Animal 18 (2024) 101321

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

The effect of early administration of antibiotics or feeding a diet containing coccidiostats on the level of their accumulation in liver and the redox status of turkeys

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ARTICLE INFO

Article history:
Received 10 January 2024
Revised 28 August 2024
Accepted 29 August 2024
Available online 4 September 2024

Keywords:
Antioxidant
Doxycycline
Enrofloxacin
Monensin
Tissue

ABSTRACT

Early administration of antibiotics may worsen the functioning of the turkeys' antioxidant system. It was also assumed that the longer the time of administration of an antibiotic, e.g. a coccidiostat, the greater the risk of its accumulation in the liver. The study aimed to determine whether early administration of antibiotics or feeding a diet containing coccidiostats causes accumulation in the liver and whether it affects the deterioration of the antioxidant system, and whether preventive vaccinations can intensify it. A total of 3 080 female turkeys were randomly allocated to eight groups. The experiment had a two-factorial design, with four treatments (C, M, E, D) and two groups of birds (vaccinated +, unvaccinated −). The C group did not receive the coccidiostat or antibiotics. Group M was administered monensin at 90 mg/kg feed for 56 days of life. Group E received enrofloxacin at 10 mg/kg BW, and group D received doxycycline at 50 mg/kg BW, added to drinking water, for the first 5 days of life. One-day-old turkeys from groups C+, M+, E+, and D+ were administered live-attenuated vaccines against turkey rhinotracheitis and Newcastle disease by coarse spray; 28-day-old birds were administered a subcutaneously injected inactivated vaccine against *Ornithobacterium rhinotracheale*. Turkeys from groups C-, M-, E-, and D- were not vaccinated. It was determined that as a result of administration of enrofloxacin or doxycycline until the 5th day of life, biotransformation of these antibiotics occurred in the liver until the 56th day of life of the turkeys, which was confirmed by their lower level than the Maximum Residue Level. Because the concentration of monensin in the liver of turkeys gradually increased with the extension of the time of its administration in the diet, it is probable that discontinuing its addition a day before the slaughter of birds will result in the presence of this coccidiostat in the liver of turkeys. Despite the accumulation of monensin in the liver of turkeys, this coccidiostat did not increase oxidative reactions in the organism of turkeys. Vaccination of turkeys can reduce oxidative reactions and apoptosis in the body. However, the effect of the redox system reaction is different immediately after vaccination, which is due to the mechanism of action of the immune system. If it is necessary to administer an antibiotic in the early rearing period, the effects of doxycycline on the organism's immunity including antioxidant defence will be less severe than those of enrofloxacin.

Implications

Despite the ban on the use of antibiotics as growth promoters, the possibility of administering coccidiostats, which are antibiotics, has been left open. The need to administer antibiotics in the early rearing period can have many negative effects. In the case of antibiotic administration in the first 5 days of life, their presence is not detected in the liver of 8-week-old turkeys, and long-term administration of monensin causes its accumulation in direct proportion to the time of use. It has been established that the disruption of antioxidant defence is less severe in the case of administration of doxycycline than enrofloxacin.

Introduction

The current challenge facing poultry production, including turkey production, is the need to limit the antibiotics used, justified for many reasons (Śmialek et al., 2023). The European Union has obliged member states to reduce the use of antibiotics in animal

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https://doi.org/10.1016/j.animal.2024.101321
1751-7311/

production by 50% by 2030. This is one of the goals of the European Green Deal as part of the fight against antimicrobial resistance (European Medicines Agency, 2023). Gaining new knowledge about the biological effects of early use of antibiotics and continuous use of coccidiostats in young turkeys (up to 8–12 weeks of age) is one of the most important ways to achieve this goal. There is a lack of empirical evidence that such treatment, including "prophylactic" administration of antibiotics in therapeutic doses already in the 1st week of life of turkeys (usually from the 2nd day of life), does not interfere with the physiological mechanisms of transferring maternal immunity to chicks and acquiring their defence against pathogens. We assume that by potentially limiting the transfer of maternal antibodies in turkey poults' first courses of life, the birds' immune system may be weakened. Maternal antibodies constitute humoral immunity, which acts through antibodies produced by stimulated B lymphocytes, which, by binding to the antigen, initiate the phagocytosis reaction by phagocytic cells. During phagocytosis, phagocytic cells produce free radicals, and the intensity of this process may modify systemic redox reactions (Splettstoesser and Schuff-Werner, 2002). In EU countries, a ban on the use of antibiotics as growth stimulants was introduced in 2006 (Regulation EC, 2003). However, the possibility of continuous use of coccidiostats in the feed, including ionophores, which are also antibiotics, has been left. According to Rajendran et al. (2018), apart from its antibacterial effect, monensin has antifungal, anti-parasitic, antiplasmodial, anti-viral, anti-trypanosomiasis, anti-toxoplasmosis and antitileishmaniasis properties. Monensin can be used until turkeys are 16 weeks old, and the withdrawal period is only 1 day (WHO, 2009). Paradoxical situations have arisen when the use of a specific antibiotic as a growth stimulator is prohibited and allowed as a coccidiostat. A commonly used method of preventing coccidiosis in birds is the constant administration of coccidiostats in feed mixtures, but their combined administration with another antibiotic may result in biological reactions in the body resulting from their interaction. The effect of such an interaction in the body may be completely different than the effect of each antibiotic individually (Madadi et al., 2014).

Data available in the literature prove that research on the use of coccidiostats in poultry is mainly focused on assessing the effectiveness of their use in the context of acquiring specific immunity in birds against coccidiosis. Drug treatment does not eliminate coccidia and may facilitate or interfere with the immune response against coccidia (Chapman, 2008, Kadykalo et al., 2018). However, there is no information on how long-term administration of coccidiostats affects the body's biological reactions, primarily their accumulation in tissues and, consequently, the response to the redox status. Research conducted by Gbylik-Sikorska et al. (2016) indicates that long-term administration of enrofloxacin or doxycycline to chickens may result in the accumulation of these antibiotics in chicken tissues. Research conducted by Elamaran et al. (2015) shows that the administration of enrofloxacin to chickens for 5 days (38–42 days of age) induced oxidative stress in the chickens' body, which was manifested by an increase in the activity of superoxide dismutase, catalase and glutathione peroxidase as well as the content of glutathione reduced and malondialdehyde in the liver (Elamaran et al., 2015). Stimulation of oxidative reactions in the body due to the administration of xenobiotics is unfavourable because it may result in a weakening of the antioxidant potential, induction of oxidative stress, and consequently, deterioration of the immunity and growth performance of birds. It is worth emphasising that the intensification of oxidative reactions may also result in deterioration of the quality of poultry meat.

