Research Article

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Microalgae *Schizochytrium limacinum* as an alternative to fish oil in enriching table eggs with n-3 polyunsaturated fatty acids

Zlata Kralik,[a,b*] Gordana Kralik,[b,c] Manuela Grčević,[a,b] Danica Hanžek[a,b] and Polonca Margeta[a,b]

Abstract

BACKGROUND: The research deals with the addition of microalgae *Schizochytrium limacinum* as an alternative to fish oil in a feed-mixture for laying-hens and its effect on the deposition of n-3 polyunsaturated fatty acids (n-3-PUFAs) [α-linoleic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] in the lipids of egg yolks. In the study, 240 Tetra SL laying hens housed in enriched cages were used, divided into six groups, each in five repetitions. Groups E1, E3 and E5 were given 0.5%, 1.0% and 1.5% microalgae and groups E2, E4 and E6 were given feed mixtures with 0.5%, 1.0% and 1.5% fish oil. The mixtures were modified on the basis of 17% of the crude protein and 11.7 MJ ME kg^{-1}.

RESULTS: The results showed satisfactory disposal of n-3 PUFA in egg yolks of laying-hens fed mixtures with the addition of either fish oil or microalgae. Eggs of E1, E3 and E5 groups contained in 100 g: 321.07 mg, 361.60 mg and 399.34 mg n-3 PUFA, respectively ($P < 0.001$). Eggs of E2, E4 and E6 groups contained in 100 g: 346.25 mg, 346.17 mg and 369.02 mg n-3 PUFA, respectively ($P < 0.001$). By increasing the content of fish oil or microalgae in feed-mixtures for laying hens, the ratio of n-6/n-3 PUFA in egg yolk lipids ($P < 0.001$) was decreased.

CONCLUSION: Our results justified the usage of the microalgae *Schizochytrium limacinum* in the enrichment of table eggs with n-3 fatty acids as an alternative feed to fish oil.

Keywords: eggs; microalgae; fish oil; n-3 PUFA; DHA

INTRODUCTION

Eggs can be enriched with different nutricines for the benefit of human health. For certain people high concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in blood plasma are associated with decreased appearance of coronary heart disease, inflammation, diabetes and cancer of breast, prostate and colon.[1–4] Romieu *et al.*[5] and Von Schacky[6] pointed out that EPA and DHA belong to bioactive components and contribute to human health, particularly in reducing cardiac diseases as arrhythmia, stroke and high blood pressure. Moreover, a positive effect on depression, rheumatoid arthritis and asthma was noticed.[7,8] Harris *et al.*[9] and Dalle Zotte *et al.*[10] indicate the effective enrichment of eggs with α-linoleic acid (ALA) if laying hens are fed with flaxseed oil or seed. There are no higher EPA and DHA concentrations in the product because the poultry has limited ALA conversion capabilities in EPA and DHA. The addition of fish oil to the feed has an effective impact on enriching eggs with n-3 polyunsaturated fatty acids (n-3-PUFAs), nevertheless, its dosage is limited because of an undesirable odor.[11] Some species of microalgae ferment high concentrations of DHA[12,13] and can be used in poultry feeding.

The purpose of our research was to investigate the alternative action of fish oil and microalgae *Schizochytrium limacinum* in feed for laying hens on the fatty acid profile, with a special reference to the n-3 PUFA content.

MATERIAL AND METHODS

Laying hens and feed

In the study, 240 40-week-old Tetra SL laying hens were used in six experimental groups (E1, E2, E3, E4, E5 and E6). In each experimental group the 40 laying hens (five repetitions; eight hens per replication) were housed in enriched cages. The experimental period lasted 21 days. Hens were given feed and water *ad libitum*. The composition of the feed-mixture was balanced at the level of 17% crude protein and 11.7 MJ ME kg^{-1}. Soybean

* Correspondence to: Zlata Kralik, Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia. E-mail: zlata.kralik@fazos.hr

a Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

b Scientific Center of Excellence for Personalized Health Care, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

