Antioxidant status in the blood, liver, and muscle tissue of turkey hens receiving a diet with alfalfa protein concentrate

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ABSTRACT This study aimed to investigate the impact of the oxidative potential of turkeys fed a diet with alfalfa protein concentrate (**APC**), used throughout the rearing period or periodically at 2-wk intervals. The research material consisted of 6-wk-old BIG 6 turkey hens kept in pens, 5 birds per pen in 6 replicates. The experimental factor was the addition of APC to the diet in the amount of 15 or 30 g/kg of diet. APC was administered in 2 ways: birds received a diet with APC throughout the experiment or periodically. In the latter case, the birds received the diet with APC for 2 wk, and then for 2 wk they received the standard diet without APC. Levels of nutrients in the diet; flavonoids, polyphenols, tannins, and saponins in APC; uric acid, creatinine, bilirubin, and some antioxidants in the blood; and enzyme parameters in the blood and tissues of turkeys were determined. The use of APC in the diet stimulated antioxidant processes, which could be seen in the values of the pro-oxidant−antioxidant parameters of the tissues and blood plasma of turkeys. The significant reduction in the H_2O_2 level ($P = 0.042$) and slight reduction in the MDA level ($P = 0.083$), accompanied by an increase in catalase ($P = 0.046$) activity in the turkeys continuously receiving APC in the amount of 30 g/kg of diet, as well as the increase in plasma antioxidant parameters (vitamin C, $P = 0.042$ and FRAP, $P = 0.048$) in these birds, reflects improvement in their antioxidant status. Thus continuous use of the APC supplement in the amount of 30 g/kg of diet proved to be a better feeding practice to optimize oxidative potential than periodic inclusion of APC.

Key words: alfalfa concentrate, antioxidants, turkeys, blood, muscle

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INTRODUCTION

Optimal poultry nutrition is based on the use of diets that are balanced in terms of energy, protein and amino acids, minerals, and vitamins. In recent years, a great deal of research has focused on alternative feed supplements of natural origin (Grashorn, 2010; Gheisar and Kim, 2018). Such supplements may include phytobiotic preparations made from alfalfa (*Medicago sativa*). Alfalfa is considered a fodder plant, but it has medicinal properties, including antioxidant properties (Seguin et al., 2004). It contains many interesting compounds, such as saponins, flavonoids, xanthophylls, tannins, and polyphenols, which may modify the oxidative potential of birds (Sen et al., 1998; Francis et al., 2002; Avato et al., 2006). Polyphenols have also been shown to interact with the metabolism of the skeletal system in humans and animals (Skiba et al., 2021).

Previous studies on pigs (Pietrzak and Grela, 2015, 2016), poultry (Karwowska et al., 2010; Czech et al., 2012; Grela et al., 2020), goats (Szymanowska et al., 2017), lambs (Ognik et al., 2012), fish (Rechulicz et al., 2014), and dairy calves (Mohyeldin, 1983) indicate that alfalfa protein concentrate is an effective ingredient in animal nutrition, improving weight gain, increasing the digestibility of certain nutrients, and reducing excretion of nitrogen in the feces and urine (Grela et al., 2008). Laying hen eggs were shown to have a health-promoting fatty acid profile, more intense yolk color, and a reduced cholesterol level (Grela et al., 2020). Positive production effects and improvement in the quality of animal products suggest that alfalfa protein concentrate (**APC**) supplementation may influence metabolism and

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indicators of the oxidative potential of animal blood and tissues.

The aim of the study was to assess the oxidative potential of turkeys fed a diet with APC, used throughout the rearing period or periodically at 2-wk intervals.

MATERIALS AND METHODS

The experimental procedures used throughout this study were approved by the Second Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland (Resolution no. 8/2013 of 22.01.2013).

Experimental Design

The experimental birds were 6-wk-old BIG 6 turkey hens kept from the age of 6 to 20 wk in pens on straw, 5 birds per pen in 6 replicates (30 birds per group). The experimental factor was the addition of APC to the diet in the amount of 15 or 30 g/kg of diet. APC was administered in 2 ways: birds received a diet with APC throughout the experiment (W) or periodically (P). In the latter case, the birds received the diet with APC for 2 wk (wk 6−8), and then for 2 wk they received the standard diet without APC. The periodic use of APC was dictated by the fact that some phytobiotics have been shown to have more beneficial effects when treatment is interrupted rather than continuous, and when active compounds with biostimulatory properties are administered for too long, they may induce immunosuppression in animals (Grashorn, 2010; Gheisar and Kim, 2018).

The birds were reared in standard conditions (controlled temperature, humidity, and concentrations of gases NH_3 and H_2S).

Animal Diets

During rearing (1−35 d), all birds were fed a commercial diet of corn and soybean meal, formulated to contain adequate levels of all essential nutrients, as recommended by standards (1994). The experiment was begun when the birds reached the age of 6 wk, using complete diets: Grower 1, Grower 2, Finisher 1, and Finisher 2 (Table 1). The turkeys received pelleted feed in accordance with NRC recommendations (NRC, 1994). The experiment included a control group (**C**) and 4 experimental groups: Ew-15 and Ep-15 (15 g ACP per kg of diet administered continuously or periodically) and Ew-30 and Ep-30 (30 g ACP per kg of diet administered continuously or periodically).

The composition of the diets for the animals in the control and experimental groups is given in Table 1. APC was produced by a cooperative from Planchez in the Department of Marne, which is part of the France Luzerne concern. The content of nutrients in 1 kg of APC used in our experiment was as follows: total protein 554 g, lysine 30.5 g. methionine + cystine 15.4 g, crude fat 104 g, calcium 33 g, phosphorus 7.9 g, metabolic

Table 1. Ingredients (g/kg) and nutrient composition of experimental diets for turkeys (air-dry basis).

