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RESEARCH ARTICLE

Modification of the effects of aflatoxin B_1 on the glutathione system and its regulatory genes by zeolite

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ABSTRACT

The purpose of the present study was to use oxidative stress markers for investigating the effect of zeolite (315 mg/kg of complete feed) in the case of aflatoxin B_1 contamination (92 μg/kg complete feed). In a 21-day feeding trial with broiler chickens, oxidative stress parameters such as conjugated dienes, conjugated trienes, malondialdehyde, reduced glutathione content and glutathione peroxidase activity were not changed significantly by supplementation with this mycotoxin absorbent. The relative gene expression of transcription factors *KEAP1* and *NRF2* was not modified by the absorbent either. Still, the expression of *GSS*, *GSR* and *GPX4* genes increased significantly due to the aluminosilicate supplementation. The results suggest that zeolite reduced lipid peroxidation in the blood plasma but not in the red blood cell haemolysate or the kidney. The relative expression of the genes encoding the glutathione redox system also changed as a result of zeolite supplementation, but these changes were not found at the protein level.

KEYWORDS

aflatoxin B_1, glutathione redox system, broiler chickens, lipid peroxidation, gene expression, zeolite

INTRODUCTION

Aflatoxins (AFs), produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*, are unavoidable natural contaminants of feedstuffs and basic foods (Abrar et al., 2013). The most important AF in terms of toxic potency and occurrence is aflatoxin B_1 (AFB_1), which is mutagenic and carcinogenic to both humans and livestock and is classified as a Group I human carcinogen (IARC, 2002). Aflatoxin contamination in complete feeds in Europe showed high variation during the last decade. Between 2006 and 2007, the rate of AFB_1-positive feed samples was 8%, with 47 μg/kg as mean and 311 μg/kg as highest contamination (Binder et al., 2007). Between 2009 and 2011, the rate of AFB_1-positive feed samples was 24.5%, with 3.5 μg/kg being the mean and 52 μg/kg the highest values (Rodrigues and Naehrer, 2012). In 2019, the rate of samples positive for AFB_1 was 8%, with 10 μg/kg as the mean and 237 μg/kg as the highest concentrations (BIOMIN, 2020).

Poultry are sensitive to AF (Fink-Gremmels, 1999); however, the sensitivity to the acute effects of AFB_1, expressed as LD_{50} values, ranges from 0.4 mg/kg in day-old ducklings to 6.8 mg/kg in day-old chicks (Diaz and Murcia, 2019). The adverse effects caused by the

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consumption of AFB_1-contaminated feed include a reduction in feed utilisation and efficiency, reduced growth rate, and changes in body and organ weights (Han et al., 2008). Sirajudeen et al. (2011) reported that AFB_1 induced liver cell injury and increased peroxidation in the liver and kidney of chickens, accompanied by a significant decrease in total antioxidant capacity.

After entering the body, AFB_1 is metabolised by the CYP450 enzyme system into a highly unstable AFB_1-exo-8,9-epoxide (AFBO) molecule which reacts with cellular macromolecules, including DNA, RNA, proteins and phospholipids, thus inducing various genetic, metabolic, signalling, and cell structure disruptions (Zhuang et al., 2016; Rushing and Selim, 2017). Nevertheless, increasing evidence shows that the toxicity of AFB_1 is due to the induction of reactive oxygen species (ROS) generation, which causes oxidative stress (Marin and Taranu, 2012). Although it remains unknown whether the mycotoxins promote lipid peroxidation directly through the enhancement of ROS formation or the enhancement of tissue sensitivity to peroxidation results from compromised antioxidant defence, it appears that both processes are present in parallel (Yilmaz et al., 2017). Lipid peroxidation products have a long half-life; thus, they can diffuse into the lipid bilayer of membranes and cause oxidative damage even in cells far from their site of formation (Shen et al., 1994). However, our previous study revealed that aflatoxin B_1 has no time- or dose-dependent effect on oxidative stress parameters and on the relative expression of genes encoding enzymes of the glutathione redox system in chicken liver. Conjugated dienes (CD) and conjugated trienes (CT), acting as markers of lipid peroxidation, showed higher values in chickens fed 182 μg AFB_1/kg of diet, while the amounts of thiobarbituric acid reactive substances (TBARS) were increased after feeding 17 μg AFB_1/kg of diet. Glutathione content was lower at day 14 in the group fed an AFB_1-contaminated (92 μg/kg) feed. Glutathione peroxidase 4 activity was increased at days 7 and 21 in the 92 μg AFB_1/kg of diet group but decreased at day 14 in groups fed contaminated diets with 92 or 182 μg AFB_1 levels per kg of feed. The *GPX4* gene was downregulated at day 7 in the 92 μg/kg of feed group, but overregulated at days 14 and 21 in the 182 μg AFB_1/kg of feed group. Expression of the *GSS* gene was downregulated at day 14 in the 17 μg AFB_1/kg of feed group but overregulated at day 21 in chickens fed 17 and 92 μg AFB_1/kg of feed. Expression of the *GSR* gene was downregulated at days 7 and 21 in all treatment groups, but on day 14 an induction was observed in the group fed 182 μg AFB_1/kg of feed (Kövesi et al., 2020).

