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Effects of humic acid diet on the serum biochemistry and oxidative status markers in pheasants

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Abstract: This study investigated the effect of different concentrations of humic acids (HAs) on the selected serum biochemistry parameters and oxidative status markers in common pheasants (*Phasianus colchicus*). The control birds were fed a diet with no HA additives, while the birds in the experimental groups were fed diets containing HAs at the level of 0.5% (EG1), 0.75% (EG2) and 1.0% (EG3) from 1 to 90 days of age. The blood sodium concentration decreased ($P < 0.01$) in the group fed by 1.00% HAs compared to the control birds. The concentration of potassium in the EG2 group increased ($P < 0.05$) in comparison to the control group. The EG1 group showed a higher ($P < 0.01$) serum glucose than the EG2 group. Significant differences ($P < 0.05$; $P < 0.01$) were also found between the experimental groups (EG1 *vs* EG2, and EG2 *vs* EG3) in the cholesterol concentrations. The birds in the experimental groups showed lower ROS (reactive oxygen species) and MDA (malondialdehyde) production. An opposite effect was observed in the TAC (total antioxidant capacity), where its values significantly increased in the experimental groups. The diet supplementation affected the enzymatic antioxidant system of the fattened pheasants, and so the HAs exhibited an antioxidant potential in these birds.

Keywords: food additives; *Phasianus colchicus*; blood serum indicators; oxidative stress; antioxidant enzymes

Humic acids (HAs) are natural organic compounds resulting from the chemical and biological decomposition of organic matter and the synthetic activity of microorganisms. Nowadays, HAs are mainly used in agriculture, industry, environmental protection and medicine (Ayuso et al. 1997; Valuev et al. 2003; Mikkelsen 2005; Pena-Mendez et al. 2005; Efimova et al. 2012; Mvila et al. 2016). In the agricultural sector, HAs have a significant impact on the soil quality and productivity, physical properties, moisture con-

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tent and fertility (Stevenson 1994; Jones and Bryan 1998). An important role of HAs lies in their ability to adsorb pollutants from the environment, such as water, soil and sludge (Khan 1972; Tang et al. 2014; Genc-Fuhrman et al. 2016; Rath et al. 2019). HAs are currently used in veterinary medicine because of their protective action on the intestinal mucosa as well as their antiphlogistic, adsorptive, antitoxic and antimicrobial activity. As such, HAs represent an alternative for the treatment of diarrhoea, dyspepsia and acute intoxication (EMEA 1999).

The high economic interest encourages producers to use alternative types of food additives in farm animal production which, however, need to be tested first (Vizzari et al. 2014; Andrejcakova et al. 2016; Russo et al. 2019; Shah et al. 2020). Studies on the use of HAs in livestock nutrition have confirmed their positive effects on animal growth, yield and reproduction (Ji et al. 2006; Agazzi et al. 2007; Cusack 2008; Wang et al. 2008; Gasparovic et al. 2017; Sladecek et al. 2018). The beneficial effects of HAs on selected production indicators, such as the increased gains, feed conversion, egg production, egg weight and the decreased mortality have been confirmed in poultry (Kocabagli et al. 2002; Yoruk et al. 2004; Kucukersan et al. 2005; Avci et al. 2007; Ozturk et al. 2010; Samudovska and Demeterova 2010; Ozturk et al. 2012; Supriyati et al. 2015; Arpasova et al. 2016; Lala et al. 2016).

Serum biochemistry and oxidative status markers are important for the interpretation of the physiology or health status in living organisms. Based on the lack of a comprehensive evidence on the behaviour of HAs *in vivo*, the present study was designed to address two aims: (1) to investigate the effect of different concentrations of HAs in the diet on the selected serum biochemistry indicators as a reflection of the overall health status, and (2) to study the effect of HAs on the oxidative status markers in pheasants.

