Effects of chestnut wood extract and flubendazole on small intestinal morphometry and mucin layer and peripheral blood leukocytes in the pheasant (*Phasianus colchicus*)

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Abstract

The study examined leukocytes and subpopulation of lymphocytes in peripheral blood, lymphoproliferative activity, morphology of intestine including mucus barrier in pheasant (*Phasianus colchicus*) after treatment with flubendazole (Flimabend®) and natural extract from sweet chestnut wood (Farmatan®). A total of 24 six-month-old female pheasants were divided into 4 groups (n = 6). The FL group received flubendazole *per os* 100 mg/g suspension in 1.43 mg of active substance/kg body weight during 7 days of experiment. The CW group received chestnut wood extract *per os* at a 0.2% concentration for 6 h per day during 5 days (experimental days from 3 to 7). The FL+CW group received a combination of doses administered in the same way as for the first two groups. The control C group received administration with no active substance. The results in our trial demonstrated a mild inflammatory effect on leukocytes, lymphocytes, heterophils, eosinophils ($P < 0.05$), and an increase of CD8+ cells in peripheral blood after administration of flubendazole ($P < 0.05$). On the other hand, administration of chestnut wood extract containing tannins revealed an anti-inflammatory effect on immunocompetent cells ($P < 0.05$). Enhanced lymphoproliferative activity of T and B lymphocytes was observed in chestnut wood extract groups ($P < 0.001$). Increased total jejunal thickness of mucus ($P < 0.001$) and increased production of MUC-2 ($P < 0.01$) was found in the CW group. Body weight was not different between the trial groups. Results indicate that chestnut wood extract can be administered as a preventive immunomodulatory substance reducing inflammatory process as well as an adjuvant in treatment with anthelmintics in pheasants.

Farmatan, benzimidazole, free living birds, immunity, jejunum

Benzimidazoles are the most extensive group of broad-spectrum anthelmintics, which are synthetic substances derived from a single chemical structure (Salahuddin et al. 2017). The primary mechanism of action of benzimidazoles is to disrupt the energy metabolism of parasites by binding to their cytoskeletal protein β-tubulin, thereby disrupting the polymerization of the microtubular matrix important for the normal function of eukaryotic cells (Taylor et al. 2016).

Excessive use of anthelmintics without knowledge of the parasite species spectrum, the intensity of infection and lack of detected level of resistance resulted in the emergence of anthelmintic resistance (Várady et al. 2011; Dolinská et al. 2014).

Plant extracts and their purified derivates can be used not only in the prevention and treatment of diseases, but they also demonstrate promising results in the field of nutrition, where they improve the performance of animals by stimulating and optimizing digestive processes (Redondo et al. 2014). Tannins can be included among plant extracts that can be successfully used as additives in poultry nutrition (Redondo et al. 2014; Karaffová et al. 2018; Levkut et al. 2019). Tannins are intermediate metabolites of the plant synthesis (Zawadzki et al. 2010). They are water soluble plant polyphenol compounds with

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a relatively high molecular weight and a sufficient amount of phenolic hydroxyl and other suitable groups, which form solid complexes with proteins and other macromolecules (Cowan 1999; Ashok and Upadhyaya 2012).

Experiments with hydrolysable tannins obtained from sweet chestnut showed a better utilization of nitrogen from feed with a significantly better nitrogen balance (Antongiovanni et al. 2007) and a beneficial effect on better feed conversion with reduced total ammonia production (Biagi et al. 2010). In addition to a positive effect of tannin on the body weight and feed conversion in broiler chickens, Jamroz et al. (2009) also found an improvement in the quality of droppings. Tannins can have a beneficial effect on digestion. During this process, tannins precipitate proteins on the surface of intestinal mucosa and thus form stable complexes that act as a protective barrier, preventing infections of intestinal mucosa and water loss through the intestinal wall. Additionally, tannins reduce intestinal motility (Javornik et al. 2019).

Chestnut wood containing tannins is often used in veterinary practice as an additive with the anthelmintic flubendazole to treat birds and improve animal performance. We suggest that prolonged administration of flubendazole can modulate the immune response in free living birds including pheasants. Therefore, our goal was focused on evaluation of count and functional indicators of peripheral blood leukocytes and their subpopulations in the pre-laying period, i.e. in winter, when preventive deworming is carried out. In addition, our objective was to study morphometry of the intestine and mucus intestinal quantity.

