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Original article

Influence of lycopene and astaxanthin in feed on metabolic parameters of laying hens, yolk color of eggs and their content of carotenoids and vitamin A when stored under refrigerated conditions

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Abstract

Orientating investigations were carried out in order to test the influence of oil extracts of lycopene (20, 40 and 60 mg/kg feed) and astaxanthin (10, 20 and 30 mg/kg feed) as feed additives on the metabolic parameters (glucose, creatinine, cholesterol) and enzyme activities (alanine aminotransferase, ALT; aspartate transaminase, AST) of laying hens. Eggs from these hens were stored at refrigerator temperatures of 4°C and 12°C for up to 30 days and analyzed for vitamin A, carotenoid and yolk color. 45 laying hens (Hy-Line W36 cross, 23 weeks of age) were divided in three groups of 15 birds each (control, lycopene fed group, astaxanthin fed group). Blood samples were taken from the hens and laid eggs were collected on days 31, 61, and 91 of the study. The eggs were stored for 30 days in refrigerators. Both lycopene and astaxanthin increased the content of glucose in serum ($P<0.05$). The content of creatinine and cholesterol, and the activity of ALT, AST and alkaline phosphatase varied dose-dependently. With the exception of cholesterol, metabolite concentrations in the serum of laying hens fed different lycopene and astaxanthin doses did not exceed clinically accepted physiological levels. The carotenoid content and color of the egg yolks from laying hens fed astaxanthin was significantly higher ($P<0.05$) compared to lycopene fed birds. Refrigerator storage of the eggs did not affect carotenoid content and egg yolk color compared to freshly laid eggs. Both feed additives showed a favorable effect on the metabolism of laying hens and the enrichment of egg yolks with carotenoids, astaxanthin significantly more ($P<0.05$) than lycopene.

Key words: feed additives, antioxidants, laying hens, blood biochemical parameters, egg yolk quality

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Table 1. Experiment design with basic data on hen groups, duration of feeding periods, diets and control group.

Groups	Diets		
	(1–30 days)	(31–60 days)	(61–90 days)
Control	BD[1] + 0.33 g/kg of refined sunflower oil	BD[2] + 0.66 g/kg of refined sunflower oil	BD[3] + 1.0 g/kg of refined sunflower oil
Lycopene diet	BD[1] + 20 mg/kg of lycopene (LP20)	BD[2] + 40 mg/kg of lycopene (LP40)	BD[3] + 60 mg/kg of lycopene (LP60)
Astaxanthin diet	BD[1] + 10 mg/kg of astaxanthin (AST10)	BD[2] + 20 mg/kg of astaxanthin (AST20)	BD[3] + 30 mg/kg of astaxanthin (AST30)

Note: BD – basic diet (Table 2), the same superscripts [1, 2, 3] show the same content of refined sunflower oil in the diet

Introduction

Proteins are essential components of human diets. Eggs are an important source of proteins and popular with consumers. They are inexpensive, easy to prepare in many dishes and also extremely nutritious (Miranda et al. 2015). Many consumers find eggs with yellow-orange yolks more preferable (Nys 2000).

The pigmentation of egg yolks is due to carotenoids, mainly xanthophylls, present in such plant feed as annatto and paprika, algae extracts and fungal synthesis products as well as animal and synthetic carotenoids, like canthaxanthin (red pigment) and ethyl ester of beta-apo-8-carotenic acid (yellow pigment) (Nolan et al. 2016, Marounek and Pebriansyah 2018). Recently, lycopene and astaxanthin have become the most widespread substances used in poultry farming for color improvement of poultry skin and egg yolks (Olson et al. 2008, Honda et al. 2020). Lycopene is a non-oxygenated acyclic carotenoid, which gives red color to tomatoes and their products and has a strong antioxidant effect (Rissanen et al. 2002). Astaxanthin is a ketocarotenoid, a product of β-carotene oxidation. It is a common pigment present in algae, salmon, and some birds (Sila et al. 2015). Its orange-red color is of significant commercial value due to the antioxidant and coloring properties (Komatsu et al. 2017, Lu et al. 2019) of such seafood as salmon, lobster and crabs. It can neutralize free radicals with antioxidant activity 500 times higher than that observed in vitamin E (Camera et al. 2009, Nishigaki et al. 2010). It also improves functioning of the immune system, reduces the intensity of aging and is well-known for its anti-cancer effects (Nys 2000).

The ability of hens to produce eggs containing significant amounts of high quality proteins, minerals, vitamins and lipids (Zdrojewicz et al. 2016, Heflinet et al. 2018) antioxidants, e.g. vitamins A and E, selenium (Nimalaratne et al. 2016, Yoo et al. 2016) and other nutraceuticals (Abeyrathne et al. 2018, Damaziak et al. 2018) as well as carotenoids, including lycopene and astaxanthin (Gervasi et al. 2017, Harada et al. 2017) is of great importance for functional properties of food products.

