Effects of supplementation with α-tocopherol, ascorbic acid, selenium, or their combination in linseed oil-enriched diets on the oxidative status in broilers

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ABSTRACT A trial was conducted to investigate whether, and if so to what extent, the combined supplementation of vitamin E, vitamin C, and selenium was superior to their sole supplementation concerning the oxidative stress induced by a high n-3 dietary polyunsaturated fatty acids (PUFA) intake in broilers. Four hundred 21-day-old Ross 308 male broilers were allocated to 5 experimental groups fed the following linseed oil (5%)-enriched finisher diets: Cont (no supplement), +E (200 IU vitamin E/kg feed), +C (250 mg vitamin C/kg feed), +Se (0.2 mg selenium/kg feed), or +ECSe (concentrations as in the sole supplementation, combined). Analyses of malondialdehyde (MDA), vitamin C, and α- and γ-tocopherols in plasma, antioxidant capacity of water- (ACW) and lipid- (ACL) soluble compounds in serum, and glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities in whole blood were performed. In breast muscle, analyses of MDA, α- and γ-tocopherols, ACW, selenium, and fatty acid (FA) composition were carried out. Birds fed the combination of antioxidants showed reduced average daily gain (ADG) and average daily feed intake (ADFI) and, as +E, lower MDA and γ-tocopherol, together with raised α- tocopherol levels in plasma and lower MDA and raised α- tocopherol levels in breast muscle compared to the control. The combination of antioxidants in the +ECSe group raised GPx activity in whole blood compared to the control. In conclusion, results indicated that vitamin E is the most effective antioxidant to alleviate oxidative stress caused by high dietary PUFA and that the supplementation with additional vitamin C and selenium did not have clear synergistic effect.

Key words: vitamin E, vitamin C, selenium, oxidative stress, broiler

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INTRODUCTION

Broilers demand a high-energy concentration in their feed, which is achieved through supplementation of feeds with various fats and oils. Moreover, there is an increasing demand for functional foods, in which supplementation with n-3 polyunsaturated fatty acids (**PUFA**)-rich plant oils is becoming an accepted practice to ensure a favorable fatty acid (**FA**) composition of meat. However, it is often overlooked that n-3 PUFA are very susceptible to oxidation and can contribute to a higher oxidative stress, also due to an enlarged production of reactive oxygen species (**ROS**) in vivo (Gladine et al., 2007; Voljč et al., 2011). Supplementation with n-3 PUFA oils can cause an imbalance between reductants and oxidants in vivo and consequently induce damage to different macromolecules such as DNA, proteins, and lipids, which can lead to impaired activity, apoptosis, and necrosis of different cells. Additionally, a high dietary intake of n-3 PUFA led to high levels of them in meat and tissues, thereby increasing the susceptibility of the animal products to oxidative deterioration (Estévez, 2015).

Studies have shown that a high dietary n-3 PUFA inclusion affects the oxidative status of broilers and can cause a pronounced oxidative stress (Estévez, 2015). This can negatively affect many in vivo lipid, protein, and DNA oxidative stress parameters, such as the integrity of lymphocyte DNA, plasma, liver, and breast muscle malondialdehyde (**MDA**) (Voljč et al., 2011; Estévez, 2015), plasma and liver α- and γ-tocopherol, as well as genes involved in the oxidative stress response (Tomažin et al., 2014). To alleviate oxidative stress in vivo and in animal products, the sole dietary

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supplementation of different antioxidants such as vitamins E and C and selenium were effectively used, also in the case of high dietary n-3 PUFA (Eder et al., 2005; Taulescu et al., 2011 Voljč et al., 2011; Skrivan et al., 2012; Voljč et al., 2013; Konieczka et al., 2015; Raederstorff et al., 2015; Akbarian et al., 2016). Linseed oil was used as a source of high n-3 PUFA in trials reporting the effects of various antioxidants in broilers (Walton et al., 2001; Bou et al., 2005; Smet et al., 2008). In addition, Voljč et al. (2013) showed that due to high PUFA intake-induced oxidative stress, recommendations for vitamin E for Ross broilers (Aviagen, 2014) did not seem to be enough to alleviate the negative effects of a large amount of n-3 PUFA in diets, and therefore higher antioxidant levels were suggested.

High environmental temperatures also have been studied as one of the most important causes of oxidative stress for a long time (Gursu et al., 2004; Akbarian et al., 2016). Even though poultry can endogenously synthesize vitamin C, it was shown that under heat stress, dietary supplementation with vitamin C could alleviate the oxidative stress (Leeson and Summers, 2001). This suggested that the endogenous synthesis of vitamin C may not be sufficient in all stress conditions. As it has been known for a long time, vitamins E, C, and selenium have important roles in the antioxidative defense network, and can work efficiently only in synergism. Vitamin E inhibits free radical-mediated lipid peroxidation, but is oxidized and depleted fast, if not regenerated by vitamin C. The antioxidant activity of vitamin C is restored by selenium-containing enzymes (Fang et al., 2002; Lykkesfeldt and Svendsen, 2007).