c Nutricin j.d.o.o., Darda, Croatia

oil, rapeseed oil and linseed oil as well as fish oil were used as energy sources. In order to significantly influencing the fatty acid profile of egg yolks, we added different levels and sources rich in n-3 PUFA feed-mixtures for laying hens. Feed-mixtures differed by the source of n-3 fatty acids. In experimental groups E1, E3 and E5 microalgae *Schizochytrium limacinum* was used, with commercial name All-G Rich® produced by the Alltech company (Nicholasville, KY, USA), with high concentrations of DHA, while in groups E2, E4 and E6 unrefined fish oil was used. The proportions of these fatty acid sources in feed-mixtures for laying hens were 5 g kg^{-1}, 10 g kg^{-1} and 15 g kg^{-1}. Among other oils in the mixtures was soybean oil whose ratio varied by groups depending on the addition of algae or fish oil, while rapeseed and linseed oil were added to all mixtures in the same proportions (12 g kg^{-1} and 13 g kg^{-1}, respectively) as well as other feeds that were present in the mixtures. The oils used in the research were purchased from the following companies: Nutrifit d.o.o. (Zagreb, Croatia) – linseed oil, Sardina (Postira, Croatia) – fish oil, Čepin Oil Factory d.d. (Čepin, Croatia) – rapeseed oil, and Agrofood d.o.o. (Zagreb, Croatia) – soybean oil. The nutrient content in the mixtures was determined using the Croatian reference methods standards for the following analyses: moisture HRN ISO 6496[14]; ash HRN EN ISO 5984[15]; crude protein HRN ISO 5983-2,[16] fat HRN ISO 6492;[17] crude fiber HRN EN ISO 6865, modified.[18] The composition of the feed mixtures used in the experiment is shown in Table 1.

All groups of hens were housed in cages in the same building, in a controlled climate, a length of light was 16 h a day. Chemical analyses of mixtures, fatty acids profile in microalgae and oils, as well as in feed-mixtures were carried out on two samples.

Table 2 shows the fatty acid profile of microalgae and soybean, rapeseed, flaxseed and fish oil, based on our own research.

Fatty acid profile of the feed for laying hens is shown in Table 3.

Fatty acid analysis

Fatty acids were determined on a total of 60 egg yolks, i.e. on ten yolks per each experimental group. Preparation and analysis of samples was performed according to the method of Csapo *et al.*[19]

Table 1. Composition of diets

	Experimental groups					
Ingredients (g kg^{-1})	E1	E2	E3	E4	E5	E6
Maize	491.9	491.9	491.9	491.9	491.9	491.9
Alfalfa	15.0	15.0	15.0	15.0	15.0	15.0
Roasted soybean	33.3	33.3	33.3	33.3	33.3	33.3
Soybean meal	210.0	210.0	210.0	210.0	210.0	210.0
Sunflower meal	50.0	50.0	50.0	50.0	50.0	50.0
Yeast	5.0	5.0	5.0	5.0	5.0	5.0
Salt	3.3	3.3	3.3	3.3	3.3	3.3
Limestone	106.8	106.8	106.8	106.8	106.8	106.8
Mono-calcium phosphate	13.3	13.3	13.3	13.3	13.3	13.3
Methionine	1.5	1.5	1.5	1.5	1.5	1.5
Sal-CURB™ [a]	3.3	3.3	3.3	3.3	3.3	3.3
Nanofeed-zeolite[b]	3.3	3.3	3.3	3.3	3.3	3.3
Premix[c]	13.3	13.3	13.3	13.3	13.3	13.3
Rapeseed oil	12.0	12.0	12.0	12.0	12.0	12.0
Linseed oil	13.0	13.0	13.0	13.0	13.0	13.0
Soybean oil	20.0	20.0	15.0	15.0	10.0	10.0
Fish oil	–	5.0	–	10.0	–	15.0
Microalgae	5.0	–	10.0	–	15.0	–
Total	1000	1000	1000	1000	1000	1000
Chemical analysis of feed-mixtures for laying hens (g kg^{-1})[d]						
Moisture	82	82	86	87	82	84
Ash	193	144	134	118	154	151
Crude protein	176.1	176.7	170.1	175.2	170.5	173.2
Fat	77	84	78	85	73	82
Crude fibers	34	31	31	33	27	32
Energy value of feed-mixtures						
Megajoules of metabolizable energy per kilogram (MJ ME kg^{-1})	11.77	11.72	11.71	11.81	11.81	11.81

[a] Sal-CURB™ feed additive used to control contamination of the feed mixture with salmonella (Kemin Industries, Des Moines, IA, USA).
[b] Nanofeed-zeolite is a natural mineral functional additive for animal feed which is added in an amount of 0.2 to 0.4% (EUROPA CHEMICA GRUPA d.o.o., Osijek, Croatia).
[c] Premix (Schaumann Agri d.o.o., Koprivnica, Croatia; content per kilogram of diet: calcium, 4.389 g; vitamin A, 11083.42 IU; vitamin D_3, 2770.92 IU; vitamin E, 111.06 mg; vitamin K_3, 2.26 mg; vitamin B_1, 2.00 mg; vitamin B_2, 4.99 mg; panthotenic acid, 7.85 mg; niacin, 27.93 mg; choline chloride, 443.42 mg; vitamin B_6, 2.66 mg; vitamin B_{12}, 12.77 mg; biotin, 94.43 mg; folic acid, 0.94 mg; vitamin C, 25.27 mg; iron, 33.25 mg; copper, 5.52 mg; zinc, 69.16 mg; manganese, 77.61 mg; iodine, 1.00 mg; selenium yeast, 0.47 mg; antioxidant (apo-ester, 1.13 mg; canthaxanthin, 3.33 mg).
[d] Reference methods applied for chemical analysis of feed: moisture HRN ISO 6496;[14] ash HRN EN ISO 5984;[15] crude protein HRN ISO 5983-2;[16] fat HRN ISO 6492;[17] crude fiber HRN EN ISO 6865, modified.[18]