Currently, a significant number of sources of natural lycopene and astaxanthin are known, but the differences in their chemical composition and origin as well as composition of the basic diets show great discrepancies regarding their impact on metabolic processes in different bird species. Furthermore, the ability of vitamin A and carotenoids to accumulate in egg yolks at different temperatures, is not yet well understood. Therefore, it seemed useful to investigate the effects of lycopene and astaxanthin on the color of chicken egg yolks in relation to important metabolic parameters of hens in order to establish effective doses and suitable timing of use of these carotenoids in chicken diets, as well as to recommend storage regimes considering the lowest possible losses of these biologically active substances.

Materials and Methods

Study design

The experiment was conducted in a vivarium at the Faculty of Veterinary Medicine, at the National University of Life and Environmental Sciences of Ukraine. There were 45 laying hens of Hy-Line W36 14 weeks of age kept in a room with adjustable ventilation. Before the study, the chickens were weighed and divided into 3 groups, each consisting of 15 birds, according to the principle of groups-analogues. The study started after an adaptive period in the peak of egg laying when the hens were 23 weeks of age. It was divided into three stages and lasted 90 days. Table 1 shows the design of the study.

Lycopene was obtained from 6% oil extract containing tomatoes (LycoRed, Israel), while 10% oil extract of astaxanthin, obtained from the biomass of algae (Haematococcus pluvialis -ALGAE Technologies, Israel) was the source of astaxanthin. The laying hens were fed a basic commercial diet, the composition of which is shown in Table 2.

The laying hens were fed complete feed, on average, 91 g and 97 g per head per day for 1 to 30 days and for 31 to 90 days, respectively. The experimental diets

Table 2. Composition of the basic diet for laying hens.

Composition	Content of components, g/100 g
Maize	50.085
Wheat	9.000
Soybean meal	17.900
Sunflower cake	9.600
Limestone	11.400
Monocalcium phosphate	1.000
Salt	0.230
Intox (sorbent)	0.100
Methionine	0.130
Proactivo[1]	0.100
Mineral complex Rovimix[2]	0.100
Lysine	0.160
Millersheim III 150[3]	0.015
Sodium sulfate	0.130
Choline chloride	0.030
Vitamin complex Rovimix[4]	0.020
Chemical composition, g/100 g	
Moisture	10.40
Crude protein	16.22
Metabolic energy, kcal/kg	2912.00
Calcium	4.76
Total phosphorus	0.77
Sodium	0.20

Note: [1] – 1 kg of Proaktivo contains: bacteria *Bacillus subtilis and Bacillus licheniformis, not less than* 1 x 10^{12} CFU, bacteria *Enterococcus faecium, not less than* 5 x 10^{10} CFU, fermentation products of *Lactococcus Lactis*, *Bacillus subtilis, Bacillus licheniformis* 100 g, xylanase 600000 units, protease 500 units, cellulase 20000 units, milk thistle meal 20 g, acidity regulator 10 g, betaine 10 g, chitosan 0.1 g, yeast cell walls (mannanoligosaccharides) 100 g, natural aluminosilicates – up to 1 kg;
[2] – 1 kg of mineral complex Rovimix contains: Iron (Fe) 35 000.00 mg, Iodine (J) 1 200.00 mg, Cobalt (Co) 100.00 mg, Copper (Cu) 10 000.00 mg, Zinc (Zn) 85 000.00 mg, Manganese (Mn) 90 000.00 mg, Selenium (Se) 250.00 mg Limestone ($CaCO_3$) to 1 000.00 g;
[3] – 1 kg of Millerzyme III 150 contains: beta-glucanase 26500 units, xylanase 26500 units, cellulase 6000 units, mannanase 200 units, beta-glucosidase 40 units, beta-xylosidase 8 units, amyloglucosidase 32 units, protease 12 units, phytase 500 units;
[4] – 1 kg of Vitamin complex Rovimix contains: Vitamin A 26 666 667.00 IU, Vitamin D_3 11 000 000.00 IU, Vitamin E 66 670.00 mg, Vitamin K_3 8 330.00 mg, Vitamin B_1 8 330.00 mg, Vitamin B_2 18 330.00 mg, Vitamin B_6 13 330.00 mg, Vitamin B_{12} 76.67 mg, Niacin 100 000.00 mg, Pantothenic Acid 26 670.00 mg, Folic acid 3 000.00 mg, Antioxidant/Luctanox 120.00 mg, Biotin 250.00 mg, Limestone ($CaCO_3$) to 1 000.00 g

were prepared every 4 days, the feed mixture was blended and stored in airtight containers made of food-grade plastic. The laying hens were kept in experimental cages in accordance with the requirements of Directive 99/74 EC. They were exposed to daylight for 16 h (light intensity was 30 lux) and 8 hours of uninterrupted darkness. The air temperature was maintained between 21°C and 23°C and relative humidity was maintained between 60% and 62%. The conditions for keeping and feeding the laying hens were provided as required in the Management Guide for W-36 commercial layers.