Information on the combined effects of antioxidants is limited, especially in n-3 PUFA-induced oxidative stress and surprisingly never includes more than 2 antioxidants. For instance, Cinar et al. (2014) showed that dietary supplementation with a combination of vitamins E and C resulted in better protection in copper-induced oxidative stress than the individual supplementation at the same level. Sahin et al. (2002) obtained similar results on hens reared at a high ambient temperature, as a combination of vitamins E and C lowered serum MDA to a greater extent than supplementing these vitamins separately. In addition, lower concentrations of MDA in the serum, liver, heart, and kidney were shown in Japanese quails under heat stress fed a combination of vitamin C and folic acid than when supplementing these antioxidants individually (Sahin et al., 2003b). In the same way, Harsini et al. (2012) showed that the combination of selenium and vitamin E was more effective in antioxidative protection in broilers under heat stress than their individual use. Similarly, Skrivan et al. (2012) showed that a combination of vitamin C and selenium lowered TBARS in broilers fed a PUFA diet more than the supplementation of only selenium. Differently, Konieczka et al. (2015) showed that the combination of dietary vitamin E and selenium is as efficient as vitamin E alone in the case of oxidative stress caused by high n-3 PUFA. Similarly, Taulescu et al. (2011) showed that a selenium-supplemented feed did not lower lipid oxidation measured as thiobarbituric acid reactive substances (**TBARS**) in comparison to the sole vitamin E supplementation in broilers. In relation to the role of selenium in the antioxidant network, it is important to consider that organic forms of selenium can be better absorbed from the gut in comparison to inorganic forms (Mahima et al., 2012), and thus more efficient in the reduction of oxidative stress (Ahmad et al., 2012).

To the best of our knowledge, no studies on the effect of combined supplementation of vitamins E, C, and selenium under conditions of oxidative stress have been performed in broilers or in other species. Most of the research is aimed to heat stress alleviation, and not to the stress caused by high dietary n-3 PUFA levels. Even though the physiological responses to heat or to oxidative stress caused by high n-3 PUFA are comparable, different strategies to alleviate them were suggested (Estévez, 2015).

Therefore, the objective of the present study was to determine whether, and if so to what extent, the supplementation with a combination of vitamin E, vitamin C, and selenium was superior in antioxidant activity than the sole supplementation of these nutrients in diets rich in n-3 PUFA. For this purpose, different markers of oxidative stress in blood and breast muscle were evaluated.

MATERIALS AND METHODS

The trial was performed at the experimental farm of the Faculty of Agriculture, University of Novi Sad (Novi Sad, Serbia). All the procedures were conducted according to the ethical norms of the EU Convention for the protection of vertebrate animals used for experimental and other scientific purposes, confirmed by Serbian authorities.

Birds and Dietary Treatments

A total of 400 day-old male Ross 308 broiler chicks was randomly divided into 5 groups with 4 replicate pens of 20 each following a complete block design. The birds were individually labeled, penned on a straw litter, and reared under a standard lightning program. Temperature was kept at 32°C during the first wk, at 28°C during the second wk, at 26 °C during the third wk, at 23 °C during the fourth wk, and at 21°C thereafter.

Chickens were fed the commercial starter diet from the 1st to the 12th d of the trial, and the commercial grower diet from the 13th to 20th d, according to the nutrition specifications for Ross 308 (Aviagen, 2014). From the 21st to the 40th d of the trial, finisher diets were enriched with 5% of cold-pressed linseed oil and supplemented with: no additives (Cont), 200 IU vitamin E/kg (+E), 250 mg vitamin C/kg (+C), 0.20 mg selenium/kg (+Se), or combined 200 IU vitamin E, 250 mg vitamin C, and 0.20 mg selenium/kg feed (+ECSe). Rovimix E50 (DSM, Heerlen, the Netherlands) as

Table 1. Ingredients and calculated nutrient content of the starter, grower, and finisher diets.

	Starter	Grower	Finisher
Composition of feed mixtures[3] (g/kg)			
Maize	421	490	537
Wheat	50.0	30.0	40.0
Wheat bran	60.0	/	/
Soya meal	285	155	320
Linseed oil[1]	/	/	50.0
Full fat soybean (extruded)	137	280	10.0
Mineral-vitamin-amino acid supplement[2]	10.0	10.0	10.0
Calculated nutrient content			
Metabolizable energy (MJ/kg)	12.5	13.0	13.4
Protein (g/kg)	230.0	216.5	196.9
Lysine (g/kg)	14.4	9.3	15.0
Methionine (g/kg)	6.8	4.4	4.7
Calcium (g/kg)	9.6	13.0	8.8
Phosphorus, available (g/kg)	4.8	5.2	4.2

[1]100 g of oil contains: 10.1 g SFA, 18.6 g MUFA, 66.9 g PUFA, 54.8 g n-3 PUFA, 12.1 g n-6 PUFA, <0.5 mg α-tocopherol, 29.8 mg γ-tocopherol, and 1.52 mg δ-tocopherol.

