0.044$). For groups C and E2, the difference in TBARS values within groups was not recorded ($P>0.05$). Analysis of the results showed that oxidation occurs more rapidly in the eggs of the modified

Table 6. **Content of fatty acids in mg/100 g of edible part of eggs (n=5 per group)**

Fatty acid	[1]Feeding treatments/Statistical parameter ($\bar{x}$)			P value
	C	E1	E2	
Myristic (C14:0)	15.87±1.19	17.41±2.05	16.41±1.99	0.414
Pentadecanoic (C15:0)	2.41±2.20^{ab}	0.00±0.00^{b}	4.97±2.78^{a}	*0.008*
Palmitic (C16:0)	1488.69±26.32	1413.97±55.77	1481.60±70.34	0.092
Heptadecanoic (C17:0)	16.87±0.63	16.68±1.88	19.21±2.88	0.132
Stearic (C18:0)	683.91±36.33^{a}	622.25±15.07^{b}	604.32±23.67^{b}	*<0.001*
Heneicosanoic (C21:0)	14.41±3.35^{a}	11.29±1.23^{b}	12.22±1.51^{b}	*0.002*
ΣSFA	2225.17±48.17^{a}	2081.61±56.07^{b}	2138.73±65.48^{b}	*0.006*
Palmitoleic (C16:1)	86.96±4.64	96.07±8.38	97.87±22.04	0.436
cis-10-heptadecenoic (C17:1)	8.33±0.57	8.15±4.98	6.27±6.12	0.737
Oleic (C18:1 cis 9)	2477.71±112.86^{b}	2767.43±70.66^{a}	2585.46±71.36^{b}	*<0.001*
Elaidic (C18:1 trans 9)	85.55±22.21	106.14±1.81	96.00±19.07	0.199
Cis-11-eicosenoic (C20:1)	13.73±1.57	13.81±0.94	12.79±0.96	0.358
ΣMUFA	2672.31±101.83^{b}	2991.62±77.91^{a}	2798.41±103.97^{b}	*<0.001*
Linoleic (C18:2 n-6)	1624.13±136.04^{a}	1321.01±99.22^{b}	1441.68±107.49^{b}	*0.004*
γ-linolenic (18:3 n-6)	8.49±0.59^{a}	1.79±4.01^{b}	8.23±1.49^{b}	*<0.001*
Eicosadienoic (C20:2 n-6)	12.24±0.23	9.07±5.30	12.72±2.85	0.237
Arachidonic (20:4 n-6)	137.80±5.05^{a}	102.43±8.27^{b}	112.27±16.63^{b}	*<0.001*
Σn-6 PUFA	1782.67±140.09^{b}	1434.30±108.38^{b}	1574.92±125.77^{b}	*0.003*
α-linolenic (C18:3 n-3)	71.73±10.03^{b}	177.33±17.65^{a}	161.00±18.92^{a}	*<0.001*
Eicosapentaenoic (C20:5 n-3)	5.70±0.44	10.37±1.04	7.88±4.54	0.054
Docosahexaenoic (C22:6 n-3)	89.90±4.42^{c}	152.25±12.11^{b}	166.55±9.19^{a}	*<0.001*
Σn-3 PUFA	167.33±10.72^{b}	339.96±25.48^{a}	335.43±18.70^{a}	*<0.001*
n-6/n-3 PUFA	10.65±0.52^{a}	4.23±0.38^{b}	4.72±0.60^{b}	*<0.001*

C, control, 5% soybean oil; E1, 0.5% fish oil + 0.5% microalgae; E2, 0.75% fish oil + 0.75% microalgae

Table 7. **TBARS values (μg MDA/g of egg yolk; n=6 per group)**

Group – feeding treatment (FT)	Fresh eggs TBARS ($\bar{x}$±s$\bar{x}$)	Stored eggs TBARS ($\bar{x}$±s$\bar{x}$)	P value
C	1.453±0.17	1.397±0.2^{b}	0,669
E1	1.455±0.16^{B}	1.745±0.21^{aA}	0,044
E2	1.508±0.37	1.829±0.22^{a}	0,136
P value	0,929	0,020	

C, control, 5% soybean oil; E1, 0.5% fish oil + 0.5% microalgae; E2, 0.75% fish oil + 0.75% microalgae; FT, feeding treatment; ST, storage time

[a, b] letters above numbers represent the difference between the values shown in the columns

[A, B] letters above numbers represent the difference between the values shown in the rows

composition (E1 and E2) than in conventional eggs (C). Thus, we believe that oxidation is faster because of the higher content of unsaturated fatty acids in the eggs of the experimental groups, which are more susceptible to oxidation.