Ethical statement

Ethical committee approval. The study protocols and experimental procedures were approved by the Commission on Bioethics National University of Life and Environmental Sciences of Ukraine. Keeping chickens in the vivarium at the Faculty of Veterinary Medicine, at the National University of Life and Environmental Sciences of Ukraine was conducted under in accordance to Council Directive 1999/74/EC. The data acquisition was performed in accordance Law of Ukraine „On protection of Animals from Cruelty" No 3447-IV from 04.08.2017 and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes of 1986.

Table 3. Effects of astaxanthin at a dose of 10 mg/kg and lycopene at a dose of 20 mg/kg of feed on the level of some metabolites in the serum of laying hens; x±SD, n=5.

Indicators	Control	LP20	AST10
Glucose, mmol/L	7.96 ± 0.28[a]	10.53 ± 0.22[b]	12.82 ± 0.40[c]
Total protein, g/L	39.48 ± 0.26[a]	42.84 ± 0.68[a]	41.14 ± 0.47[a]
Creatinine, µmol/L	69.07 ± 0.37[a]	72.18 ± 0.70[b]	68.64 ± 0.61[a]
Cholesterol, mg/dl	269.20 ± 2.77[a]	222.98 ± 1.14[b]	216.18 ± 5.67[b]
Phosphorus, mmol/L	1.40 ± 0.05[a]	1.42 ± 0.03[a]	2.29 ± 0.05[b]
Calcium, mmol/L	1.39 ± 0.05[a]	1.33 ± 0.13[a]	2.11 ± 0.08[b]
ALAT, IU/L	9.08 ± 0.19[a]	10.90 ± 0.22[b]	8.19 ± 0.19[c]
AST, IU/L	164.06 ± 5.42[a]	115.82 ± 2.23[b]	140.26 ± 1.38[c]
Alkaline phosphatase, I/L	834.56 ± 3.90[a]	539.16 ± 7.87[b]	564.06 ± 1.64[b]

Note: different lowercase letters [a, b, c] indicate values that differed in one row of the Table ($p<0.05$) according to the results of comparison using the Tukey test with Bonferroni correction

Table 4. Effects of astaxanthin at a dose of 20 mg/kg and lycopene at a dose of 40 mg/kg of feed on the level of some metabolites in the serum of laying hens; x±SD, n=5.

Indicators	Control	LP40	AST20
Glucose, mmol/L	8.76 ± 0.28[a]	13.06 ± 0.16[b]	12.94 ± 0.29[b]
Total protein, g/L	43.68 ± 0.53[a]	42.45 ± 1.71[a]	41.00 ± 0.74[a]
Creatinine, µmol/L	70.61 ± 1.10[a]	73.74 ± 1.22[a]	73.38 ± 1.48[a]
Cholesterol, mg/dl	108.83 ± 4.25[a]	151.64 ± 2.47[b]	212.68 ± 3.80[c]
Phosphorus, mmol/L	2.79 ± 0.16[a]	2.90 ± 0.07[a]	2.21 ± 0.10[a]
Calcium, mmol/L	3.56 ± 0.19[a]	3.13 ± 0.06[a]	2.07 ± 0.05[b]
ALAT, IU/L	9.00 ± 0.28[a]	11.09 ± 0.15[b]	8.05 ± 0.14[a]
AST, IU/L	149.94 ± 4.77[a]	93.71 ± 1.75[b]	139.80 ± 4.30[a]
Alkaline phosphatase, I/L	617.48 ± 5.47[a]	600.97 ± 4.35[a]	558.22 ± 4.64[b]

Note: see Table 3.

Blood sample collection and lab analysis

On days 31, 61, and 91 of the experiment, 5 laying hens from each group were selected for tests from the axillary vein taken between 8.00-10.00 am. Serum for biochemical studies was obtained by sparing centrifugation at 2000 rpm for 10 minutes and then frozen at -20°C.

The serum of laying hens was examined for glucose, total protein, cholesterol, creatinine, inorganic phosphorus, total calcium and the activity of ALT, AST and alkaline phosphatase using reagents purchased from Pointe Scientific Inc. (USA) and a Pointe 180 semi-automatic (Poland).