Our previous research on the discussed research topic showed that the constant use of monensin improved turkey BW gain and feed efficiency compared to doxycycline treatment (Mikulski et al., 2022). In turn, histopathological changes in immunocompetent organs were observed in turkeys that were constantly administered monensin or briefly doxycycline or enrofloxacin, and the most visible changes were in the case of constant use of monensin (Smagieł et al., 2023). Therefore, determining the impact of antibiotic administration on the redox status and their possible accumulation in the liver, as the tissue responsible for the biotransformation of xenobiotics, is important in the context of a better understanding of the previously noted histopathological changes.

It was assumed that early administration of antibiotics may worsen the functioning of the turkeys' antioxidant system. Feeding birds with mixtures containing a coccidiostat, which is also an antibiotic, may cause similar effects. Additionally, it was assumed that the longer the antibiotic administration time, the greater the risk of its accumulation in the liver. The aim of the study was to determine whether early administration of antibiotics or feeding a diet containing coccidiostats causes accumulation in the liver and whether it affects the deterioration of the antioxidant system, and whether preventive vaccinations can intensify it.

Material and methods

Birds and housing

The experiment was conducted in the Animal Research Laboratory of the Department of Poultry Science, University of Warmia and Mazury in Olsztyn (Poland). The protocol for this study was approved by the Local Ethics Committee for Animal Experiments in Olsztyn, Poland (Approval No. 47/2021; Olsztyn. Poland), and the animals were cared for under guidelines comparable to those laid down by EU Directive 2010/63/EU. The study was carried out in compliance with the ARRIVE guidelines. Every effort was made to minimise the suffering of the animals used in the experiment.

In the presented experiment, 3 080 one-day-old Hybrid Converter female turkeys were used. The birds were divided randomly into eight groups, each consisting of seven pens with 55 birds per pen. Each pen had an area of 10 m^2 and was lined with litter. Environmental conditions were under the recommendations of Hybrid Turkeys (2020), controlled automatically, adjusted to the age of the birds, and identical for all turkeys. The experiment was conducted for 8 weeks from 1 to 56 days of life. During two feeding phases (weeks 1–4 and 5–8), birds were fed a diet formulated based on Hybrid Turkeys, (2020) to meet the nutritional requirements of commercial turkeys at a given rearing stage. The detailed composition of the diets was presented in Supplementary Material (Supplementary Table S1) and Mikulski et al. (2022) and Smagieł et al. (2023). Feed and water were available for turkeys *ad libitum*.

Experimental design

The experiment used a two-factor design with four treatments (C, M, E, D) and two groups of birds (vaccinated, +, unvaccinated, −). Turkeys from group C did not receive the coccidiostat monensin added to their feed or antibiotics added to their drinking water. Birds from group M received monensin (Coxydin 200, Huvepharma Polska, Warsaw, Poland) in an amount of 90 mg/kg of feed for 56 days. Birds from group E received the addition of enrofloxacin (Enrofloxacin 10%, Biowet, Drwalew, Poland) to drinking water for first 5 days of life in an amount of 10 mg E/kg BW, while birds from group D received the addition of doxycycline (Doxylin CT WSP 433 mg/g, Dopharma Research B.V., Raamsdonksveer, Netherlands) into drinking water for first 5 days of life in an amount of 50 mg D/kg BW.

In four experimental groups (C+, M+, E+, D+), 1-day-old turkeys were administered live-attenuated vaccines against turkey rhino-

tracheitis (**TRT**) (Poulvac TRT; Zoetis) and Newcastle disease (**ND**) (Nobilis ND clone 30; MSD Animal Health) by coarse spray, and 28-day-old birds were administered a subcutaneously injected inactivated vaccine against omitobacteriosis (Ornitin, Phibro, Poland). Turkeys from groups C-, M-, E-, and D- were not vaccinated. Vaccinated birds were kept in a separate area of the same building and were handled by different people to prevent cross-contamination.

Sample collection and investigations

During the experiment, the BW of turkeys and feed consumption were recorded on a pen basis for 56 days of life. Daily feed intake and the feed conversion ratio were calculated. Mortality rates were recorded daily.

At 7 and 56 d of age, blood samples were collected from seven birds from each group (one bird per replicate). At 1, 3, 5, 7, and 56 d of life, one bird per replicate pen (seven birds per treatment) was euthanised by cervical dislocation (Close et al., 1997). Liver samples were collected from seven birds from each group.

Sample preparation and liquid chromatography- mass spectrometry analysis

Samples were prepared by simple protein precipitation with acetonitrile. For liver analysis, 200 mg of tissue was mixed up with 200 μL of water and 300 mg of ceramic beads (1.4 mm dimension) in 2 mL Bead Mill Tubes and homogenised using a soft tissue program (Bead Mill Max Homogeniser, VWR International LLC, Randor, USA). Then, 1 000 μL of acetonitrile was added. Before the homogenisation and precipitation step, an equivalent amount of internal standard as in the calibration standards was added (final concentration 50 ng of nigericin in mL/mg of the sample). The precipitated sample was centrifuged at 14 000 rpm for 5 min, and the supernatant was transferred into autosampler vials and immediately analysed.

The concentration of monensin, doxycycline, and enrofloxacin was determined using a high-performance liquid chromatograph (ExionLC AD, AB Sciex, Framingham, MA, USA) coupled with a mass spectrometer (QTRAP 6500+, AB Sciex, Framingham, MA, USA). Chromatographic separation of sample supernatants was carried out on a Kinetex Biphenyl (100 mm × 3 mm, 2.6 μm particle size) column (Phenomenex, Torrance, CA, USA). Column temperature was set at 40 °C. The mobile phase flow rate was 0.4 mL/min, and the injection volume was 5 μL. The mobile phase consisted of water containing 0.1% v/v of formic acid (component A) and ACN containing 0.1% v/v of formic acid (component B). Mobile phase gradient conditions: 0.0–0.5 min 45% B, 0.5–3.0 min 45–95% B, 3.0–4.5 min hold 95% B, 4.5–4.6 min 95–45% B, and 4.6–6.0 min 45% B. Electrospray ionisation (ESI) in the positive ion mode was used. The ion source parameters and MRM transitions (e.g., precursor (Q1), product ions (Q2), collision energy (CE), and retention times) are listed in the Supplementary Materials (Supplementary Table S2). Analyst 1.7.2 software (AB Sciex, Framingham, MA, USA) was used to control the LC/MS/MS system. SCIEX OS Version 2.1.6.59781 (AB Sciex, Framingham, MA, USA) was used for data processing. An internal standard calibration method for the quantification of enrofloxacin, doxycycline, and monensin was applied. Nigericin was used as an internal standard. Mobile phase constituent (LC-MS grade) and standards were purchased from Sigma Aldrich (St. Louis, MO, USA).