Materials and Methods

Ethical approval

The project of the clinical study was approved by the Ethics Committee No. EKVP/2021-07.

Experimental design

The experiment was conducted on a commercial pheasant (*Phasianus colchicus)* fattening farm. The pheasants came from the stock flock hatched in an artificial hatchery at the pheasant farm (approx. 500 animals) where the experiment was done.

Twenty four 6-month-old female pheasants were included in the trial. The pheasants were weighed, labelled and randomly divided into four groups of 6 pheasants each (n = 6): C (control), CW (chestnut wood extract), FL (flubendazole), and FL+CW (flubendazole with chestnut wood extract). The pheasants were housed in four identical floor pens with the same direction and covered area (0.12 m^2/pheasant). Wooden barriers separated the different groups of pheasants. The animals had free access to water and feed (Farmavel s.r.o., Martin, Slovak Republic) in the form of granules without coccidiostats. The diet composition in 100% dry matter was as follows: crude protein 18.25%, methionine 0.42%, crude fat 2.55%, calcium (Ca) 0.91%, crude fibre 4.18%, sodium (Na) 0.18%, crude ash 4.46%, phosphorus (P) 0.62%, and lysine 0.89%. Additional substances were vitamin A 10.000 IU/kg, vitamin D3 3.000 IU/kg, vitamin E 101.00 mg/kg, manganese 91.00 mg/kg, zinc 71.00 mg/kg, iron 40, 00 mg/kg, copper 15.00 mg/kg, anhydrous calcium iodate 2.00 mg/kg, and sodium selenite 0.30 mg/kg.

Sweet chestnut wood (*Castanea sativa* Miller) extract (Farmatan® liquid, Tanin, Sevnica d.d., Slovenia) was added to water of the CW group of pheasants at a 0.2% concentration for 6 h (8.00 h till 14.00 h) over 5 d (experimental d 3 to 7). Pheasants in the FL group received individually the anthelmintic flubendazole (Flimabend®, KRKA, Novo mesto, Slovenia) 100 mg/g suspension *per os* at 1.43 mg of active substance/kg body weight during 7 d of the experiment (experimental d 3 to 9). The FL+CW group received chestnut wood extract and flubendazole in the same way and for the same period as the FL and CW groups. Group C served as negative control and was fed a diet without chestnut wood extract or flubendazole. Two days after administration of chestnut wood extract and flubendazole (experimental d 11), all pheasants were weighed and killed by cervical dislocation. Intestinal samples were collected during necropsy.

White blood cell count

Leukocytes were counted in a haemocytomer using Fried-Lukačová solution (475 µl of solution, 25 µl of blood). Differential cell counts of 100 cells per slide were evaluated by light microscopy at × 1,000 magnification using blood smears stained with Hemacolor® (Merck, Darmstadt, Germany). The total number of different subtypes of white blood cells was evaluated according to the pattern:

Total leukocyte count × proportion of differential cells counted / 100 = absolute levels in $g \cdot l^{-1}$.

Evaluation of adherent jejunal mucin

The samples were processed with modification of the methods of Smirnov et al. (2004) and Thompson and Applegate (2006). Briefly, 1 cm long samples were cut lengthwise and gently washed with 0.9% NaCl and then they were subsequently immersed in 160 mmol·l^{1} saccharose and buffered with 50 mmol·l^{-1} sodium acetate (both from Centralchem, Bratislava, Slovak Republic) adjusted with 36–38% HCl to pH 5.8. After this, the samples were washed with 250 mmol·l^{-1} saccharose for 15 min and again for 45 min. Finally, the tissue was immersed in a 10 g·l^{-1} solution of docusate sodium (Sigma-Aldrich, Taufkirchen, Germany) overnight at room temperature, centrifuged at 700 *g* and evaluated in triplets spectrophotometrically at 620 nm (Opsys MR™, Dynex Technologies Inc., Sullyfield, USA). Alcian blue solution was used as a standard. The amount of absorbed dye was evaluated at µg of Alcian blue on 1 cm^2 of the intestinal tissue.