MATERIAL AND METHODS

Experimental design and animal management

The experiment was performed on pheasants (*Phasianus colchicus*) (n = 200). The pheasants were divided into four groups (control and experimental groups – EG1, EG2, EG3; n = 50 in each group). The experiment lasted 90 days and it started with one-day-old birds and finished with ninety-day-old birds. The birds were housed in pens on deep litter. Each group was housed separately with the same controlled temperature and light conditions until they were 42 days old. Thereafter, the birds were acclimated and housed in the outer partially covered aviaries from 49 days of age. They were fed a standard complete feed mixture for pheasant fattening. The feeding period lasted until they were 90 days old. The birds of the EG1, EG2 and EG3 groups received HAs (dry matter content of 85%; the humic acid content in the dry matter, a min. of 62 %, of that, a min. 48% of free humic acids; the fulvonic acids in the dry matter, a min. of 9%; the minerals in the dry matter, a min. of 9%) added to the feed and mixed thoroughly in different concentrations (EG1 – 0.5%; EG2 – 0.75%; EG3 – 1.00% HAs). The control group received a diet without any additives. The diets were also different from Day 1 till Day 35 of the feeding (starter) and from Day 36 till Day 90 of the feeding (grower). The ingredients and nutrient composition of the diets are shown in Table 1. The feed and water were provided *ad libitum*. The pheasants were healthy, and their condition was judged as good during the experiment. The conditions of animal care, manipulations and use corresponded with the instructions of the Ethics Committee of the Slovak University of Agriculture in Nitra, Protocol No. 48/2013.

Blood and serum sampling

After the end of the experiment, the birds (5 males and 5 females from each group) were euthanised by decapitation in the fixation equipment and blood samples were collected. No additive tubes were used for the biomarker tests. The blood samples were allowed to coagulate in tubes. The blood serum was separated from the coagulum by centrifugation at 1 006 *g* for 20 min and the obtained serum was stored at –20 °C until further analyses at the Department of Animal Physiology (SUA in Nitra).

Serum biochemistry analyses

The serum concentrations of the analysed indicators (calcium – Ca, phosphorus – P, magnesium – Mg, total protein – TP, glucose, aspartate aminotransferase – AST, alanine aminotransferase – ALT,

https://doi.org/10.17221/174/2019-VETMED

Table 1. The diet composition of the feed mixture: starter (day 0–35) and grower (day 36–90)

Ingredients	Starter	Grower
Crude protein (%)	29.0	24.7
Crude fat (%)	1.6	1.9
Crude fibre (%)	3.9	3.3
Ash matter (%)	7.3	9.6
Lysine (%)	1.4	1.0
Methionine (%)	0.6	0.5
Nitrogen free extract (%)	58.2	60.5
Organic matter (%)	92.7	90.4
Crude Starch (%)	35.8	37.0
Crude saccharides (%)	6.3	6.9
Calcium (%)	1.5	0.9
Phosphorus (%)	0.87	0.66
Sodium (%)	0.15	0.14
Magnesium (g/kg)	3.5	3.9
Potassium (g/kg)	14.7	13.0
Copper (mg/kg)	30.0	18.0
Iron (mg/kg)	80.0	48.0
Manganese (mg/kg)	121.0	72.0
Zinc (mg/kg)	141.0	84.0
Selenium (mg/kg)	0.60	0.40
Vitamin A (IU/kg)	20 101.00	12 060.00
Vitamin D_3 (IU/kg)	5 025.00	3 015.00
Vitamin E (α-tokoferol) (mg/kg)	50.00	30.00

alkaline phosphatase – ALP, cholesterol, triacylglycerol – TG, bilirubin – Bili, and creatine kinase – CK) were measured using DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) commercial kits and the Randox RX Monza (Randox Laboratories, Crumlin, UK) semi-automated chemistry analyser. The other minerals (sodium – Na, potassium – K, and chloride ions – Cl^-) were analysed using an EasyLyte Analyzer (Medica, Bedford, MA, USA) with an ion-selective electrode (Massanyi et al. 2014; Kovacik et al. 2017). The albumin (Alb) concentration was measured using an ALB BioLa Test (PLIVA-Lachema, Brno, Czech Republic) commercial kit and a Genesys 10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) spectrophotometer (Kovacik et al. 2019). The concentration of the serum globulin (Glob) was calculated by subtracting the serum albumin values from the total protein values. The albumin to globulin ratio was calculated using the formula:

$$A/G\ ratio = Albumin/(Total\ protein - Albumin) \quad (1)$$

Measurements of oxidative status markers

The reactive oxygen species (ROS) production in each sample was measured by the chemiluminescence assay based on a luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) probe (Kashou et al. 2013) using a Glomax Multi$^+$ Combined Spectro-Fluoro-Luminometer (Promega Corporation, Madison, WI, USA) (Tvrda et al. 2016). The results of the ROS are expressed as RLU/s/g TP (relative light units/second per g of total protein). The total antioxidant capacity (TAC) of the samples was measured using an improved chemiluminescence antioxidant assay which utilises the horseradish peroxidase conjugate and luminol (Muller et al. 2013). The chemiluminescence was quantified on 96-well plates in ten consecutive one-minute long cycles using the Glomax Multi$^+$ Combined Spectro-Fluoro-Luminometer (Promega Corporation, Madison, WI, USA) (Kovacik et al. 2019). The results are expressed as Eq. μmol Trolox/g TP (Eq. micromoles of Trolox per gram of total protein).

The superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured using the Randox commercial kits (Randox Laboratories, Crumlin, UK) and the Randox RX Monza (Randox Laboratories, Crumlin, UK) semi-automated chemistry analyser (Tvrda et al. 2016). The results are expressed as IU/g TP (units per gram of total protein).

The catalase (CAT) enzymatic activity was measured according to Beers and Sizer (1952) by tracking the decrease of hydrogen peroxide (H_2O_2) at 240 nm (Tvrda et al. 2016). The obtained values are expressed as IU/mg TP (international units per milligram of total protein). The lipid peroxidation (LPO) was measured with the help of the TBARS (Thiobarbituric Acid Reactive Substances) assay, modified for a 96-well plate and ELISA (enzyme-linked immunosorbent assay) reader (Tvrda et al. 2016). The lipid peroxidation was expressed through the malondialdehyde (MDA) production. The MDA concentration is expressed as μmol/g TP (micromoles per gram of total protein).

Statistical analyses

The obtained data were subjected to statistical analysis using the GraphPad Prism v6.01 (GraphPad Software, Inc., San Diego, California,

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USA) and STATGRAPHICS Centurion© (StatPoint Technologies, Inc., Warrenton, VA, USA). The data were checked for normality using a D'Agostino & Pearson omnibus normality test before the statistical analyses.

The effect of the humic acids on the serum biochemistry and oxidative status markers was analysed using the analyses of variance (ANOVA) followed by Tukey's multiple comparison test (the means ± standard deviations are reported). The data for the serum chemistry and oxidative status parameters in the blood of the pheasants were analysed using a factorial ANOVA, with the effect of the diet and sex as the fixed effects. The results of the analyses were considered significant at $P < 0.05$; $P < 0.01$ and $P < 0.001$.

RESULTS

Mineral profile of pheasant blood serum

The data collected from the assessment of serum minerals (Ca, P, Mg, Na, K, and Cl^-) are shown in Table 2. The concentration of Na in the EG3 group was significantly lower ($P < 0.01$) in comparison to the Control group. There were also significant ($P < 0.05$) changes observed in the K concentration between the control group and the EG2 group. On the other hand, no significant differences in the other mineral (Ca, P, Mg, and Cl^-) concentrations occurred in the serum samples between the treated groups. However, the experimental groups (EG1, EG2, EG3) tended to have higher Ca and P concentrations.

Energetic profile of pheasant blood serum

The changes in the energetic profile of the pheasants following the experimental treatments are presented in Table 3. Compared to the control pheasants, no significant differences were found in the experimental groups.

However, the EG1 group exhibited significantly higher ($P < 0.01$) glucose concentrations than the EG2 group. Significant differences ($P < 0.05$; $P < 0.01$) were also observed in the cholesterol concentrations between the experimental groups (EG1 *vs* EG2, and EG2 *vs* EG3). No significant changes were found in the serum TG concentrations ($P > 0.05$).