The activities of antioxidant enzymes, such as glutathione peroxidase (GPx), decreased as an effect of AFB_1 exposure, which can be explained by the conversion of free radicals into less harmful or harmless metabolites. Simultaneously, the amount of co-substrate, in this case reduced glutathione (GSH), also decreased, which might have resulted in lower enzyme activity, too (Kodama et al., 1990).

Malondialdehyde (MDA) is a significant meta-stable end product of lipid peroxidation, which is formed via the degeneration of certain primary and secondary lipid peroxidation products (Janero, 1990). A high MDA level was found in the liver and kidney of chicks given 150–300 μg/kg of feed AFB_1 for 21 days (Ozen et al., 2009). In another study with chickens given 300 μg/kg of feed AFB_1 for 21 days, increased levels of MDA and decreased levels of GSH were found in the liver and kidney (Karaman et al., 2010).

Numerous feed additives are proposed for use against the toxic effects of aflatoxin B_1 (Fouad et al., 2019). Among them, mycotoxin absorbents, such as charcoal (Yamauchi et al., 2014), zeolite and bentonite (Prasai et al., 2018) and calcium aluminosilicates (Chen et al., 2014) can prevent the absorption of aflatoxins from the gastrointestinal tract and their entry to the liver. Aluminosilicates, hydrated sodium calcium silicates, or yeast cell wall-derived glucomannan can prevent the adverse effects of aflatoxin B_1 in broiler chicken, including impairment of the production traits and some clinical and biochemical parameters of the blood plasma (Nazarizadeh and Pourreza, 2019). Several previous researches demonstrated the effect of different adsorbents on the level of ROS or lipid peroxidation parameters, such as MDA. For instance, aluminosilicate (bentonite) significantly decreased the ROS level in Japanese quail, as measured by 2′-7′-dichlorofluorescein assay, or MDA content in the liver and blood serum in a long-term (20-day) feeding trial with aflatoxin-contaminated feed (Migliorini et al., 2017).

The aim of this study was to investigate the effect of zeolite as mycotoxin absorbent on the changes of some lipid peroxidation and glutathione redox markers and on the relative expression of the *GPX4* gene and several transcription factors of GSH synthesis as well as glutathione disulphide (GSSG) reduction, caused by aflatoxin B_1 exposure. We hypothesised that aflatoxin B_1 induces oxidative stress and, on the other hand, activates the antioxidant defence. These changes may be affected by feed supplementation with a mycotoxin absorbent, which was zeolite in this study.

MATERIALS AND METHODS

Birds and experimental design

A total of 60 Cobb 540 broilers were obtained from a commercial hatchery and were kept on deep litter with a natural light regimen (12 L/12 D). Chickens were randomly allocated to three experimental groups (control, AFB_1 and AFB_1 + zeolite (ZE); n = 20 in each), resulting in similar average body weights. The experiment was started at 21 days of age, and the experimental diets were fed for 21 days. The basal diet was a commercial chicken grower complete feed (Vitafort Ltd., Dabas, Hungary) without mycotoxin sequesters and coccidiostats. The basal diet contained aflatoxins at levels lower than the limit of quantification (LOQ) (0.1 μg/kg for AFB_1, 0.2 μg/kg for AFB_2, 0.5 μg/kg for AFG_1 and 0.5 μg/kg for AFG_2). The nutrient content of the diet met the requirements for broiler chickens (Hungarian Feed Code, 2014). Before starting the feeding of experimental

diets, feed deprivation of 12 h was applied. The mycotoxin absorbent was aluminosilicate (zeolite) containing 62% clinoptilolite and 15% mordenite. The zeolite content of the diet was 315 mg/kg. The amount of zeolite selected was based on a previous study, which suggested that adverse effects can be found at high levels (5–10 mg/kg of feed), but none of the studies showed changes in the antioxidant status of the animals (Elliott et al., 2020).

