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THE INFLUENCE OF DIETARY REPLACEMENT OF SOYBEAN MEAL WITH HIGH-TANNIN FABA BEANS ON GUT-BONE AXIS AND METABOLIC RESPONSE IN BROILER CHICKENS*

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Abstract

Faba bean (FB) seeds can be a good protein-energy component in animal feed. However, the presence of anti-nutritional substances is a negative feature of FB seeds. The aim of this study was to examine the influence of different levels of unprocessed FB seeds in feed on the gut-bone axis and metabolic profile in broilers. Ninety six, 1-day-old Ross 308 broiler chickens were randomly selected to one of the 3 dietary treatments (32 chickens in each, divided into 8 pens with 4 birds per each pen): the control group fed standard diet with soybean meal and without FB seeds, group I fed 8/15% (starter/grower) of high-tannin FB seeds, and group II fed 16/22% of high-tannin FB seeds. Bone mechanical examination, hematological and serum biochemical analysis as well histomorphometry of small intestine and liver tissue were performed. The intake of high-tannin FB seeds, irrespective of their amount, did not alter the bone geometric, mechanical and densitometric parameters nor influenced basal hematological parameters, however it resulted in: decreased serum concentration of total cholesterol and calcium; a reduced longitudinal myenteron of small intestine; increased mucosa and villus epithelium thickness, villus length, thickness and absorptive surface in duodenum; increased number of active crypts in jejunum; unchanged collagen area, intercellular space, and total cell number in the liver; decreased number of multinuclear hepatocyte cells. Moreover, the livers of birds fed the higher dose of high-tannin FB seeds had lymphocytic infiltrates in portal tracts and sinusoids. Feeding of unprocessed high-tannin FB seeds exerted an influence on the gastrointestinal tract by increased absorptive surface. In conclusion, the dietary inclusion of unprocessed high-tannin FB seeds had no negative effects on broiler growth, tibial bone mechanical properties and intestinal characteristics. Unprocessed high-tannin FB seeds may be used in broiler diets, but their dietary levels should not be higher than those discussed.

Key words: broiler chickens, high-tannin faba bean, intestine histomorphometry, liver tissue, bones

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The use of soybean meal, especially from genetically modified plants, prompts a search for other protein sources that could be alternatively introduced into animal diet (Bederska-Łojewska et al., 2017). Moreover, Europe's unfavorable climate to cultivate soy gives an additional reason for finding an alternative protein sources for imported soy. Since faba bean (FB) seeds (*Vicia faba* L. *minor*) have a favorable chemical composition, they can be a good protein-energy component in animal feed. They are characterized by high protein content (26–30%) and a high concentration of starch (~40%), and can therefore be useful in monogastric animal feeding, including poultry (Tufarelli and Laudadio, 2015; Przywitowski et al., 2016). On the other hand, the presence of anti-nutritional substances like non-starch polysaccharides, alkaloids, lectins, phytic acid, saponins, enzyme inhibitors, and condensed polyphenols (tannins) limits the absorption of other nutrients. The content of tannin in seeds of commonly cultivated FB varieties ranges from 5 to 10 g/kg of dry matter, although there are also tannin-free varieties (Vilariño et al., 2009; Crépon et al., 2010; Shams Shargh and Ahani Azari, 2010). The role of tannins as enzyme inhibitors of digestive enzymes (i.e. trypsin, α-amylase, and lipase) through the formation of a tannin-enzyme (protein) complex has been well investigated and documented in *in vitro* studies (Vilariño et al., 2009). Thus, the presence of tannins in the diet is related to numerous nutritional problems and has many negative effects on a range of animal species. They cause a reduction in feed consumption, nitrogen and protein digestibility, growth, and egg weight in chickens (Longstaff and McNab, 1991). Tannins could also alter the bone formation process as they exert an adverse effect on mineral availability and absorption of minerals, including Ca, P, Na, Mg, Fe and K (Houshmand et al., 2015). Other effects include hepatotoxicity, toxic nephrosis and damage of intestinal mucosa (Bilić-Šobot et al., 2016). Furthermore, lectins present in FB seeds influence immune function in gut. They reduce the production of gut hormones, interfere with microbiome in the gut lumen, and disturb digestive processes by binding to receptors of epithelial cells of the intestinal mucosa (King et al., 1983; Gatel, 1994). Moreover, FB seeds contain about 73–99 g/kg of crude fiber, mostly non-starch polysaccharides (Crépon et al., 2010). Dietary fiber plays a significant role in transit time of chyme, increasing the incidence of enteric disorders and influencing the secretion of gastric and pancreatic juices (Gdala, 1998; Jiménez-Moreno et al., 2009). The amount of dietary fiber affects the total production of short chain fatty acids, including butyrate, which have important implications for metabolism and the structure and function of intestinal epithelial cells. On the other hand, dietary fiber, including that from FB seeds, can increase the loss of minerals, e.g. Zn, Ca, Mg, Fe, and Cu (Gdala, 1998). Moreover, chickens fed a diet containing a high level of dietary fiber show lower serum concentrations of triglycerides and phospholipids, as well as total cholesterol, triglycerides, and phospholipids in the high-density lipoprotein fraction (Eder et al., 1996).

Considering the presence of numerous anti-nutritive factors in FB seeds, it is expected that diet introducing unprocessed high-tannin FB seeds could negatively affect gut structure and bone development. Numerous studies have been performed to check the feeding value of FB for poultry, but the influence on the gut-bone axis has not yet been described.

The aim of the study was to examine the effects of the replacement of soybean meal with FB seeds with a high content of tannins on the gut-bone axis. Serum lipidogram and other biochemical parameters were taken as the criteria in assessment of the effects on metabolism. Possible damaging effects of unprocessed high-tannin FB seeds were monitored by determination of the activity of selected indicator enzymes (ALAT and AspAT). To our knowledge, there is no information on the influence of diets containing unprocessed high-tannin FB seeds on gut and bone development as well as physiological and histological consequences in broiler chickens.

Material and methods

The experimental procedures used throughout this study were approved by the Local Ethics Committee on Animal Experimentation in Lublin, Poland.

Experimental design, birds and diets

A total of 96 1-day-old Ross 308 broiler chickens with initial average body weight of 49.6±0.26 g were used in the study. The birds were randomly selected to one of the 3 dietary treatments, 32 chickens in each, divided into 8 pens with 4 birds per pen. All birds were held in battery cages under standard rearing conditions. Air temperature was set at the optimal level depending on the birds' age. Initial temperature (33°C) was reduced by 2°C weekly, until the final value of 24°C. The birds had a constant access to fresh water and were fed *ad libitum* in accordance with the stage of the production (Table 1).