Table 2. The effect of humic acids on the serum mineral profile of the pheasants. The results are presented as the Mean ± SD

Indicator	Diet			
	Control	EG1	EG2	EG3
Calcium (mmol/l)	2.41 ± 0.15	2.46 ± 0.04	2.52 ± 0.11	2.51 ± 0.08
Phosphorus (mmol/l)	1.89 ± 0.25	2.05 ± 0.33	2.13 ± 0.17	2.03 ± 0.24
Magnesium (mmol/l)	1.00 ± 0.11	1.12 ± 0.25	0.98 ± 0.08	0.96 ± 0.14
Sodium (mmol/l)	159.4 ± 2.81^{A}	157.3 ± 2.23	156.4 ± 2.02	155.3 ± 1.73^{B}
Potassium (mmol/l)	3.34 ± 0.72^{a}	4.29 ± 0.89	4.57 ± 0.53^{b}	4.26 ± 0.87
Chlorides (mmol/l)	119.4 ± 3.29	119.5 ± 2.03	118.2 ± 1.96	117.6 ± 2.09

^{a,b}The means within a row with different superscript letters differ significantly ($P < 0.05$); ^{A,B}The means within a row with different superscript letters differ significantly ($P < 0.01$)

Table 3. The effect of humic acids on the energetic profile of the pheasants. The results are presented as the Mean ± SD

Indicator	Diet			
	Control	EG1	EG2	EG3
Glucose (mmol/l)	17.58 ± 0.68	18.35 ± 0.58^{A}	17.12 ± 0.55^{B}	17.42 ± 0.89
Cholesterol (mmol/l)	3.34 ± 0.53	3.03 ± 0.26^{a}	$3.86 \pm 0.51^{b,A}$	2.80 ± 0.72^{B}
Triacylglycerol (mmol/l)	1.02 ± 0.40	1.24 ± 0.47	0.98 ± 0.51	0.74 ± 0.45

^{a,b}The means within a row with different superscript letters differ significantly ($P < 0.05$); ^{A,B}The means within a row with different superscript letters differ significantly ($P < 0.01$)

https://doi.org/10.17221/174/2019-VETMED

Nitrogenous and hepatic profile of pheasant blood serum

The activity of the hepatic enzymes (AST, ALT, and ALP), creatine kinase and concentrations of the total protein, albumin, globulin, and bilirubin in the experimental groups are summarised in Table 4. No significant differences in these variables were observed between the dietary treated groups. Yet, the non-significant reduction of the TP, globulin, ALT, and CK, as well as the non-significant increase of the A/G ratio, AST, ALP (except EG3 group), and bilirubin were found in the experimental groups.

Oxidative status markers

The HA effects on the blood oxidative status markers are presented in Figure 1. The pheasants receiving HAs were characterised by a lower dose-dependent production of reactive oxygen species/ROS in comparison to the control birds. An opposite effect was observed in the total antioxidant capacity/TAC. Its values were significantly increased with the increasing HA levels in the diet. The effect of the supplementation on the enzymatic antioxidant system (SOD, CAT, GPx) was also confirmed. Along with the experimental diet, a significant impact of the HAs on the

Table 4. The effect of humic acids on the nitrogenous and hepatic profile of the pheasants. The results are presented as the Mean ± SD

Indicator	Diet			
	Control	EG1	EG2	EG3
Total protein (g/l)	35.08 ± 1.99	32.12 ± 3.42	34.75 ± 3.59	34.86 ± 3.19
Albumin (g/l)	15.63 ± 0.47	14.80 ± 1.27	15.54 ± 1.59	16.75 ± 1.96
Globulin (g/l)	19.46 ± 1.49	17.32 ± 2.85	19.21 ± 3.10	18.11 ± 2.64
A/G ratio	0.81 ± 0.09	0.87 ± 0.16	0.83 ± 0.16	0.94 ± 0.19
AST (μkat/l)	7.56 ± 0.80	8.08 ± 1.88	7.61 ± 0.74	7.67 ± 0.98
ALT (μkat/l)	0.18 ± 0.05	0.17 ± 0.05	0.16 ± 0.03	0.15 ± 0.02
ALP (μkat/l)	30.85 ± 6.51	31.30 ± 5.36	31.15 ± 5.92	30 46 ± 6.09
Bilirubin (μmol/ l)	21.88 ± 0.60	22.51 ± 0.97	22.58 ± 0.98	22.89 ± 0.85
Creatine kinase (μkat/l)	40.83 ± 9.36	29.86 ± 15.46	28.16 ± 7.97	32.48 ± 12.93