Egg sample collection and lab analysis

On days 30-31, days 60-61 and days 90-91 of the experiment all laid eggs were collected from each group of laying hens (control, LP20 and AST10, control and LP40, AST20 and control and LP60, AST30) and 5 from each group (of the same size and weight) were selected and examined for the content of vitamin A, carotenoids and color of the yolks on the day of laying. The remaining eggs, collected on days 30-31, days 60-61 and days 90-91 from each group of chickens (control, LP20, AST10, control and LP40, AST20 and control and LP60, AST30), were divided into two parts: one stored in a refrigerator at 4°C, and the other in an egg storage house at 12°C. The duration of storage of all eggs was 30 days. 5 eggs (of the same size and weight) were then selected from each group to determine the content of vitamin A, carotenoids and yolk color.

The total content of carotenoids present in the egg yolks was determined using the method described by Amaya (2004). Samples of yolks weighing 0.5 g were extracted into 5 ml of bottled hydroxytoluene (BHT) (0.05%) in cold acetone (4°C) and stirred for 1 h 30 min. After 15 min. of centrifugation at 3000 rpm, the supernatant was transferred to another tube containing 7 ml of petroleum ether. 20 ml of distilled water was then slowly added to the test tube. After separation of the two phases, 10 ml of distilled water was added for 1 h.

Table 5. Effects of lycopene at a dose of 60 mg/kg and astaxanthin at a dose of 30 mg/kg of feed on the level of some metabolites in the serum of laying hens; x±SD, n=5.

Indicators	Control	LP60	AST30
Glucose, mmol/L	8.09 ± 1.45[a]	12.24 ± 0.30[b]	11.86 ± 0.16[b]
Total protein, g/L	42.39 ± 0.74[a]	45.94 ± 1.14[a]	45.41 ± 2.20[a]
Creatinine, µmol/L	82.56 ± 1.51[a]	104.49 ± 4.51[b]	44.61 ± 1.66[c]
Cholesterol, mg/dl	277.70 ± 4.39[a]	322.02 ± 2.01[b]	336.29 ± 5.23[b]
Phosphorus, mmol/L	2.89 ± 0.55[a]	2.92 ± 0.40[a]	2.52 ± 0.21[a]
Calcium, mmol/L	6.00 ± 0.72[a]	5.32 ± 0.21[a]	5.28 ± 0.10[a]
ALAT, IU/L	9.20 ± 0.35[a]	9.47 ± 0.66[a]	8.53 ± 0.31[a]
AST, IU/L	114.12 ± 26.03[ab]	113.61 ± 3.86[a]	67.90 ± 1.99[b]
Alkaline phosphatase, I/L	307.81 ± 1.88[a]	294.02 ± 9.87[a]	559.02 ± 6,70[b]

Note: see Table 3.

Table 6. Effects of lycopene at a dose of 20 mg/kg and astaxanthin at a dose of 10 mg/kg of feed on the content of vitamin A, carotenoids and egg yolk color of laying hens; x±SD, n=5.

Indicators	Control	LP20	AST10
	Freshly laid eggs		
Vitamin A, mg/kg	6.08±0.48[a]	6.88±0.29[a]	8.00±0.86[a]
Carotenoids, mg/kg	10.72±0.17[a]	12.94±0.66[b]	17.01±0.84[c]
Coloring of yolks, points	5.40±0.27[a]	7.80±0.42[b]	11.00±0,35[c]
After 30 days of storage at 4° C			
Vitamin A, mg/kg	5.84±0.52[a]	6.86±0.31[a]	7.86±1.01[a]
Carotenoids, mg/kg	12.24±0.36[a]	14.39±0.65[b]	16.33±1.02[b]
Coloring of yolks, points	5.20±0.22[a]	7.80±0.22[b]	11.00±0,35[c]
After 30 days of storage at 12° C			
Vitamin A, mg/kg	5.46±0.26[a]	5.84±0.12[a]	6.10±0.10[a]
Carotenoids, mg/kg	10.88±0.25[a]	14.20±0.80[b]	19.90±0.71[c]
Coloring of yolks, points	5.40±0.27[a]	7.60±0.27[b]	11.20±0.42[c]

Note: see Table 3.

The aqueous phase was discarded. The ether phase was transferred to another tube and the absorbance was measured using a PD-303S spectrophotometer (APEL, Japan).

The content of vitamin A was measured in 0.5 g of egg yolk ground in a porcelain mortar containing anhydrous sodium sulfate (10 g), 10 ml of acetone was added, stirred and extracted for 10 minutes. After filtration, the volume of the extract was adjusted to 20 ml and evaporated in a water bath under vacuum. The dry residue was dissolved in 5 ml of chloroform, transferred to a test tube and layered with 1 ml of boron trifluoride ester, and the contents of the tubes were shaken for 3-5 s. After 30 s, the first measurement was performed, and after 1 min the second measurement of optical density was made using a PD-303S spectrophotometer (APEL, Japan) at a wavelength of 610 nm.