Discussion

In this study, the aim was to determine the increase of n-3 PUFA content in table eggs using specially designed feeding treatments (C=5% soybean oil; E1=0.5% fish oil + 0.5% microalgae *Schizochytrium limacinum* (SL) and E2=0.75% fish oil + 0.75% microalgae). The aim was also to determine the effect of feeding treatments and storage time on egg quality and freshness. The results show that by using a feeding treatment with a lower level of fish oil and the microalgae *Schizochytrium limacinum* resulted in an increase in n-3 PUFA in eggs that meets the prescribed n-3 PUFA content for eggs that can be declared enriched. Furthermore, the results of the study show that eggs from feeding treatments with the addition of the microalgae *Schizochytrium limacinum* have a more intense yolk color, which is a very

important indicator of egg quality for consumers.. Egg quality is influenced by various factors, which can be divided into genetic and non-genetic factors (Shin *et al*., 2012; Wolc *et al*., 2012). Non-genetic factors include feeding, hens' rearing system, and egg storage conditions. The most important storage factors are temperature, storage time, relative air humidity, and the amount of CO_2 in the room where the eggs are stored (Silversides and Scott, 2001, Samli *et al*., 2005; Shin *et al*., 2012). Lee *et al*. (2016) indicated that during storage, certain egg quality indicators gradually decrease (egg weight, shell weight, HU, albumen height and viscosity, and shell thickness) while some increase (pH of yolk and albumen, yolk color, and shell density). In our study, we have also determined the trend in the changes of some of these indicators.

For all the listed indicators, the values changed more rapidly when the storage temperature was higher (Lee *et al*., 2016). Although the SI is not considered an egg quality factor, it can affect the thickness and strength of the shell. Therefore, this indicator is important for egg quality assessment (Ikegwu *et al*., 2016). According to Duman *et al*. (2016), eggshell index can be classified into three classes: sharp eggs (shape index lower than 72), normal or standard eggs (shape index from 72 to 76), and round eggs (shape index higher than 76). These authors observed a negative correlation between the SI and shell thickness and strength for eggs with SI below 72, while a positive correlation was observed for standard eggs. In our study, we used eggs with SI characteristic for standard eggs, which also had optimal values for shell strength and thickness (C＝3.09 kg/cm^2 and 0.442 mm; E1＝2.90 kg/cm^2 and 0.414 mm, and E2＝2.85 kg/cm^2 and 0.401 mm). The shell thickness and strength values in our groups were higher than those of standard eggs mentioned by the aforementioned authors (2.69 kg/cm^2 and 0.349 mm). Kralik *et al*. (2005) observed that addition of the n-3 PUFA-rich oil to the laying hens' feed increased the weight of the egg yolk, as well as the thickness and weight of the shell. Their results are in partial agreement with those of ours, as we have also recorded higher yolk weight in the group of hens that consumed feed supplemented with fish oil. In our study, the shell quality indicators were significantly influenced by storage time (shell strength $P＝0.032$ and shell thickness $P＜0.001$) and the interaction of storage time and feeding treatment (shell thickness $P＜0.001$).

Several studies have reported that storage conditions (storage time and temperature) primarily affect internal egg quality (HU, albumen height, yolk color, and pH of albumen and yolk), whereas in most cases storage conditions do not affect egg shell quality (Silversides and Scott, 2001; Lee *et al*., 2016). However, Samli *et al*. (2005) observed that storage time has no statistically significant effect on shell thickness ($P＝0.467$), while storage temperature combined with storage time significantly affected shell thickness ($P＝0.041$).

In a study on the effect of the level and type of fish oil in the feed for laying hens on the content and profile of fatty acids in yolks, Cachaldora *et al*. (2006) observed that higher levels of fish oil (60 g/kg in the feed for laying hens) reduced the shell thickness. In agreement with the above results, we showed that the use of fish oil in hen feed, and in combination with egg storage time, significantly affected the thickness of the shell in our study ($P＜0.001$). Analysis of the results of this study indicated that the shell strength and thickness in the eggs of the E1 and E2 experimental groups are lower than those of the control group ($P＞0.05$). Studies have shown that the addition of different seeds and oils to hen feed for increasing the omega-3 fatty acid content affects the shell quality (reduced strength and thickness). Phytic acid, found in many grains, soybean, and legumes, can disrupt the absorption of various minerals, including calcium, which is required for the calcification of the shell, thereby reducing its quality. Based on the factors mentioned above, we assumed that the effect of the interaction of feeding treatment and storage time on eggshell thickness in our study is actually related to the nutrition of the hens. Omar *et al*. (2014) did not detect any significant changes in egg weight and yolk share in eggs after using fish oil in the mixtures for laying hens, which is in agreement with the results reported by Ceylan *et al*. (2011). Jin *et al*. (2011) confirmed that egg shell weight and albumen weight decreased during storage ($P＜0.001$), as was the case in our study. Grobas *et al*. (2001) and Gűclű *et al*. (2008) indicated that different oils can affect egg weight. In our study, this property was affected by the duration of egg storage ($P＜0.01$).