A/G = albumin/globulins ratio; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase

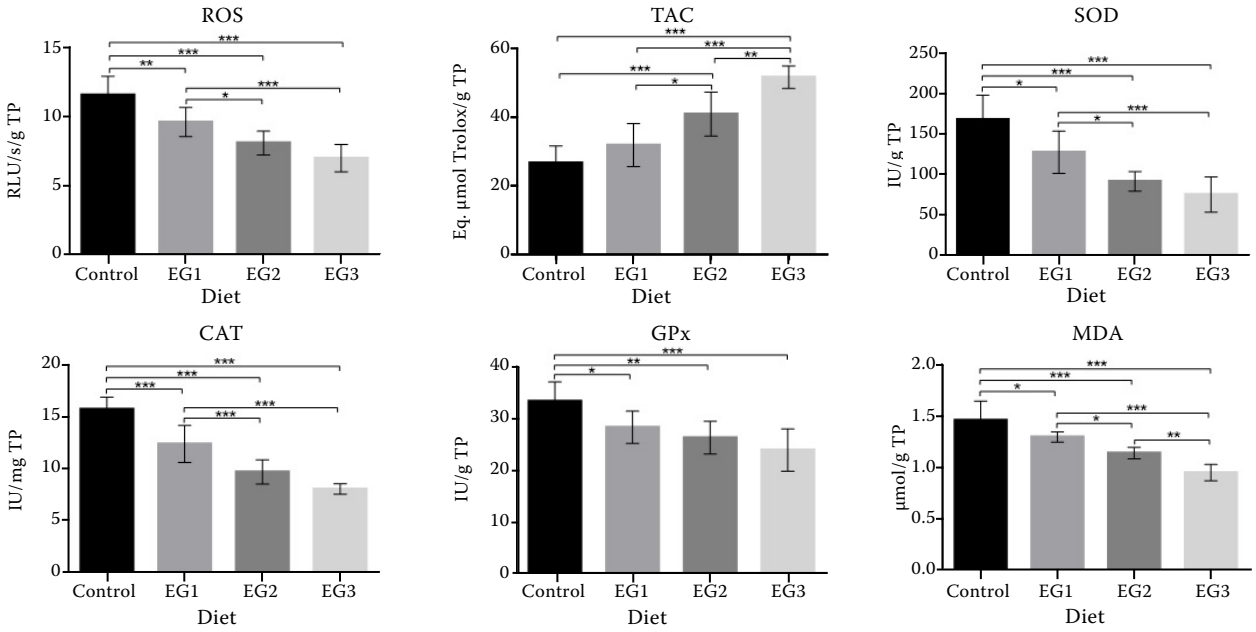

Figure 1. The oxidative status markers in the blood serum of the pheasants. The results are presented as the Mean ± SD. Significantly different between groups at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

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MDA (malondialdehyde) production as a biomarker of the lipid peroxidation was found. The results (*P*-values) of the factorial ANOVA for the monitored biomarkers depending on the sex and diet are shown in Table 5. The comparison between the male and female pheasants revealed that the content of Mg, K, Cl^-, Chol, TG, ALP and bilirubin were significantly affected. The results of the factorial ANOVA also confirmed a non-significant sex × diet effect.

DISCUSSION

HAs have been tested in animal nutrition for several years. The main objectives of the previous studies evaluated the effects of HAs on the growth performance and feed conversion (Kocabagli et al. 2002), carcass traits (Karaoglu et al. 2004), meat quality (Wang et al. 2008; Ozturk et al. 2010) or mortality (Yoruk et al. 2004) in different species of livestock and/or poultry. The findings of these studies partly confirmed the positive effect of HAs on the growth and production traits. However, studies focused on animal health while achieving economic interests by using different feed mixes are relatively sparse.