The color intensity of the yolks was determined using the BASF Yolk color Fan (Germany), on a scale of 1 to 15, from 1 (light yellow) to 15 (dark orange) (Vuilleumier 1969).

Statistical analysis

The data obtained in the study were analyzed statistically using the ANOVA program. The normality of data distribution was confirmed using the program R-3.6.3 for Windows (R Development Core Team 2020). The difference between the values in the groups was determined using the Tukey test. The difference was considered significant at $p<0.05$ (taking into account the Bonferroni correction).

Table 7. Effects of lycopene at a dose of 40 mg/kg and astaxanthin at a dose of 20 mg/kg of feed on the content of vitamin A, carotenoids and egg yolk color of laying hens; x±SD, n=5.

Indicators	Control	LP40	AST20
	Freshly laid eggs		
Vitamin A, mg/kg	7.42 ± 0.74[a]	7.60 ± 0.60[a]	7.64 ± 0.62[a]
Carotenoids, mg/kg	10.77 ± 0.46[a]	14.28 ± 0.53[b]	17.69 ± 1.16[c]
Coloring of yolks, points	6.00 ± 0.35[a]	8.80 ± 0.42[b]	12.40 ± 0.27[c]
After 30 days of storage at 4° C			
Vitamin A, mg/kg	5.62 ± 0.38[a]	5.84 ± 0.30[a]	6.86 ± 0.28[b]
Carotenoids, mg/kg	10.33 ± 0.25[a]	11.62 ± 0.41[b]	18.34 ± 0.40[c]
Coloring of yolks, points	6.20 ± 0.22[a]	9.20 ± 0.42[b]	12.80 ± 0.42[c]
After 30 days of storage at 12° C			
Vitamin A, mg/kg	6.66 ± 0.56[a]	6.10 ± 0.43[a]	6.46 ± 0.51[a]
Carotenoids, mg/kg	10.52 ± 0.38[a]	11.84 ± 0.68[a]	18.42 ± 0.81[b]
Coloring of yolks, points	6.40 ± 0.27[a]	9.20 ± 0.42[b]	13.00 ± 0.35[c]

Note: see tab. 3.

Table 8. Effects of lycopene at a dose of 60 mg/kg and astaxanthin at a dose of 30 mg/kg of feed on the content of vitamin A, carotenoids and egg yolk color of laying hens; x±SD, n=5.

Indicators	Control	LP60	AST30
	Freshly laid eggs		
Vitamin A, mg/kg	5.33 ± 0.20[a]	6.28 ± 0.59[a]	6.95 ± 0.70[a]
Carotenoids, mg/kg	9.80 ± 0.52[a]	11.43 ± 2.14[a]	17.30 ± 0.59[b]
Coloring of yolks, points	6.60 ± 0.27[a]	9.80 ± 0.42[b]	13.80 ± 0.42[c]
After 30 days of storage at 4° C			
Vitamin A, mg/kg	5.82 ± 0.12[a]	6.66 ± 0.50[a]	7.42 ± 0.60[b]
Carotenoids, mg/kg	7.72 ± 0.38[a]	13.07 ± 1.45[b]	15.21 ± 0.86[b]
Coloring of yolks, points	6.60 ± 0.27[a]	9.80 ± 0.42[b]	14.00 ± 0.35[c]
After 30 days of storage at 12° C			
Vitamin A, mg/kg	5.90 ± 0.24[a]	6.88 ± 0.25[a]	7.16 ± 0.40[a]
Carotenoids, mg/kg	7.61 ± 1.07[a]	8.25 ± 0.29[a]	15.43 ± 1.08[b]
Coloring of yolks, points	6.40 ± 0.27[a]	10.00 ± 0.50[b]	14.20 ± 0.42[c]

Note: see Table 3.

Results

Effects of lycopene and astaxanthin supplements added to the diets of laying hens on biochemical parameters of serum

Supplementation of laying hens with lycopene oil extracts at doses of 20, 40 and 60 mg/kg and astaxanthin at doses of 10, 20 and 30 mg/kg for 30 days increased the concentration of glucose in the serum ($p<0.05$) as compared with controls (Tables 3 5).

Astaxanthin at doses of 10 and 20 mg/kg and lycopene at doses of 20 and 40 mg/kg of the diet did not affect the total protein content and caused insignificant fluctuations in the content of creatinine in the serum of laying hens (Tables 3, 4).

Increasing the dose of lycopene in the diet of laying hens to 60 mg/kg increased ($p<0.05$) the level of creatinine in the serum, while astaxanthin at a dose of 30 mg/kg of feed decreased its content as compared with the control ($p<0.05$) and with the addition of lycopene ($P<0.05$) (Tables 3, 5).