Feeding period	Grower 2			Grower 3			Finisher 1			Finisher 2		
Feeding weeks	6−9			10−13			14−16			17−20		
Diet	C	E-15	E-30	C	E-15	E-30	C	E-15	E-30	C	E-15	E-30
Wheat	482.5	482.5	482.5	488.6	488.6	488.6	577.7	577.7	577.7	576.7	576.7	576.7
Triticale	0	0	0	100	100	100	100	100	100	150	150	150
Rapeseed cake	50	50	50	60	60	60	90	90	90	100	100	100
Alfalfa protein concentrate (APC)	0	15	30	0	15	30	0	15	30	0	15	30
Soybean meal	370	355	340	265	250	235	150	135	120	100	85	70
Soya oil	50	50	50	40	40	40	40	40	40	37	37	37
Limestone	15	15	15	17	17	17	15	15	15	11	11	11
Dicalcium phosphate	25	25	25	22	22	22	20	20	20	18	18	18
Salt	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Choline chloride, 75%	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Acidifier[1]	2	2	2	2	2	2	2	2	2	2	2	2
Mineral premix[2]	1	1	1	1	1	1	1	1	1	1	1	1
Vitamin premix[3]	1.5	1.5	1.5	1.4	1.4	1.4	1.3	1.3	1.3	1.3	1.3	1.3
Chemical analysis, g/kg												
Dry matter	899.8	902.3	898.4	899.4	901.8	900.8	900.6	903.1	900.2	898.6	900.4	900.6
Crude protein	234.7	235.5	236.5	221.4	219.2	220.1	184.7	185.1	185.7	173.9	174.6	177.7
Crude fiber	19.9	19.8	19.6	20.7	20.5	20.5	21.3	21.2	21.1	22.8	22.6	22.3
Ether extract	50.8	50.9	51.2	51.6	51.7	51.8	53.8	53.9	53.9	51.6	51.8	5.13
Crude ash	59.2	59.5	59.9	58.3	59.1	59.3	55.3	55.6	55.8	54.4	54.6	54.8
Calcium	12.5	12.6	12.6	12.6	12.7	12.7	11.9	11.8	11.9	11.6	11.7	11.7
Available phosphorus	7.21	7.23	7.23	6.41	6.42	6.41	6.27	6.31	6.32	5.49	5.52	5.54
Metabolizable energy, MJ[4]	12.56	12.57	12.57	12.97	12.98	12.98	13.34	13.34	13.35	13.42	13.43	13.43

C: control group; E15 = E_F or E_P-15: diet with 15 g APC in 1 kg; E30 = E_F or E_P-30: diet with 30 g APC in 1 kg.

[1]Acidifier—citric acid, fumaric acid, phosphoric acid (62%).

[2]Mineral premix contained in 1 g: I, 2 mg; Se, 200 μg; Cu, 20 mg; Fe, 50 mg; Mn, 120 mg; Zn, 100 mg; and Ronozyme P (6-phytase − 10 FYT) and Ronozyme WX (1,4-beta-xylanase − 3.5 FXU).

[3]Vitamin premix contained in 1 g: A, 1,000 IU; D3, 3,500 IU; E, 80 mg; K, 3 mg; folic acid, 2 mg; pantothenic acid, 20 mg; nicotinic acid, 50 mg; vitamin B_1, 3 mg; vitamin B_2, 5 mg; vitamin B_6, 5 mg; vitamin B_{12}, 15 μg; biotin, 200 μg.

[4]Metabolizable energy corrected for zero nitrogen balance—ME_N (kcal/kg) = 14.7 × CP + 32.9 × EE + 17.2 × starch + 14.9 × sugars.

energy 17.5 MJ. The detailed content of APC nutrient is given in a previous paper (Grela and Pietrzak, 2014; Pietrzak and Grela, 2015). During the study birds from all groups received complete diets ad libitum and had unlimited access to drinking water.

Experimental Procedures

Blood samples were taken from 6 turkeys (1 per pen with the average initial (8-wk) body weight of birds in the pen) from each group 5 times: at 8, 11, 14, 17, and 20 wk of age. Blood was always collected from the same animals (1 turkey from each pen) under veterinary supervision. For 12 h before blood collection the animals had no access to feed. Blood samples were collected from the wing vein into sterile tubes containing heparin as an anticoagulant for analysis of biochemical and antioxidant status parameters.

At 20 wk of age, 12 birds representing the average body weight per pen and group were selected from each treatment for muscle (pectoral and femoral) and liver sample collection. The birds were then euthanized and their tissues were removed. The skin was removed from the muscles, which were packed into individual sealed plastic bags and kept frozen at −20°C until chemical analysis.

Chemical Analysis

The feed samples were analyzed for contents of basic nutrients according to standard AOAC procedures (AOAC, 2011). Calcium content was determined in an ASA SOLAR 939 UNICAM flame spectrophotometer, and phosphorus content by spectrometry according to AOAC (2011). The content and composition of the main components of the alfalfa protein concentrate were analyzed by HPLC on an HP Agilent 1100 liquid chromatograph coupled with a LC/MSD mass detector (Agilent Technologies, Waldbronn, Germany).

Flavonoids were quantified by a spectrophotometric method described in *Polish Pharmacopoeia VI* (Polish Pharmacopoeia VI, 2003). The percentage content of flavonoids was expressed as quercetin equivalents.

$$X = \frac{A \cdot 0.875}{m}$$

where A is the absorbance of solution and m is the sample weight (g).

Total phenolic content was determined by spectrophotometry using Arnow's reagent according to *Polish Pharmacopoeia VI* (Polish Pharmacopoeia VI, 2003).

Tannin content was determined by spectrophotometry according to the method described in *Polish Pharmacopoeia VI* (Polish Pharmacopoeia VI, 2003), in which the aqueous extract obtained is treated with hide powder, which binds tannins from the solution. The procedure consists of 2 stages: 1) spectrophotometric determination of the aqueous extract with phosphorus-molybdenum-tungsten reagent prior to adsorption on reference hide powder and 2) spectrophotometric determination of the aqueous extract with phosphorus-molybdenum-tungsten reagent after adsorption on standard hide powder.