Table 1. Feed ingredients and nutritive value of the diet

Item	Starter			Grower		
	control	group I	group II	control	group I	group II
1	2	3	4	5	6	7
Ingredient (%)						
Maize	49.92	44.59	39.37	55.29	45.24	41.22
Faba bean seeds	-	8.00	16.00	-	15.00	22.00
Soybean meal	41.50	38.00	34.50	36.00	29.50	26.00
Rapeseed oil	4.70	5.50	6.20	5.00	6.50	7.00
L-lysine	-	-	-	0.02	0.02	0.02
DL-methionine	0.22	0.24	0.26	0.19	0.23	0.24
Limestone	1.30	1.33	1.36	1.31	1.35	1.37
Monocalcium phosphate	1.50	1.48	1.45	1.32	1.28	1.27
Salt	0.36	0.36	0.36	0.37	0.38	0.38
Premix*	0.50	0.50	0.50	0.50	0.50	0.50
The nutritional value of mixtures						
[b] ME (MJ/kg DM)	12.77	12.77	12.75	13.08	13.08	13.05
[a] Crude protein (g/kg)	223	223	223	203	203	203
[a] Crude fiber (g/kg)	26.01	29.40	32.81	25.32	31.68	34.64
[a] Lysine (g/kg)	12.91	13.11	13.32	11.70	12.09	12.16
[a] Methionine (g/kg)	5.78	5.82	5.87	5.28	5.29	5.29

Table 1 – contd.

1	2	3	4	5	6	7
[a] Methionine + cysteine (g/kg)	9.73	9.67	9.61	8.95	8.77	8.66
[a] Threonine (g/kg)	9.19	9.11	9.03	8.36	8.21	8.08
[a] Tryptophan (g/kg)	2.92	2.85	2.78	2.62	2.49	2.41
[a] Ca (g/kg)	9.79	9.82	9.82	9.26	9.25	9.24
[a] P (g/kg)	6.99	7.04	7.07	6.42	6.52	6.56
[b] P bioavailable (g/kg)	4.56	4.56	4.54	4.05	4.06	4.05
[a] Na (g/kg)	1.67	1.65	1.64	1.70	1.69	1.69

* – 1 kg of mixture contained (starter/grower) vitamins: A – 13500/10000 IU, D_3 – 10000/3000 IU, E – 80/50 mg, K – 4/3, mg, B_1 – 3/2 mg, B_2 – 8.75/7 mg, B_6 – 5/4 mg, B_{12} – 24/27.5 µg, PP – 70/70 mg, B_5 – 25/14 mg, B_9 – 2.00/1.50 mg, H – 0.20/0.15 mg, B_t – 500/500 mg; microelements: Fe – 80/80 mg, Mn – 100/100 mg, Zn – 80/60 mg, Cu – 9/8 mg, I – 1.25/1.00 mg, Se – 0.275/0.25 mg, Co – 0.30/0.25 mg, Ca – 1.311/1.28g.

[a] – analyzed values.

[b] – calculated values.

There were three experimental diets in the experiment: the control group was fed a standard mixture based on soybean meal and without FB seeds, group I was fed a mixture containing 8%/15% (starter/grower) of high-tannin FB seeds (*cv*. Granit, with flowers displaying a large black spot on the wings, and a seeds cover of beige-brown colour), and group II was fed a mixture containing 16/22% of high-tannin FB seeds in the starter/grower mixtures, respectively. All diets within the feeding phases were isonitrogenous and isoenergetic. The FB seeds were crushed into about 4 mm pieces using a shredding machine and mixed with the other components of the fodder and given to birds in loose form.

During the whole study period, chickens were weighed weekly, while feed consumption was monitored daily. These data were used to calculate the body weight gain, feed intake, and feed conversion ratio.

At the end of the experiment, 8 birds randomly selected (1 bird form each pen) from each group were weighed and slaughtered. Ten hours before the slaughter, birds were fasted, and had only free access to water.

Feed analysis

The nutrient composition of the diets as well as content of crude protein and fiber in FB seeds were analyzed using standard methods. Dry matter (DM) was measured using oven-drying AOAC 930.15 method (AOAC, 2011). Crude protein content was determined on the basis of the nitrogen content according to the Kjeldahl method. Crude fiber content was determined on the basis of weight method using sulphuric acid and potassium hydroxide (AOAC, 2011).

The Ca content in the feed samples was determined using the FAAS technique (AOAC, 2011) while the total phosphorus was determined colorimetrically with molybdovanadate reagent and the optical density was measured in a spectrophotometer at 430 nm (AOAC, 2011). P bioavailable was calculated basing on non-phytate P (NPP) and phytate P, and it was assumed that NPP, together with P from non-plant sources are completely available to poultry (NRC, 1994).

The composition of amino acids was determined by ion-exchange chromatography. However, cysteine and methionine were oxidized to cysteic acid and methionine sulphone respectively prior to hydrolysis. Oxidation was performed at 0°C with a performic acid/phenol mixture. Excess oxidation reagent was decomposed with sodium disulphite. Then, the oxidized and unoxidized samples were hydrolyzed with hydrochloric acid for 23 hours. The hydrolysate was adjusted to pH 2.20. The amino acids were separated by ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline). For the determination of tryptophan, the samples were hydrolyzed under alkaline conditions with saturated barium hydroxide solution and heated to 110°C for 20 hours and determined by HPLC with fluorescence detection.

The content of anti-nutritional factors in FB seeds was analyzed with the colorimetric method for tannins (BN-90/9160-42, 1990) and the method described by Kakade et al. (1974) for trypsin inhibitors. The FB seeds contained 88.35% of dry matter (DM), which contained 24.71% of crude protein, 6.82% of crude fiber, 1.02% of tannins, and 0.15% of trypsin inhibitors.

Metabolizable energy values (MJ/kg DM) were calculated by Fisher and McNab (1987) method.

Hematological and serum biochemical analyses

Blood samples were collected carefully for hematological and blood serum biochemical analyses. After clotting at room temperature, the samples were immediately centrifuged and serum was frozen at –80°C for further analysis. Immediately after collecting from each chicken, other blood samples were transferred to a glass tube containing EDTA and mixed. The assay of the blood samples was carried out in the laboratory immediately after the blood was collected. Manual counting of total red and white blood cells was carried out. The hemoglobin concentration (Hb) and packed cell volume (PCV) were determined on a hematological analyzer ABACUS Junior Vet (Diatron, Vienna, Austria).

Total protein, glucose, uric acid, creatinine, lipid profile (total cholesterol, LDL – low density lipoproteins, HDL – high density lipoproteins and TG – triglycerides), aspartate aminotransferase (AspAT), alanine aminotransferase (ALAT), amylase (AMY), lactate dehydrogenase (LDH), P, Ca, Fe were determined with the colorimetric method using a biochemical analyzer (Mindray BS-120, Bio-Medical Electronics Co., Ltd, Shenzhen, China) and sets of ready-made biochemical reagent kits (Alpha Diagnostics, Warsaw, Poland).