Intestinal flush

The caudal part of the jejunum was excised, and 5 ml of warm flush solution (M Tris/glycine buffer with 0.25% Tween 20, pH 7.00, Sigma-Aldrich) was injected into intestinal lumen with a syringe. The solution was aspirated and injected several times to flush the secretion from the intestinal wall, then the content was collected into a syringe and expelled into a tube.

ELISA

Chicken MUC-2 ELISA kit (Kamiya Biomedical Company, Seattle, USA) was used for detection of total MUC-2. Ninety-six-well microtitre plates were coated with affinity purified anti-chicken MUC-2 antibody. After incubation, each plate was washed, and 50 µl of substrate solution was added into each well. The samples were diluted 1:5 in phoshate-buffered saline (PBS) pH 7.0–7.2 and added at 100 µl doses into pre-designated wells in duplicates. Then, 10 µl of balance solution and 50 µl of conjugate binding with horseradish peroxidase in stabilizing buffer were applied into the plate wells, and incubated at 37 °C for 1 h. The reaction was stopped with 50 µl of stop solution. Then the plates were incubated at 37 °C for 10 to 15 min, and absorbance was measured spectroscopically at 450 nm on a microplate reader (Revelation Quicklink, Opsys MR, Dynex technologies, Sullyfield, USA). A calibration curve prepared according to the manufacturer's protocol was done for interpretation of results.

Flow cytometry

Histopaque gradient sedimentation (1.077 g/ml, Sigma-Aldrich) according to Boyum (1974) was used for separation of peripheral blood lymphocytes. At the preliminary test mouse anti-chicken monoclonal antibodies CD8α FITC and IgMµ heavy chain FITC (Southern Biotech, Birmingham, USA) showed positivity to pheasant lymphocytes and they were used for immunophenotyping of lymphocytes by direct imunofluorescent method. The control antibody, polyclonal goat-anti mouse FITC-conjugated immunoglobulin F(ab')$_2$ fragment (Dako, Glostrup, Denmark) was used at a working dilution 1:50 with PBS. After separation the lymphocytes were washed twice with PBS. Fifty µl of cellular suspension (1 ×10^6 lymphocytes in PBS) and 2 µl of specific or control MoAbs were mixed and incubated in dark at 22 °C for 15 min. After being stained, the cells were washed once in 0.5 ml PBS, and resuspended in 0.2 ml of PBS with 0.1% paraformaldehyde. Measurement and analysis of stained cells was performed on the FACS system (Becton Dickinson, Heidelberg, Germany) provided with a 15 mV argon ion laser. The analysis examined a dot plot of the leukocytes obtained by the forward scatter (FSC) and side scattering (SSC) of the physical character of the lymphocyte population. Gates were drawn around lymphocytes based on 90° and forward-angle light scatter. The fluorescence data were collected on at least 10.000 lymphocytes using the Becton Dickinson CellQuest programme. The results are therefore expressed at the relative percentage of the lymphocyte subpopulation which was positive for a specific MoAb. The absolute values were obtained as follows: Absolute count of lymphocytes × relative percentage of subpopulation lymphocytes/100.

Phagocytic assay

Phagocytic activity of polymorphonuclears and monocytes and phagocytic index was assessed by flow cytometry using whole heparinized blood. A commercial kit (Phagotest, OrphegenPharma, Heidelberg, Germany) was used for measuring phagocytosis. The kit contains fluorescein-labelled opsonized bacteria (*Escherichia coli*-FITC) and reagents to measure the overall percentage of polymorphonuclears and monocytes which ingest one or more bacteria per cell and the index of phagocytic activity shows the number of phagocytic bacteria per cell.

Cells were analysed by flow cytometry using blue-green excitation light (488 nm argon-ion laser and CellQuest Software). Granulocytes were measured after gating the relevant leukocyte cluster using a scatter plot (lin FSC vs lin SSC) after collecting 5,000 leukocytes per sample and analysing the green fluorescence histogram. The Geo Mean value indicated medium intensity of fluorescence which means the index of phagocytic activity.