The L-canavanin content in APC was determined by HPLC as described in (Weissberger et al., 1984), and coumestrol content by HPCE (Moravcova et al., 2002).

The content of selected biochemical parameters in the blood plasma, that is, uric acid, creatinine, and bilirubin, was determined by spectrophotometry using tests from Cormay.

Blood plasma and tissue homogenates from the turkeys were analyzed by spectrophotometry for activity of antioxidant enzymes: superoxide dismutase (**SOD**) by the adrenaline method (Greenwald, 1985), modified for a wavelength of 320 nm (for greater selectivity of transition reaction products), and catalase (**CAT**) according to Bartosz (2004). Total glutathione (**GSH + GSSG**) was determined according to Akerboom and Sies (1981) and Weitzel et al. (1989).

Other antioxidant system parameters analyzed were the total antioxidant potential of the plasma (ferric reducing ability of plasma (**FRAP**)) according to Benzie and Strain (1996) vitamin C content in the plasma and tissue homogenates according to Omaye et al. (1979), and hydrogen peroxide ($\mathbf{H_2O_2}$) according to Gay and Gębicki (2000, 2002).

The plasma and tissue homogenates of the experimental animals were also analyzed for the concentration of the lipid peroxidation product malondialdehyde (**MDA**), the end product of oxidation of lipid tissues, according to Ledwożyw et al. (1986).

Statistical Analysis

All parameters were analyzed statistically, and the significance of differences between means in groups was determined by 2-way analysis of variance for orthogonal data using Tukey's test, with significance levels of 0.05 and 0.01. A 2-way analysis was carried out to assess the significance of the feeding period (F) and APC supplement (D) using the general linear model (**GLM**) procedure in StatSoft software (version Statistica 12.5). The analysis model was as follows:

$$Y_{ijk} = \mu + F_i + D_j + (F\,x\,D)_{ij} + e_{ijk}$$

where F_i is the feeding period ($i = 1, 2$), D_j is the APC supplement ($j = 1, 2, 3, 4$), $(F \times D)_{ij}$ is the interaction between feeding period and APC supplement effects, and e_{ijk} is the residual error.

RESULTS

The use of APC in diets for growing turkeys did not cause significant changes in the chemical composition or energy value of the diets with 15 or 30 g of APC per kg of diet (Table 1). Therefore the diets can be considered to provide equal amounts of energy and protein.

Table 2. Content of flavonoids, polyphenols, tannins, and saponins in alfalfa protein concentrate (APC).

Flavonoids:	g/kg DM
7-*O*-[*β*-D-glucuronopyranosyl(1-2)-*O*-*β*-D-glucuronopyranosyl]-4'-*O*-*β*-D-glucuronopyranoside apigenin	0.102 ± 0.008
4'-*O*-*β*-D-glucuronopyranoside apigenin	0.097 ± 0.011
7-*O*-[*β*-D-glucuronopyranosyl(1-2)-*O*-*β*-D-glucuronopyranoside] apigenin	0.079 ± 0.008
7-*O*-[2-*O*-pheruloylo- *β*-D-glucuronopyranosyl(1-2)-*O*-*β*-D-glucuronopyranosyl]-4'-*O*-*β*-D-glucuronopyranoside apigenin	0.028 ± 0.005
7-*O*-*β*-D-glucuronopiranosyl-4'-*O*-[2'-*O*-p-kumaroyl-o-*β*-D-glukopiranosyl(1-2)-*O*-*β*-D-glucuronopyranoside] apigenin	0.044 ± 0.003
7-*O*-*β*-D-luteolin glucuronopyranoside	0.105 ± 0.009
7-*O*-{2-*O*-pheruloyl-[*β*-D-glucuronopyranosyl(1-3)]-*O*-*β*-D-glukuronopyranosyl(1-2)]-*O*-*β*-D-glukuronopyranoside} apigenin	0.241 ± 0.023
7-*O*-*β*-D-glucuronopyranoside apigenin	0.531 ± 0.019
7-O-{2-O-p-coumaroyl-[*β*-D-glucuronopyranosyl(1-3)]-O-*β*-D-glucuronopyranosyl(1-2)]-O-*β*-D-glucuronopyranoside} apigenin	0.632 ± 0.037
7-O-{2′-O-feruloyl-[*β*-D-glucuronopyranosyl(1-3)]-O-*β*-D-glucuronopyranosyl(1-2)]-O-*β*-D-glucuronopyranoside} chrysoeriol	0.349 ± 0.012
7-O-{2′-O-feruloyl-[*β*-D-glucuronopyranosyl(1-3)]-O-*β*-D-glucuronopyranosyl(1-2)]-O-*β*-D-glucuronopyranoside} tricine	7.95 ± 0.236
Tricin 7-O-*β*-D-glucuronopyranoside	3.06 ± 0.104
7-O-[2′-O-feruloyl-*β*-D-glucuronopyranosyl(1-2)]-O-*β*-D-glucuronopyranoside] tricine	2.08 ± 0.073
Sum of flavonoids	15.29 ± 0.241
Phenolic acids, g/kg DM	11.12 ± 1.23
Tannins, g/kg DM	1.46 ± 0.09
Saponins	g/kg DM
Zanoside tridesmoside	0.93 ± 0.013
3 Glc Glc, 28 Ara Rha Xyl of medicagenic acid	0.67 ± 0.009
3 GlcA, 28 Ara Rha Xyl of medicagenic acid	5.43 ± 0.281
3 Glc, 28 Ara Rha Xyl of medicagenic acid	0.49 ± 0.003
Soyasaponin I	3.45 ± 0.173
Sum of saponins	10.94 ± 0.652
L-canavanine, mg/kg DM	3.16 ± 0.38
Total coumestrol, mg/kg DM	53.2 ± 6.4

In the total flavonoid content (15.3 g/kg DM) the compounds with the highest share were tricine: 7-O-{2′-O-feruloyl-[*β*-D-glucuronopyranosyl(1-3)]-O-*β*-D-glucuronopyranosyl(1-2)]-O-*β*-D-glucuronopyranoside} tricine (7.95 g/kg DM), tricin 7-O-*β*-D-glucuronopyranoside (3.06 g/kg DM), and 7-O-[2′-O-feruloyl-*β*-D-glucuronopyranosyl(1-2)]-O-*β*-D-glucuronopyranoside] tricine (2.08 g/kg DM) (Table 2).