Gas chromatography was performed on a Bruker 430-GC apparatus (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. Characteristic operating conditions were: injector temperature: 220 °C, detector temperature: 230 °C, helium flow: 25 mL min^{-1}. The oven temperature was graded: from 50 to 225 °C: 6.0 °C min^{-1}, 21 min at 225 °C. To identify individual fatty acids in the chromatogram, a fatty acid standard mixture Supelco 37 Component FAME Mix (Supelco Inc., Bellefonte, PA, USA) was used. Portions of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA), as well as n-6 PUFA and n-3 PUFA were shown as a g 100 g^{-1} of total fatty acids in microalgae, oils and feed-mixtures, and in mg 100 g^{-1} in eggs.

The egg yolk color

For the egg yolk color analysis fresh eggs of L-class (egg mass of 63 to 73 g) were taken. The color was analyzed on a total of 150 fresh eggs and 150 eggs stored for 28 days in a refrigerator at 4 °C. The yolk color was determined on a total of 50 eggs per group, of which 25 were fresh and 25 were stored eggs. Yolk color was measured by the automatic device Egg Multi-Tester EMT-5200 (Robotmation Co., Ltd, Tokyo, Japan).

Statistical analysis

The research results were processed with Statistica.[20] In the analysis of results descriptive statistics and analysis of variance (ANOVA) were used, and if the *P* values were statistically significant, the differences between groups were tested by Fisher's least significant difference (LSD) test.

RESULTS

The results of the analysis showed in Table 2 that the microalga composition contained significant concentrations of SFA (68.58%), as well as n-3 PUFA (23.23%) with a high proportion of DHA (21.33%) in total fatty acids. Soybean oil is rich in MUFA and PUFA (28.45% and 49.42%). Rapeseed oil contains 65.55% MUFA, 19.73% n-6 PUFA and 7.48% ALA. Linseed oil contains a significant amount of MUFA and ALA (23.90% and 47.91%). Fish oil is rich in MUFA, ALA and DHA (54.18%, 6.33% and 5.23%). Soybean oil, rapeseed oil and linseed oil do not contain EPA or DHA. Optimal n-6/n-3 PUFA ratio was recorded in the microalga preparation (0.17%), followed by fish oil (0.91%).

The mixtures E1 and E2 (Table 3) contained 13.07% and 12.81% n-3 PUFA, where E1 mixture had a higher content of DHA than the E2 mixture (0.59%: 0.40%). Mixtures for E3 and E4 groups of hens contained 14.61% and 13.22% n-3 PUFA, also with a

Table 2. The fatty acid profile in microalgae and in oils (g 100 g^{-1} of total fatty acids)

Fatty acids	Microalgae	Soybean oil	Rapeseed oil	Linseed oil	Fish oil
Lauric (C12:0)	0.19	0.00	0.00	0.00	0.00
Myristic (C14:0)	5.62	0.00	0.00	0.00	2.15
Pentadecanoic (C15:0)	1.90	0.00	0.00	0.00	0.00
Palmitic (C16:0)	57.18	10.31	4.35	7.20	9.40
Heptadecanoic (C17:0)	0.58	0.00	0.00	0.00	0.00
Stearic (C18:0)	2.24	6.12	1.67	4.08	2.87
Heneicosanoic (C21:0)	0.00	0.00	0.00	0.00	1.13
Arachidic (C20:0)	0.37	0.00	1.23	0.58	0.00
Behenic (C22:0)	0.20	0.00	0.00	0.00	0.00
Tricosanoic (C23:0)	0.16	0.00	0.00	0.00	0.00
Lignoceric (C24:0)	0.14	0.00	0.00	0.00	0.00
ΣSFA	68.58	16.44	7.25	11.87	15.54
Palmitoleic (C16:1)	0.31	0.00	0.22	0.48	2.78
cis-10-Heptadecenoic (C17:1)	0.05	0.00	0.00	0.00	0.00
Oleic (C18:1 cis 9)	3.45	26.89	62.31	22.35	40.25
Elaidic (C18:1 trans 9)	0.34	1.56	3.01	1.07	3.15
Eicosenoic (C20:1)	0.00	0.00	0.00	0.00	4.82
Erucic (C22:1)	0.00	0.00	0.00	0.00	3.19
Nervonic (C24:1)	0.16	0.00	0.00	0.00	0.00
ΣMUFA	4.31	28.45	65.55	23.90	54.18
Linoleic (C18:2 n-6)	2.97	49.42	19.73	16.32	14.41
Eicosadienoic (C20:2 n-6)	0.13	0.00	0.00	0.00	0.00
Arachidonic (C20:4 n-6)	0.79	0.00	0.00	0.00	0.00
Σn-6 PUFA	3.89	49.42	19.73	16.32	14.41
α-Linolenic (C18:3 n-3)	1.47	5.69	7.48	47.91	6.33
Eicosatrienoic (C:20:3 n-3)	0.00	0.00	0.00	0.00	0.51
EPA (C20:5 n-3)	0.43	0.00	0.00	0.00	3.81
DHA (C22:6 n-3)	21.33	0.00	0.00	0.00	5.23
Σn-3 PUFA	23.23	5.69	7.48	47.91	15.88
n-6/n-3 PUFA	0.17	8.69	2.64	0.34	0.91