[2]Calculated to meet the mineral and vitamin requirement for finisher diets for broiler Ross 308, per kilogram of feed: vitamin A (10,000 IU), vitamin D (4,500 IU), vitamin E (50 IU), vitamin K (3 mg), biotin (0.18 mg), choline (1600 mg), folic acid (1.9 mg), niacin (60 mg), pantothenic acid (18 mg), vitamin B_1 (2.5 mg), vitamin B_2 (6.5 mg), vitamin B_6 (3.2 mg), vitamin B_{12} (0.017 mg), Cu (16 mg), I (1.25 mg), Fe (20 mg), Mn (120 mg), Se (0.25 mg), and Zn (110 mg).

[3]Feed mixtures also contain sodium chloride, limestone, monocalcium phosphate, sodium bicarbonate, l-lysine-HCl, dl-methionine, and l-threonine to meet the requirements (Aviagen, 2014), and phytase (DSM, Heerlen, the Netherlands).

vitamin E supplement (dl-α-tocopheryl acetate), Rovimix Stay-C35 (DSM, Heerlen, the Netherlands) as vitamin C supplement, and Alkosel R397 (Lallemand, Rexdale, ON, Canada) as Se supplement (L(+) selenomethionine and other selenoproteins) were used.

The ingredients and calculated nutrient content of the starter, grower, and finisher diets are presented in Table 1.

Samples of finisher feeds were taken to determine the proximate and mineral compositions, α- and γ-tocopherol, vitamin C (only in the supplemented groups), MDA, antioxidant capacity of lipid- (**ACL**) soluble compounds, and antioxidant capacity of water- (**ACW**) soluble compounds (Table 2), as well as fatty acid composition (Table 3).

Experimental Procedure and Sample Collection

Water and feed were provided ad libitum, and live weight gain and feed consumption were recorded weekly. At the age of 40 d, 19 d after the division of the experimental groups and feeding experimental diets, 12 animals per group were sacrificed by cervical dislocation. Samples of blood and breast muscle were collected for analyses. Breast muscle samples were stored in zip-type bags, tightly closed, and stored at –80°C for a maximum storage time of 2 months. Blood samples for plasma were collected into K2 EDTA tubes (Vacuette, Greiner Bio-One, Cassina de Pecchi, Italy), separated by centrifugation (1,000 × g for 15 min at 4°C), and stored in plastic microcentrifuge tubes for analysis of

Table 2. Proximate composition, concentration of minerals, α- and γ- tocopherol, vitamin C, MDA, and antioxidant capacity of water- (ACW) and lipid- (ACL) soluble compounds of the finisher feed mixtures.

	Cont	+E	+C	+Se	+ECSe
Proximate and mineral composition of the feed mixtures[1]					
Dry matter (g/kg)	877.7	879.7	879.1	879.9	878.5
Crude protein (g/kg)	183.9	186.0	188.1	183.2	189.7
Crude fat (g/kg)	59.80	58.18	59.63	62.40	58.87
Crude fiber (g/kg)	37.44	37.52	35.23	38.03	41.55
Crude ash (g/kg)	48.23	47.19	47.09	47.23	47.15
Nitrogen free extract (g/kg)	548.3	550.8	549.0	549.01	541.18
P (g/kg)	5.87	5.89	5.89	5.27	5.87
Ca (g/kg)	7.70	7.23	7.20	7.23	7.17
Mg (g/kg)	1.06	1.05	1.06	1.03	1.07
K (g/kg)	9.65	9.51	9.65	9.44	9.83
Na (g/kg)	1.89	1.75	2.08	1.53	1.83
Se (mg/kg)	0.33	0.30	0.23	0.49	0.50
Vitamin C(mg/kg)	/	/	132.4	/	129.4
Tocopherol isomers (mg/kg)					
α-tocopherol	51.2	277.5	55.2	45.9	241.6
γ- tocopherol	31.3	34.2	31.8	31.0	35.1
MDA, ACL and ACW (μmol/kg)					
MDA	42.09	50.36	37.77	39.45	47.72
ACL	350	310	360	350	370
ACW	5240	4836	6478	5183	5802

[1]Proximate and mineral analyses of feed mixtures were determined according to official Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten methods (Naumann and Bassler, 1997). Values are means of 2 analyses per sample.

Table 3. Fatty acid composition[1] of finisher diets (g fatty acids/kg feed).

	Cont	+E	+C	+Se	+ECSe
C16:0	5.67	5.58	5.71	5.93	5.70
Σ C16:1[2]	0.09	0.09	0.09	0.09	0.09
C18:0	1.98	1.88	1.98	2.15	2.03
Σ C18:1[2]	12.09	11.75	12.11	12.91	12.06
C18:2 n-6	19.16	19.05	19.23	20.09	19.01
C18:3 n-3	17.45	16.60	17.17	17.73	16.66
SFA	8.07	7.86	8.12	8.55	8.17
MUFA	12.45	12.11	12.49	13.30	12.45
PUFA	36.64	35.65	36.40	37.81	35.66
n-3 PUFA	17.52	16.60	17.17	17.73	16.66
n-6 PUFA	19.13	19.05	19.23	20.09	19.01
n-6/n-3 PUFA	1.09	1.15	1.12	1.13	1.14

[1]Values are means of 2 analyses per sample. Only predominant and nutritionally important fatty acids are listed, but the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA are computed from all fatty acids analyzed.