Lymphoproliferative assay

Isolated lymphocytes on histopaque 1,077 density gradient were resuspended in RPMI -1640 medium (Roswell Park Memorial Institute - growth medium used in cell culture; Sigma-Aldrich) enriched with 10% bovine foetal serum and antibiotics (100 U·ml^{-1} penicillin, 100 µg·ml^{-1} streptomycin) and their concentration adjusted to 5 × 10^6 cells. The concetration of mitogens Con A (concanavalin A, LPS lipopolysaccharide, Sigma-Aldrich) was

2 μg·ml^{-1}. Testing of samples in triplets was performed in 96-well microplates (Lambda Life, Bratislava, Slovak Republic). Wells with stimulated cells were filled with 100 μl of cell suspension and 100 μl of diluted mitogens, wells without stimulation contained 100 μl of cell suspension and 100 μl of RPMI 1640 medium (Sigma-Aldrich). A blank was placed in each plate to determine the background of the reaction. Plates were incubated for 72 h at 37 °C in a CO_2 incubator (5%).

After incubation, 20 μl tetrazolium salt (0.1% MTT - reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); Sigma-Aldrich) was added to each well and incubation in CO_2 was continued for further 4 h at 37 °C. This was followed by 5 min centrifugation of the plates, suction of the supernatant, addition of 100 μl of dimethyl sulphoxide to each well (DMSO, Merck, Darmstadt, Germany), resuspension and 30 min incubation at room temperature. The measurement was performed with a spectrophotometer at a wave length of 540/630 nm. Proliferative activity expressed the stimulation index (SI) by calculation according to the formula: $SI = OD_{540}–OD_{630}$ (stimulated cells)/ $OD_{540}–OD_{630}$ (unstimulated cells).

Histology and morphometry of jejunum

Routine histological method with haematoxylin-eosin staining was used. The height and surface area of the villi in jejunal samples from five pheasants of each group (four groups) were analysed. The histological samples were microphotographed (Nikon LABOPHOT 2 with a camera adapter DS Camera Control Unit DS-U 2) and the NIS-Elements version 3.0 software (Laboratory Imaging, Prague, Czech Republic) was used. The height of the villi was measured from the basal region, which corresponded to the higher section of the crypts, the apex (μm). The total cutting surface area of separate intestinal segments included the length and breadth of villi (μm^2). The data were finally exported to MS Excel and subsequently statistically analysed.

Statistical analysis

Statistical analysis of obtained data was done by one-way analysis of variance (ANOVA) with the *post hoc* Tukey multiple comparison test using Graph Pad Software, statistical version 5.0 (USA). The differences between the mean values for the groups of pheasants were considered significant when $P < 0.05$. Values were expressed as means ± standard deviation (SD).

Results

White blood cell count

The number of leukocytes and lymphocytes (Table 1) was higher on day 11 in the FL group than in the CW group ($P < 0.05$). Density of heterophils was higher in the FL group then in the C and CW groups ($P < 0.05$). Similarly, increased number of eosinophils was found in the FL group compared to groups CW and FL+CW ($P < 0.05$). Monocytes showed only a tendency to increase in the FL group compared to groups C, CW, and FL+CW.

Table 1. Total count of the peripheral white blood cells ($g \cdot l^{-1} = 10^9 \cdot l^{-1}$) in control and experimental groups of pheasants.

	Group			
	C	FL	CW	FL+CW
Leukocytes	5.70 ± 0.97	7.70 ± 3.14^a	4.65 ± 1.16^b	6.15 ± 1.78
Lymphocytes	3.18 ± 0.79	3.10 ± 0.84^a	2.03 ± 0.31^b	2.39 ± 1.07
Heterophils	2.08 ± 0.20^b	4.47 ± 2.52^a	2.32 ± 1.14^b	3.68 ± 0.86
Eosinophils	0.11 ± 0.02	0.15 ± 0.06^a	0.10 ± 0.02^b	0.11 ± 0.04^b
Monocytes	0.11 ± 0.05	0.14 ± 0.03	0.10 ± 0.04	0.09 ± 0.03

C – control, FL – flubendazole, CW – chestnut wood extract, FL+CW – flubendazole with chestnut wood extract

^{a,b} – Means with different superscripts in the same row differ significantly at $P < 0.05$

Functions of lymphocytes and phagocytes

Values of phagocytic activity (Table 2) of leukocytes, heterophils, and monocytes were not changed between experimental and control groups. Similarly, the index of phagocytic activity (Table 2) did not show changes between the study groups.

Table 2. Values of functional indicators of peripheral blood leukocytes in control and experimental groups of pheasants.