Other biologically active compounds contained in APC included phenolic acids (11.1 g/kg DM) and saponins (10.9 g/kg DM). The level of tannins was lowest (1.46 g/kg DM). The concentration of the nonprotein amino acid L-canavanine in the alfalfa protein concentrate averaged 3.16 mg/kg DM, and the content of total coumestrol in APC averaged 53.2 mg/kg DM (Table 2).

No significant differences were found for the mean from 5 measurements of the content of uric acid, creatinine and bilirubin in the plasma of turkeys receiving a diet with APC throughout the feeding period or periodically (2 wk of diet with APC and 2 wk without APC) (Table 3). The

Table 3. Uric acid, creatinine, and bilirubin blood levels of turkey hens receiving APC in the diet during the whole experiment or periodically.

			Whole experiment		Periodically			*P* value			
Parameter	Age, weeks	C	E_W-15	E_W-30	E_P-15	E_P-30	SEM	F	D	FxE-15	FxE-30
Uric acid, *μ*mol/L	8	206.3^{c}	227.9^{b}	271.5^{a}	216.8^{bc}	227.1^{b}	15.72	0.052	0.041	0.098	0.047
	11	209.8^{b}	215.1^{b}	241.5^{a}	213.1^{b}	203.1^{b}	11.64	0.057	0.048	0.041	0.042
	14	182.3^{c}	202.1^{b}	226.8^{a}	221.5^{a}	221.7^{a}	12.02	0.385	0.035	0.048	0.218
	17	207.7^{b}	233.6^{a}	234.6^{a}	216.2^{b}	218.9^{b}	17.26	0.041	0.052	0.039	0.042
	20	172.3^{c}	276.7^{b}	328.5^{a}	241.3^{b}	307.1^{a}	15.33	0.123	0.013	0.191	0.211
	$\bar{x}$	195.7^{b}	231.1^{ab}	260.6^{a}	221.8^{ab}	235.6^{ab}	8.37	0.154	0.043	0.098	0.103
Creatinine, *μ*mol/L	8	24.15^{c}	28.33^{b}	30.44^{a}	28.82^{b}	28.09^{b}	0.697	0.107	0.029	0.305	0.027
	11	23.62^{c}	28.59^{b}	33.14^{a}	26.81^{bc}	23.66^{c}	0.809	0.035	0.021	0.204	0.028
	14	18.06^{b}	21.50^{ab}	23.62^{a}	20.94^{ab}	22.87^{a}	0.722	0.177	0.096	0.102	0.185
	17	31.05	32.50	33.29	31.92	33.73	1.838	0.225	0.158	0.287	0.262
	20	39.16^{b}	41.06^{ab}	43.37^{a}	40.80^{b}	42.05^{ab}	1.021	0.074	0.088	0.523	0.097
	$\bar{x}$	27.21^{b}	30.40^{ab}	32.77^{a}	29.86^{ab}	30.08^{ab}	0.698	0.102	0.099	0.186	0.076
Bilirubin, *μ*mol/L	8	8.27	7.53	8.28	7.74	8.17	0.311	0.712	0.059	0.521	0.634
	11	9.43^{c}	10.29^{bc}	13.76^{a}	11.18^{b}	11.21^{b}	0.356	0.196	0.047	0.101	0.032
	14	10.06^{b}	11.32^{ab}	12.36^{a}	11.31^{ab}	11.83^{ab}	0.451	0.386	0.088	0.469	0.096
	17	12.74^{b}	13.28^{ab}	14.06^{a}	13.68^{ab}	14.72^{a}	0.395	0.394	0.126	0.418	0.189
	20	11.91^{b}	10.45^{c}	12.80^{b}	10.19^{c}	14.26^{a}	0.388	0.128	0.022	0.308	0.033
	$\bar{x}$	10.48^{b}	10.57^{b}	12.25^{a}	10.82^{b}	12.03^{a}	0.218	0.382	0.039	0.341	0.474
Influence of age on analyzed parameters (*P* value)											
Uric acid		0.112	0.041	0.032	0.097	0.021					
Creatinine		0.039	0.025	0.024	0.025	0.026					
Bilirubin		0.037	0.033	0.031	0.033	0.035					

C: control group; E15 = E_W or E_P -15: diet with 15 g APC in 1 kg; E30 = E_W or E_P -30: diet with 30 g APC in 1 kg; F: feeding period (whole experiment vs. periodically); D: diet with different levels of APC supplementation (control vs. E-15 vs. E-30).

[a,b,c]Results in rows marked with different letters differ statistically significantly at $P \leq 0.05$.Abbreviation: SEM, standard error of means.

values for uric acid and creatinine were significantly higher in the experimental groups than in the controls.

Supplementation with APC throughout the experiment increased the levels of uric acid, creatinine and bilirubin, and the differences between the control group (C) and the group receiving 30 g APC per kg diet (E_w-30) were statistically significant (Table 3). No such relationship was shown for uric acid or creatinine when the ACP supplement was used periodically. The bilirubin content in the blood of turkeys receiving 30 g/kg APC periodically or during the entire rearing period was similar, and it was significantly higher than in the control group and the group receiving 15 g/kg APC. The content of creatinine and bilirubin significantly depended on the age of the turkeys, but it is difficult to demonstrate a clear linear relationship determined by the size of the APC supplement (Table 3). There was also no clear relationship for the uric acid level, especially in the control group and the group receiving 15 g/kg APC periodically.