[2]Sum of isomers.

MDA, vitamin C, and tocopherols. Vitamin C in plasma was stabilized by the immediate addition of 250 μL 10% m-phosphoric acid to 250 μL of plasma and stored in plastic microcentrifuge tubes. Blood samples for measuring ACL and ACW were taken in serum tubes and centrifuged at 2,500 × g for 10 min at 4°C. Lithium heparin (Vacuette, Greiner Bio-One, Cassina de Pecchi, Italy) tubes were used to collect whole blood to measure glutathione peroxidase (**GPx**) and superoxide dismutase (**SOD**). All samples were stored at −80°C until the analyses were carried out. The extent of lipid oxidation in individual chicken breast muscle was monitored by measuring MDA, tocopherols, ACW, and FA composition. All samples of breast muscle were sliced into

pieces, frozen in liquid nitrogen, and homogenized in a laboratory homogenizer (Grindomix GM200, Retsch GmbH and Co., Haan, Germany). Homogenized breast muscle samples were stored at –80 °C before they were analyzed.

Determination of Vitamin C

Vitamin C concentration in plasma was analyzed according to the methodology of Wehtersbach and Cigič (2007). One mL of 2% m-phosphoric acid and 10 μL tris—(2-carboxyethyl) phosphine were added to the plasma samples stabilized with m-phosphoric acid. This mixture was centrifuged 10 min at 20,000 × g, and the supernatant was filtered through 0.2 μm filters. Samples were analyzed by HPLC (Agilent, Santa Clara, CA) using Synergi 4μ Hydro- RP 80 A column (Phenomenex, Torrance, CA). The mobile phase was 2.5 mmol/L H_2SO_4, and a UV/VIS (250 nm) detector was used. The calibration was made with ascorbic acid (L-ascorbic acid, A.C.S reagent, Sigma-Aldrich, St Louis, MO).

Determination of Vitamin E (α- and γ-tocopherol)

Concentrations of tocopherols in plasma were measured according to the previously described method by Voljč et al. (2011). Tocopherols in feed and meat samples were analyzed according to the method of Grebenstein and Frank (2012). Briefly, samples (feed: 150 to 200 mg and homogenized muscle: 1.1 to 1.3 g) were placed in glass tubes with screw caps and processed as described by the authors, except that 4 mL of hexane were used to extract tocopherols after saponification. Dried hexane extracts were dissolved in 1.5 mL of ethanol (vortex, 15 min) and filtered. Extracted tocopherols from plasma, feed, and muscle samples were analyzed by reversed-phase HPLC using a Prodigy 5-μm ODS2 column (250 mm × 4.6 mm × 5 μm, Phenomenex, Torrance, CA). The mobile phase consisted of methanol, and the flow rate of the mobile phase was 1.5 mL/min. An Agilent (Santa Clara, CA) HPLC equipped with a 1260 Infinity FLD fluorescence detector was used at excitation and emission lengths of 280 and 330 nm, respectively. The calibration was made using analytical standards of tocopherols (Tocopherol set, Calbiochem, Merck, Darmstadt, Germany). Accurate concentrations of stock standard solutions used for calibration were determined spectrophotometrically using molar absorption coefficients adapted from Müller et al. (2010) using Cary 100 UV-Vis spectrophotometer.

Determination of Selenium

Selenium was determined according to the methodology of García et al. (2005) and the European norm EN 14,627 by hydride generation atomic absorption spectrometry with standard addition method, according to recommended analytical conditions (PerkinElmer Inc., Waltham, MA). An acid digestion in a closed vessel device with temperature control microwave heating was used to digest the samples (Milestone Inc, Shelton, CT). MHS10 hydride system and atomic absorption spectrophotometer 1100B were used (PerkinElmer Inc., Waltham, MA).

Determination of Glutathione Peroxidase and Superoxide Dismutase Activities

GSH-Px activity in blood hemolysates was determined spectrophotometrically with an automated biochemical analyser RX-Daytona (Randox Laboratories, Crumlin, UK) using the commercial Ransel kit (Randox Laboratories, Crumlin, UK), which is based on the method of Paglia and Valentine (1967). Blood hemolysates were diluted 41-fold before being analyzed with Ransel Diluent (Diluting agent, Randox Laboratories, Crumlin, UK). GSH-Px activity was expressed as units per gram of hemoglobin (U/g Hgb). SOD activity in blood hemolysates was determined spectrophotometrically with an automatic biochemical analyser RX Daytona (Randox Laboratories, Crumlin, UK), using a commercially available Ransod kit (Randox Laboratories, Crumlin, UK), which is based on the original method of McCord and Fridovich (1969). Before the analyses, samples of hemolysates were diluted 1:200 with the Ransod Sample Diluent (0.01 mmol/L phosphate buffer, pH 7.0; Randox Laboratories, Crumlin, UK). The activity was expressed as U/g Hgb. Hemoglobin concentration was determined by the cyanmethemoglobin method using an automated hematological analyser ADVIA 120 (Siemens, Munich, Germany) (Paglia and Valentine, 1967).