Tissue collection and histomorphometrical analysis

Intestinal tract analysis

Two 10-mm-long segments of the intestine from the duodenum (1 cm distal to the pylorus) and 50% of the total length of jejunum were taken from each bird, then opened along the mesenteric border, cleaned in PhS and pinned flat without stretching on histological cassettes (Tomaszewska et al., 2012 a). The liver samples were collected from the same lobe from each bird. All samples were fixed in 4% buffered formaldehyde (pH 7.0) for 24 h, dehydrated in graded ethanol solutions, then fixed

and embedded in paraffin in a tissue processor (STP 120, Waltham, MA, USA). Further, 4 μm thick tissue samples, cut with a microtome (Microm HM 360, Microm, Walldorf, Germany), were subjected to histology as described previously (Śliwa et al., 2005; Tomaszewska et al. 2012 a, 2014). Masson's trichrome staining was used to differentiate the small intestine wall layers (Suvara et al., 2013).

A microscope (AXIOVERT 200M, Carl Zeiss, Jena, Germany) equipped with a CCD camera (AxioCam HRc; Carl Zeiss, Jena, Germany) was used to collect microscopic images of the different intestinal structures. The structure of the liver tissue and small intestine wall was examined using graphic analysis software (Olympus cellSens Version 1.5; Olympus, Tokyo, Japan) (Dobrowolski et al., 2012; Tomaszewska et al., 2017 a).

The following morphometric parameters in the intestine were analyzed: the thickness of mucosa, submucosa, and myenteron (longitudinal and transversal lamina); villus epithelium thickness, crypt depth (the invagination between adjacent villi from the bottom of the crypt to the base of villi); crypt width (measured in the middle of the crypt depth); the crypts number (active, showing mitoses and having an open internal space and access to the intestinal lumen; inactive, showing no mitoses and Paneth cells, having a closed internal space; total, inactive plus active crypts); villus length (from the tip of the villi to the villous-crypt junction); villus thickness (measured in the middle of villus height); the number of villi; small intestine absorptive surface (Tomaszewska et al., 2017 a). Only vertically oriented villi and crypts were analyzed.

Microscopic observations allowed identifying and assessing normal structures such as portal triads and terminal hepatic venules, necessary for the evaluation of the lobular architecture as well as lobular architecture and small hepatocytes (as characteristic of regeneration). Moreover, following parameters were measured: total number of cells per mm^2; total hepatocyte number per mm^2; non-hepatocyte cells number per mm^2; hepatocyte nuclei number per mm^2; number of mononuclear hepatocytes per mm^2; and multinucleated hepatocytes number per mm^2 (Śliwa et al., 2009; Tomaszewska et al., 2015 a).

To localize Meissner and Auerbach plexuses immunohistochemical reaction with mouse monoclonal antibody to 200-kD neurofilament heavy subunit neuronal marker (Sigma-Aldrich, St. Louis, MO, USA, dilution 1:40) was performed. After deparaffinizing and rehydration, antigen retrieval was achieved by boiling sections of duodenum and jejunum in the citrate buffer (pH 6.0) in a microwave oven (3×5 min, 700 W). Then, the samples were incubated for 30 minutes in mixture of a 3% hydrogen peroxide and methanol (1:1) to block endogenous peroxidase activity. After blocking in normal serum, the sections were incubated with the first antibody at 4ºC overnight, then incubated (30 min) with the second antibody: biotinylated rabbit polyclonal to mouse immunoglobulin (Sigma-Aldrich, St. Louis, MO, USA, dilution 1:200) with streptavidin/HRP (DacoCytomation, Glostrup, Denmark, dilution 1:300) and developed in a 3,3'-diaminobenzidine tetrahydrochloride (DAB) (DacoCytomation, Glostrup, Denmark) for 15 minutes at room temperature. Negative control sections for immunohistochemical staining were prepared by omitting the primary antibody. Finally, all the samples were counterstained with Mayer's hema-

toxylin. Microscopic images of immunohistochemistry reactions were subjected to further analysis and regarding neurofilament detection the following variables were analyzed: the cross section area of the nerve ganglion; sphericity, perimeter, mean Feret diameter, and the minimal and mean diameter of the ganglion. The Feret diameter was defined as the distance between the two parallel planes restricting the object perpendicular to that direction (Tomaszewska et al., 2015 b).

Bone analysis

The tibiae from individual chickens were dissected, cleaned from the remnants, weighed, wrapped in gauze soaked in isotonic saline and kept frozen (–25°C) until further examination. The bone mechanical properties were determined after 4 hours thawing at room temperature using the three-point bending test of bone midshaft on a universal testing machine (Zwick Z010, Zwick GmbH & Company KG, Ulm, Germany). The bone was loaded in the anterior-posterior plane with a displacement rate of 10 mm/min until fracture (Tomaszewska et al., 2016 a). During the test, the applied force and the resulting bone deflection were registered continuously. After the bending test, external and internal diameters of the mid-diaphyseal cross-section (both in medial-lateral and anterior-posterior plane) were measured with a digital caliper. The geometric parameters (cortical cross section area, cortical index, and the vertical cortical index, the cross-sectional moment of inertia and radius of gyration) were calculated as described previously (Muszyński et al., 2017, 2018 a). The determined mechanical properties were as follow: the yield strength, bending moment, elastic stress, elastic strain, elastic energy, Young modulus, the ultimate strength, work to fracture, toughness modulus and ultimate strain (Tomaszewska et al., 2017 b; Muszyński et al., 2018 b). All mechanical properties were determined from the force-displacement curves using the Origin software (OriginLab, Northampton, MA, USA).

After evaluating the geometric properties, the bones were subjected to the measurement of bone mineral density and bone mineral content (Blicharski et al., 2017). The analysis was performed using a DXA densitometer (Discovery W, Hologic Inc., Marlborough, MS, USA) for the whole bone and separately for the middle section of the midshaft and distal and proximal parts, including both trabecular and cortical bone compartments. After the measurements of mineral content the bones were defatted in ethanol and acetone mixture (1:1) and dried for 24 h at 105°C. The bone tissue density of dried midshaft fragments of bone was measured using the gas pycnometer (AccuPyc 1330, Micromeritics, Nocross, GA, USA) as described previously (Tomaszewska et al., 2015 c).

Finally, bones were mineralized in a muffle furnace at 500°C (Tomaszewska et al., 2017 c), the bone ash percentage and the ash weight to bone volume ratio were calculated. The content of main mineral components (Ca, P) in bone was determined by atomic absorption spectrometry (Solar 939, Unicam, Kassel, Germany) (AOAC, 2011). The bone ash and Ca, P content were expressed as a percentage of the crude ash.