Production of mycotoxin, artificial mycotoxin contamination of the feed and determination of mycotoxin content in feeds

AFs were produced by an *Aspergillus flavus* strain isolated by Dobolyi et al. (2013) on artificially infected corn substrate. The strain was identified and deposited in the Microbiological Collection of the University of Szeged (SZMC) with the accession number SZMC 20750. The measured AFB_1 concentration of the mould-infected corn substrate was 4.694 mg/kg of dry matter.

An appropriate amount of corn substrate containing AFs was mixed with the basal diet. The measured AF content of the diets is given in Table 1. The aflatoxin contamination level was calculated as the median of mean and highest values from the last decade in feed commodities in Europe, as it was previously mentioned, namely 47 µg/kg mean and 311 µg/kg highest (Binder et al., 2007), 3.5 µg/kg mean and 52 µg/kg highest (Rodrigues and Naehrer, 2012) and 10 µg/kg mean and 237 µg/kg highest (BIOMIN, 2020).

The AF content of the inoculate and the experimentally contaminated feeds was determined from three replicate samples (20 g each), which were taken from five different points of the batch (10 g each) and thoroughly homogenised before preparing the analytical samples. The samples were analysed after extraction with acetonitrile: water (9:1, v/v), immunoaffinity clean-up was done with Aflaprep® column (R-Biopharm, Darmstadt, Germany) and after reversed-phase isocratic (acetonitrile: methanol: water; 8:27:65, v/v/v) HPLC method with fluorescence detection (Khayoon et al., 2010). The LOQ of the determination was 1.0 µg/kg for AFB_1, 1.0 µg/kg for AFB_2, 0.5 µg/kg for AFG_1 and 0.5 µg/kg for AFG_2.

Table 1. Aflatoxin content of the experimental diets (µg/kg)

Diet	AFB_1	AFB_2	AFG_1	AFG_2
Control	<1.0	<1.0	<0.5	<0.5
Aflatoxin	97.0	<1.0	<0.5	<0.5
Aflatoxin + ZE	92.0	<1.0	<0.5	<0.5

ZE: zeolite.

Measurement of feed intake and mortality

Feed intake was measured daily in each group, and the average daily aflatoxin intake was calculated based on the AFB_1 content of the feed. Mortality was checked daily in each experimental group.

Sampling and determination of lipid peroxidation and antioxidant parameters

At the start of the experiment, two birds each were taken out from each group. These six randomly selected birds were euthanised and served as absolute control (day 0). On days 7, 14 and 21 of mycotoxin exposure, 6 birds from each group were sampled. The birds were euthanised by cervical dislocation. Whole blood was collected on ice and, post mortem, liver and kidney were removed and collected on ice. The blood plasma was separated by centrifugation (2,500 *g*, 10 min), and red blood cells were haemolysed with a nine-fold volume of redistilled water. All samples were stored at −70 °C until analysed. For gene expression studies, portions of the liver were frozen in liquid nitrogen immediately after sampling and stored at −70 °C until analysis to prevent RNA degradation.

The markers of lipid peroxidation (CD, CT and MDA), as well as the concentration of GSH and the activity of GPx, were determined from blood plasma, red blood cell haemolysates, liver and kidney samples as described in our previous study (Balogh et al., 2019).

RNA isolation, reverse transcription and qPCR

RNA isolation, reverse transcription and qPCR measurements were performed as described previously (Kövesi et al., 2019). Briefly, total RNA extraction was performed with Nucleozol Reagent (Macherey-Nagel, Düren, Germany) from 10 mg liver tissue homogenate based on the instructions of the manufacturer. RNA samples were treated with DNase I (Thermo Fisher Scientific, San Jose, CA, USA) to avoid genomic DNA contamination. Agarose gel electrophoresis was performed to check the quality and integrity of RNA, and the absorption ratio of 260:280 nm higher than

Table 2. Primers of target (*GPX4*, *GPX3*, *GSS*, *GSR*, *NRF2*, *KEAP1*) and endogenous housekeeping (*GAPDH*) genes