Determination of MDA

Concentrations of MDA in plasma and muscle samples were measured following the previously described methods (Voljč et al., 2011), except that an Agilent HPLC (Santa Clara, CA) equipped with a 1260 Infinity FLD fluorescence detector was used in the chromatographic analysis.

Determination of Antioxidant Capacity of Water- and Lipid-Soluble Compounds

ACW and ACL compounds in blood plasma were measured using a photochemiluminiscence method by PhotoChem (Analytik Jena, Jena, Germany). The quantification of the antioxidative capacity of the samples was determined by comparison with a standard (the calibration curves were made using ascorbic acid for ACW and trolox for ACL). Two hundred μL of serum and 200 μL of methanol were centrifuged (15,000 × g, 10 min, 4°C) for ACL analyses, while

Table 4. Body weight, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) during the experimental period.

	Cont	+E	+C	+Se	+ECSe	SEM	*P*-value
Body weight (g)							
Day 20	760	766	769	784	782	14.18	0.716
Day 27	1242	1203	1219	1272	1261	27.71	0.389
Day 35	1860	1820	1853	1895	1828	31.60	0.499
Day 40	2394	2312	2370	2372	2276	31.91	0.089
ADG (g)							
1st to 20th d	33.5	34.4	34.6	35.3	34.8	0.616	0.868
21st to 40th d	89.7^{a}	85.9^{a,b}	89.0^{a,b}	88.2^{a,b}	83.5^{b}	1.46	0.041
1st to 40th d	60.3	58.2	59.7	59.7	57.3	0.819	0.094
ADFI (g)							
1st to 20th d	49.3	48.1	49.0	49.1	48.6	0.499	0.460
21st to 40th d	155.3^{a}	147.8^{b,c}	154.1^{a,b}	152.8^{a-c}	146.9^{c}	1.65	0.005
1st to 40th d	98.2^{a}	94.1^{b}	97.5^{a,b}	97.0^{a,b}	94.0^{b}	0.877	0.005
FCR							
1st to 20th d	1.41	1.40	1.42	1.39	1.40	0.0156	0.744
21st to 40th d	1.73	1.72	1.73	1.73	1.76	0.0210	0.812
1st to 40th d	1.63	1.62	1.64	1.63	1.64	0.0148	0.853

$^{a–c}$Different superscript letters in each row indicate significant differences ($P < 0.05$).

50 μl of serum and 400 μL of water were used in the case of ACW. To analyze ACW in breast muscle, approximately 300 mg of homogenized muscle samples were mixed with 600 μl of 2% m-phosphoric acid, left for 10 min, and centrifuged (20,000 x g, for 10 min at 4 °C). The supernatants were analyzed using the ACL-Kit or ACW-Kit protocols (Analytik Jena, Jena, Germany).

Fatty Acid Composition of Feed and Breast Muscle

Fatty acids in feed and breast muscle samples were transmethylated in situ, in accordance with Park and Goins (1994). For the separation of fatty acid methyl esters (FAME), an Agilent 6890 CG (Agilent, Santa Clara, CA) equipped with an Omegawax 320 column (30 m × 0.32 mm i.d. × 0.25 μm, Supelco, Bellefonte, PA), and a flame-ionization detector was used. The chromatographic conditions were as follows: carrier gas (helium, flow 2.0 ml/min), initial temperature (T): 185 °C, ramp: 1 °C/min, final T: 215 °C, injector T: 250 °C, detector T: 280 °C, and split ratio: 20 : 1. Separated FAME were identified by retention time comparison, and results were calculated using response factors derived from chromatographic standards of known composition (Nu411, Nu68A, Nu85, Nu-Chek Prep, Inc., Elysian, MN).

Statistical Analyses

Data were analyzed by the mixed procedure from the SAS software (Ver. 9.4; SAS Institute Inc., Cary, NC,). Least square means (**LSMEANS**) are shown in the results, and the differences were determined by a Tukey-Kramer multiple comparison test. Dispersion was expressed as the standard error of the mean (**SEM**). In the statistical model, the group was included as a fixed effect, and the replication pen as a random effect. Statistical significance was considered at $P < 0.05$.

RESULTS

Body Weight Gain and Feed Consumption

Animals adapted well to the experimental conditions. No differences in the body weight were observed among groups (Table 4). Also, the average daily gain (**ADG**) was not different among groups except from the 21st to the 40th day, when group +ECSe had a lower ADG (for 6.9%) ($P < 0.05$) than Cont. The average daily feed intake (**ADFI**) was lower in the +ECSe group than in the Cont group from the 21st to the 40th d and from the 1st to the 40th d (5.4% and 4.3%, respectively), as well as from the 1st to the 40th d in group +E (4.2%). No differences among groups were detected concerning the feed conversion ratio (**FCR**).

Markers of Oxidative Stress in Blood

No differences in the content of vitamin C in plasma, ACW in serum, or SOD activity in whole blood were observed among groups (Table 5). However, +E (p = 0.059) and +ECSe groups had a 25 and 29% lower MDA concentration in plasma, respectively, in relation to Cont. Similarly, +E and +ECSe lowered γ-tocopherol in plasma by 50 and 46%, respectively, but raised α-tocopherol by 188 and 206% with respect to Cont. Furthermore, supplementation with vitamin C in +C group increased ACL in serum by 14.1%, and supplemental selenium in +ECSe raised GPx by 44.3%, when compared to Cont.