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. The content of fatty acids in feed-mixtures for laying hens (g 100 g^{-1} of total fatty acids)

	Experimental groups[a]					
Fatty acid	E1	E2	E3	E4	E5	E6
Caproic (C6:0)	0.00	0.026	0.00	0.075	0.00	0.013
Caprylic (C8:0)	0.00	0.025	0.00	0.058	0.00	0.013
Lauric (C12:0)	0.014	0.015	0.017	0.00	0.026	0.018
Myristic (C14:0)	0.239	0.278	0.355	0.300	0.568	0.591
Pentadecanoic (C15:0)	0.081	0.059	0.121	0.054	0.199	0.064
Palmitic (C16:0)	11.39	10.72	11.96	10.70	13.73	10.42
Heptadecanoic (C17:0)	0.096	0.093	0.101	0.101	0.121	0.108
Stearic (C18:0)	4.371	4.235	4.014	3.912	3.717	3.857
Arachidic (C20:0)	0.469	0.466	0.442	0.497	0.431	0.448
Heneicosanoic (C:21:0)	0.235	0.361	0.170	0.331	0.086	0.469
Behenic (C22:0)	0.330	0.323	0.297	0.344	0.276	0.289
Tricosanoic (C23:0)	0.047	0.032	0.043	0.00	0.049	0.00
Lignoceric (C24:0)	0.241	0.221	0.205	0.238	0.186	0.233
ΣSFA	17.52	16.85	17.73	16.61	19.39	16.52
Myristoleic (C14:1)	0.010	0.016	0.00	0.00	0.00	0.00
Palmitoleic (C16:1)	0.137	0.293	0.139	0.332	0.148	0.746
cis-10-Heptadecenoic (C17:1)	0.067	0.073	0.068	0.00	0.069	0.092
Oleic (C18:1 cis 9)	29.06	32.22	28.74	31.70	27.97	32.25
Elaidic (C18:1 trans 9)	0.780	1.532	0.698	1.430	0.507	0.870
Eicosenoic (C20:1)	0.459	0.720	0.447	0.655	0.450	1.317
Erucic (C22:1)	0.00	0.021	0.00	0.00	0.017	0.101
Nervonic (C24:1)	0.00	0.00	0.029	0.078	0.050	0.086
ΣMUFA	30.51	34.87	30.12	34.19	29.21	35.46
Linoleic (C18:2 n-6)	38.89	35.42	37.49	35.91	35.43	33.36
Eicosadienoic (C20:2 n-6)	0.00	0.015	0.012	0.021	0.017	0.047
Dihomo-γ-linolenic (C20:3 n-6)	0.00	0.014	0.00	0.018	0.016	0.014
Arachidonic (C20:4 n-6)	0.00	0.013	0.029	0.028	0.062	0.044
Σn-6 PUFA	38.89	35.46	37.53	35.98	35.52	33.46
α-Linolenic (C18:3 n-3)	12.35	11.96	13.41	12.14	13.80	12.95
Eicosatrienoic (C:20:3 n-3)	0.00	0.094	0.036	0.00	0.022	0.159
EPA (C20:5 n-3)	0.126	0.352	0.097	0.409	0.071	0.707
DHA (C22:6 n-3)	0.595	0.400	1.074	0.665	1.971	0.738
Σn-3 PUFA	13.07	12.81	14.61	13.22	15.86	14.55
ΣUSFA/SFA	4.74	4.93	4.64	5.02	4.15	5.05
n-6/n-3 PUFA	2.97	2.77	2.56	2.72	2.24	2.30

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA, unsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
[a] E_1, 5 g microalgae and 20 g soybean oil kg^{-1} of diet; E_2, 5 g fish oil and 20 g soybean oil kg^{-1} of feed; E_3, 10 g microalgae and 15 g soybean oil kg^{-1} of feed; E_4, 10 g fish oil and 15 g soybean oil; E_5, 15 g microalgae and 10 g soybean oil kg^{-1} of feed; E_6, 15 g fish oil and 10 g soybean oil kg^{-1} of feed.