The amount of added APC (15 or 30 g/kg) and the period of administration (continuous vs. periodical) were not shown to affect the plasma level of GSH + GSSG (Table 4). Only at wk 11 did continuous APC administration significantly ($P = 0.037$) increase GSH + GSSG content compared to periodical administration. However, this parameter was statistically significantly influenced by the age of the turkeys, as it was nearly twice as high at 20 wk of age as at 2 wk after the start of the experiment. The content of vitamin C in the plasma of turkeys depended on the amount of APC in the diet, and the differences between the control (C) and the group receiving 30 g/kg APC (E_w-30 and E_p-30) were statistically significant. Neither the period of administration of the diet supplemented with APC nor the age of the turkeys was shown to affect the level of vitamin C (Table 4). It is worth noting the increase in total antioxidant potential (FRAP) in the plasma, especially in the groups receiving 30 g/kg APC vs. the controls. The FRAP value was not influenced by the age of the birds (Table 4).

The addition of 30 g APC per kg of diet throughout the rearing period increased SOD and CAT activity and reduced the content of H_2O_2, while no such relationships were noted in the case of periodic use of APC (Table 5). The activity of CAT ($P = 0.045-0.065$) and SOD ($P = 0.034-0.043$) decreased as the age of the birds increased, while H_2O_2 content did not differ significantly between ages. The MDA content in the plasma was not significantly determined by the amount of APC in the diet, the period of administration, or the age of the turkeys (Table 5).

Activity of SOD in the muscles and liver of turkey hens (Table 6) was dependent on the amount of added APC and also indicated an interaction of the supplement with the period of administration (continuous vs. periodical). Activity of SOD decreased in both the breast and thigh muscles as the share of APC in the diet increased, irrespective of how the diet was administered, but the reverse was noted for the liver, especially in the case of periodic inclusion of 30 g APC per kg of diet. Activity of CAT was higher in the femoral muscle than in the pectoral muscle and did not depend on the amount of APC in the diet. In the liver, a significant interaction was shown between the amount of APC and periodic inclusion of the additive,

Table 4. Blood levels of some antioxidant parameters of turkey hens receiving APC in the diet during the whole experiment or periodically.

			Whole experiment		Periodically			*P* value			
Parameter	Age, weeks	C	E_W-15	E_W-30	E_P-15	E_P-30	SEM	F	D	FxE-15	FxE-30
GSH + GSSG, μmol/L	8	0.127	0.128	0.124	0.132	0.126	0.007	0.795	0.224	0.342	0.678
	11	0.221^{b}	0.264^{ab}	0.295^{a}	0.244^{b}	0.203^{c}	0.015	0.037	0.185	0.103	0.021
	14	0.262	0.276	0.267	0.282	0.274	0.024	0.603	0.093	0.205	0.316
	17	0.224	0.240	0.279	0.239	0.271	0.018	0.594	0.189	0.503	0.408
	20	0.254	0.251	0.284	0.241	0.281	0.023	0.524	0.102	0.352	0.602
	$\bar{x}$	0.218	0.232	0.250	0.228	0.231	0.015	0.411	0.108	0.298	0.087
Vitamin C, mg/L	8	0.141^{b}	0.164^{ab}	0.195^{a}	0.153^{b}	0.211^{a}	0.010	0.694	0.042	0.325	0.309
	11	0.184	0.188	0.187	0.182	0.192	0.011	0.867	0.296	0.515	0.412
	14	0.186	0.213	0.203	0.210	0.206	0.012	0.892	0.125	0.546	0.687
	17	0.185	0.190	0.196	0.192	0.199	0.009	0.886	0.168	0.714	0.614
	20	0.178^{b}	0.184^{ab}	0.186^{ab}	0.188^{ab}	0.204^{a}	0.011	0.294	0.054	0.687	0.063
	$\bar{x}$	0.175^{b}	0.188^{ab}	0.197^{a}	0.185^{ab}	0.202^{a}	0.009	0.668	0.042	0.642	0.396
FRAP, μmol/L	8	70.58^{c}	72.95^{c}	98.50^{a}	74.05^{c}	84.53^{b}	2.06	0.063	0.014	0.198	0.031
	11	73.71	76.70	79.14	78.62	84.54	2.16	0.191	0.078	0.326	0.108
	14	61.41^{b}	68.69^{ab}	66.63^{ab}	72.47^{a}	76.12^{a}	2.32	0.104	0.062	0.092	0.085
	17	66.01	65.54	64.95	67.50	69.86	2.59	0.204	0.201	0.189	0.107
	20	73.45	72.90	72.12	73.10	77.15	2.14	0.286	0.316	0.246	0.132
	$\bar{x}$	69.03^{b}	71.36^{ab}	76.27^{a}	73.15^{ab}	78.44^{a}	1.42	0.205	0.048	0.186	0.184
Influence of age on parameters (*P* value)											
GSH +GSSG		0.018	0.025	0.021	0.022	0.019					
Vitamin C		0.091	0.076	0.104	0.102	0.197					
FRAP		0.207	0.164	0.058	0.153	0.072					

C: control group; E15 = E_W or E_P-15: diet with 15 g APC in 1 kg; E30 = E_W or E_P-30: diet with 30 g APC in 1 kg; F: feeding period (whole experiment vs. periodically); D: diet with different levels of APC supplementation (control vs. E-15 vs. E-30).

^{a,b,c}Results in rows marked with different letters differ statistically significantly at $P \leq 0.05$.Abbreviations: FRAP, total antioxidant potential of plasma; GSH, the reduced glutathione; GSSG, the oxidized glutathione; SEM, standard error of means.

Table 5. Selected antioxidant enzymes and pro-oxidative blood parameters of turkeys hens receiving APC in the diet during the whole experiment or periodically.