Table 5. Plasma, serum, and whole blood parameters at the end of the supplementation period.

	Cont	+E	+C	+Se	+ECSe	SEM	*P*-value
MDA (nmol/mL plasma)	0.44^{a,b}	0.33^{b,c}	0.45^{a}	0.42^{a-c}	0.31^{c}	0.026	0.002
Vitamin C (μg/mL plasma)	18.23	17.74	18.82	16.80	17.84	0.82	0.547
α-tocopherol (μg/mL plasma)	9.17^{b}	26.43^{a}	8.71^{b}	6.88^{b}	28.05^{a}	2.18	<0.001
γ-tocopherol (μg/mL plasma)	1.12^{a}	0.58^{b}	1.16^{a}	0.90^{a,b}	0.63^{b}	0.111	0.003
ACW (nmol/mL serum)	375.0	378.5	466.6	449.6	411.7	31.47	0.186
ACL (nmol/mL serum)	324.5^{b}	352.8^{a,b}	370.4^{a}	330.1^{b}	354.3^{a,b}	9.37	0.014
GPx in whole blood (U/gHGB)	213.7^{b}	211.9^{b}	237.4^{b}	249.4^{b}	308.4^{a}	9.80	<.0001
SOD in whole blood (U/gHGB)	1159.3	1007.4	1035.9	990.24	990.8	46.5	0.098

$^{a-c}$Different superscript letters in each row indicate significant differences ($P < 0.05$).

Table 6. Content of MDA, α- and γ- tocopherol, ACW and Se in breast muscle.

	Cont	+E	+C	+Se	+ECSe	SEM	*P*-value
MDA (nmol/100 g)	118.7^{a}	60.2^{b}	116.5^{a}	92.1^{a,b}	66.8^{b}	10.9	0.002
α-tocopherol (μg/100 g)	287.3^{b}	773.3^{a}	275.6^{b}	278.7^{b}	950.1^{a}	56.4	<0.001
γ-tocopherol (μg/100 g)	71.6	57.6	67.8	71.0	56.7	4.62	0.076
ACW (μmol AA*/100 g)	22.6	23.1	25.8	23.1	24.6	1.65	0.647
Se (μg/100 g)	11.7^{b}	N.A.$^{\#}$	N.A.$^{\#}$	22.2^{a}	24.0^{a}	1.82	0.001

*AA—ascorbic acid; $^{\#}$ N.A.—not analyzed.
^{a,b}Different superscript letters in each row indicate significant differences ($P < 0.05$).

Markers of Oxidative Stress in Breast Muscle

Results of α- and γ-tocopherol contents in breast muscle (Table 6) revealed that the supplementation with α-tocopherol (groups +E and +ECSe) significantly rose α-tocopherol in breast muscle (169 and 231%, respectively) compared to Cont. In addition, MDA concentration was reduced by 49 and 44% in groups +E and +ECSe, respectively, in comparison to Cont. The antioxidant capacity of water-soluble antioxidants did not differ between groups, but the concentration of selenium was higher by 91 and 106% in +Se and +ECSe groups, respectively.

Fatty Acid Composition of Breast Muscle

No differences in the FA composition of breast muscle were observed among animals fed the different experimental diets (Table 7).

DISCUSSION

Broilers exposed to a stressful environment, e.g., high temperatures or increased dietary n-3 PUFA, have higher demands for dietary antioxidants as protection from oxidative stress. Numerous studies showed that the sole supplementation with vitamin E (Panda and Cherian, 2014), vitamin C, and selenium (Estévez, 2015) improved the antioxidative status in vivo. Creating n-3 PUFA-enriched products, and, as a consequence, oxidative stress, requires additional antioxidants in the feed (GfE, 1999; Leeson and Summers, 2001). The practical recommendations for broilers (Aviagen, 2009) are already taking into account the basic need for extra supplementation with antioxidants in the diet, regarding the level and type of fat. The present recommendations are 55 to 80 IU of vitamin E/kg (Aviagen, 2014) instead of the 10 IU/kg recommended by NRC (1994). Normally, extra vitamin E varying from 2.5 IU to 3.7 IU per g of PUFA is recommended in the feed (Leeson and Summers 2001; Barroeta, 2007). Thus, we supplemented a basal diet containing 50 IU of vitamin E with an additional 200 IU, as proposed before for similar dietary PUFA contents (Barroeta, 2007; Voljč et al. 2011). For vitamin C and Se, to the best of our knowledge, there are no supplementation recommendations regarding high dietary n-3 PUFA. For vitamin C, the general recommendation for birds reared under stress conditions is 250 to 300 ppm (Leeson and Summers, 2001), which is similar to our supplementation level (200 ppm). Selenium was adjusted to the upper law restriction in the EU (0.5 mg/kg) (European Union, 2004).