higher DHA content in a mixture in which microalgae composition was incorporated (1.07% and 0.66%, respectively). The E5 and E6 mixtures contained a higher concentration of n-3 PUFA than the previous ones (15.86% and 14.55%, respectively). The E5 group of hens was fed with a mixture containing 1.97%, and the E6 group of hens with a mixture containing 0.74% of DHA in total fatty acids. EPA concentration was higher in the groups that contained fish oil in the feed (E2, E4 and E6) in comparison to feed containing microalga. The results show that mixtures with microalgae (E1, E3 and E5) contain more n-6 PUFA, ALA, DHA and n-3 PUFA than mixtures containing fish oil (E2, E4 and E6). In feed mixtures with increasing microalga or fish oil concentrations, the n-6/n-3 PUFA ratio ($P < 0.001$) in egg yolks was reduced from 4.63 to 3.58, and from 4.34 to 3.89, respectively.

Table 4 shows the results of fatty acid content in mg 100 g^{-1} of eggs. Statistically significant differences ($P < 0.05$) were determined in the content of SFAs as follows – myristic acid (C18:0), pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). Regarding values of MUFAs, statistically significant differences between the investigated groups were observed in values of *cis*-10-heptadecenoic acid (C17:1), elaidic acid (C18:1 trans 9) and *cis*-11-eicosenoic acid (C20:1). Statistically significant differences ($P < 0.05$) were also observed in the values of arachidonic acid (C20:4 n-6) and eicosadienoic acid (C20:2 n-6). Statistically significant differences between the investigated groups were also observed in the values of DHA and n-3 PUFA, as well as in the ratio of n-6/n-3 PUFA.

The level of the applied algae sources or fish oil influenced significantly the values of myristic (C14:0), heptadecanoic (C17:0) and arachidonic (20:4 n-6) fatty acids ($P < 0.05$). For myristic and heptadecanoic fatty acids, it has been observed that increasing the level of algae or fish oil in feed-mixtures for laying hens from 0.5%

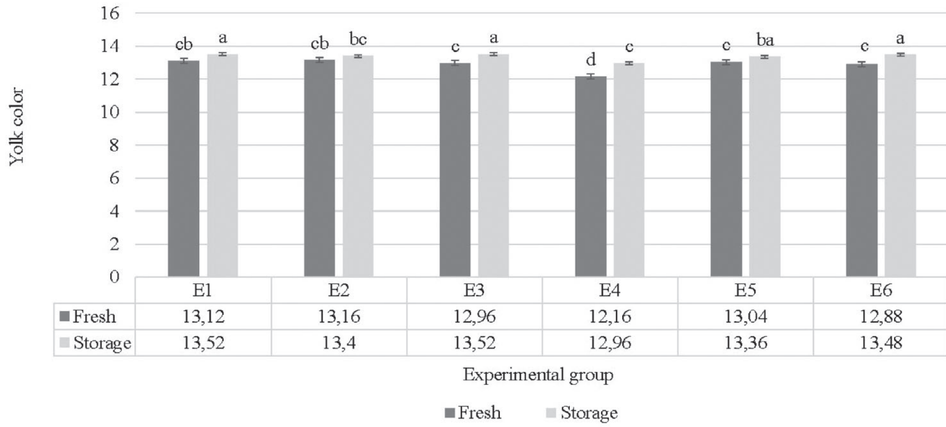

Figure 1. The values of egg yolk color of fresh and stored eggs. E_1, 5 g microalgae and 20 g soybean oil kg^{-1} of diet; E_2, 5 g fish oil and 20 g soybean oil kg^{-1} of feed; E_3, 10 g microalgae and 15 g soybean oil kg^{-1} of feed; E_4, 10 g fish oil and 15 g soybean oil; E_5, 15 g microalgae and 10 g soybean oil kg^{-1} of feed; E_6, 15 g fish oil and 10 g soybean oil kg^{-1} of feed; each group consisted of forty 40-week-old Tetra SL hybrid laying hens which were fed experimental diets for 3 weeks; $n = 25$ eggs per group; eggs were analyzed as fresh (1 day after collection on the farm) and stored (after 28 days of storage in a refrigerator at 4 °C); a,b,c,d values with different superscripts differ significantly at $P < 0.05$.