RNA extraction from the blood and quantitative real-time PCR

The total RNA extraction from the blood of turkeys was performed using the RNeasy Protect Animal Blood Kit (Qiagen, Wrocław, Poland) following the manufacturer's recommendation. The quantity of isolated RNA samples was assessed spectrophotometrically with a UV–VIS Nabi spectrophotometer (MicroDigital Co. Ltd., Gyeonggi, Republic of Korea), and its integrity was confirmed through agarose gel electrophoresis (0.8% concentration). To synthesise complementary cDNA, 1 μg of total RNA underwent reverse transcription using the NG dART RT kit (EURX Ltd., Gdańsk, Poland), following the manufacturer's protocol.

Specific primers (sequences presented in Table 1) designed for assessing inducible nitric oxide synthase (iNOS) gene expression were created utilising Primer 3 software from the Whitehead Institute (Cambridge, MA, USA), and synthesised by Genomed (Warsaw, Poland). The Real-time PCR method was performed on a Quantabio thermocycler (VWR International LLC, Radnor, PA, USA) using a universal solution for quantitative real-time PCR (SG qPCR Master Mix, EURX Ltd., Gdańsk, Poland). The amplification was conducted for 35 cycles: denaturation at 95 °C for 10 s, annealing at 58–59 °C for 15 s, and elongation at 72 °C for 20 s. The experiment was normalised to β-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) reference genes. The target gene relative mRNA level analysis was evaluated using the $2 - \Delta Ct$ method.

Analysis of redox parameters in serum

The serum levels of 8-isoprostane (cat. no. QY-E80208), advanced oxidation protein products (cat. no. QY-E80192), 8 – hydroxydeoxyguanosine (**8-OHdG**) (cat. no. QY-E80001), NADPH oxidase (**NOX**) (cat. no. QY-E80199), cyclohydrolase 1(**GCH1/GTP**) (cat. no. QY-E80195), catalase (cat. no. QY-E80173), oxoguanine glycosylase 1 (**OGG1**) (cat. no. QY-E80159), caspase 3 (**Casp 3**) (cat. no. QY-E80111), caspase 8 (**Casp 8**) (cat. no. QY-E80112), nitric oxide (cat. no. QY-E80065) and activity of myeloperoxidase (cat. no. QY-E80209), superoxide dismutase (cat. no. QY-E80142) were determined in serum of 7-day-old and 56-day-old turkeys, using Qayee-Bio diagnostic kits (Qayee Biotechnology Co., Ltd., Shanghai, China). According to Qayee-Bio for all tested parameters repeatability: the plate CV was less than 15%, and accuracy: standard linear regression correlation coefficient R with the expected concentration value was greater than or equal to 0.9900. Total antioxidant status (**TAS**) was determined using a Randox TAS kit (cat. no. NX2332, Randox Laboratories Ltd., Warszawa, Polska).

Statistical analysis

An individual bird (n = 7) was considered as the experimental unit in analyses of antibiotic levels in the liver, and other parameters in the serum of turkeys. The data were analysed by two-way ANOVA with the GLM procedure to examine the main effects of antibiotics used (C, M, E, D), the applied challenge (vaccinated vs unvaccinated; V effect), and their interaction. When the model was significant, Tukey's HSD test was performed to separate treatment means. The results were presented as means and pooled SEs of the mean (SEM). The statistical analysis was performed using STATISTICA software version 13.1 (2017) at a significance level of $P < 0.05$ (TIBCO Software Inc., 2017).

Results

Antibiotic levels in turkey liver

The conducted research shows that the use of a diet containing monensin resulted in a time-dependent increase in the level of this coccidiostat in the livers of turkeys, amounting to 0.75 ± 0.61 μg/kg on the 1st day of life vs 32 ± 16 μg/kg on the 56th day of life, respectively (Fig. 1). In the livers of turkeys that received doxycycline for the first 5 days of life, the level of this antibiotic was

Table 1
The sequences of all using primers for turkey genes.

Gene	Primer	Sequence (5′-3′)	Melting temperature (°C)	Product size (nt)	GenBank access no.
iNOS	Forward	CAACTCTCACAAAGACGCGG	59	91	NM_001303213
	Reverse	TTTGTGTGATGTGGGAACGC			
ACTB	Forward	TACCCCATTGAACACGGCAT	58	96	NM_001303173
	Reverse	CTCCTCAGGGGCTACTCTCA			
GAPDH	Forward	AGGATACACAGAGGACCAGGTTG	58	71	NM_001303179
	Reverse	CCGCATCAAAGGTGGAGGAATG			

iNOS - inducible nitric oxide synthase; *ACTB* - β-actin; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase.

13572.2 ± 11397.4 μg/kg on the 1st day of life and increased to 49109.61 ± 612.5 μg/kg on the 5th day of life. The level of this antibiotic in turkey livers decreased in the following days of life, reaching the value of 3.92 ± 0.78 μg/kg on day 56 (Fig. 2). Similarly, in the case of enrofloxacin, the level of this antibiotic in the liver increased in the first 5 days of life, when the birds received this antibiotic (3157.48 ± 973.4 μg/kg on the 1st day of life vs 9526.5 4 ± 227.1 μg/kg on the 5th day of life), and then on the 56th day of life, the level of this antibiotic in turkey livers was 2.86 ± 0.07 μg/kg (Fig. 3).

Interaction antibiotic × vaccination

The conducted research showed an antibiotic × vaccination interaction in 7- and 56-day-old turkeys in the case of analysis of iNOS gene expression ($P < 0.001$, both) and the case of analysis of nitric oxide level ($P = 0.007$ and $P < 0.001$, respectively) in the blood (Table 2 and Table 3). An antibiotic × vaccination interaction was also found in 7-day-old turkeys when analysing the levels of advanced oxidation protein products, 8-OHdG, NOX, TAS ($P < 0.001$, respectively), Casp 3 ($P = 0.008$) and Casp 8 ($P = 0.018$) in blood serum (Table 4). Statistical analysis of the results obtained from blood tests of 56-day-old turkeys also showed an antibiotic × vaccination interaction in the case of the levels of advanced oxidation protein products ($P < 0.001$), 8-OHdG ($P = 0.001$), myeloperoxidase ($P = 0.029$), NOX ($P = 0.012$), Casp 8 ($P < 0.001$), TAS ($P = 0.001$) (Table 5). The observed interactions indicate that the tested blood parameters were influenced by both early antibiotic administration and vaccination.