	Group			
	C	FL	CW	FL+CW
	Phagocytic activity (%)			
Leukocytes	38.40 ± 12.02	41.60 ± 10.42	44.00 ± 8.20	48.50 ± 15.53
Heterophils	37.90 ± 12.98	40.20 ± 10.01	42.30 ± 8.49	47.40 ± 15.69
Monocytes	38.70 ± 14.20	44.70 ± 17.97	37.00 ± 8.20	43.60 ± 12.56
	Index of phagocytic activity			
Leukocytes	694 ± 120.80	641 ± 129.80	683 ± 158.60	589± 227.80
Heterophils	601 ± 109.40	583 ± 116.30	631 ± 129.20	565 ± 223.60
Monocytes	938 ± 229.30	1155 ± 418.70	997 ± 478.70	1052 ± 210.40

C – control, FL – flubendazole, CW – chestnut wood extract, FL+CW – flubendazole with chestnut wood extract

Phenotyping of lymphocytes

The number of CD8+ cells was higher ($P < 0.05$) in the FL group than in the CW and FL+CW groups (Table 3). On the other hand, a decrease of IgM+ lymphocytes was found in the FL, FL+CW ($P < 0.05$), and the CW group ($P < 0.001$) compared to control.

Table 3. Actual count of lymphocyte subpopulations ($g \cdot l^{-1} = 10^9 \cdot l^{-1}$) measured by flow cytometry.

	Group			
	C	FL	CW	FL+CW
CD8	0.140 ± 0.053	0.240 ± 0.070^{a}	0.110 ± 0.300^{b}	0.080 ± 0.060^{b}
IgM	0.080 ± 0.029^{a}	0.039 ± 0.018^{b}	0.025 ± 0.010^{c}	0.038 ± 0.017^{b}

C – control, FL – flubendazole, CW – chestnut wood extract, FL+CW – flubendazole with chestnut wood extract

$^{ab, ac}$ - Means with different superscripts in the same row differ significantly at $P < 0.05$ and $P < 0.01$, respectively

Lymphoproliferative activity

Stimulation of T lymphocytes with concanavalin A showed an increased stimulatory effect in the FL+CW ($P < 0.01$) and CW ($P < 0.001$) groups compared to groups C and FL (Fig. 1). Lipopolysacharide stimulated B lymphocytes (Fig. 2) showed increased stimulatory effect in the CW group ($P < 0.05$) compared to the groups with flubendazole.

Morphometry of jejunum

The height of villi (Fig. 3) was lower in the CW group than in the FL and C groups ($P < 0.05$). Depth of crypt (Fig. 4) was lower in the CW group compared to the FL group ($P < 0.05$) and the FL+CW group ($P < 0.01$). Cut surface of villi (Fig. 5) showed increased values in the FL group ($P < 0.01$) compared to the CW group.

Quantification of mucin in the intestinal content

Thickness of the mucin layer in the jejunum (Fig. 6) was increased in the CW group compared to the FL+CW ($P < 0.05$) and FL and C groups ($P < 0.001$). On the other hand, the MUC-2 concentration (Fig. 7) was increased in the CW ($P < 0.01$) and FL+CW ($P < 0.001$) groups compared to the C group.

Body weight

Body weight did not show significant differences between groups (Fig. 8).

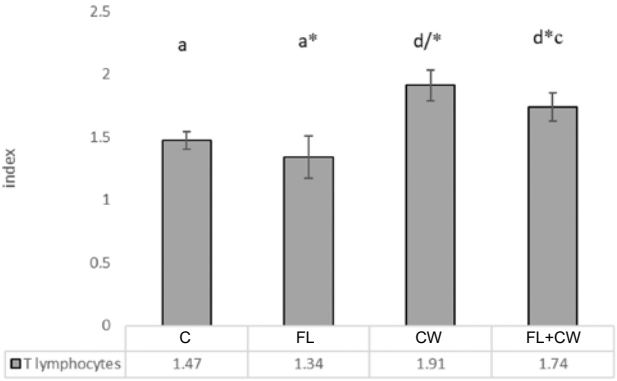

Fig. 1. Proliferation of T lymphocytes. Means with different superscripts between columns differ significantly at $^{ac}P < 0.01$, $^{ad*}P < 0.001$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