to 1.5% increases their content in 100 g of eggs ($P = 0.021$ and $P = 0.034$, respectively). Thus, the content of myristic fatty acid in the mixtures with the addition of algae increases from 15.17 mg (E1) to 18.5 mg (E5), and heptadecanoic fatty acid from 16.1 mg (E1) to 17.98 mg (E5). In eggs of laying hens fed diet supplemented with fish oil content of myristic fatty acid increases from 15.86 mg (E2) to 16.99 mg (E4) and 18.9 mg (E6), and heptadecanoic fatty acid from 15.85 mg (E2) to 16.95 mg (E6). In the case of arachidonic fatty acid, it was noticed that with the increase of algae or fish oil in the mixtures its content in 100 g of eggs decreases (E1, 109.45 mg; E3, 108.87 mg; E5, 102.63 mg or E2, 109.58 mg; E4, 109.21 mg; E6, 93.64 mg). The level of algae or fish oil in the mixtures also had a significant effect on the n-3 PUFA content ($P < 0.001$). Lower content of n-3 PUFA was observed in groups with addition of 0.5% algae or fish oil (E1, 321.07 mg; E2, 346.25 mg) compared to groups with added 1% and 1.5% algae or fish oil in mixtures (E3, 361.60 mg; E5, 399.17 mg or E4, 346.17 mg; E6, 369.02 mg). Although rapeseed and flaxseed oil were used as a good source of ALA in hens feed-mixtures, we assume that fish oil and microalgae had a statistically significant effect on total n-3 PUFAs as the proportion of rapeseed and flaxseed oil in the mixtures was constant.

The level of algae or fish oil in the mixtures also influenced the n-6/n-3 PUFA ratio, which was more favorable in groups with higher algae or fish oil content (E5, 3.58; E6, 3.89). The source of fatty acids influenced the content of pentadecanoic (C15:0), heptadecanoic (C17:1), elaidic (C18:1, trans 9), eicosenoic (C20:1, cis 11) and eicosadienoic (C20:6) fatty acids. Eggs originating from laying hens that consumed the mixture with the addition of algae had higher values of these fatty acids than eggs of laying hens fed diet supplemented with fish oil.

The difference in the DHA values was influenced by the source ($P = 0.021$) as well as the content ($P = 0.008$) of n-3 fatty acids in the feed-mixture for laying hens. Thus, significantly better DHA deposition was observed in the group where hens were fed diet with algae compared to mixtures with fish oil. Furthermore, it has been noticed that by increasing the proportion of algae or fish oil the DHA content in 100 g of eggs was increased ($P < 0.001$).

The interaction between the levels of the added ingredients (0.5%, 1% and 1.5%) and the source (microalgae or fish oil) was significant for the content of eicosenoic acid (C20:1, cis 11, $P = 0.013$).

Figure 1 shows the values of egg yolk color of fresh eggs and eggs stored in the refrigerator at 4 °C for 28 days. Yolk color was influenced by feeding treatment and time of yolk color analysis ($P < 0.001$), while the effect of interaction was not significant ($P = 0.067$).

Increased level of the added fish oil to a laying hen feed-mixture affects the reduction of the color intensity of the egg yolk (the egg yolk becomes lighter), while the mixture with the addition of algae causes the decrease of the yellow color but the difference is not significant. The data shows that fresh and stored eggs of laying hens that consumed the mixture with the addition of algae have more intensive yolk color compared to eggs of hens fed mixtures with added fish oil. The results suggest that the algae added to the feed mixture of laying hens as a source of n-3 PUFA with an attempt to increase the content of n-3 PUFA in eggs, is an excellent source of pigment that affects the color of egg yolks.

DISCUSSION

Carrillo *et al.*[21] indicate that the amount of EPA and DHA in eggs depends on the type of algae used. Lemahieu *et al.*[22] indicate that for the enrichment of egg yolks with n-3 PUFA different types of microalgae can be used – some increase EPA or DHA, some increase both fatty acids. Significant is their impact on the color intensity of the egg yolk, which was confirmed also with our research.

In the poultry industry the most used mixtures for laying hens are sunflower, soybean and rapeseed oil,[23,24] which is reflected in an undesirable n-6/n-3 PUFA ratio. Fish oil is rich in essential fatty acids such as EPA and DHA, but a higher concentration of fish oil in feed for laying hens may cause undesirable organoleptic properties of eggs, and for that reason some researchers have used flaxseed and rapeseed oil in combination with fish oil.[23,25–27]

Lešić *et al.*[28] investigated fatty acid composition in chicken eggs on the Croatian market. The study included three producers during 2014 and 2015. Among the PUFAs, the dominant were n-6 PUFAs (linoleic acid 97%). EPA and DHA were not found in egg yolks. The most abundant fatty acids were oleic, palmitic and linoleic. The ratio of n-6/n-3 PUFA, depending on the egg producer, was 43.30, 62.96 and 36.69. Samman *et al.*[29] found that n-6/n-3 PUFA ratio in conventional eggs was 11.03, in n-3 PUFA enriched eggs 2.17

Table 4. Content of fatty acids in mg 100 g^{-1} of eggs (edible part); $n = 10$ per group