Effects of antibiotics and/or a coccidiostat

Compared to the control group, higher BW and higher BWG ($P = 0.006$, both) were found in 56-day-old turkeys with lower feed

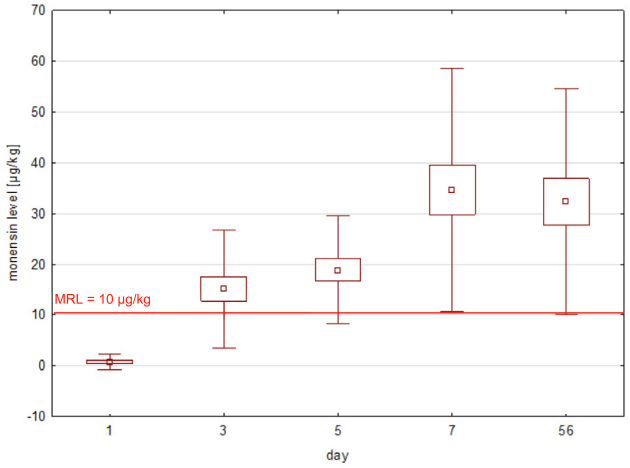

Fig. 1. Changes in monensin level in turkey liver (μg/kg of tissue) during the subsequent days of the experiment (n = 7) MRL - Maximum Residue Level.

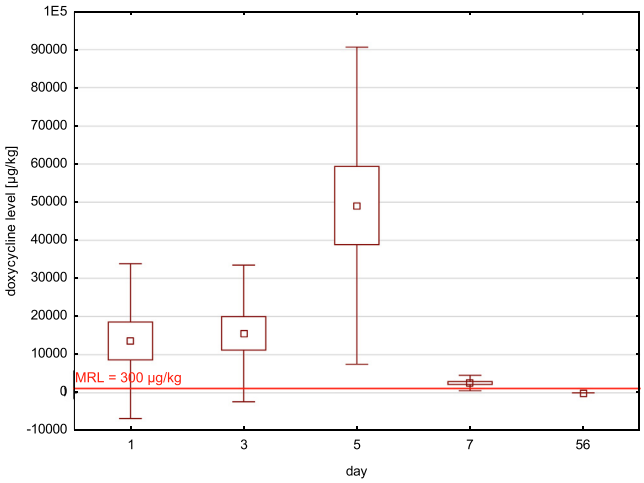

Fig. 2. Changes in doxycycline level in turkey liver (μg/kg of tissue) during the subsequent days of the experiment (n = 7) MRL - Maximum Residue Level.

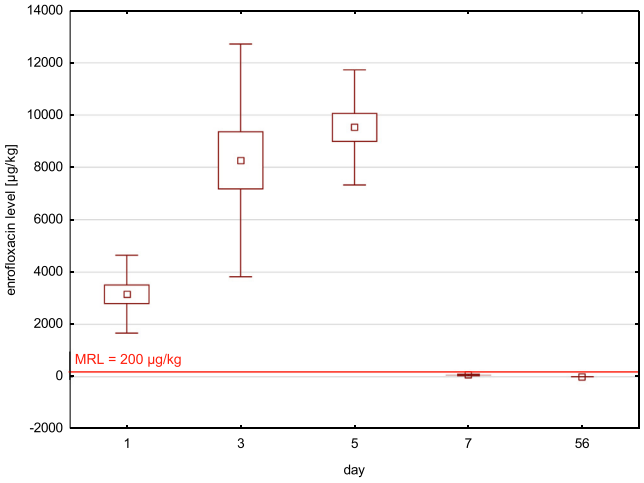

Fig. 3. Changes in enrofloxacin level in turkey liver (μg/kg of tissue) during the subsequent days of the experiment (n = 7) MRL - Maximum Residue Level.

conversion ratio ($P < 0.001$) in the group of turkeys receiving monensin supplements to feed (Table 6).

The conducted research showed increased catalase activity ($P < 0.001$) in the blood of 7-day-old turkeys receiving doxycycline. Administration of enrofloxacin and doxycycline to turkeys resulted in a reduction in OGG1 levels ($P = 0.006$) in the blood of 7-day-old turkeys (Table 4). A decrease in the level of 8-isoprostanes was found in the blood serum of 56-day-old turkeys receiving doxycycline in the first days of life ($P = 0.047$). Early administration of enrofloxacin resulted in an increase in GCH1/GTP levels ($P = 0.001$) and a decrease in catalase activity ($P = 0.027$) in the blood serum of 56-day-old turkeys (Table 5).

Table 2
iNOS expression and NO level in the blood of turkeys at 7 days of age (n = 7).

	Antibiotic[1]				Vaccine[2]		SEM	*P*-value		
Item	C	M	E	D	–	+		Antibiotic (A)	Vaccine (V)	A × V interaction
iNOS expression	0.571[a]	0.514[c]	0.554[b]	0.549[b]	0.565[a]	0.529[b]	0.005	<0.001	<0.001	<0.001
NO (μmol/L)	4.043[a]	2.887[b]	3.780[ab]	3.551[ab]	3.978[a]	3.152[b]	0.161	0.022	0.003	0.007

iNOS - inducible nitric oxide synthase; NO - nitric oxide.
[1] Treatment: C - untreated control; M - treated with monensin; E - treated with enrofloxacin; D – treated with doxycycline.
[2] Unvaccinated (–) or vaccinated (+).
[a,b,c] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Table 3
iNOS expression and NO level in the blood of turkeys at 56 days of age (n = 7).

	Antibiotic[1]				Vaccine[2]		SEM	*P*-value		
Item	C	M	E	D	–	+		Antibiotic (A)	Vaccine (V)	A × V interaction
iNOS expression	0.573[c]	0.589[b]	0.603[a]	0.581[bc]	0.576[b]	0.597[a]	0.004	<0.001	<0.001	<0.001
NO (μmol/L)	8.388	8.730	9.518	8.857	8.478[b]	9.268[a]	0.192	0.084	0.014	<0.001

iNOS - inducible nitric oxide synthase; NO - nitric oxide.
[1] Treatment: C - untreated control; M - treated with monensin; E - treated with enrofloxacin; D - treated with doxycycline.
[2] Unvaccinated (–) or vaccinated (+).
[a,b,c] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Table 4
Redox parameters in blood plasma of turkey at 7 days of age (n = 7).