Dietary supplementation with combinations of vitamins E and C (Cinar et al., 2014), as well as of vitamin E and selenium (Harsini et al., 2012), was previously used as an effective antioxidant against copper-induced toxicity and heat stress in broilers, respectively. Also, in relation to the oxidative stability of broiler meat, the combination of vitamin C and selenium resulted superior to the sole supplementation (Skrivan et al., 2012). However, no additional benefit was observed in feeding additional Se in combination with high vitamin E (150 IU per kg of diet) on the oxidative stability of frozen stored breast meat from broilers fed high PUFA diets (Konieczka et al., 2015). To the best of our knowledge, the combined use of vitamin E, vitamin C, and selenium has never been tested on the oxidative stress caused by a high n-3 PUFA intake and other possible oxidative stressors.

In the present study, dietary supplementation with individual vitamin E, vitamin C, and selenium did not affect the final body weight of the animals. The

Table 7. Fatty acid profile[1] in breast muscle (g fatty acids/100 g total fatty acids).

	Cont	+E	+C	+Se	+ECSe	SEM	*P*-value
C16:0	18.68	18.47	18.75	18.86	18.71	0.24	0.830
Σ C16:1[2]	2.56	2.69	3.01	2.77	2.79	0.30	0.864
C18:0	8.52	8.19	8.27	8.54	8.66	0.44	0.933
Σ C18:1[2]	24.7	24.4	25.9	24.6	25.6	0.85	0.672
C18:2 n-6	19.76	20.68	19.27	19.63	19.23	0.46	0.213
C18:3 n-3	13.09	12.61	12.20	12.31	11.46	0.78	0.652
C18:4 n-3	0.160	0.165	0.163	0.158	0.155	0.011	0.974
C20:4 n-6	2.71	3.05	2.87	3.12	3.23	0.36	0.841
C20:5 n-3	1.43	1.58	1.61	1.58	1.61	0.14	0.855
C22:4 n-6	0.341	0.420	0.425	0.454	0.469	0.052	0.465
C22:5 n-3	2.07	2.58	2.33	2.45	2.53	0.24	0.579
C22:6 n-3	0.890	0.945	0.851	0.878	1.05	0.110	0.710
SFA	29.8	28.5	28.9	29.5	29.4	0.62	0.622
MUFA	27.8	27.5	29.3	27.8	28.8	1.13	0.744
PUFA	42.2	44.1	41.8	42.9	42.0	0.78	0.281
n-3 PUFA	18.31	18.65	17.94	18.10	17.56	0.41	0.453
n-6 PUFA	24.0	25.4	23.9	24.8	24.4	0.67	0.495
n-6/n-3 PUFA	1.32	1.37	1.33	1.37	1.38	0.06	0.915
LC PUFA	9.24	10.49	10.12	10.47	11.06	1.03	0.774
n-3 LC PUFA	5.12	5.87	5.62	5.68	6.02	0.53	0.778
n-6 LC PUFA	4.13	4.62	4.49	4.79	5.04	0.52	0.765
n-6/n-3 LC PUFA	0.806	0.794	0.788	0.846	0.834	0.034	0.552

[1]Only predominant and nutritionally important fatty acids are listed, but the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) are computed from all fatty acids analyzed.
[2]Sum of all isomers.

combination of antioxidants lowered the ADG and ADFI of the animals from the 21st to the 40th d and from the 1st to the 40th d of the experimental period. The same trend was shown for group E, which does not comply with other trials that showed that vitamin E (Gao et al., 2010) and vitamin C (Sahin et al., 2003a; Lohakare et al., 2005) improved performance traits in broilers. In spite of this, in a trial carried out by Özkan et al. (2007), broiler performance was not affected by the supplementation with vitamin E or selenium. Differently, Konieczka et al. (2015) showed that the combination of vitamin E and selenium negatively affected the growth of broilers fed high n-3 PUFA levels. Overall, in the present study, the combined concentration of antioxidants might have exceeded the physiological requirements, and so the final body weight was negatively affected, as described before (Konieczka et al., 2015). Adversely as it was shown by Bouayed and Bohn (2010), who stated that an excessive consumption of dietary antioxidants in humans could have pro-oxidative effects, no oxidative status parameters in plasma or muscle were affected by the combination of antioxidants.

Vitamin E is one of the most important dietary antioxidants in poultry feed. In our study, the dietary supplementation with α-tocopherol resulted in an increased α-tocopherol concentration in plasma, which is a well-known fact (Morrissey et al., 1997; Voljč et al., 2013). Consequently, the MDA concentration in plasma was inversely correlated with the inclusion of vitamin E in the feed, which is in accordance with results from Voljč et al. (2013), while it effectively protected lipids from peroxidation (Raederstorff et al., 2015). It should be noted that MDA is a relatively unspecific marker of lipid peroxidation, and is largely formed also ex vivo. On the other hand, MDA assay is still one of the best predictors of oxidative stress, since it is normally very well connected to other markers (Lykkesfeldt and Svendsen, 2007). Interestingly, the content of γ-tocopherol in plasma was negatively affected by the inclusion of α-tocopherol in the diet, which is not in agreement with Bottje et al. (1997), who showed that the inclusion of α-tocopherol did not influence the level of γ-tocopherol in tissues of broilers. The same conclusion was drawn by Tomažin et al. (2013). On the other hand, Voljč et al. (2011) showed that dietary α-tocopherol lowered the plasmatic content of γ-tocopherol. Since authors used different sources and concentrations of vitamin E, the reasons for the differences in the absorption and metabolism of tocopherols compared to other studies are difficult to explain. Vitamin E supplementation did not raise the content of ACL in serum. This is a very interesting result, since the content of α-tocopherol in plasma in group +E was higher, and so we believe that there should be another lipid soluble substance that substituted the activity of vitamin E in the blood, but further research is needed to elucidate it. In contrast, Tomažin et al. (2013) showed that the dietary inclusion of 67 mg/kg of RRR-α-tocopherol raised ACL content in plasma 38% in comparison to control.