Fatty acid	Experimental groups/statistical parameter ($\bar{x}$)[1]							Impact/P value[3]		
	E1	E2	E3	E4	E5	E6	SE[2]M	Level	Source	Interaction
Myristic (C14:0)	15.17^{b}	15.86^{ab}	17.51^{ab}	16.99^{ab}	18.05^{a}	18.09^{a}	0.89	0.025	0.923	0.796
Pentadecanoic (C15:0)	3.67^{a}	0.00^{b}	3.77^{a}	0.00^{b}	5.60^{a}	0.00^{b}	1.00	0.555	<0.001	0.555
Palmitic (C16:0)	1430.23	1362.98	1433.53	1461.08	1474.93	1443.04	36.49	0.212	0.431	0.435
Heptadecanoic (C17:0)	16.01^{b}	15.85^{b}	16.42^{ab}	15.72^{b}	17.98^{a}	16.95^{ab}	0.61	0.034	0.217	0.771
Stearic (C18:0)	625.72	607.71	594.00	597.55	595.58	592.50	13.98	0.217	0.613	0.734
Heneicosanoic (C21:0)	12.00	12.28	12.88	12.26	11.37	9.37	1.19	0.171	0.430	0.637
ΣSFA	2102.08	2014.68	2078.13	2103.62	2123.53	2079.98	35.69	0.461	0.239	0.298
Palmitoleic (C16:1)	95.36	97.32	107.38	112.56	105.10	96.85	7.73	0.221	0.953	0.667
cis-10-Heptadecenoic (C17:1)	10.27^{a}	3.48^{b}	8.12^{ab}	3.73^{b}	9.10^{ab}	8.17^{ab}	2.01	0.407	0.021	0.359
Oleic (C18:1 cis 9)	2729.52	2763.34	2692.21	2667.05	2651.96	2741.34	33.91	0.144	0.249	0.259
Elaidic (C18:1 trans 9)	89.78^{b}	109.20^{a}	107.97^{a}	108.21^{a}	101.69^{ab}	112.35^{a}	4.67	0.156	0.014	0.143
cis-11-Eicosenoic (C20:1)	13.44^{bc}	14.84^{b}	14.86^{b}	14.63^{b}	12.27^{c}	16.49^{a}	0.67	0.744	0.002	*0.013*
ΣMUFA	2938.38	2988.18	2930.54	2906.18	2880.12	2975.20	36.65	0.444	0.191	0.278
Linoleic (C18:2 n-6)	1361.45	1379.49	1353.72	1372.10	1328.02	1320.48	38.96	0.456	0.764	0.929
γ-Linolenic (18:3 n-6)	3.81	2.86	3.45	3.22	2.72	2.54	1.67	0.888	0.744	0.968
Eicosadienoic (C20:2 n-6)	11.22^{bc}	6.42^{c}	11.33^{bc}	11.47^{a}	11.12^{bc}	6.60^{bc}	1.64	0.287	0.041	0.330
Arachidonic (20:4 n-6)	109.45^{a}	109.58^{a}	108.87^{a}	109.21^{a}	102.63^{ab}	93.64^{b}	4.27	0.021	0.423	0.471
n-6 PUFA	1485.93	1498.35	1477.38	1491.42	1444.49	1423.26	40.93	0.320	0.958	0.888
α-Linolenic (C18:3 n-3)	170.59	199.14	191.04	190.63	198.82	207.52	9.70	0.179	0.134	0.329
EPA (C20:5 n-3)	8.92	4.44	7.41	6.26	6.48	9.26	2.31	0.870	0.664	0.309
DHA (C22:6 n-3)	141.56^{b}	142.66^{b}	163.15^{b}	148.91^{b}	194.04^{a}	152.24^{b}	9.07	0.008	0.021	0.076
n-3 PUFA	321.07^{c}	346.25^{bc}	361.60^{b}	346.17^{bc}	399.34^{a}	369.02^{ab}	12.04	<0.001	0.492	0.077
n-6/n-3 PUFA	4.63	4.34^{ab}	4.09^{bc}	4.34^{ab}	3.58^{d}	3.89^{cd}	0.15	<0.001	0.524	0.145

[1] E_1, 5 g microalgae and 20 g soybean oil kg^{-1} of diet; E_2, 5 g fish oil and 20 g soybean oil kg^{-1} of feed; E_3, 10 g microalgae and 15 g soybean oil kg^{-1} of feed; E_4, 10 g fish oil and 15 g soybean oil; E_5, 15 g microalgae and 10 g soybean oil kg^{-1} of feed; E_6, 15 g fish oil and 10 g soybean oil kg^{-1} of feed.
[2] SEM, standard error of arithmetic mean.
[3] Impact: the level of addition to the mixture 5 g kg^{-1}, 10 g kg^{-1} and 15 g kg^{-1}.
Source: microalgae or fish oil; interaction level × source.
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA, unsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
Means in the same rows followed by different lower-case superscript letters differ significantly ($P < 0.05$).

and in organic eggs 11.19. The results of these authors, related to enriched eggs, are close to the results in our work.