	Antibiotic[1]				Vaccine[2]		SEM	*P*-value		
Item	C	M	E	D	–	+		Antibiotic (A)	Vaccine (V)	A × V interaction
8-isoprostanes (pg/mL)	221.9	202.1	217.8	211.8	211.3	215.5	6.550	0.748	0.759	0.436
AOPP (ng/mL)	91.33	84.76	87.67	95.51	93.87[a]	85.77[b]	2.077	0.075	0.009	<0.001
8-OHdG (ng/mL)	13.27[ab]	11.72[b]	14.43[ab]	16.23[a]	12.27[b]	15.56[a]	0.636	0.009	0.001	<0.001
MPO (U/L)	95.83	88.61	96.11	92.22	76.39[b]	110.00[a]	3.456	0.732	<0.001	0.510
NOX (pg/mL)	440.2[ab]	422.3[b]	468.8[a]	377.8[c]	450.7[a]	403.8[b]	8.622	<0.001	<0.001	<0.001
GCH1/GTP (ng/mL)	5.869	5.890	6.320	6.536	5.737[b]	6.570[a]	0.195	0.524	0.032	0.333
SOD (U/mL)	252.3	239.6	255.3	250.1	251.1	247.6	3.252	0.376	0.601	0.769
CAT (ng/mL)	15.81[b]	16.99[b]	17.18[b]	23.42[a]	16.94[b]	19.76[a]	0.773	<0.001	0.032	0.129
OGG1 (pg/mL)	601.9[a]	595.0[ab]	572.5[b]	570.3[b]	597.8[a]	572.0[b]	4.375	0.006	0.001	0.298
Casp 3 (pg/mL)	956.4	891.0	871.8	826.5	997.8[a]	775.0[b]	24.42	0.075	<0.001	0.008
Casp 8 (ng/mL)	119.1	120.3	106.7	119.5	121.2[a]	111.6[b]	17.58	0.073	0.025	0.018
TAS (mmol trolox/L)	0.989[a]	0.719[b]	0.735[b]	0.728[b]	0.862[a]	0.723[b]	0.034	0.001	0.008	<0.001

AOPP - advanced oxidation protein products; 8-OHdG - 8-hydroxy-2′ -deoxyguanosine; MPO - myeloperoxidase; NOX - NADPH oxidase; GCH1/GTP - cyclohydrolase 1; SOD - superoxide dismutase; CAT - catalase; OGG1 - oxoguanine glycosylase 1; Casp 3 - caspase 3; Casp 8 - caspase 8; TAS – total antioxidant status.
[1] Treatment: C - untreated control; M - treated with monensin; E - treated with enrofloxacin; D - treated with doxycycline.
[2] Unvaccinated (–) or vaccinated (+).
[a,b,c] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Table 5
Redox parameters in blood plasma of turkey at 56 days of age (n = 7).

	Antibiotic[1]				Vaccine[2]		SEM	*P*-value		
Item	C	M	E	D	–	+		Antibiotic (A)	Vaccine (V)	A × V interaction
8-isoprostanes (pg/mL)	499.9[a]	453.3[ab]	425.3[ab]	388.0[b]	473.7[a]	409.6[b]	15.11	0.047	0.026	0.479
AOPP (ng/mL)	54.21[a]	47.78[a]	46.64[a]	34.98[b]	51.67[a]	40.14[b]	1.857	<0.001	<0.001	<0.001
8-OHdG (ng/mL)	80.01[a]	51.85[c]	66.71[ab]	63.34[bc]	69.12[a]	61.83[b]	2.468	<0.001	0.050	0.001
MPO (U/L)	104.44	97.50	94.44	96.11	114.17[a]	82.08[b]	4.687	0.827	<0.001	0.029
NOX (pg/mL)	392.7[ab]	483.3[a]	419.2[ab]	351.1[b]	438.7[a]	384.5[b]	14.78	0.004	0.031	0.012
GCH1/GTP (ng/mL)	5.307[b]	6.167[ab]	7.421[a]	6.385[ab]	5.722[b]	6.918[a]	0.212	0.001	0.001	0.590
SOD (U/mL)	295.9	288.8	284.5	283.2	294.5	281.7	3.661	0.624	0.092	0.921
CAT (ng/mL)	431.8[a]	412.6[ab]	382.7[b]	387.7[ab]	432.9[a]	374.5[b]	7.723	0.027	<0.001	0.252
OGG1 (pg/mL)	949.5	904.0	873.5	968.7	990.2[a]	857.7[b]	4.079	0.246	0.001	0.234
Casp 3 (pg/mL)	1112.4	1109.9	1108.0	1017.5	1165.9[a]	1008.0[b]	19.76	0.124	<0.001	0.366
Casp 8 (ng/mL)	30.55	32.82	32.84	29.23	32.96[a]	29.76[b]	0.835	0.191	0.025	<0.001
TAS (mmol trolox/L)	1.280	1.336	1.343	1.328	1.297	1.347	0.028	0.804	0.313	0.001

AOPP - advanced oxidation protein products; 8-OHdG - 8-hydroxy-2′ -deoxyguanosine; MPO - myeloperoxidase; NOX - NADPH oxidase; GCH1/GTP - cyclohydrolase 1; SOD - superoxide dismutase; CAT - catalase; OGG1 - oxoguanine glycosylase 1; Casp 3 - caspase 3; Casp 8 - caspase 8; TAS – total antioxidant status.
[1] Treatment: C - untreated control; M - treated with monensin; E - treated with enrofloxacin; D - treated with doxycycline.
[2] Unvaccinated (–) or vaccinated (+).
[a,b,c] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Table 6
The effect of different antibiotics and vaccination on the growth performance of 56 d turkeys (n = 7).

	Antibiotic[1]				Vaccine[2]		SEM	*P*-value		
Item	C	M	E	D	–	+		Antibiotic (A)	Vaccine (V)	A × V interaction
BW 56 d (kg)	4.702[b]	4.808[a]	4.722[ab]	4.676[b]	4.855[a]	4.599[b]	0.022	0.006	<0.001	0.841
Average BWG (kg)	4.632[b]	4.738[a]	4.652[ab]	4.606[b]	4.785[a]	4.529[b]	0.022	0.006	<0.001	0.842
FCR (kg/kg)	1.751[a]	1.703[b]	1.762[a]	1.769[a]	1.752	1.740	0.006	<0.001	0.248	0.148
DFI (g)	142.9	141.8	144.4	143.5	147.3[a]	139.0[b]	0.749	0.305	<0.001	0.331
Mortality (%)	2.99	3.90	2.99	4.94	4.29	3.12	0.507	0.784	0.232	0.358

BWG - BW gain; FCR - feed conversion ratio; DFI - daily feed intake.
[1] Treatment: C - untreated control; M - treated with monensin; E - treated with enrofloxacin; D - treated with doxycycline.
[2] Unvaccinated (–) or vaccinated (+).
[a,b] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Effects of vaccination

It was found that vaccination of turkeys resulted in worse BW gain of turkeys from 1 to 56 days of age ($P < 0.001$). Lower daily feed intake was also found in vaccinated turkeys ($P < 0.001$) (Table 6).