Statistical analysis

Data were analyzed using Statistica 12 software (StatSoft, Inc., Tulsa, OK, USA). The distribution of the variables was tested for normality using the Shapiro-Wilk's

test, equality of variance was tested by the Brown-Forsythe test. Then, data were analyzed by one-way ANOVA followed by Tukey's HSD *post-hoc* test. For all tests, $P<0.05$ was considered statistically significant.

Results

Growth traits of broiler chickens and liver weight

Body weight, body weight gain, total feed intake, and total feed conversion ratio were not affected by the dietary treatment at the end of individual production stages (starter and grower) as well as at the end of the whole study (Table 2).

Table 2. Body weight and rearing results of broiler chickens

Item	Control	Group I	Group II
Final body weight, 35 day (g)	1925±57	1876±37	1864±129
Body weight gain, starter period, 1–21 days (g)	733±36	730±44	716±29
Body weight gain, grower period, 22–35 days (g)	1142±55	1116±23	1102±99
Total body weight gain, 1–35 days (g)	1876±57	1826±37	1814±129
Mean daily feed intake, starter period, 1–21 days (g)	49±2	48±4	48±3
Mean daily feed intake, grower period, 22–35 days (g)	139±6	135±8	135±10
Total feed intake, 1–35 days (g)	2965±75	2904±15	2893±87
Feed conversion ratio, starter period, 1–21 days ($g/kg_{b.w.}$)	1410±44	1400±23	1432±46
Feed conversion ratio, grower period, 22–35 days ($g/kg_{b.w.}$)	1705±39	1736±32	1699±42
Total feed conversion ratio, 1–35 days ($g/kg_{b.w.}$)	1582±54	1591±29	1599±81

Data given are mean ± SD.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

The birds fed diet containing the higher amount of high-tannin FB seeds (group II) had heavier livers compared to the control group ($P<0.01$). However, the relative liver weight (liver weight/body weight*100%) of birds from group II did not differ from the ratios noted in the control and group I (Table 3).

Table 3. Effect of faba beans on the absolute and relative weight of liver obtained from broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Absolute weight (g)	32.78±3.11 a	36.55±3.54 ab	38.93±2.44 b
Relative weight (%)	1.70±0.32	1.95±0.28	2.08±0.29

Data given are mean ± SD, a, b – mean values in rows with different letters differ significantly at $P<0.05$.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

Hematological parameters and blood serum biochemistry

There was no effect of high-tannin FB seeds, irrespective of their level in the diet, on the basal hematological parameters (Table 4).

Table 4. Effect of faba beans seeds on basal hematological parameters in blood obtained from broiler chicken at the age of 35 days

Item	Control	Group I	Group II
RBC (10^{12}/L)	1.84±0.13	1.79±0.07	1.83±0.15
WBC (10^{9}/L)	20.46±2.27	20.05±1.00	18.42±1.42
Hb (mmol/L)	7.72±0.39	7.59±0.33	7.74±0.16
PCV (L/L)	0.34±0.02	0.32±0.02	0.33±0.02

Data given are mean ± SD; RBC – red blood cells; WBC – white blood cells; Hb – hemoglobin; PCV – packed cell volume.

control – the control group fed standard diet without FB seeds.

group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.

group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

Table 5. Effect of faba beans seeds on blood serum parameters obtained from broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Total cholesterol (mmol/L)	3.61±0.11 b	3.02±0.12 a	3.04±0.21 a
LDL (mmol/L)	0.78±0.24 b	0.53±0.06 a	0.59±0.11 ab
HDL (mmol/L)	2.25±0.94	2.15±0.24	2.25±0.32
TG (mmol/L)	0.442±0.092	0.444±0.080	0.426±0.067
ALAT (U/L)	11.3±3.9	10.9±6.0	12.2±3.1
AspAT (U/L)	387±39	403±86	442±50
LDH (U/L)	1521±286	1889±514	1488±305
AMY (U/L)	652±162	665±189	586±141
Creatinine (μmol/L)	15.47±3.09	12.37±2.74	14.85±2.03
Glucose (mmol/L)	12.5±1.0	12.2±0.3	12.1±1.5
Total protein (g/L)	25.5±4.3	25.7±2.8	28.3±1.6
Uric acid (mmol/L)	0.230±0.007	0.216±0.048	0.291±0.088
Ca (mmol/L)	2.30±0.07 b	1.97±0.10 a	2.05±0.17 a
P (mmol/L)	2.52±0.42 b	2.39±0.45 ab	2.03±0.13a
Fe (μmol/L)	16.69±3.04 ab	13.39±2.83 a	18.80±3.40 b

Data given are mean ± SD, a, b – mean values in rows with different letters differ significantly at $P<0.05$; AMY – amylase; ALAT – alanine aminotransferase; AspAT – aspartate aminotransferase; LDH – lactate dehydrogenase; TG – triglycerides; HDL – high density lipoproteins, LDL – low density lipoproteins; Ca – calcium; P – phosphorus; Fe – iron.

control – the control group fed standard diet without FB seeds.

group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.

group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

The concentrations of total cholesterol and Ca were lower for FB groups compared to the control group ($P<0.01$ in both cases, Table 5). The concentration of LDL was lower in group I compared to the control group ($P<0.05$), but LDL did not differ

between group II and control group. The highest concentration of Fe was detected in group II; it differed significantly from the lowest concentration noted in group I ($P<0.05$). The serum concentration of P decreased only in group II, compared to the control group ($P<0.05$). No other changes in serum parameters were observed.

Gastro-intestinal tract morphology

The intake of high-tannin FB seeds, irrespective of their amount, resulted in a narrower longitudinal myenteron in the duodenum, compared to the control birds ($P<0.05$, Table 6). The transversal lamina thickness was reduced in group II, compared to the controls and group I, where widening was observed ($P<0.001$). The intake of high-tannin FB seeds led to reduction of the submucosa thickness in the dose-dependent manner ($P<0.001$). In turn, the mucosa and villus epithelium thickness, villus length and thickness as well as intestine absorptive surface increased in both groups fed high-tannin FB seeds, compared to the control group ($P<0.001$ for all). Moreover, the increase in the villus epithelium and villus thickness was dependent on the amount of high-tannin FB seeds in the diet. Furthermore, the crypt depth and width decreased in group II ($P<0.05$, $P=0.01$, respectively), while the crypt depth increased in group I, compared to the other groups. The total and inactive crypt number increased in group II, compared to group I ($P<0.01$ in both cases). Moreover, the inactive crypt number in the duodenum in group I was lower, compared to the control group.