Gene	Forward (5′ 3′)	Reverse (5′ 3′)	GenBank accession number
GAPDH	TGACCTGCCGTCTGGAGAAA	TGTGTATCCTAGGATGCCCTTCAG	NM_204305.1
KEAP1	CATCGGCATCGCCAACTT	TGAAGAACTCCTCCTGCTTGGA	XM_025145847.1
NRF2	TTTTCGCAGAGCACAGATAC	GGAGAAGCCTCATTGTCATC	NM_205117.1
GPX4	AGTGCCATCAAGTGGAACTTCAC	TTCAAGGCAGGCCGTCAT	NM_001346448.1
GSS	GTACTCACTGGATGTGGGTGAAGA	CGGCTCGATCTTGTCCATCAG	XM_425692.6
GSR	CCACCAGAAAGGGGATCTACG	ACAGAGATGGCTTCATCTTCAGTG	XM_015276627.2

2.0 was accepted. cDNA production was implemented with RevertAID Reverse transcriptase (Thermo Fisher Scientific, San Jose, CA, USA) based on a standard protocol.

The primers (Table 2) used for the quantification of relative mRNA transcription of *GPX4*, *GSS*, *GSR*, Kelch-like ECH-Associated Protein 1 (*KEAP1*), and Nuclear Factor Erythroid 2 p45-Related Factor 2 (*NRF2*), and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were as described previously (Balogh et al., 2019). *GAPDH* can be used as a housekeeping gene because some previous studies with mycotoxins in broiler chickens (Yarru et al., 2009; Salem et al., 2018) did not show an effect on its relative expression in oxidative stress.

The threshold cycle (Ct) of the target genes (*NRF2*, *KEAP1*, *GPX4*, *GSS* and *GSR*) and the endogenous housekeeping control gene (*GADPH*) was determined by StepOne™/StepOnePlus™ Software v2.2 (Thermo Fisher Scientific, San Jose, CA, USA). The delta Ct values (ΔCt), delta-delta Ct values (ΔΔCt) and relative quantification ($RQ = 2^{-\Delta\Delta Ct}$) values were calculated by the formula described by Livak and Schmittgen (2001).

Statistical analyses

The normality of distribution was confirmed by the Shapiro–Wilk test, and homogeneity of variance was tested with Bartlett and Browne–Forsythe tests. Data with these conditions were analysed by one-way ANOVA. The significance of differences between groups was evaluated using a *post-hoc* Tukey test ($P < 0.05$). Analyses were performed with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± standard deviation (SD).

Ethical issues

The experiment was carried out in conformity with the Hungarian Animal Protection Act and according to the relevant EU rules. The experimental protocol was authorised by the Department of Food Chain Safety, Land Register, Plant and Soil Protection, and Forestry of the Pest County Government Office (Hungary) with the permission number PE/EA/1964-7/2017.

RESULTS

There were no clinical signs of toxicity and mortality in the trial. The average daily AFB_1 intake calculated from the feed intake was nearly the same in the groups fed aflatoxin-contaminated or aflatoxin-contaminated and mycotoxin absorbent containing diets (Table 3).

Markers of the initial phase of lipid peroxidation, CD and CT did not change significantly in the liver as an effect of the treatments (data not shown). The termination marker of lipid peroxidation processes, TBARS expressed as MDA, was significantly lower in the blood plasma on day 7 but significantly higher on days 14 and 21 of AFB_1 treatment as compared to the control. On the 21st day of exposure, the MDA level was significantly lower in the AFB_1 + mycotoxin adsorbent group than in the AFB_1-treated one (Table 4). As the effect of AFB_1 treatment, MDA content in red blood cell haemolysates showed significantly lower values on day 21 than in the control and the AFB_1 + mycotoxin adsorbent group. In the liver, only trend-level changes were observed during the experiment (data not shown). In contrast, the MDA content of the kidney showed significantly higher

Table 3. Effect of diets containing aflatoxin B_1 or aflatoxin B_1 and zeolite on the average daily AFB_1 intake of chickens (μg/day)

Group	Days 1–7	Days 8–14	Days 15–21
Control	<1.00	<1.00	<1.00
Aflatoxin	13.87	13.16	12.32
Aflatoxin + zeolite	11.50	10.86	11.22

Table 4. Effect of AFB_1 and AFB_1 + zeolite containing diets on malondialdehyde content in blood plasma, red blood cell haemolysates and kidney homogenates (mean ± SD; $n = 6$)