Vaccination of turkeys resulted in an increase in myeloperoxidase and catalase activity ($P < 0.001$, both) and GCH1/GTP levels ($P = 0.032$) in the blood serum of 7-day-old turkeys. At the same time, a decrease in the level of OGG1 ($P = 0.001$) in blood serum was found in vaccinated 7-day-old turkeys (Table 4). As a result of the vaccination of turkeys, a decrease in the level of 8-isoprostanes ($P = 0.026$) was found in the blood serum of 56-day-old turkeys. It was also found that vaccination of turkeys resulted in an increase in GCH1/GTP levels ($P = 0.001$) and a decrease in catalase activity and Casp 3 levels ($P < 0.001$, both), (Table 5).

Discussion

In EU countries, a ban on the use of antibiotics as growth stimulants was introduced in 2006. However, the possibility of continuous use of coccidiostats in the feed, including ionophores, which are also antibiotics, has been left. Constant administration of coccidiostats (antibiotics) in feed mixtures is a method of preventing coccidiosis in birds. Paradoxical situations have arisen where the use of a specific antibiotic as a growth stimulator is prohibited, but monensin is allowed as a coccidiostat.

The conducted research showed that when monensin was constantly administered in the diet of turkeys, birds in the initial period of rearing (1–56 d) grew slightly better. However, during the later rearing period, the BW gains of turkeys from this group were equal to those of the control group, as presented in the publication by Mikulski et al. (2022). Research conducted by Elwinger et al. (1998) showed that the monensin ionophore induces a growth-stimulating effect, which can be attributed to its antibacterial effect. The literature review presented by Rajendran et al. (2018) confirms the pleitropic biological effects of monensin, including antibacterial, antiviral, antiparasitic, and even anticancer properties. Although there are reports of the inability of monensin to improve the growth of healthy birds, in the case of infected birds, the presence of monensin in the diet by modifying the intestinal microflora does not result in worse growth results (Robinson et al., 2019). Early administration of enrofloxacin or doxycycline did not affect the growth performance of turkeys.

Literature data show that this coccidiostat is safe for turkeys and turkey meat consumers, it is quickly biotransformed, which means that after use, only a short withdrawal period of 3–7 days is required for slaughter birds (JECFA, 2021). It is worth noting, however, that the current assessment of the safety of this coccidiostat rarely involved the analysis of redox status indicators at the molecular level. Coccidiostats generally induce the formation of reactive oxygen species, which have killing properties against the pathogen to be combated. Therefore, there is a risk that excessive production of free radicals during the administration of monensin may not only affect the pathogen against which they are directed but also the cells of the host organism, inducing unfavourable oxidative reactions. Available literature indicates that monensin sodium is easily absorbed in the gastrointestinal tract of monogastric animals, metabolised mainly in the liver, and then secreted into the bile and eliminated in the faeces (JECFA, 2021). Anadón and Martínez-Larrañaga (2014) showed that the highest concentrations of monensin residues are detected in the liver and lower concentrations in the fat and muscles of poultry in the period from 0 to 2 days after drug discontinuation. Our research shows that the concentration of monensin in the liver of turkeys gradually increased with increasing the time of its administration to the birds in the diet. Referring to the obtained values to the current values of Maximum Residue Level (**MRL**) – 10 μg/kg in the liver (EMA, 2013), it should be stated that during the period of monensin administration, its level in the liver exceeded three times the MRL value.

It is worth noting, however, that the last measurement of the concentration of this coccidiostat was performed in young birds on the 56th day of life without a withdrawal period, and standard rearing of turkeys for slaughter is carried out for 105–112 days. The liver is the organ where the biotransformation of antibiotics takes place. The recorded comparable values of monensin concentration in the liver of 7- and 56-day-old turkeys indicate the effective biotransformation of this coccidiostat. The withdrawal period for this coccidiostat is approximately 7 days before slaughter. It can therefore be assumed that with an appropriate withdrawal period, the concentration of monensin in the liver will decrease to values consistent with the MRL. There is therefore a need to precisely determine whether the 7-day withdrawal period for administering monensin to turkeys is sufficient, considering that the WHO even allows this period to be shortened to 1 day.

Enrofloxacin is an antibiotic from the fluoroquinolone group that is commonly used to treat bacterial infections in various animal species, including poultry. After oral administration, enrofloxacin is rapidly and almost completely absorbed from the gastrointestinal tract and then distributed throughout the body (Acaröz and Sözbilir, 2020; Slana et al., 2014). Particularly, high concentrations of enrofloxacin are found in the lungs, liver, kidneys, skin, bones, and lymphatic system (Anadon et al., 1995). Interestingly, the concentration of enrofloxacin and its metabolite – ciprofloxacin in tissues is 2–3 times higher than in serum (Knoll et al., 1999). Anadon et al., (1995) based on the results of studies in which chickens were orally administered enrofloxacin at a dose of 10 mg/kg per day for 4 consecutive days, found that although enrofloxacin is slowly removed from the body, on the 12th day after cessation administration, its residues were recorded only in the liver of chickens, and its average concentration in this

organ was only 0.025 +/- 0.003 mg/g. Our research also shows that the level of enrofloxacin in the liver of turkeys increased in the 1st 5 days of life when the birds received this antibiotic (48 μg/kg on the 1st day of life vs 95263.54 μg/kg on the 5th day of life), and then, on the 56th day of life, the level of enrofloxacin in turkey livers was 2.86 μg/kg, which is a very low value to the permissible MRL values for poultry livers of 200 μg/kg. It can therefore be concluded that this antibiotic is very well metabolised in the body and eliminated from it. Therefore, the low content of enrofloxacin in the liver observed on the 56th day of the life of turkeys allows us to assume that its early administration while maintaining an appropriate withdrawal period does not pose a risk to the consumer of turkey meat. It can be assumed that since the content of enrofloxacin in the liver remains very low, and this organ is most exposed to the accumulation of antibiotics as it is responsible for their biotransformation, the content of enrofloxacin in the more frequently consumed skeletal muscles will be even lower and therefore safer than in the liver. This is consistent with the results of the study by Hassan et al. (2019), who showed that the frequency of enrofloxacin residues is much higher in the liver than in the breast muscle, thigh, or stomach of chickens (43.33 vs 13.33, 20, 30%, respectively).