Table 6. Effect of faba beans on histomorphometrical parameters of duodenum in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Myenteron thickness (μm)			
longitudinal lamina	66.4±12.2 b	56.0±10.3 a	53.2±12.5 a
transversal lamina	131.5±35 b	147.6±20.0 c	120.8±28.2 a
Submucosa thickness (μm)	37.3±7.1 c	33.7±12.9 b	28.1±6.3 a
Mucosa thickness (μm)	1488±176 a	1564±352 b	1590±315 b
Villus epithelium thickness (μm)	13.7±4.3 a	19.7±3.0 b	26.2±4.4 c
Villus length (μm)	1063±136 a	1311±317 b	1359±221 b
Villus thickness (μm)	65.6±9.8 a	91.1±12.0 b	106.5±15.3 c
Total number of villi (mm)	11.0±2.5	10.5±3.7	10.6±1.7
Crypt depth (μm)	88.6±23.0 b	101.1±32.0 c	67.0±19.3 a
Crypt width (μm)	38.1±6.3 b	37.9±5.2 b	32.5±6.1 a
Intestine absorptive surface (μm^2)	26.7±4.4 a	29.7±8.8 b	30.7±6.6 b
Crypts number (mm)			
Active crypts	2.3±1.0	3.1±1.3	3.0±0.8
Inactive crypts	15.1±3.2 b	11.0±1.7 a	17.1±2.0 b
Total	17.4±3.3 ab	14.1±0.8 a	20.0±2.5 b

Data given are mean ± SD, a, b, c – mean values in rows with different letters differ significantly at $P<0.05$.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

The intake of high-tannin FB seeds led to the reduction of the longitudinal myenteron ($P<0.001$) and submucosa thickness ($P<0.05$) in the jejunum depending on their amount in the diet (Table 7), while reduction of the mucosa thickness was observed only in group II ($P<0.001$). The intake of high-tannin FB seeds, irrespective of their amount, resulted in a lower villus epithelium thickness ($P<0.001$), compared to the control birds. On the other hand, the transversal lamina thickness was reduced in group I and increased in group II, compared to the control group ($P<0.01$). Furthermore, the villus thickness increased in group I but decreased in group II, compared to the other groups ($P=0.01$). The intake of high-tannin FB seeds resulted in reduction of the crypt width in group I compared to the other groups ($P<0.05$). Although a total crypt number did not change, the number of active crypts increased in both groups fed high-tannin FB seeds ($P<0.001$). Moreover, the inactive crypt number in the jejunum in group I decreased, compared to the control group ($P<0.01$).

Table 7. Effect of faba beans on histomorphometrical parameters of jejunum in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Myenteron thickness (μm)			
longitudinal lamina	49.0±20.2 c	30.9±5.8 a	43.6±7.0 b
transversal lamina	119.0±29.5 b	103.1±12.7 a	135.6±32.3 c
Submucosa thickness (μm)	35.1±7.7 c	30.9±6.7 b	17.7±4.5 a
Mucosa thickness (μm)	1439±183 b	1394±271 b	1144±54 a
Villus epithelium thickness (μm)	22.7±10.8 b	10.8±1.7 a	14.4±2.7 a
Villus length (μm)	1071±172	1072±164	1055.7±85
Villus thickness (μm)	83.2±22.5 b	96.9±14.9 c	65.1±17.7 a
Total number of villi (mm)	11.0±2.5	10.9±0.8	10.8±1.2
Crypt depth (μm)	94.0±29.3	93.9±35.7	93.3±34.8
Crypt width (μm)	40.5±8.8 b	36.1±9.9 a	40.6±10.7 b
Intestine absorptive surface (μm^2)	24.3±5.5	24.1±4.4	25.8±5.5
Crypts number (mm)			
Active crypts	2.3±1.0 a	6.0±0.6 b	5.4±0.7 b
Inactive crypts	15.1±3.2 b	8.2±3.6 a	10.7±2.3 ab
Total	17.4±3.3	14.2±3.6	16.1±2.8

Data given are mean ± SD; a, b, c – mean values in rows with different letters differ significantly at $P<0.05$.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

The intake of high-tannin FB seeds did not influence the innervations of the duodenum and jejunum wall in the birds (Table 8 and Table 9).

In liver tissue, the intake of high-tannin FB seeds did not change the collagen area, intercellular space, and total cell number (Table 10). However, the number of multinuclear hepatocyte cells decreased in birds fed high-tannin FB seeds irrespective of their amount in the diet ($P<0.05$), while the number of non-hepatocyte cells increased only in group I ($P<0.001$). On the other hand, the total number of

hepatocytes, hepatocyte nuclei, and mononuclear hepatocytes increased in group I, compared to group II ($P<0.05$ in all cases).

Table 8. Effect of faba beans on histomorphometrical parameters of nerve plexuses in duodenum in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Auerbach plexus			
Area (μm^2)	2847±1850	3156±2283	2075±1490
Perimeter (μm)	225±98	236±97	181±71
Mean Feret diameter (μm)	67.9±29.4	71.8±28.8	54.7±21.0
Min. diameter (μm)	40.4±12.1	40.4±11.2	36.3±9.2
Mean diameter (μm)	55.2±15.8	57.4±17.0	47.6±14.2
Sphericity	0.343±0.209	0.310±0.181	0.421±180
Meissner plexus			
Area (μm^2)	1301±707	2019±1380	1476±951
Perimeter (μm)	176±65	249±120	187±83
Mean Feret diameter (μm)	53.5±19.5	74.5±35.6	56.5±24.8
Min. diameter (μm)	20.3±4.6	18.5±7.1	20.5±5.0
Mean diameter (μm)	35.5±8.7	38.4±14.2	36.8±11.3
Sphericity	0.125±0.092	0.083±0.127	0.135±0.109

Data given are mean ± SD.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

Table 9. Effect of faba beans on histomorphometrical parameters of nerve plexuses in jejunum in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Auerbach plexus			
Area (μm^2)	1584±1164	2396±1839	1751±672
Perimeter (μm)	192±116	247±184	210±135
Mean Feret diameter (μm)	56.7±33.9	73±52	53±10
Min. diameter (μm)	25.3±7.3	34±11	32.7±8
Mean diameter (μm)	39.1±8.4	51±22	47.5±13.7
Sphericity	0.231±0.171	0.288±0.215	0.279±0.131
Meissner plexus			
Area (μm^2)	260±238	414±301	664±578
Perimeter (μm)	84.5±57.9	96.0±39.2	116±53
Mean Feret diameter (μm)	25.1±17.3	28.5±11.6	35.1±15.9
Min. diameter (μm)	7.2±2.4	11.9±4.5	14.6±4.8
Mean diameter (μm)	14.2±4.9	19.8±6.6	24.9±10.2
Sphericity	0.141±0.145	0.216±0.214	0.144±0.091