The results obtained from the present study reveal interactions between the dietary HA supplementation and the physiological/health status of the pheasants.

Table 5. The results of the factorial ANOVA for the serum chemistry and oxidative status markers in relation to the sex; the results are presented as the Mean ± SD

Biomarker	Sex		Significance	
	male	female	sex × diet	sex
Calcium (mmol/l)	2.47 ± 0.13	2.47 ± 0.08	ns	ns
Phosphorus (mmol/l)	1.97 ± 0.18	2.08 ± 0.31	ns	ns
Magnesium (mmol/l)	0.95 ± 0.08	1.08 ± 0.19	ns	*
Sodium (mmol/l)	156.60 ± 2.20	157.50 ± 2.99	ns	ns
Potassium (mmol/l)	3.68 ± 0.74	4.54 ± 0.78	ns	***
Chlorides (mmol/l)	117.30 ± 2.11	120.10 ± 1.95	ns	***
Glucose (mmol/l)	17.61 ± 0.70	17.63 ± 0.92	ns	ns
Cholesterol (mmol/l)	3.50 ± 0.53	3.02 ± 0.67	ns	*
Triacylglycerol (mmol/l)	0.82 ± 0.45	1.17 ± 0.45	ns	*
Total protein (g/l)	33.62 ± 3.12	34.78 ± 3.27	ns	ns
Albumin (g/l)	15.76 ± 1.66	15.59 ± 1.67	ns	ns
Globulin (g/l)	17.86 ± 2.79	19.19 ± 2.33	ns	ns
A/G ratio	0.91 ± 0.19	0.82 ± 0.11	ns	ns
AST (μkat/l)	7.32 ± 0.89	8.14 ± 1.26	ns	ns
ALT (μkat/l)	0.15 ± 0.03	0.18 ± 0.05	ns	ns
ALP (μkat/l)	33.00 ± 4.51	28.88 ± 6.14	ns	*
Bilirubin (μmol/l)	22.15 ± 0.53	22.78 ± 1.08	ns	*
Creatine kinase (μkat/l)	36.38 ± 11.36	29.28 ± 12.44	ns	ns
ROS (RLU/sec/g TP)	9.21 ± 2.02	8.94 ± 2.08	ns	ns
TAC (Eq. μmol Trolox/g TP)	38.06 ± 11.00	37.38 ± 11.12	ns	ns
SOD (IU/g TP)	120.00 ± 39.90	110.70 ± 45.75	ns	ns
CAT (IU/mg TP)	11.21 ± 3.41	11.77 ± 3.04	ns	ns
GPx (IU/g TP)	27.91 ± 5.33	27.97 ± 4.54	ns	ns
MDA (μmol/g TP)	1.24 ± 0.23	1.19 ± 0.21	ns	ns

A/G = albumin/globulins ratio; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; CAT = catalase; GPx = glutathione peroxidase; MDA = malondialdehyde; ROS = reactive oxygen species; SOD = superoxide dismutase; TAC = total antioxidant capacity

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

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Mineral profile indicators

The concentrations of two mineral indicators (Na and K) were significantly affected by the HAs. In general, a decrease of Na and an increase of K were observed. Sodium fulfils the basic physiological functions such as the maintenance of the acid-base balance, it is responsible for determining the extracellular fluid volume and optimal osmotic pressure, it also participates in muscle cell contractions and adrenal gland functions. Potassium is associated with the maintenance of the acid-base balance, optimal osmotic balance, and with the activation of a range of intracellular enzymes, it participates in the protein and carbohydrate metabolism, normal heart function and cell membrane permeability (Balos et al. 2016; Doneley 2016; Samour 2016). Avci et al. (2007) did not find any effect of HAs on the mineral profile of Japanese quails except for an increased Ca content. Rath et al. (2006) tested HAs in the diets of broiler chickens. In their experiment, a significant decrease in the Ca and P concentrations in the blood serum was observed, contrary to the present study. The Ca concentration increase could be considered as a positive effect for their roles in the bone metabolism and structure (Ozturk et al. 2012). On the other hand, Samudovska and Demeterova (2010) reported a potential effect of HAs depending on the length of administration in broiler chickens. Higher concentrations of Ca and lower concentrations of P occurred after 14 days when compared to the control group, and lower concentrations of Ca and higher concentrations of P were observed after 35 days in the experimental groups in comparison to the control. Overall, we may consider the effects of HAs on the mineral profile to be positive, as the detected mineral concentrations are comparable with reference ranges in previous studies (Nazifi et al. 2012; Dzikamunhenga et al. 2017). Similar to the present study, no significant effect of sex on the Ca and P concentrations was reported by Nazifi et al. (2012) who studied 20-week-old male and female ring-necked pheasants. The serum Mg, K and Cl^- concentrations measured in the present study were significantly higher in the females.