The use of astaxanthin supplements at a dose of 10 mg/kg or lycopene at a dose of 20 mg/kg in the diet of laying hens reduced serum cholesterol ($P<0.05$) as compared with the control (Table 3). In contrast, increasing the content of astaxanthin to 20 and 30 mg/kg or lycopene in the diet of laying hens to 40 and 60 mg/kg reduced cholesterol content in the serum as compared to the control ($p<0.05$) (Tables 4, 5).

Lycopene supplements in the diet of chickens did not affect the level of inorganic phosphorus and total calcium, but reduced ($p<0.05$) the activity of alkaline phosphatase in the serum only at a dose of 20 mg/kg of feed (Table 3, 5). Astaxanthin at a dose of 10 mg/kg of feed increased ($p<0.05$), and at doses of 20 and 30 mg/kg of feed did not affect the content of inorganic phosphorus and total calcium in the serum of chickens (Table 3, 5).

The activity of liver enzymes in the serum of chickens varied depending on the dose of astaxanthin or lycopene added to the feed. In particular, astaxanthin at a dose of 10 mg/kg reduced ($p<0.05$), and at doses of 20 and 30 mg/kg feed did not affect the activity of ALT and reduced ($p<0.05$) the activity of AST in serum compared to the control. Lycopene at doses of 20 and 40 mg/kg increased ($p<0.05$) ALT activity and decreased ($p<0.05$) AST, and at a dose of 60 mg/kg of feed did not affect these indicators.

Effects of lycopene and astaxanthin supplements in the diets of laying hens on the content of vitamin A, carotenoids and egg yolk color

Astaxanthin supplementation of laying hens at doses of 10, 20 and 30 mg/kg or lycopene at doses of 20, 40 and 60 mg/kg for 30 days did not affect the accumulation of vitamin A, but contributed to an increase ($p<0.05$) in the content of carotenoids in the yolks eggs as compared to the control. Astaxanthin had a significantly greater effect on the color of the yolks ($p<0.05$) than lycopene (Tables 6, 7, 8).

Storage of eggs of laying hens supplemented with astaxanthin or lycopene in different doses at both 4°C and 12°C for 30 days did not significantly affect the content of vitamin A, carotenoids and yolk color as compared to freshly laid eggs (Table 6, 8).

Discussion

Effects of lycopene and astaxanthin supplements added to the diets of laying hens on biochemical parameters of serum

Addition of oil extracts of astaxanthin (at doses of 10, 20 and 30 mg/kg) and lycopene (at doses of 20, 40 and 60 mg/kg) to the diet of laying hens for 30 days increased the concentration of glucose in serum significantly, as compared to the control. Similar results were found in the serum of broiler chickens when lycopene was used at a dose of 100 mg/kg of feed (Mezbani et al. 2020). Clinical studies on humans show that astaxanthin can improve glucose metabolism and reduce blood pressure in patients suffering from type 2 diabetes mellitus (Mashhadi et al. 2018). However, in our study the use of astaxanthin oil extract in healthy laying hens had the opposite effect. This might be due to quite big differences in physiology and metabolism and needs further research.

Changes in the creatinine level of the serum of laying hens fed diets enriched with carotenoids were not dose-dependent in our study. Both lycopene and astaxanthin doses and length of the feeding period (30 days and 59 days) did not result in any negative effects on creatinine concentrations in the serum of laying hens in our study.

Enrichment of the diets for laying hens with lycopene at a dose of 20 mg/kg and astaxanthin at a dose of 10 mg/kg resulted in a hypocholesterolemic effect as compared to the control. These data are in concurrence with the data obtained using lycopene supplements administered to quails at doses from 50 up to 200 ppm (Sahin et al. 2006). Most researchers focus their studies on the hypocholesterolemic activity of both lycopene and astaxanthin in animals and humans. This is associated with the ability of lycopene to reduce triglycerides and total cholesterol in quail serum and egg yolks (Sahin et al. 2006). Lycopene doses within the ranges from 20 to 80 mg/kg of the diet reduced triglycerides and total cholesterol in the serum of breeding hens (Sun et al. 2014a). The addition of synthetic lycopene to compound feed for laying hens at doses of 10 and 20 mg/kg of feed for 4 weeks did not affect either the cholesterol content or the lipid profile of serum (An et al. 2019). It is also reported that astaxanthin added to pigs' feed at doses of 1.5 and 3 ppm did not affect the cholesterol content of pork (Yang et al. 2006). The intensity of the effect of lycopene and astaxanthin on lipid metabolism was associated with the duration of their use in the diets for the hens used in our study. It is worth noting that lycopene affected serum cholesterol and high-density lipoprotein cholesterol for 35 days, but this effect was not observed on days 21 and 28 in breeding hens (Sun et al. 2014b).