In the present study, the plasmatic concentration of vitamin C was not affected by any of the supplements used. It is well known that galliform birds synthesize vitamin C in the kidney, but the endogenous synthesis can be limited in young birds. Therefore, it is not known if the dietary supplementation can be beneficial, especially in earlier stages of life (Leeson and Summers, 2001). We assume that pharmacokinetics are constant

in vivo, as it was shown in humans (Padayatty et al., 2004). In line with this, plasmatic vitamin C was not affected by the dietary supplementation. On the other hand, in animals not able to synthesize vitamin C endogenously (guinea pigs), the supplementation with vitamin C did not affect vitamin C or E in plasma and tissues (Keller et al., 2004). It is important to remark that vitamin C supplementation (+C) increased ACL in serum compared to Cont. As it is well known, vitamin C can regenerate vitamin E (Kagan et al., 1992), and therefore higher serum concentrations of vitamin E, and consequently of ACL, could be expected. Furthermore, there was no difference in ACW, although a trend towards a higher value was noticed in group +C. Jakowski et al. (2016) exposed that turkeys fed diets enriched with different antioxidant supplements showed a change in the level of ACW in plasma, and so additional research on this topic should be carried out.

Dietary supplementation with selenium and the combination of antioxidants resulted in an increased GPx activity in serum. This result was expected and in accordance with Chen et al. (2013). The activity of SOD was not affected by the supplements, which agrees with Voljč et al. (2013), who showed that α-tocopherol did not change SOD activity.

The inclusion of α-tocopherol in feeds raised the content of α-tocopherol but lowered that of γ-tocopherol in breast muscle. Also, γ-tocopherol concentration in muscle was affected by the supplementation with α-tocopherol in the same way as in plasma. This could be explained by the faster metabolism of γ-tocopherol when the α-tocopherol intake is increased and by the competition for intestinal transporter proteins, as it is the case in humans (Wolf and Phil, 2006), since several factors affect absorption and metabolism. Results of tocopherol analyses were also in compliance with Voljč et al. (2013), who observed higher α- but lower γ-tocopherol contents in breast muscle of α-tocopherol supplemented broilers. Further research is needed to support the knowledge on the role of tocopherols in vivo, as results showed that γ-tocopherol concentration is lowered by the supplementation with α-tocopherol.

The positive effects of the feed supplementation with vitamin E on the protective effects against lipid oxidation observed in this study were consistent with results of previous experiments (Voljč et al., 2013; Hu et al., 2015). Supplementation with vitamin E and the combination of antioxidants lowered the content of MDA in breast muscle. Moreover, we did not detect differences in the lipid peroxidation measured as MDA in breasts when diets were supplemented with vitamin C and selenium, which is in agreement with previous trials (Pena et al., 2008; Habibian et al., 2016).

In the present study, there were no effects of the dietary antioxidants on the content of ACW in breast muscle, which was unexpected, since vitamin C is a water-soluble vitamin, and so it should contribute to an amplified antioxidant capacity.

Results showed that supplements did not affect the FA composition of breast muscle. Vitamin E is a key lipophilic antioxidant and can contribute to the protection against the peroxidation of PUFA as reported in humans (Raederstorff et al., 2015), and therefore differences in the FA composition were expected. Our results comply with those of Nam et al. (1997) and Tomažin et al. (2013), who reported a lack of effect of dietary vitamin E on the FA composition of breast muscle in broilers fed n-3 PUFA. On the other hand, Haug et al. (2007) showed that high dietary selenium level in broilers fed rapeseed and linseed oil increased the concentration of long chain FA in thigh muscle, which does not agree with our results. The FA composition is variable and could be affected by many internal and external factors, and thus further research on this topic would be of high interest.

To conclude, additional antioxidants such as vitamins E and C, selenium, or their combination, did not influence muscle FA composition, vitamin C in plasma, ACW in serum, or GPx and SOD in whole blood. The inclusion of high amounts of α-tocopherol in diets led to high concentration of α-tocopherol in plasma and breast muscle, and since α-tocopherol protects lipids from oxidation, lower plasma and breast muscle MDA concentrations were detected in comparison to the control group. Overall, the recommendations for the dietary antioxidant content in feed by Ross (Aviagen, 2014) are adequate except for vitamin E. Even though our results showed that the inclusion of additional supplements had no synergistic effect on the assessed parameters, it would be of great interest to carry out further research under different environmental conditions to fully elucidate possible synergistic effects of dietary antioxidants such as vitamin E, vitamin C, and selenium in broilers.

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