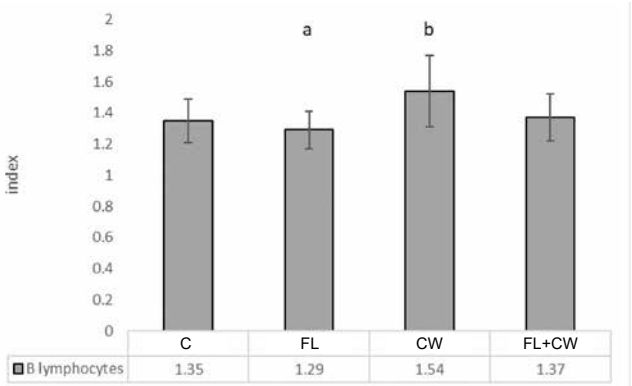

Fig. 2. Proliferation of B lymphocytes. Means with different superscripts between columns differ significantly at $^{ab}P < 0.05$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

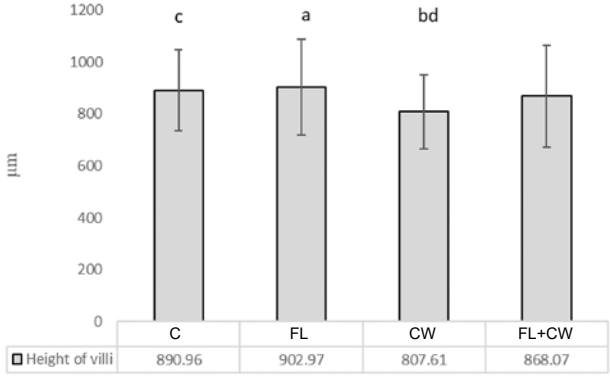

Fig. 3. The height of jejunal villi (µm). Means with different superscripts between columns differ significantly at $^{ab}P < 0.05$; $^{cd}P < 0.05$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

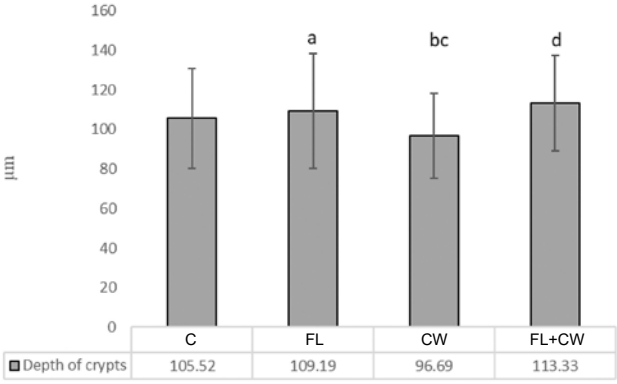

Fig. 4. The depth of jejunal crypts (µm). Means with different superscripts between columns differ significantly at $^{ab}P < 0.05$; $^{cd}P < 0.01$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

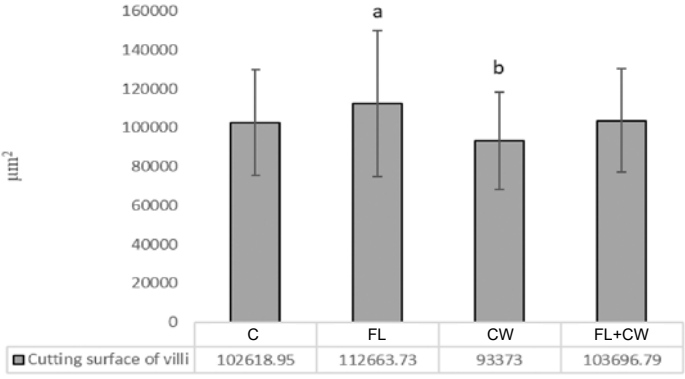

Fig. 5. Cutting surface of jejunal villi (µm^2). Means with different superscripts between columns differ significantly at $^{ab}P < 0.01$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