The increases in lycopene content to 40 and 60 mg/kg and astaxanthin to 20 and 30 mg/kg added to the diets for laying hens also resulted in a hypercholesterolemic effect. This effect was observed especially with lycopene added at a dose of 60 mg/kg and astaxanthin at a dose of 30 mg/kg of feed when cholesterol in chickens increased beyond normal levels – 52 148 mg/dl (Basmacioglu and Ergul 2005). It is worth noting that the level of cholesterol in the serum of hens in the control group also increased during this period when compared with range values. This was likely due to the age of our hens reaching the peak of egg production, caused by vitelogenesis and transport of cholesterol into the egg yolk (Hrabčáková et al. 2014), duration of the sup-

plementation period, increased transport of carotenoids into egg yolk in lipoproteins (Gawecki et al. 1977), and the use of synthesis of bile acids required for emulsification and absorption of carotenoids in the small intestine. It is wellknown that during digestion in the duodenum, carotenoids are incorporated with other lipids (cholesterol and phospholipids) and products of lipid digestion (free fatty acids, monoacylglycerols, lysophospholipids) into mixed micelles (Reboul 2013), which are important in the process. It seems likely that one of the reasons for the increased levels of cholesterol in the serum of laying hens in our study was the use of lycopene and astaxanthin supplements in the form of oil extracts, which consequently facilitated transport of these carotenoids across plasma membranes. There are sufficient data confirming a significant role of plasma membrane cholesterol in transmembrane protein function (Story et al. 2010).

The dose of lycopene in the diet for laying hens in our experiment did not affect the level of inorganic phosphorus and total calcium, and the activity of alkaline phosphatase decreased in serum only at a dose of 20 mg/kg of lycopene. Astaxanthin used in our experiment at a dose of 10 mg/kg of feed increased, while the doses of 20 and 30 mg/kg of feed did not affect the content of inorganic phosphorus and total calcium in the serum of laying hens. The results obtained in the present study are in agreement with the data obtained in other studies (Hwang et al. 2018) on the antiosteoporotic effect of astaxanthin on bone mass in mice with ovariectomy. Administration of astaxanthin (5 or 10 mg/kg) for 6 weeks inhibited the increase in serum calcium, inorganic phosphorus and alkaline phosphatase activity in mice (Hwang et al. 2018).

The levels of ALT and AST enzymes in the blood serum characterizing the functional state of the liver are important criteria for assessing the safety of feed additives for poultry. Lycopene supplements affect the activity of liver enzymes in the serum of chickens in different ways. Changes in the activity of liver enzymes, in particular ALT and AST, in the serum of laying hens, caused by dietary supplements containing different doses of astaxanthin or lycopene in our study did not show any negative effects of carotenoids on the liver. Reduced activities of the liver enzymes in the serum of laying hens were within physiological parameters and indicated the absence of degenerative changes in the liver resulting from the use of lycopene or astaxanthin. This was due to the high antioxidant effects of lycopene preventing cell destruction in the liver caused by free radicals and singlet oxygen (Inoue et al. 2017). As has been shown, lycopene addition to compound feed at a dose of 100 mg/kg of feed reduced the content of malonic dialdehyde in the serum of broiler chickens (Mezbani et al. 2020). Enrichment of lamb's diets with lycopene doses of 50, 100 and 200 mg/kg of feed not only reduced the serum content of malonic dialdehyde, but also linearly increased the level of vitamin E, total antioxidant capacity and activity of catalase, glutathione peroxidase and superoxide dismutase thereby improving the antioxidant status of the body (Jiang et al. 2015). Astaxanthin is also a strong antioxidant (Kobori et al. 2017) and can quench free radicals (superoxide anion, hydrogen peroxide, singlet oxygen, etc.) in both the inner and outer layers of the cell membrane, unlike most antioxidants, the action of which is concentrated in the inner (for example, vitamin E and β-carotene) or the outer side of the membrane (for example, vitamin C) (Sztretye et al. 2019). The latter not only determines the clinical condition of laying hens, but also egg productivity and other indicators determining their quality and biological value.

In general, astaxanthin supplements in the diets for laying hens had a greater effect on the levels of the metabolites in the serum of the birds than lycopene supplements despite significantly lower amounts added to the diets.

Effects of lycopene and astaxanthin supplements in the diets of laying hens on the content of vitamin A, carotenoids and egg yolk color

Lycopene or astaxanthin supplementation in the diets for laying hens at different doses, for 30 days, respectively did not affect vitamin A content, but, as we expected, they increased carotenoids in egg yolks, as compared to the control, due to lack of provitamin activity in the two carotenoids (Ruhl 2013). In this case, the addition of astaxanthin (belonging to xanthophylls) to the diet of laying hens showed a more pronounced effect than the addition of lycopene (Tables 6, 8). This was due to higher bioavailability of xanthophylls, including astaxanthin, to the body, and the presence of hydroxyl groups in the molecule, which increases their solubility in micellar structures (Reboul et al. 2006, Mapelli-Brahm et al. 2017) and the ability of laying hens to secrete excess xanthophylls with egg yolks (Skibsted 2012).