Jin *et al*. (2011) observed brighter egg yolks in eggs stored for 28 days compared to those stored for 7 days in the refrigerator ($P＜0.003$). These results are consistent with the observations of Fasiangova and Borilova (2017), as well as those of Kralik *et al*. (2017). Scheideler *et al*. (2010) stated that the pH values of fresh albumen is in the range of 7.6−8.5 and for yolks around 6.0, which increase during egg storage to ＞ 9 in the albumen and up to 6.9 in the yolk. The albumen becomes less dense after the hydrolysis of ovomucin, which affects the height of albumen (indicated by HU) used in the freshness display of eggs. Batkowska *et al*. (2016) suggested that egg storage affects the increase in egg air space, egg weight loss, and increase in the albumen pH value. They showed that the pH of yolks ranged from 6.57 to 6.75 after 28 days of storage in the refrigerator, which is similar to the pH values of 6.09−6.31 observed in this study. Papas *et al*. (2005) and Silversides and Scott (2001) indicated that HU is maximum after egg oviposition and decreases during storage. The results of this study, as well as those of Batkowska *et al*. (2016), are in agreement with the results of the aforementioned authors. According to Rakonjac *et al*. (2014), lysozyme, ovomucoid, and cystatin are biologically active proteins in the albumen and affect the preservation of egg freshness.

Ao *et al*. (2015) used 1%, 2%, and 3% microalgae (All-G-Rich^{TM}, Alltech Inc.) in the mixtures for laying hens. The content of DHA increased linearly with the increase in the proportion of microalgae in the mixture. The authors observed that 248 mg DHA was present in the eggs of the control group, whereas 509 mg, 717 mg, and 776 mg/100 g

DHA was present in the egg yolk of the three experimental groups. Ao *et al*. (2015) concluded that addition of microalgae in the mixture for laying hens can enable the production of DHA-enriched eggs without any negative effect on their quality. Ceylan *et al*. (2011) fed laying hens with mixtures containing 1.5% and 3.0% sunflower oil, fish oil, flaxseed oil, and canola oil. The eggs from hens that were fed sunflower oil had lighter egg yolk color than the other egg yolks. ALA content increased after adding 3% flaxseed oil and canola oil in the mixtures for laying hens, and the addition of fish oil increased the deposition of DHA in the egg yolks. Ceylan *et al*. (2011) consider that the nutritional quality of a product can be evaluated by the ALA:LA and n-6/n-3 PUFA ratios. ALA can be desaturated and elongated to DHA in the liver of laying hens. ALA from flaxseed oil can act as a DHA precursor if the feed does not contain sufficient DHA (Da Silva *et al*., 2009). Kralik *et al*. (2008), Ceylan *et al*. (2011), and Nain *et al*. (2012) concluded that the fatty acid profile may be modified by adding selected feed (oils) rich in n-3 PUFA and determined the following order for decreasing the n-6/n-3 PUFA ratio: flaxseed oil > fish oil > canola oil > sunflower oil. Janječić *et al*. (2018) added 0.5 and 1.0% *S. limacinum* microalgae in the mixture for laying hens (A control, B 0.5% microalgae, and C 1.0% microalgae). The addition of 0.5 and 1.0% microalgae resulted in higher levels of n-3 PUFA in the eggs (B 1.23%, C 1.56%, A 0.82%) and the n-6/n-3 PUFA ratios were 14.89: 22.29 and 11.45: 22.29, respectively. These results are not consistent with the results of our and other studies. Kaewsutas *et al*. (2016) investigated the effect of adding microalgae and fish oil on the DHA content in eggs. Laying hens were fed with the following mixtures: control group, 4% fish oil; 1st experimental group, 1% microalgae; 2nd experimental group, 2% microalgae. Higher DHA content was observed in egg yolks from hens fed 1% and 2% microalgae than the control eggs from hens fed 4% fish oil ($P<0.05$). Feeding with 2% microalgae in the mixture resulted in DHA content of 114 mg/egg, and the n-6/n-3 PUFA ratio was reduced to optimal limits. European food safety experts (International Life Sciences, 2010) believe that consumption of 250 mg/day EPA + DHA is sufficient for the daily requirement. According to EU Directives and the EPA and DHA content, eggs produced in E1 and E2 groups can be considered high source of omega-3 fatty acids.

Compared to the control group, Dunn-Hurrocks *et al*. (2011) and Kralik *et al*. (2012) observed higher lipid oxidation intensity in eggs when laying hens were fed fish oil or canola oil, which was similar to our observations made during egg storage. Gajčević *et al*. (2009) and Mohiti-Asli *et al*. (2008) observed that the addition of antioxidants in food decelerates oxidation and increases the freshness persistence of eggs.

The results of our study on the use of different concentrations (0.5% and 0.75%) of fish oil and microalgae *S. limacinum*, and their comparison to the results obtained after using the soybean oil control, showed that lower concentrations of fish oil and *S. limacinum* are sufficient for enrichment of eggs with n-3 PUFA. We also observed that feeding treatments do not impair the quality of eggs that, according to the indicators, remain within the optimal limits for table eggs. Furthermore, we noticed that the addition of microalgae to the feed for laying hens positively affected yolk color, which might be because of the carotenoids content in microalgae. In future work related to this topic, it is necessary to carry out research regarding the influence of these feeding treatments on the sensory properties of eggs.

Acknowledgments

This study was supported by the European Structural and Investment Funds grant for the Croatian National Scientific Center of Excellence for Personalized Health Care (grant #KK.01.1.1.01.0010) and by the Ministry of Science and Education of the Republic of Croatia.

Conflicts of Interest

The authors declare no conflict of interest.

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