Parameter	Age, weeks	C	Whole experiment		Periodically		SEM	*P* value			
			E_W-15	E_W-30	E_P-15	E_P-30		F	D	FxE-15	FxE-30
SOD, U/mL	8	34.15	35.21	34.5	34.29	34.29	0.452	0.415	0.514	0.283	0.657
	11	27.65^{b}	30.87^{ab}	34.41^{a}	29.68^{ab}	29.68^{ab}	0.514	0.092	0.041	0.261	0.054
	14	22.58$^{\beta}$	24.42^{ab}	27.19^{a}	23.65^{ab}	23.65^{ab}	0.376	0.196	0.058	0.106	0.053
	17	22.54^{b}	24.10^{a}	25.39^{a}	19.45^{c}	18.42^{c}	0.436	0.039	0.104	0.022	0.014
	20	23.21	24.19	24.98	22.65	22.65	0.304	0.094	0.398	0.057	0.062
	$\bar{x}$	26.03^{b}	27.76^{ab}	29.31^{a}	25.94^{b}	25.74^{b}	0.346	0.087	0.118	0.068	0.023
CAT, U/mL	8	5.02^{b}	5.16^{b}	6.36^{a}	5.22^{b}	5.27^{b}	0.139	0.054	0.034	0.243	0.039
	11	4.65^{b}	5.23^{a}	5.12^{a}	4.62^{b}	4.68^{b}	0.129	0.041	0.086	0.026	0.028
	14	4.19	4.23	4.32	4.08	4.18	0.093	0.104	0.162	0.125	0.124
	17	4.60	4.73	4.66	4.53	4.68	0.100	0.289	0.295	0.092	0.461
	20	3.71^{b}	4.28^{ab}	4.53^{a}	4.04^{ab}	4.05^{ab}	0.052	0.068	0.042	0.196	0.092
	$\bar{x}$	4.43^{b}	4.73^{ab}	4.99^{a}	4.50^{b}	4.57^{b}	0.077	0.044	0.046	0.108	0.039
H_2O_2, μmol/L	8	6.85^{a}	6.39^{ab}	5.37^{c}	6.20^{b}	6.15^{b}	0.162	0.106	0.025	0.201	0.001
	11	7.85^{a}	7.51^{a}	6.07^{b}	7.45^{a}	7.41^{a}	0.108	0.102	0.131	0.134	0.001
	14	7.02^{ab}	6.73^{b}	6.52^{b}	7.62^{a}	7.56^{a}	0.226	0.033	0.097	0.031	0.039
	17	7.13^{a}	6.44^{ab}	6.25^{b}	7.12^{a}	7.24^{a}	0.110	0.059	0.144	0.085	0.024
	20	6.92^{a}	6.33^{b}	6.17^{b}	6.43^{b}	6.34^{b}	0.081	0.194	0.042	0.379	0.186
	$\bar{x}$	7.15^{a}	6.68^{ab}	6.08^{b}	6.96^{a}	6.94^{a}	0.067	0.043	0.042	0.101	0.005
MDA, μmol/L	8	1.60^{a}	1.30^{b}	1.29^{b}	1.79^{a}	1.67^{a}	0.049	0.041	0.069	0.041	0.039
	11	1.67	1.48	1.46	1.43	1.36	0.053	0.162	0.076	0.244	0.206
	14	1.54	1.41	1.37	1.40	1.34	0.022	0.283	0.109	0.687	0.305
	17	1.50	1.31	1.29	1.32	1.24	0.027	0.415	0.102	0.637	0.392
	20	1.45	1.37	1.31	1.41	1.37	0.021	0.364	0.204	0.374	0.299
	$\bar{x}$	1.55	1.36	1.34	1.46	1.40	0.018	0.285	0.083	0.272	0.284
Influence of age on parameters (*P* value)											
SOD		0.038	0.041	0.043	0.039	0.034					
CAT		0.046	0.065	0.045	0.054	0.056					
H_2O_2,		0.105	0.096	0.172	0.095	0.102					
MDA		0.123	0.119	0.201	0.094	0.078					

C: control group; E15 = E_W or E_P-15: diet with 15 g APC in 1 kg; E30 = E_W or E_P-30: diet with 30 g APC in 1 kg; F: feeding period (whole experiment vs. periodically); D: diet with different levels of APC supplementation (control vs. E-15 vs. E-30).

[a,b,c]Results in rows marked with different letters differ statistically significantly at $P \leq 0.05$.Abbreviations: CAT, catalase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; SEM, standard error of means; SOD, superoxide dismutase.

with the highest CAT activity shown in group E_P-30 (Table 6). The MDA level showed a tendency to decrease in both muscles and in the liver as the amount of APC in the diet increased, and in the femoral muscle this parameter was significantly the lowest in group E_P-30. The diets with APC increased the level of vitamin C in the muscles and liver, but only when administered continuously. No such relationship was shown in the case of periodic inclusion of APC in the diet, and in the liver there was even a decrease in the level of vitamin C, irrespective of the amount of APC in the diet.

DISCUSSION

Alfalfa protein concentrate, as a source of valuable protein in the amount of 55 to 60% as well as

Table 6. Antioxidant parameters in the tissues of turkey hens receiving APC in the diet during the whole experiment or periodically.