Data given are mean ± SD.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

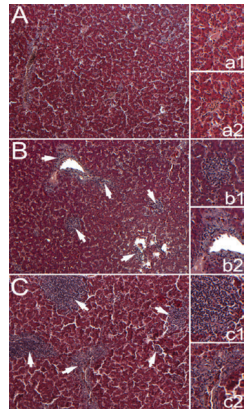
Table 10. Effect of faba beans on histomorphometrical parameters of liver in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Intercellular space (%)	7.78±4.48	6.99±5.28	7.87±4.56
Collagen area (%)	0.55±0.33	0.42±0.43	0.59±0.63
	Number of cells (mm^2)		
Total cell	10916±1348	13052±1689	10652±1496
Total hepatocyte	9271±701 ab	9852±1123 b	8782±713 a
Total hepatocyte nucleus	9483±856 ab	10035±1346 b	8936±895 a
Mononuclear hepatocyte	9059±693 ab	9669±724 b	8628±745 a
Multinuclear hepatocyte	212.76±34.77 b	183.62±51.03 a	154.76±28.59 a
Non-hepatocyte cells	1645±278 a	3200±1124 b	1870±242 a

Data given are mean ± SD; a, b – mean values in rows with different letters differ significantly at $P<0.05$.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

Histopathological analysis of the liver tissue

The histological examination revealed no marked changes or degenerative changes in the control group. Normal structure of liver parenchyma with preserved portal and triad architecture was observed. On the other hand, the intake of high-tannin FB seeds resulted in focal inflammation with dose-dependent severity (Figure 1). Animals fed the higher doses of high-tannin FB seeds had greater and more numerous inflammation foci. However, no marked signs of edema or hyperemia were observed in any group. Leukemic small lymphocytic infiltrates were present mostly in portal tracts and, in a lower amount, in sinusoids.



A – the control group; B – group I fed 8/15% (starter/grower) of high-tannin FB seeds; C – group II fed 16/22% (starter/grower) of high-tannin FB seeds. No local lymphocytic infiltrates in portal tracts and sinusoids are present in the control group (a1, a2). Leukemic lymphocytic infiltrates (indicated by white arrows) are present in portal tracts and sinusoids in livers belonging to birds from group I (b1, b2) and group II (c1, c2). Magnification x200.

Figure 1. Representative images of MT staining carried out on formaldehyde-fixed sections from liver tissue of chickens fed different amount of the seeds of faba beans introduced into the starter/grower mixtures

Bone morphology, mechanical and geometric parameters

There was no effect of high-tannin FB seeds, irrespective of their level in the diet, on the weight and length of tibiae and calculated geometric or mechanical parameters (Table 11).

Table 11. Effect of faba beans on basal morphology, mechanical and geometric parameters of tibia in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Bone general properties			
Bone weight (g)	17.5±2.3	16.60±1.7	17.30±2.8
Bone length (mm)	104.3±3.5	103.30±3.7	103.9±2.9
Bone weight/bone length (g/mm)	0.168±0.019	0.160±0.014	0.165±0.023
Bone geometric properties			
Cross section area (mm^2)	42.7±4.5	43.3±6.1	38.3±6.3
Mean relative wall thickness	1.038±0.188	0.994±0.221	0.845±0.161
Cortical index (%)	49.6±3.9	49.9±4.0	46.1±4.2
Moment of inertia (mm^4)	207±40	209±51	187±46
Index of gyration (mm)	2.19±0.11	2.20±0.16	2.19±0.14
Bone mechanical properties			
Yield strength (N)	167±21	161±11	175±36
Ultimate strength (N)	331±38	346±48	345±39
Elastic stress (MPa)	34.0±5.7	33.8±7.1	39.3±10.5
Bending moment (N·m)	1.79±0.18	1.68±0.13	1.70±0.20
Elastic energy (mJ)	38.9±4.7	34.1±4.9	36.1±7.3
Work to fracture (mJ)	470±108	446±91	474±103
Young Modulus (GPa)	2.85±0.50	3.04±0.33	3.13±0.44
Elastic strain (%)	1.16±0.14	1.07±0.13	1.23±0.28
Ultimate strain (%)	5.72±0.55	5.08±0.65	5.31±1.02
Toughness modulus (mJ/mm^3)	2.82±0.65	2.52±0.34	2.83±0.44

Data given are mean ± SD.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin FB seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

Bone ash, Ca and P content, and densitometric analysis

The intake of high-tannin FB seeds induced no changes in the ash and Ca content as well as in all densitometric parameters of tibiae (Table 12). However, the P concentration increased ($P<0.001$) and, subsequently, the Ca/P ratio decreased ($P<0.01$) and the ash weight/volume ratio increased in group II ($P<0.001$), compared to the other groups.

Discussion

The inclusion of unprocessed high-tannin FB seeds to the diet for broiler chickens, irrespective of their level in the starter/grower diet, provided similar rearing results in all groups (Table 2). Our results are in agreement with a study performed

on male broiler chickens fed diet containing 0, 6, 12, and 18% unprocessed FB for 42 days (Shams Shargh and Ahani Azari, 2010). Another study shows that dietary inclusion of dehulled-micronized FB seeds to broiler chickens from 14th to 49th day of age also has no effect on growth performance parameters (Laudadio et al., 2011). Another study, performed on turkeys for 56 days, with FB seeds shows that there is no influence on growth (Mikulski et al., 2017). In contrast, Diaz et al. (2006) have observed decreased feed conversion efficiency and reduced body weight in broilers fed extruded FB seeds in 48% of the diet. Thus, it was recommended, that dietary inclusion of FB should not exceed the level of 200 g/kg (20%) of poultry diet (Smulikowska and Rutkowski, 2005; Shams Shargh and Ahani Azari, 2010; Nalle et al., 2010). However, the overall effects of dietary FB depend both on the species, genotype, and age of birds as well as on the FB cultivar.

Table 12. Effect of faba beans on ash, Ca and P content, and densitometric parameters of tibia in broiler chicken at the age of 35 days

Item	Control	Group I	Group II
Ash (%)	35.1±4.5	34.0±3.6	36.8±3.9
Ash weight/volume (g/cm^3)	0.87±0.09 a	0.86±0.07 a	0.96±0.05 b
Ca (g/kg)	442±12	443±22	441±26
P (g/kg)	178±2a	180±4a	201±11b
Ca/P ratio	2.49±0.08 b	2.47±0.10 b	2.20±0.18 a
Bone tissue density (g/cm^3)	1.74±0.07	1.73±0.07	1.76±0.07
Bone mineral density (g/cm^2)			
Total	0.174±0.014	0.169±0.025	0.170±0.25
Distal part	0.207±0.031	0.196±0.026	0.194±0.023
Midshaft	0.167±0.023	0.160±0.034	0.162±0.031
Proximal part	0.195±0.025	0.178±0.028	0.195±0.033
Bone mineral content (g)			
Total	1.371±0.203	1.349±0.197	1.458±0.287
Distal part	0.352±0.139	0.317±0.158	0.312±0.112
Midshaft	0.479±0.166	0.486±0.128	0.505±0.098
Proximal part	0.216±0.105	0.275±0.152	0.271±0.156

Data given are mean ± SD; a, b – mean values in rows with different letters differ significantly at $P<0.05$.
control – the control group fed standard diet without FB seeds.
group I – the group fed 8/15% (starter/grower) of high-tannin B seeds.
group II – the group fed 16/22% (starter/grower) of high-tannin FB seeds.