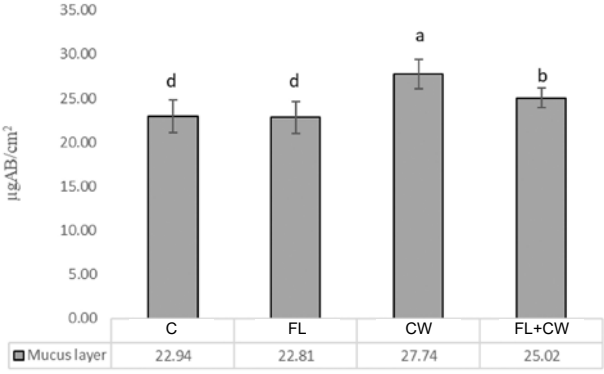

Fig. 6. Thickness of mucus layer in jejunum (µgAB·cm^{-2}). Means with different superscripts between columns differ significantly at $^{ab}P < 0.05$, $^{ad}P < 0.001$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

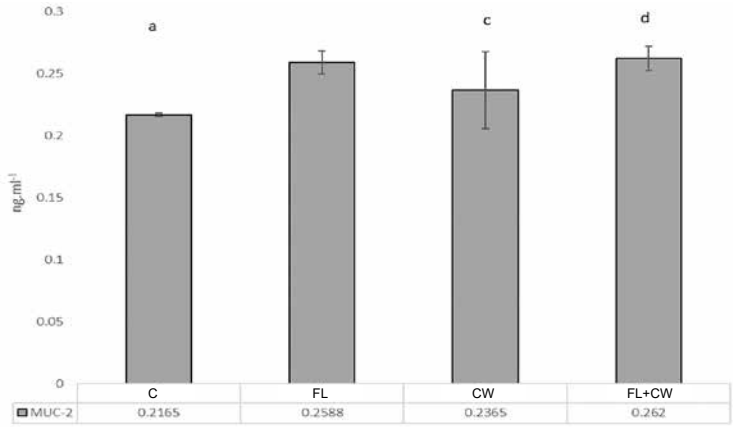

Fig. 7. Concentration of MUC-2 in jejunum (ng·ml^{-1}). Means with different superscripts between columns differ significantly at $^{ac}P < 0.01$, $^{ad}P < 0.001$. Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

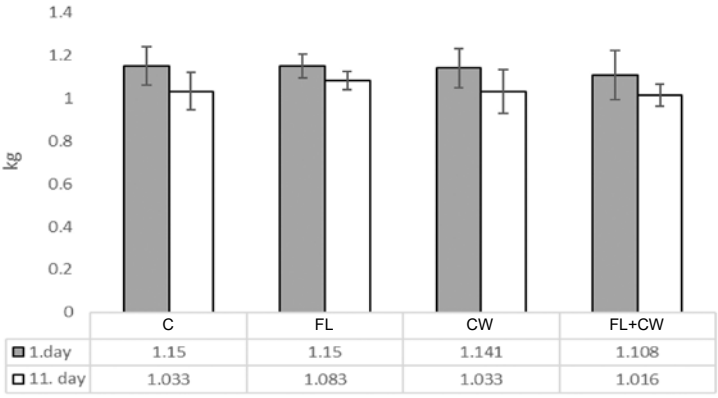

Fig. 8. Body weight (kg). Groups of pheasants: C (control), FL (flubendazole), CW (chestnut wood extract), FL+CW (flubendazole with chestnut wood extract)

Discussion

Benzimidazoles and their derivates play a very important role as anthelmintic agents. In addition, benzimidazole derivates show pharmacological activities such as antimicrobial, antiviral, anticancer, and anti-inflammatory ones (Salahuddin et al. 2017). After the ban on the use of antibiotics in livestock feed and with the increasing number of resistant pathogens, alternative feed additives including microbes (Li et al. 2006), organic acids (Kim et al. 2005), herbal extracts (Shan et al. 2007), and mannan oligosaccharides have been developed (Kogan and Kocher 2007).

These ingredients primarily inhibit the growth of pathogenic bacteria. Among the various plant extracts, tannins represent an important group that is often reported to have effects similar to antibiotics. Tannins belong to the group of secondary metabolites that contribute to the plant defence mechanism against herbivores (Brus et al. 2018).

Several nutritional experiments were conducted on broiler chickens to test different concentrations of tannin extracts from horse chestnut (Schiavone et al. 2008; Xiong et al. 2016; Karaffová et al. 2018). However, in the available literature we did not find information on the effect of the horse chestnut extract on the intestinal system of pheasants as free-living birds or on its modulation of the immune response after administration of the anthelmintic flubendazole. Flimabend at a dose of 1.43 mg of active substance/kg of live weight and Farmatan with a 0.2% tannin content were chosen for this trial as it was indicated by previous experiment (Levkut et al. 2019).