Tissue	Parameter	C	Whole experiment		Periodically		SEM	*P* value			
			E_W-15	E_W-30	E_P-15	E_P-30		F	D	FxE-15	FxE-30
Pectoral muscle	SOD U/mg of protein	9.61^{a}	8.36^{b}	6.94^{c}	7.12^{c}	6.13^{d}	0.204	0.041	0.035	0.036	0.041
	CAT U/mg of protein	6.78	6.62	6.99	6.57	6.66	0.319	0.377	0.429	0.794	0.695
	MDA nmol/mg of protein	0.66	0.55	0.56	0.60	0.61	0.029	0.148	0.105	0.521	0.574
	Vitamin C mg/g of tissue	0.107^{b}	0.119^{ab}	0.132^{a}	0.101^{b}	0.102^{b}	0.005	0.038	0.057	0.084	0.039
Femoral muscle	SOD U/mg of protein	8.54^{a}	8.50^{a}	7.49^{b}	7.45^{b}	7.36^{b}	0.208	0.195	0.048	0.037	0.312
	CAT U/mg of protein	9.25	9.74	10.04	9.47	9.54	0.123	0.236	0.272	0.685	0.114
	MDA nmol/mg of protein	0.64^{a}	0.61^{a}	0.53^{ab}	0.54^{ab}	0.49^{b}	0.022	0.094	0.071	0.108	0.073
	Vitamin C mg/g of tissue	0.182^{b}	0.198^{ab}	0.219^{a}	0.191^{ab}	0.211^{a}	0.011	0.305	0.063	0.363	0.359
Liver	SOD U/mg of protein	10.81^{b}	10.58^{b}	12.33^{ab}	11.22^{b}	13.02^{a}	0.401	0.134	0.043	0.352	0.114
	CAT U/mg of protein	16.73^{b}	16.79^{b}	17.26^{b}	17.60^{ab}	18.32^{a}	0.601	0.108	0.035	0.054	0.034
	MDA nmol/mg of protein	0.28	0.24	0.26	0.25	0.25	0.021	0.895	0.273	0.612	0.638
	Vitamin C mg/g of tissue	0.056^{ab}	0.058^{ab}	0.069^{a}	0.049^{b}	0.048^{b}	0.013	0.036	0.084	0.056	0.021

C: control group; E15 = E_W or E_P-15: diet with 15 g APC in 1 kg; E30 = E_W or E_P-30: diet with 30 g APC in 1 kg; F: feeding period (whole experiment vs. periodically); D: diet with different levels of APC supplementation (control vs. E-15 vs. E-30).

[a,b,c,d]Results in rows marked with different letters differ statistically significantly at $P \leq 0.05$.Abbreviations: CAT, catalase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; SEM, standard error of means; SOD, superoxide dismutase.

xanthophylls in the amount of 1,250 to 2,000 mg/dm^3, some vitamins, and polyunsaturated fatty acids, is a valuable supplement in livestock feed (Grela and Pietrzak, 2014). The content of phenolic acids, saponins and coumestrol in the APC used in this study was similar to the values given for this product by the EFSA (2009). The concentration of the nonprotein amino acid L-canavanine in APC (3.16 mg/kg) is about double the amount in soy flour (2.1 mg/kg), but much lower than that found in other common foods such as lentil flour (2,800 mg/kg) (EFSA, 2009).

Experiments in ruminants (Ognik et al., 2012; Szymanowska et al., 2017), and pigs (Pietrzak and Grela, 2015, 2016) have shown that diet supplementation with APC beneficially influences growth performance and animal health. The positive effects on animal health are attributed not only to the presence of protein of high biological value, but also to numerous bioactive substances, such as vitamins (A, B_1, B_6, B_{12}, C, E, and K), provitamin A (β-carotene), flavonoids, omega-3 fatty acids, and mineral compounds. In the present study, many polyphenolic compounds were identified in APC, especially flavonoids, but also saponins, which have immunomodulatory (Liu et al., 2011) and antioxidant (Aziz et al., 2006) properties. The antioxidant activity of flavonoids depends on the order of the functional groups in the structure of the compound. Compounds containing hydroxyl groups, which include 7-O-{2′-O-feruloyl-[β-D-glucuronopyranosyl(1-3)]-O-β-D-glucuronopyranosyl (1-2)]-O-β-D-glucuronopyranoside} tricine and tricin 7-O-β-D-glucuronopyranoside, exhibit a range of antioxidant properties, including scavenging of free oxygen radicals and metal chelation (Pandey et al., 2012). The positive stimulatory effect of flavonoids on the antioxidant system has been demonstrated by North et al. (2019) as well as in the present study. The significantly higher ($P \leq 0.05$) SOD activity in the blood of turkeys from the group continuously receiving a 30 g/kg APC supplement in comparison to the control group, and the similar relationship in the case of catalase, may indicate that APC stimulates antioxidant processes, and in this way protects against reactive oxygen species. These components not only can damage the structure of proteins, lipids, and DNA, but can also cause gastrointestinal disorders (Aviello and Knaus, 2017).

The above-mentioned assumptions about the antioxidant properties of APC administered continuously in the amount of 30 g/kg are also explained by the fact that it reduced lipid peroxidation products, that is, H_2O_2 ($P < 0.05$) and MDA ($P > 0.05$), relative to the control. A similar reaction was not observed in the birds receiving a diet with ACP periodically (E_P-30). The reduction in lipid peroxidation products may have been linked to the above-mentioned presence of flavonoids. According to Harborne and Williams (2000) the antioxidant properties of flavonoids are manifested in part in their ability to inactivate oxygen radicals. Radicals that are most easily scavenged by flavonoids include superoxide anion radical, hydroxyl radical, singlet oxygen, and lipid radicals (Harborne and Williams, 2000). The reaction of flavonoids with hydroxyl radical, which reacts easily with aromatic compounds and is a product of the decomposition of H_2O_2, is particularly efficient. This makes it possible to inhibit enzymes associated with free radical formation, such as lipooxygenase, cyclooxygenase, and xanthine oxygenase. This is confirmed by Sychrová et al. (2022) who showed that the presence of flavonoids in the diet may enhance the functioning of the antioxidant system, resulting in a reduction in MDA and LOOH, which are biomarkers of endogenous lipid peroxidation and radical-induced damage. Supported by exogenous antioxidants, the endogenous antioxidant system can compensate for excessive production of lipid peroxides and protect against the consequences of oxidative stress.