A major constraint on the use of FB in poultry diet is the presence of anti-nutritional factors that could depress poultry performance (Vilariño et al., 2009; Shams Shargh and Ahani Azari, 2010). Tannins from FB reduce feed intake in birds and cause the formation of an enzyme-tannin complex, which reduces protein digestibility (Gatel, 1994). Grosjean et al. (2000) have showed that energy digestibility is significantly affected by the tannin content of FB in adult cockerels. Similarly, another study with male broiler chickens fed diet containing 500 g of unprocessed FB seeds with 9.9 g/kg of tannins shows that energy digestibility is negatively correlated with tan-

nins (Longstaff and McNab, 1991). Helsper et al. (1996) have observed a significant reduction in feed intake in broilers fed 500 g/kg of high-tannin FB. Contrarily, Jansman et al. (1993) did not detect effects on the metabolism in chickens fed 300 g/kg of FB in the diet.

On the other hand, poultry performance parameters depend additionally on digestion of non-starch polysaccharides, changes in the viscosity of digesta in intestines, availability of digestive enzymes, and the amount of absorbed nutrients. All these parameters influence the growth, development, vital organ structure, and intestine function (Palander et al., 2006). It seems that impaired body weight, feed intake, or feed conversion ratio should result in changes in the intestine structure and function. There are many reports concerning performance parameters, but there is no information on the damaging or any effect of unprocessed high-tannin FB seeds on histomorphological parameters of intestine mucosa or liver tissue. Available reports have been published at least 20 years ago. For example, Ortiz et al. (1994) have showed a relationship between decreased body weight gain, feed intake, mortality, and histological changes in the intestines and liver in chickens due to the addition of tannin-containing extracts of FB. Histological analysis revealed structural alterations in the ileum and liver. Atrophy and shortening of villi as well as hyperplasia and hypertrophy of goblet cells are described. Histopathological examination of the liver shows extensive vacuolization of hepatocytes without fat degeneration (Ortiz et al., 1994). Vohra et al. (1966) have reported thickening of mucosa in the esophagus and the crop when chicks are fed diet containing 50 g of vegetable tannic acid/kg. Similar morphological alteration, mainly in the jejunum, has been observed by Rubio et al. (1989). Besides shortening of villi and an increase in enterocyte proliferation with degeneration (swollen enterocytes), increased intestinal transit in growing chickens fed unprocessed FB (500 g/kg) is noted. Moreover, the relative weight of the pancreas increases in birds fed diets containing 250, 350, or 500 g of unprocessed FB seeds /kg; however, the intestinal tract of these chickens fed the lowest amount of FB seeds does not differ histologically from the control birds. There is no alteration in the length of villi (Rubio et al., 1989). Another study also demonstrates changes in pancreas weight, which is enlarged in male broiler chickens fed diets containing vegetable tannins at levels of 25 and 50 g/kg. This is probably the response of the intestinal tract to the presence of tannins, but the liver remains unaffected (Ahmed et al., 1991). In contrast with these findings, Sell et al. (1985) have observed no histopathological alteration in the intestinal tract of chickens and laying hens fed high-tannin sorghum grains, similar to that present in FB seeds. In general, the intestinal sections are morphologically normal and there is only a slight reduction in the depth of the crypt and intestinal wall (Sell et al., 1985). The levels of high-tannin FB or tannins used alone in all these experiments are not set at practical dietary levels for broiler feeding. Thus, it seems that tannins or even other anti-nutritional factors contained in FB seeds are in sufficient amounts to cause loss of mucosa and breakdown of the intestinal tract (Mitjavila et al., 1977). On the other hand, Kumar et al. (2007) do not reveal such negative effects of feeding unprocessed high-tannin plants on vital organs, including liver and intestine tissue, as presented above. Our broiler chickens fed FB seeds in the amount of 16/22% in the starter and grower period,

respectively, had enlarged livers, although the relative weight did not differ among the groups. One study confirms this observation, but the increase in the liver size in the chickens is related to feeding with non-starch polysaccharides also present in FB, leading to more enhanced activity of the liver, which produces more bile (Salih et al., 1991). Moreover, the histological observation carried out in our study revealed other opposite effects, e.g. elongation and widening of villi as well as augmentation of mucosa thickness and the absorptive surface of the intestine. This was probably an adaption to the presence of anti-nutritional factors in FB seeds and decreased digestion. Moreover, the alteration of the myenteron depending on the amount of FB seeds in the diet can lead to improper motor activity of the intestine in our chickens. Additionally, it could be linked with modification of the intestinal flora due to the introduction of FB seeds to the diet and could influence the host immune system due to the changes in the number of active and inactive crypts. Paneth cells, i.e. specialized epithelial cells located in crypts, secrete antimicrobial peptides. The increase in the inactive crypt number could suggest some problem with the local immune system. Our data, for the first time, showed that high-tannin FB seeds introduced into the diet at the level of 8/15% or 16/22% in the starter/grower mixture, respectively, did not influence wall innervation, since there were no changes in the morphological parameters of Auerbach or Meissner plexuses.

Besides, depressed growth, death and hemolytic anemia, interference with mineral availability could be found in chickens fed plant diet containing anti-nutritional factors (Wiryawan and Dingle, 1990). Both biochemical and hematological parameters are significant indicators of health status in all animals, including poultry, and are an obligatory tool in the diagnosis and treatment of many diseases. Observations reported by Emiola et al. (2013) have demonstrated that feeding with mucuna bean (20%) reduced not only basal hematological but also biochemical parameters in broiler chickens. The reduction of RBC, Hb, or PCV is a result of dysfunction of blood hematopoiesis and the drop of WBC, which leads to dysfunction of defense capabilities in the organism. A decrease in serum total protein in birds is probably linked with inhibition of the utilization of protein in the intestinal tract (Myer et al., 1982). Moreover, the level of hepatic enzymes increases due to elevated amino acid catabolism and liver damage (Emiola et al., 2013). The effect of the inclusion of high-tannin FB seeds in the broiler diet on basal hematology or biochemical parameters is rarely described, thus it is difficult to discuss our results. Abbel-Monein (2013) has reported that broiler chickens fed diet introducing 8%, 16%, and 24% of green beans for 35 days of their life do not exhibit changes in recorded blood parameters or in blood glucose, total protein, and creatinine, which were in a normal range. On the other hand, the diet containing green beans reduces blood cholesterol and triglyceride concentration in 35-day-old broiler chickens. Probably, this is caused by the high level of fiber in green beans (Abbel-Monein, 2013). Similarly, Burr et al. (1985) have reported the same effect of fiber on serum cholesterol. Other authors also note a cholesterol decreasing effect of different types of beans, which is attributed to an increase in rapid excretion of intestinal cholesterol and bile acids. There is also no effect of green beans on ALAT and AspAt (Moundras et al., 1997; Carew et al., 2003).