Analogous observations can also be made for doxycycline because, in the livers of turkeys that received doxycycline for the 1st 5 days of life, the level of this antibiotic was 1357.2 μg/kg on the 1st day of life and increased to 49109.61 μg/kg on the 5th day of life. However, the level of this antibiotic in the livers of turkeys decreased in the following days of life, reaching a value of 3.92 μg/kg on day 56, which is not much higher, but even comparable to the level of enrofloxacin in the liver. Moreover, Mestorino et al. (2018) administered doxycycline to broiler chickens at a dose of 10 mg/kg in drinking water for 5 days and also noted that the concentration of this antibiotic in muscles, liver, kidneys and skin/-fat usually falls below the MRL value (i.e. 100 μg/kg for muscles, 300 μg/kg for skin and fat, 300 μg/kg for the liver and 600 μg/kg for the kidneys) already on the 7th day after the end of antibiotic therapy. Due to their chemical structure, ionophores cause Ca^{2+} accumulation in cells and may also promote lipid peroxidation as a result of intensifying oxidative stress, which is undoubtedly one of the unfavourable side effects of its use (Ekinci et al., 2023). Available literature indicates that the ability of monensin to cause oxidative stress is because it affects mitochondria by reducing the mitochondrial membrane potential and disturbing the morphology of mitochondria, thereby inducing excessive production of free radicals (Charvat and Arrizabalaga, 2016). The main molecular mechanism of ionophore toxicity is disruption of the ion concentration gradient in cells, resulting in a change in pH and loss of mitochondrial membrane potential, which ultimately leads to disruption of the electron transport chain, oxidative phosphorylation, and ATP production, as well as increased production of reactive oxygen species (Ekinci et al., 2023; Charvat and Arrizabalaga, 2016). Moreover, Ketola et al. (2010) indicate that the use of monensin induces a transcriptional profile characteristic of the response to oxidative stress. It seems that the cells most sensitive to the adverse effects of reactive oxygen species are cardiac and skeletal muscle cells, which is probably because they are characterised by very high metabolic activity (Ekinci et al., 2023). However, our research does not indicate that the use of monensin worsened the redox status of the tested birds. This may probably be because the level of coccidiostat used was so low that these changes were not visible.

Available literature indicates that antibiotics such as enrofloxacin or doxycycline can also induce the production of reactive oxygen species, which is related to their mechanism of action (Xu et al., 2022; Liu et al., 2023; Badawy et al., 2021; Grabowski et al., 2022; Shan et al., 2022). Enrofloxacin, an antibiotic from the fluoroquinolone group, works by inhibiting bacterial topoisomerase II (DNA gyrase), an enzyme necessary for bacterial DNA replication, transcription, and repair (Grabowski et al., 2022). During this process, enrofloxacin can generate reactive oxygen species that can damage DNA, proteins, and lipids, leading to bacterial cell death, but also deterioration of host cell functioning (Badawy et al. 2021). Studies on carp liver cells have shown that this antibiotic can induce apoptosis of hepatocytes in a mitochondria-dependent manner, negatively affect the level of selected biochemical parameters related to the redox status, such as lactate dehydrogenase and malondialdehyde, and reduce the total potential mitochondrial membrane (DJm), as well as increase the production of reactive oxygen species (at a dose of 200 μg/ml) and reduce the total antioxidant capacity (Liu et al., 2015). Moreover, there is an assumption that the process of deethylation of enrofloxacin to ciprofloxacin by cytochrome P450 microsomal enzymes, which occurs during its biotransformation in the liver, results in the release of free radicals, which may then lead to a decrease in the efficiency of the antioxidant system and increased lipid peroxidation (Giergiel and Posyniak, 2016). The biocidal effect of doxycycline is related to its ability to inhibit protein synthesis by binding to bacterial ribosomes (Chopra et al., 1992). Although the mechanism of its oxidative action is not as good as in the case of monensin or enrofloxacin, it is known that sufficiently high doses of doxycycline may also result in increased induction of free radicals, probably resulting from its ability to interfere with the energy metabolism of the cell (Tan et al., 2017). Interestingly, some reports indicate that the use of pharmacologically appropriate doses of doxycycline reduces oxidative stress, thereby inhibiting lipid peroxidation and inflammatory reactions, thanks to which it can be successfully used in the treatment of many diseases characterised by chronic inflammation (Clemens et al., 2018). The results of our research showed increased catalase activity in the blood of 7-day-old turkeys as a result of doxycycline administration in the first 5 days of life. This enzyme is an important element of the antioxidant defence system of cells of aerobic organisms, and the mechanism of its action involves the degradation of hydrogen peroxide into water and oxygen (Gebicka and Krych-Madej, 2019). It can therefore be assumed that the observed increased catalase activity in the blood of 7-day-old chicks resulted from the increased synthesis of free oxygen radicals and hydrogen peroxide, which required immediate neutralisation. This assumption may be confirmed by the fact that the increase in catalase activity in the blood of young turkeys receiving doxycycline was also accompanied by a decrease in the level of OGG1. OGG1 is an enzyme that plays a key role in repairing DNA damage by excising damaged bases (base excision repair). OGG1 recognises and removes mutagenic bases induced by reactive oxygen species, such as 8-oxoguanine (8-oxoG), replacing them with normal counterparts (Rajendran et al., 2012). Moreover, OGG1 is also involved in regulating the transcription of various oxidative stress response genes (Wang et al., 2021). The binding of OGG1 to its substrate causes DNA bending and induces allosteric DNA change, which facilitates the occupation of Nuclear Factor kappa B and the assembly of the transcription apparatus (Ba and Boldogh, 2018). Reducing the level of OGG1 may indicate an increased synthesis of free oxygen radicals inducing many DNA damages, the need for repair of which led to the depletion of the functional OGG1 enzymatic protein. In the blood serum of 56-day-old turkeys receiving doxycycline in the first days of life, a decrease in the level of 8-isoprostanes, i.e. prostaglandin F2α isomers, which are formed as a result of arachidonic acid peroxidation in a cyclooxygenase-independent reaction, was found. They are considered biomarkers of oxidative stress because their presence in the body is the result of the action of free radicals on the phospholipids of cell membranes. It is assumed that they are a much more sensitive indicator of lipid peroxidation than

malondialdehyde (Milne et al., 2011). The observed reduction in the level of 8-isoprostanes in the blood of older turkeys that received doxycycline at an early stage of life can be treated as a positive effect. As previously mentioned, there are reports that doxycycline used at a sufficiently low dose may have an antioxidant effect. Therefore, it can be assumed that the observed beneficial effect of doxycycline may be related to the fact that in the days following the end of the therapy, its level in the body of the turkey decreased, reaching a level that protected lipids against peroxidation at the end of the experiment.