Administration of the anthelmintic flubendazole in the current trial caused an increased level of total leukocytes in the peripheral blood of pheasants. Similarly, cells of agranulocytic line as lymphocytes and monocytes and granulocytic cells including eosinophils and heterophils showed higher values in peripheral blood after flubendazole administration. Increased values of all leukocytes and their groups in peripheral blood indicate increased haemopoiesis and systemic response to flubendazole administration. This fact was also confirmed by a significant increase of CD8+ lymphocytes in pheasants with flubendazole administration. CD8+ lymphocytes after interaction with major histocompatibility complex proliferate and become an armed antigen-specific cytotoxic T lymphocytes and are able to kill infected cells (Kaspers and Kaiser 2014). Karaffová et al. (2018) observed a similar shift in the level of peripheral leukocytes in broiler chickens after a 7-day administration of flubendazole. Based on the reduction of IgM+ cells in the experimental groups of our trial, we assume that the administration of chestnut wood extract and flubendazole did not affect the antibody response.

Administration of chestnut wood extract in our trial showed enhanced proliferation of T and B lymphocytes. This lymphoproliferative activity can be promising support to fight against many diseases. Recent studies have shown that tannins reveal activity against Gram-positive and Gram-negative bacteria, e.g. *Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes* and *Enterococcus faecalis* (Wangensteen et al. 2009; Kaczmarek 2020).

Administration of chestnut wood extract in our study had no stimulatory effect on the height of villi. Brus et al. (2018) observed a beneficial effect of the horse chestnut tannin extract on the intestinal epithelium in *in vitro* conditions. Moreover, tannins showed antioxidant and antiproliferative activities in colon inflammation (Hamiza et al. 2012). When a colitis-inducing agent (dextran sulphate sodium) was administered to rats both with and without a mucin defect, mucin-defective rats presented increased colonic inflammation (Van de Sluis et al. 2006).

However, administration of flubendazole increased the height of villi and depth of the crypts, which indicates that there was oedema or inflammation after administration of benzimidazole. This is supported by the increased area of the cutting surface of villi in the FL group. These data are consistent with Levkut et al. (2019) who observed an increased number of immunocompetent cells (CD3+, CD4+, CD8+ and IgM+ lymphocytes) in jejunal mucosa of chickens and an increased cutting surface after administration of flubendazole. However, we could not find suitable specific antibodies for the study of lymphocyte subpopulations in pheasants. Recently, Karaffová et al. (2018) observed increased expression of IL-1β and IL-18 in intestine of broiler chickens after administration of flubendazole.

Administration of chestnut wood extract in our experiment increased production of MUC-2 in the jejunum. Mucus is the first line of defence in the intestinal tract against invading pathogens. MUC-2 is the main mucin-producing gel that contributes to the formation of the mucus barrier (Smirnov et al. 2004; Thompson and Applegate 2006). Similarly, increased thickness of jejunal mucus was proved by Alcian blue in pheasants of the CW group. These consistent data in our trial confirm the ability of chestnut wood extract to stimulate production and excretion of strategic component of gut mucous

membrane protection (Karaffová et al. 2018). In spite of the administration of tannins and benzimidazole, the body weight of pheasants at the end (day 11) of the experiment was not significantly changed between the experimental groups and control. It indicates that the trial ought to be prolonged for the evaluation of this parameter.

In conclusion, the results of our trial demonstrated a mild inflammatory effect on leukocytes, lymphocytes, heterophils, eosinophils, and increase of CD8+ cells in peripheral blood after administration of flubendazole. On the other hand, administration of chestnut wood extract containing tannins revealed an anti-inflammatory effect on immunocompetent cells. Moreover, the enhanced lymphoproliferative activity of T and B lymphocytes in the CW group indicates a promising support to fight against diseases. Increased production of MUC-2 and increased total jejunal thickness of mucus in the jejunum of the CW group suggests suitable environment for limiting parasite growth and microbial infection. Body weight was not changed among evaluated groups of our trial. The results indicate that chestnut wood extract can be administered both as a preventive immunomodulatory substance reducing inflammatory processes and as an adjuvant in anthelmintic treatment in pheasants.

Conflict of interest

The authors declare that they have no conflict of interest.

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