The antioxidant properties of flavonoids have also been confirmed in vitro. Consumption of flavonoids has been shown to increase the body's antioxidant capacity. This is manifested primarily as an increase in the activity of antioxidant enzymes (SOD, glutathione peroxidase, and catalase), observed mainly in the liver, small intestine, and lungs, and an increase in the concentrations of low-molecular-weight antioxidants (ascorbate and α-tocopherol) (Middleton et al., 2000). Studies on the use of other flavonoid extracts have confirmed the antioxidant and immunostimulatory effects of flavonoids from alfalfa (Dong et al., 2007; Chen et al., 2016; North et al., 2019). Therefore it seems that the combination of flavonoids present in alfalfa is particularly beneficial, and further research specifically analyzing the effects of these main flavonoid components, individually and in combination, would be interesting.

The average increase in FRAP ($P < 0.05$) induced by the diet with 30 g/kg APC, administered continuously or periodically, coincided with higher plasma concentrations of uric acid, creatinine, bilirubin, and vitamin C (in the first 2-wk period of the experiment). This is because the increase in the concentration of low-molecular-weight antioxidants was an important factor determining the total antioxidant potential of the plasma and thus the ability to scavenge free radicals. According to Benzie and Strain (1996), the content of low-molecular-weight antioxidants contributing to the FRAP value, such as uric acid, vitamin C, and bilirubin, like the activity of the antioxidant enzyme SOD, indicates the beneficial effect of APC in antioxidant processes.

Beneficial effects of APC on antioxidant processes were demonstrated in an earlier study on lambs (Ognik et al., 2012). Lambs fed a diet with APC in the amount of 15 g/kg or 30 g/kg showed an increase in total antioxidant potential (FRAP) and lower catalase and SOD activity in the plasma (Ognik et al., 2012). Similar effects, in the form of increased antioxidant levels in the plasma and a decrease in the level of lipid oxidation products, were observed in poultry receiving plant extracts, containing mainly polyphenols such as flavonoids, tannins, and phenolic acids, which was confirmed in research on chickens and turkeys (Ognik et al., 2016). A positive effect on antioxidant status in the form of increased total antioxidant capacity was noted following

the addition of protein-xanthophyll concentrate (**PX**) from alfalfa to diets for turkeys (Czech et al., 2012). According to Xie et al. (2008), the proteins in alfalfa leaves are able to donate electrons or hydrogen, thereby neutralizing superoxide, hydroxyl radicals and 1,1-diphenyl-2-picrylhydrazyl radical.

Stimulation of antioxidant processes in turkeys receiving 30 g/kg APC may also have been influenced by the presence of saponins. Many studies indicate that saponins, in addition to reducing cholesterol levels, also stimulate the immune response and exert antioxidant effects, which makes them a functional nutrient (Smith and Dilger, 2018; Ku et al., 2020; Kim et al., 2021; Zaynab et al., 2021). This applies in particular to soya saponin, whose content in the analyzed APC is about 3.45 g/kg DM. It can reduce the time needed for harmful substances to come into contact with the mesentery of the small intestine, thus accelerating absorption of harmful substances, effectively reducing their toxicity (Smith and Dilger, 2018; Tian et al., 2018), and thereby removing potential radical-forming agents. In addition, by acting as antioxidants saponins inhibit cell damage caused by free radicals (Ganesan and Xiu, 2017). Soya-saponins may also reduce the rate of DNA mutation (Tin et al., 2007) and enhance the activity of killer cells (Xu et al., 2016; Fuchs et al., 2017; Kim et al., 2021).

Due to their high content of polyunsaturated fatty acids and high concentration of free iron, turkey meat and tissues are highly susceptible to oxidation (Mercier et al., 2001). Therefore products that slow down oxidation processes are highly beneficial. Many studies indicate that the scavenging activity of FRAP and DPPH has a tendency to increase in response to increased supplementation with flavonoids (Sohaib et al., 2022).

The reduced concentration of MDA in the femoral muscle of turkeys receiving diets periodically supplemented with 30 g/kg APC in comparison to the control group may be linked to the presence of flavonoids. Ognik et al. (2016) reported that the use of various flavonoid compounds, such as hesperidin, diosmin, quercetin, or resveratrol, reduces redox potential in muscle tissue (mainly in the breast muscle). Jiang et al. (2007) also found that supplementation with isoflavones increases the activity of several antioxidant enzymes, including SOD and catalase, which was confirmed in our experiment. Activity of SOD was significantly higher in the breast and thigh muscles and in the liver of turkeys receiving diets with 30 g/kg APC continuously in comparison with the control group. This may also be attributed to the presence of vitamin C, whose content in the breast muscle and liver was significantly higher in the group of birds whose diet contained 30 g/kg APC than in the control group. Vitamin C has antioxidant properties which effectively support antioxidant processes in the body and can support flavonoids in reactions scavenging free oxygen radicals. An increase in the oxidative stability of the muscle tissue in chickens receiving various levels of polyphenolic compounds (catechins) extracted from green tea was noted by Tang et al. (2001). According to Cao et al. (2012), administration of *Ginkgo biloba* did not affect the activity of SOD or glutathione peroxidase, despite a reduced level of MDA. Similar observations were made by Karwowska et al. (2010), who found that the addition of alfalfa concentrate to turkey diets did not play a significant antioxidant role in the raw meat.

CONCLUSIONS

The use of APC in the diet stimulated antioxidant processes, which could be seen in the values of the prooxidant−antioxidant parameters of the tissues and blood plasma of turkeys. The significant reduction in the H_2O_2 level and slight reduction in the MDA level, accompanied by an increase in CAT activity in the turkeys continuously receiving APC in the amount of 30 g/kg of diet, as well as the slight increase in the plasma antioxidant potential (FRAP) in these birds, reflects improvement in their antioxidant status. Thus continuous use of the APC supplement in the amount of 30 g/kg of diet proved to be a better feeding practice than periodic inclusion of APC. However, there is a need for more focused research to fully explain the mechanisms by which flavonoids exert antioxidant effects in the meat of birds.

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DISCLOSURES

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in the present study.

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