Accumulation of carotenoids in chicken egg yolks affected the intensity of their color, lycopene supplements enhanced a darker color ($p<0.05$) as compared to the control, but it was lighter ($p<0.05$) than with astaxanthin supplements. If the basic diet of chickens contains corn, the astaxanthin dose of 20 mg/kg of feed can increase the color of the yolk from 6 to 14 points. Under the same conditions, the addition of lycopene, even at a dose of 60 mg/kg, was able to increase these parameters to 10 points on a 15-point scale. The doses of lycopene and astaxanthin in the diets of laying hens

did not affect the total carotenoid content of egg yolks significantly, which was likely due to the saturation effect, since increased doses of lycopene and astaxanthin were used in the diets of laying hens for a period of 90 days. The results of our studies are in agreement with the data of a regression analysis (Olson et al. 2008), which showed that 420 mg of lycopene per 1 kg of feed should ensure its optimal inclusion in the egg yolk, but saturation of the mechanisms of absorption in the intestine was likely to reduce the efficiency of absorption. The amount of lycopene administered at a dose of 65 mg/kg of feed absorbed by the egg yolk was about 4.5%, but it decreased to 0.6% when the lycopene dose increased to 650 mg/kg of feed (Olson et al. 2008).

Storage of eggs of the lycopene or astaxanthin supplemented hens at different doses both at 4°C and at 12°C for 30 days did not have a significant effect on the content of vitamin A in the yolks in our study. This fact is important, especially for consumers in Asian (Koppel et al. 2014) or European countries (Koppel et al. 2015) who can buy eggs stored at room temperature from different producers (farmers, local markets or shops).

No significant differences in the color of yolks and the total content of carotenoids in the yolks of eggs of laying hens supplemented with different doses of lycopene and astaxanthin were observed during storage at 4°C and 12°C, as compared to freshly laid eggs. However, a significant difference ($p<0.05$) between the color of the yolks and their carotenoid content of egg yolks was found between the groups of laying hens supplemented with lycopene or astaxanthin and the control ($p<0.05$).

Storage of eggs of laying hens supplemented with different doses of lycopene and astaxanthin at 4°C and 12°C for 30 days did not affect the total carotenoid content of egg yolks in our study. These data are in agreement with the results obtained in other studies (Omri et al. 2019) in which flaxseed and tomato-red pepper mixture were used as additives in feeding of laying hens. Carotenoids in egg yolks were stable up to 6 weeks when they were refrigerated (Barbosa et al. 2011, Nimalaratne et al. 2016).

Contrasting results are also reported showing that storage of eggs at 26.5°C for 35 days reduced the total carotenoid content in the yolks from 28.55 to 22.09 µg/g, and from 28.55 to 23.57 µg/g when they were stored at 7.9°C (Barbosa et al. 2011). On the other hand, storage of eggs at 2°C for 8 weeks did not reduce the total carotenoid content of the egg yolk, but after 15 weeks of storage, it decreased from 28.55 to 27.03 µg/g (Gawecki et al. 1977). It can therefore be concluded that the shelf life of carotenoids present in yolks depends on their type, their concentration, the ratio to other components of the diet for laying hens and temperature and length of egg storage.

The results of the orientating study presented above show reliable data, which have led us to the following conclusions:

Enrichment of the diets for laying hens with lycopene oil extracts (20 – 60 mg/kg) or astaxanthin (10 30 mg/kg) increased the content of glucose in the serum, but did not affect the content of total protein, calcium and phosphorus. The effects on creatinine, cholesterol, the activity of ALT, AST and alkaline phosphatase were dose- and time-dependent. The enriched diets did not exert any negative effects on the health and welfare of the hens.

Lycopene doses of 20, 40 and 60 mg/kg and astaxanthin doses of 10, 20 and 30 mg/kg of feed did not affect the vitamin A content of the yolks of freshly laid eggs, nor those stored at 4°C and 12°C for 30 days.

Astaxanthin-supplemented diets for laying hens had a greater enriching effect on carotenoids in egg yolks and their attractive color than lycopene supplements.

The results were obtained from a relatively small number of 45 hens kept in enriched cages. Further comparative studies should include more hens kept in alternative systems.

Recommendations

Egg producers who serve markets and consumers with a preference for eggs containing higher carotenoid concentrations and intensely coloured egg yolks may consider using appropriate feed additives such as astaxanthin and lycopene.

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