There is very little information on the effect of high-tannin FB seeds on the

metabolism of the liver and their influence on the serum biochemical composition. However, our results confirmed the information presented above, since there was no influence of high-tannin FB seeds on most of the analyzed serum parameters in the 35-day-old broiler chickens in our study, except changes in the concentration of total cholesterol and LDL, which decreased probably due to the higher amount of fiber in the experimental diets compared to the common diet based on soybean meal. Furthermore, the increasing concentration of high-tannin FB seeds in the diet of our chickens did not influence the activities of AspAt, ALAT, and LDH. This may indicate the absence of changes in the liver structure. Although the intake of high-tannin FB seeds did not result in fibrosis of the liver, the increase in the number of non-hepatocyte cells might indicate that phagocytes, like Kupffer cells, accumulated due to the inflammation caused by the anti-nutritional factors (Figure 1). On the other hand, the increase in the total hepatocyte number, mononuclear hepatocyte number, multinuclear hepatocytes, and total number of hepatocyte nuclei might suggest enhanced metabolism in the liver, which could produce more bile. It should be noted that there was an increase in the liver weight.

However, contrary to mammals, AspAt activity in birds is not specific for the liver, although it usually indicates liver or muscle damage (Lewandowski et al., 1986). The diagnostic value of ALAT in poultry is poor as well. Sometimes birds with liver damage have ALAT in a normal range. A decrease in LDH may be related to liver disease or the breakdown of red blood cells (Zantop, 1997).

The increasing level of high-tannin FB seeds in the diet of our chickens did not influence the serum concentration of creatinine and uric acid. The serum creatinine concentration depends on the age, muscle mass, and kidney excretory capacity (Silva et al., 2007). Serum urea acid increases as dietary nitrogen increases and can be used to assess amino acid utilization. Serum uric acid in birds is influenced by the age, sex, reproductive state, and nutritional status (Okumura and Tasaki, 1969; Hevia and Clifford, 1977).

As mentioned above, tannins from FB can additionally form a protein-tannin complex that reduces protein digestibility. It seems that the high-tannin FB seeds used in our study did not bind sufficient amounts of dietary protein to change nitrogen metabolism and serum biochemical parameters. On the other hand, tannins present in FB seeds can bind some macro elements like Ca and Fe (Houshmand et al., 2015), whose blood serum concentration decreased in our broiler chickens. In turn, the reduction of the Fe concentration in our broiler chickens did not influence the red blood cell system and the change in the serum Ca concentration was not linked with the alteration of bone Ca content. However, the reduction of the serum P concentration resulted in the decrease in the bone P content. It could be expected that changes in the main elements of bone should result in leg abnormalities, as reported in many studies.

Some anti-nutritive factors present in plants can also reduce absorption of Ca, P, or Fe and influence bone development (Hassan et al., 2003). Abbel-Monein (2013) has reported that broiler chickens fed diet supplemented with green beans at a concentration of 8%, 16%, and 24% develop leg problems. Elkin et al. (1978) have described leg abnormalities in broiler chickens fed high-tannin sorghum grains, but

these data indicate that bowing is not a result of the influence of tannins on bone mineralization, but a change in the organic matrix, mainly in collagen synthesis. This lesion is characterized by outward bowing of legs with swelling of the hock joint without changes in the serum Ca concentration (Armstrong et al., 1973). Other study shows that bone ash and bone mineralization are not influenced by tannic acid, tannins, or feeding poultry with high-tannin sorghum (Kumar et al., 2007). Opposite effects are reported by Houshmand et al. (2015) who shows that feeding broiler chickens with high-tannin oak acorn (15%) results in lower tibia ash content and mechanical strength without changes in tibia length or weight. On the contrary, Moschini et al. (2005) have described opposite effect in chickens fed diet containing 25 or 50% of unprocessed FB seeds, because these birds have no leg problems.

The results of our study are contrary to the presented data, because broiler chickens fed unprocessed high-tannin FB seeds (8/15% or 16/22%, respectively, in the starter/grower mixture) had no visible leg lesions, and both the mechanical testing and geometric analysis revealed no changes in tibia. It can be suggested that feeding with unprocessed high-tannin FB seeds exerted an influence on the gastrointestinal tract primarily by leading to an increase in the absorptive surface, probably in response to the presence of anti-nutritive factors in FB seeds, which allowed maintaining the protein and energetic metabolism at a sufficient level, which ensured a proper bone development. Thus, the lack of either negative or even positive effects, which could be exerted by the feeding with unprocessed high-tannin FB seeds is a very promising result. Faba bean is a cheap source of protein, and introduced to the diet at proper level allows not only maintaining similar growth and body weight gain of broiler chickens as in the case of soybean meal diet, but also comparable bone development.

The results of our study support also the hypothesis of the existence of the gut-bone axis (Kowalik et al., 2005; Tatara et al., 2006; Tomaszewska et al., 2012 b, 2016 a). The gastrointestinal tract is a place where basal mineral elements and nutritional ingredients are absorbed. They are needed for bone development ensuring proper general growth and locomotor activity. There are many studies proving the existence of the gut-bone axis (Tatara et al., 2005; Śliwa et al., 2005; Śliwa, 2010; Tomaszewska et al., 2015 d, 2016 b, 2016 c). These studies indicate that the mammalian or avian gastrointestinal tract can act as a significant system influencing bone development and plays the crucial role in the process of skeleton mineralization.

Conclusion

The dietary FB inclusion (up to 15% or 22%, respectively, in the starter/grower mixture) had no effect on the production performance, hematological parameters, most of serum biochemical parameters and tibia characteristics (geometry, mineral density and mechanical strength) of the broilers. The increase of the villus length and thickness resulted in the increased absorptive surface which allowed maintaining a proper level of protein and energetic metabolism, and bone development. Concluding, unprocessed high-tannin FB seeds may be used in broiler diets, but the dietary level of inclusion should not be higher than those reported in our study. However,

more studies on the type, concentration, and possible role of dietary FB seeds in the influence on the gut-bone axis are needed.

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