Ao *et al.*[30] found that by adding microalgae for 4 weeks at 1%, 2%, and 3%, DHA levels increased linearly from 1.08 mg g^{-1} to 2.58 mg g^{-1} or 4.26 mg g^{-1}, respectively.

Linear increase of DHA in 100 g of eggs was also observed in our study in groups of hens that consumed mixtures with the addition of microalgae (from 141.56 mg to 163.15 mg or 194.04 mg).

Addition of 2% and 3% microalgae significantly increased the red color of the egg yolk. In poultry, algae are also used as a source of minerals and pigments and as a means to reduce cholesterol and may also be a natural source of antioxidants.[31,32] Chadha *et al.*[33] used *Schizochytrium* algae in two experiments. In the first experiment, 0%, 0.5%, 1% and 2% of microalgae were added to the feed-mixture for laying hens. The concentration of DHA in the control was 81 mg 100 g^{-1} of eggs and with the addition of 2% microalgae it was 188 mg 100 g^{-1}. In the second experiment 0%, 1%, 2% and 3% of microalgae were added in feed, and it was found that in experimental groups DHA content was 143 mg 100 g^{-1}, 198 mg 100 g^{-1} and 214 mg 100 g^{-1} of eggs. Our results in DHA content per 100 g of egg of laying hens in the group which received 1% algae in the feed were somewhat lower (163.15 mg) compared to the results of the earlier-mentioned authors. These authors have found an increase in a^* values and reduction in b^* values of the egg yolk color and consider that using microalgae in mixtures can increase DHA in eggs by 2.5 times in comparison to conventional eggs. The control group of eggs contained 248 mg of DHA and the experimental groups 509 mg, 717 mg and 776 mg of DHA per 100 g of egg yolk. The authors have concluded that the All-G-Rich[TH] preparation can be used in laying hens feed to increase DHA content, as well as for high-quality egg production, without adversely affecting oxidative processes during egg storage. Oxidative processes can also adversely affect the color of the egg yolk, which is a very relevant indicator of egg quality, and affects consumer attitudes. Zeller *et al.*[12] stated that microalgae contain carotenoids, particularly canthaxanthin and β-carotenes that affect the color of the egg yolk. Their observations of the effect of algae on the color of the egg yolk are consistent with the results of our research. Kaewsutas *et al.*[34] compared the use of fish oil and microalgae (1% and 2%) in the feeding of laying hens. The concentration of DHA in eggs was greater for hens that were given microalgae in a feed. The addition of 2% microalgae to feed increases DHA above 100 mg per egg and reduces the ratio of n-6/n-3 PUFA to the optimal limits. Lawlor *et al.*[11] were able to increase DHA in egg yolk lipids from 62 to 96 mg per egg and 129 mg per egg by adding microcapsulated fish oil to a feed-mixture for laying hens (2% and 4%). Hadley *et al.*[35] point out that carotenoids in microalgae act as natural antioxidants in the products. Janječić *et al.*[36] investigated the effect of the addition of microalgae *Schizochytrium limacinum* in the laying hens feed on the fatty acid profile in egg yolks. Laying hens were divided into groups: A (control), B (0.5% *Schizochytrium limacinum*) and C (1.0% *Schizochytrium limacinum*). Addition of 0.5% and 1% of microalgae affect the rise of total n-3 fatty acids and DHA. These results are in line with the results of our research and with the results of other authors cited in this article.

When analyzing the results of our research, as well as the research of Baucells *et al.*[37] and Bovet *et al.*,[38] an increase in DHA deposition in egg yolk lipids was found using microalgae as compared to fish oil ($P < 0.001$). Statistically increased concentrations of n-3 PUFA ($P < 0.001$) were found in our study, and n-6/n-3 PUFA ratios were reduced in the egg yolk lipids of laying hens fed with microalgae in comparison to fish oil. Dalle Zotte *et al.*[10] were able to lower the n/6-n-3 PUFA ratio from 11.4 to 2.0 and 2.3 when adding linseed oil and fish oil in the mixture for laying hens as compared to the control.

CONCLUSION

This article explores the use of microalgae *Schizochytrium limacinum* or fish oil in laying hen mixtures for enrichment of eggs with n-3 fatty acids. The results of the analysis show that a more efficient disposal of n-3 PUFA was found in egg yolks of laying hens fed mixtures with microalgae rather than fish oil. Microalgae concentrate increase of 1% and 1.5% in mixtures influenced higher content of DHA in egg yolks in E3 and E5 groups. The content of arachidonic acid in total fatty acids was linearly decreased with increasing DHA in egg yolks. The ratio of n-6/n-3 PUFA depended statistically highly significant ($P < 0.001$) on the level of microalgae, or fish oil. The research has confirmed the justifiability for the use of microalgae as an alternative to fish oil in the process of enriching table eggs with n-3 fatty acids.

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