Energetic profile indicators

Avci et al. (2007) reported increasing (n.s.) concentrations of all the energetic profile indicators in quails. In the present study, increased concentrations of glucose and TG in the EG1 group, and cholesterol levels in the EG2 group in comparison to the control group were observed. The decreased glucose concentrations in the EG3 group (1.00% of HAs) are comparable with previous studies in broiler chickens (Rath et al. 2006; Ozturk et al. 2012). The glucose concentrations in the present study were within the normal range in birds (Lumeji 1997; Forbes and Guzman 2017) and comparable with the values reported by Lloyd and Gibson (2006) and Dzikamunhenga et al. (2017) in pheasants, but higher than the values reported by Kececi and Col (2011) and Nazifi et al. (2012). Blood glucose elevations occur commonly due to stress or after the feed intake and, occasionally, diabetes mellitus occurs. The changed cholesterol and TG concentrations in the birds may accompany physiological or pathological conditions (Harris 2009), but it is relatively complicated to associate their concentrations with normal or abnormal findings, as they have been only recently recognised as of value in avian medicine (Doneley 2016). TG is the main lipid reserve in bird organisms (Lumeji 1997) and cholesterol is associated with liver functions/diseases, high fat diets or meat quality (as an economic trait). A non-significant increase of cholesterol and TG in quails due to HAs was described by Avci et al. (2007), which partly corresponds with our data (cholesterol increasing in the EG2 group and TG increasing in the EG1 group). On the other hand, a non-significant increase of the TG was reported by Ozturk et al. (2012), contrary to a decrease in the cholesterol in all the experimental groups of broilers (diet supplemented with humic substances). Kececi and Col (2011) found a significant effect of the sex on the TG level and a non-significant sex-related effect on the cholesterol and glucose in pheasants of different ages. Similarly, no significant effect of the sex on the glucose level was reported by Nazifi et al. (2012). No sex-related effect on the glucose concentrations was observed in the present study. The concentrations and significant differences of the cholesterol in the present study correspond to the values reported in the pheasants by Nazifi et al. (2012).

Nitrogenous and hepatic profile indicators

The concentrations and activities of the selected nitrogenous, hepatic indicators and enzymes