Similarly to the early administration of doxycycline, the early administration of enrofloxacin also negatively influenced the processes of repairing DNA damaged by the activity of reactive oxygen species in young chicks, as evidenced by the reduced level of OGG1 in the blood of 7-day-old turkey hens. Nevertheless, the unfavourable effect of early administration of enrofloxacin persisted until the end of experimental rearing, as increased levels of GCH1/GTP were found in the blood of older turkeys, along with decreased levels of catalase. GTP cyclohydrolase I (GCH1) is an enzyme that is part of the folate and biopterin biosynthetic pathways. It is responsible for the hydrolysis of guanosine triphosphate (GTP) to the form of 7,8-dihydroneopterin triphosphate (7,8-DHNP-3′-TP, 7,8-NH2-3′-TP) (Kraft et al., 2020). It has been proven that its activity may increase in response to oxidative stress (Latremoliere and Costigan, 2011). The reduction in catalase levels resulting from early administration of enrofloxacin may, in turn, be caused by the depletion of functional enzymatic protein as a result of the need to neutralise excessive amounts of hydrogen peroxide generated during the fight against free radicals generated in large amounts by antibiotic therapy.

Vaccination of birds against various pathogens also seems to have an impact on the redox status. Available literature proves that vaccination of poultry may induce oxidative stress through various mechanisms. Vaccinations, like other medical interventions, may cause stress reactions in animals' bodies, which may then lead to increased production of reactive oxygen species and other free radicals (Wyszyńska et al., 2019). As a consequence, it may also explain the deterioration in the growth performance of vaccinated turkeys observed in our studies. Vaccinations can also induce oxidative stress by inducing an immune response leading to the activation of immune cells such as T cells and macrophages. These, in turn, can produce reactive oxygen species and other free radicals as part of their biocidal activity (Wyszyńska et al., 2019). Our research showed that the vaccinations increased the levels of myeloperoxidase and catalase as well as the levels of GCH1/GTP while reducing the level of OGG1 in the blood serum of 7-day-old turkeys. An increase in the levels of myeloperoxidase and catalase in the blood serum of turkeys after vaccination may indicate increased antioxidant activity. Myeloperoxidase and catalase are enzymes that are involved in the neutralisation of reactive oxygen species, and an increase in their level in the blood may suggest that the turkey's body is responding to oxidative stress induced by vaccination, which is a positive effect because it helps protect cells from damage. An increase in the level of GCH1/GTP may suggest an intensification of the folate and biopterin biosynthetic pathways, which are important for many biological processes, including DNA synthesis and amino acid metabolism. Therefore, the observed increase in the level of this enzyme may suggest that the organism of turkeys is trying to counteract the oxidative stress caused by vaccination by increasing the production of folates and biopterin. Nevertheless, the observed reduction in the level of OGG1, which plays a key role in the repair of DNA damage, is disturbing because it suggests the depletion of the pool of functional OGG1 protein and thus deterioration of the cell's ability to repair DNA damage, which may lead to increased susceptibility to mutations and other DNA damage in young turkeys.

In the blood of 56-day-old turkeys, a decrease in the level of 8-isoprostanes, catalase, and Casp3 and an increase in the level of GCH1/GTP were found due to the vaccination. The obtained results can be interpreted as a positive effect that develops sometime after vaccination. Reducing the level of 8-isoprostanes, which are markers of oxidative stress, as well as catalase involved in the neutralisation of hydrogen peroxide, suggests a reduction in the severity of oxidative stress in the body. Caspase 3 is a key enzyme involved in the process of apoptosis (programmed cell death); hence, its reduction may indicate a lower level of apoptosis, probably resulting from less cellular damage generated by reactive oxygen species. In this case, increasing the level of GCH1/GTP can be treated as a beneficial effect, because GCH1 is an enzyme involved in the synthesis of tetrahydrobiopterin (BH4), which is necessary for the production of some neurotransmitters and is crucial for the proper immune response. In light of the above, it can be assumed that vaccination can improve the long-term health of turkeys by reducing oxidative stress and apoptosis, as well as improving immune functions. However, before the turkey's body reaches the state of adaptation and strengthening of the antioxidant status, a temporary deterioration of the antioxidant response is possible resulting from excessive generation of free radicals in response to vaccination.

Conclusion

Based on the conducted research, it was determined that as a result of administration of enrofloxacin or doxycycline until the 5th day of life, biotransformation of these antibiotics occurred in the liver until the 56th day of life of the turkeys, which was confirmed by their lower level than the MRL. Because the concentration of monensin (a coccidiostat that can be used up to 16 weeks of age) in the liver of turkeys gradually increased with the extension of the time of its administration in the diet, it is probable that discontinuing its addition a day before the slaughter of birds will result in the presence of this coccidiostat in the liver of turkeys. However, despite the accumulation of monensin in the liver of turkeys, this coccidiostat did not increase oxidative reactions in the organism of turkeys.

If it is necessary to use short-term antibiotic therapy in the early rearing period (up to the 5th day of life), it was found that the administration of doxycycline and enrofloxacin intensifies oxidative reactions in the body of turkeys, which is particularly visible in the short period after the end of their administration. Interestingly, the unfavourable effect of inducing oxidative reactions after enrofloxacin administration may persist much longer, even up to 8 weeks of age. However, after administration of doxycycline during the period when its level in the body decreases, there is a beneficial stimulation of the antioxidant system, which eliminates the oxidative reactions induced by this antibiotic.

The research shows that vaccination of turkeys can reduce oxidative reactions and apoptosis in the body. However, the effect of the redox system reaction is different immediately after vaccination (induction of oxidative reactions), which is due to the mechanism of action of the immune system.

If it is necessary to administer an antibiotic in the early rearing period, the effects of doxycycline on the organism's immunity will be less severe than those of enrofloxacin. However, the decision to administer an antibiotic should take into account the fact of a diagnosed disease where the expected health benefits outweigh the risk of weakening immunity.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101321.

Ethics approval

The protocol for this study was approved by the Local Ethics Committee for Animal Experiments in Olsztyn, Poland (Approval No. 47/2021; Olsztyn. Poland).

Data and model availability statement

The data were not deposited in an official repository.

The data that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

Acknowledgment

The authors would like to thank Marzena Mikulska from the Department of Poultry Science and Apiculture, University of Warmia and Mazury in Olsztyn for help with statistical calculations.

Financial support statement

This work was supported by the National Science Centre in Poland, Grant No. 2020/39/B/NZ9/00765.

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