https://doi.org/10.17221/174/2019-VETMED

were not modified by the HA supplementation in the pheasants in the present study. Jerabek et al. (2018) stated that the plasma TP concentrations of birds are only 50% of the mammalian TP concentration. In broilers, HAs added to the feed caused a significant decrease in the TP and albumin concentrations (Rath et al. 2006). In our study, only slight and non-significant changes in these values were observed. Contrary, an increase of such indicators following a 14-day administration and a decrease after 35 days were reported by Samudovska and Demeterova (2010). Furthermore, a decrease of CK and ALT is comparable with Rath et al. (2006), however, the activities of ALP showed an opposite tendency in the present study. The overall content of the TP in the blood serum was comparable with the previous studies (Lloyd and Gibson 2006; Kececi and Col 2011; Nazifi et al. 2012), but higher than the values presented by Avci et al. (2007). The levels of bilirubin were relatively higher than in the previous studies. Comparable with the present study, the sex-related effect on the ALP (2.058 IU/l in the male and 1.310 IU/l in the female) and the total bilirubin (8.03 µmol/l in the male and 15.73 µmol/l in the female) concentrations was reported by Nazifi et al. (2012), in pheasants (20 weeks old). Even lower values of bilirubin (3.59 µmol/l) were presented by Dzikamunhenga et al. (2017) in 6-week-old Chinese ring-neck pheasants. Bilirubin is considered to be a marker of the hepatic function; however, birds lack biliverdin reductase, thus, the secretion of biliverdin is higher than bilirubin (Lumeij and Westerhof 1987; Denbow 2015). The A/G ratio values increased in the experimental groups, which could be considered as a positive effect of the HAs, whereas a decrease in the ratio of albumin to globulins (a decreased A/G ratio) often indicates liver diseases and acute or chronic inflammatory conditions (Kawai 1973; Lumeij 1987). The values of the CK obtained in the present study were relatively low when compared to the values obtained by Dzikamunhenga et al. (2017) and Kececi and Col (2011) in pheasants. Overall, the decreasing CK activity corresponded to the study performed on broilers (Rath et al. 2006). In general, the CK is responsible for muscle disorders and myocardial infarction (Neumeier and Jockers-Wretou 1981). The plasma CK activity increases with age and could reflect tissue damage (Hocking et al. 1998) or hyperthermia and can affect the acid/base balance (Sandercock et al. 2001). Its decrease could be considered as a positive effect of the HAs on the health status.

Oxidative status/stress indicators

In medical disciplines, much attention is dedicated to natural sources of bioactive substances with antioxidant properties and their potential contribution to the development of new bioavailable drugs. The presence of active acidic groups (–COOH, –OH) in the HA macromolecules suggests their capability for antioxidant action. This capability has been linked to humic acids from different natural sources (Valuev et al. 2003; Efimova et al. 2012; Smirnova et al. 2012). In the present study, both the antioxidant enzymes and endogenous antioxidants were affected by the HAs. Moreover, the ROS production decreased with an increasing HA concentration in the diet and consequently the MDA production was significantly lower. Mammalian cells possess a family of antioxidant enzymes (first line of antioxidant defence), SOD, that convert harmful superoxide anion to hydrogen peroxide, which, in turn, is metabolised to water and oxygen by CAT and GPx (Afonso et al. 2007; Simos et al. 2012). These indicators (SOD, CAT, GPx) were significantly lower in all the experimental groups when compared with the control group which could be attributed to the chemical structure of the HAs. The quinone moieties of the HA may be the electron-accepting groups, with the resultant hydroquinones donating electrons to an ultimate electron acceptor such as iron (III) (Lovley et al. 1996). On the other hand, the TAC was significantly higher in all the experimental groups in comparison to the control group, which is why we may suppose that the HAs could increase the concentrations of the endogenous antioxidants in an animal's blood resulting in a stronger antioxidant power of the organism. The antioxidant capacity of the biological fluids can be assessed by their aggregate, cumulative action and synergic effect. This is known as the TAC (Fingerova et al. 2007). Ozkan et al. (2015) confirmed the significant antioxidant properties of HAs in rats by reducing the oxidative stress markers and enhancing the positive antioxidant effect in a cerebral ischemia. Akbas et al. (2015) reported that HAs reduced the degree of damage to the kidney and reduced the TOS (total oxidant status) with respect to the stimulated TAC

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values in the case of induced ischemia and reperfusion in rats. On the other hand, Ipek et al. (2008) did not recommend high amounts (600 mg/kg) of HAs in the diet because of an increasing oxidative stress status (decline in the TAC) in Japanese quails. However, the TAC was not affected at doses of 360 mg/kg to 480 mg/kg of HAs in their study. Nevertheless, detailed published studies on the antioxidant properties of HAs are lacking and other studies are necessary to evaluate the antioxidant potential of these compounds.

In conclusion, it could be stated that humic acids are used worldwide in numerous areas of interest. The present study demonstrated that HAs do affect the serum chemistry indicators. An important outcome of our study is the fact that the HAs exhibit an antioxidant potential. When compared to the control group, the HA enriched diet increased the concentrations of the endogenous antioxidants (TAC). Moreover, the ROS production significantly decreased with the increasing HA concentration in the diet and consequently the MDA production was significantly lower in the animal's blood.

Conflict of interest

The authors declare no conflict of interest.

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