Test parameter	Sample matrix	Experimental group	Day 0	Day 7	Day 14	Day 21
Malondialdehyde	Blood plasma*	Control	5.81 ± 0.93	7.21^{b} ± 1.73	5.62^{a} ± 0.76	4.43^{a} ± 0.46
		Aflatoxin		5.09^{a} ± 0.98	6.58^{b} ± 0.33	6.48^{b} ± 1.23
		Aflatoxin + ZE		6.26^{ab} ± 0.33	6.24^{ab} ± 0.45	4.28^{a} ± 0.68
	Red blood cell haemolysate*	Control	11.45 ± 0.94	11.78 ± 2.07	8.88 ± 0.73	8.37^{b} ± 0.49
		Aflatoxin		10.94 ± 0.61	9.91 ± 0.51	6.93^{a} ± 0.91
		Aflatoxin + ZE		12.31 ± 0.73	8.89 ± 1.25	8.44^{b} ± 0.80
	Kidney**	Control	43.85 ± 4.12	59.46 ± 19.4	39.13 ± 6.75	65.6^{ab} ± 11.57
		Aflatoxin		57.93 ± 9.47	48.94 ± 7.10	79.25^{b} ± 11.26
		Aflatoxin + ZE		61.83 ± 12.08	38.40 ± 7.36	54.48^{a} ± 11.37

ZE: zeolite.

[a,b] Different superscripts in the same column indicate significant difference from the control at $P < 0.05$ level. *μmol/ml; **μmol/g wet weight tissue.

values in the AFB_1 group than in the AFB_1 + mycotoxin adsorbent group (Table 4).

The concentration of GSH in the blood plasma decreased significantly in the group fed the AFB_1 + mycotoxin adsorbent containing diet compared to the control and the AFB_1-treated group on day 14 of exposure. Also, lower values were measured in the 10,000 *g* supernatant fraction of kidney homogenates in the group fed the AFB_1 + mycotoxin adsorbent containing diet compared to the AFB_1-treated group on the 14th day of exposure (Table 5). There were trend-level but not significant changes in the GSH content of red blood cell haemolysates and liver homogenates (data not shown).

No significant differences were found in GPx activity in the 10,000 *g* supernatant fraction of liver and kidney homogenates (data not shown). In the blood plasma, significantly higher values were measured on day 7 of AFB_1 exposure than in the control and the AFB_1 + mycotoxin adsorbent group. However, significantly lower values were measured as an effect of AFB_1 treatment on day 14 than in the control. One week later, on day 21, there were no significant differences. However, on day 21, regarding the red blood cell haemolysates, significantly lower values were observed in the AFB_1 + mycotoxin adsorbent group than in the control (Table 6).

The relative expression of the *GPx4* gene was significantly lower in both treatment groups than in the control on day 14 of exposure. Later, on day 21, there was no difference between the treatment groups and the control group; however, *GPx4* expression in the AFB_1-treated group was significantly lower than in the AFB_1 + mycotoxin adsorbent group (Table 7).

GSS and *GSR* gene expression showed the same statistically significant differences. The gene expression was significantly higher in the AFB_1 + mycotoxin adsorbent treatment group on days 7, 14 and 21. Still, significantly lower values were measured in the AFB_1-treated group than in the control for both *GSS* and *GSR* on day 21 (Table 7).

The relative expression of *KEAP1* was significantly higher in both treatment groups than in the control on day 7, but later no changes were observed. On day 7, *NRF2* gene expression was significantly higher in both treatment groups than in the control but on day 14 it was significantly lower, and then no changes were found on day 21 (Table 8).

DISCUSSION

Changes in the markers of the initial phase of lipid peroxidation (CD and CT values) suggest that the lipid peroxidation processes in the examined tissues, due to long-term aflatoxin exposure, were in their termination phase.

MDA content of the blood plasma showed significantly higher values on days 14 and 21 in the AFB_1-treated group than in the control, but it was significantly lower in the AFB_1 + zeolite group than in the AFB_1-treated one. In contrast, on day 21 the MDA content in the red blood cell haemolysates showed significantly lower values in the AFB_1-treated group than in the control and the AFB_1 + zeolite group. There were no significant changes in the MDA content of

Table 5. Effect of AFB_1 and AFB_1 + zeolite containing diets on reduced glutathione concentration in blood plasma and kidney homogenates (mean ± SD; $n = 6$)

Test parameter	Sample matrix	Experimental group	Day 0	Day 7	Day 14	Day 21
Reduced glutathione	Blood plasma*	Control	8.44 ± 0.99	8.99 ± 0.56	7.96^{b} ± 0.90	7.62 ± 0.83
		Aflatoxin		10.10 ± 1.88	7.65^{b} ± 0.54	7.48 ± 0.98
		Aflatoxin + ZE		8.59 ± 0.81	6.61^{a} ± 0.47	7.19 ± 0.69
	Kidney**	Control	6.28 ± 0.36	4.84 ± 1.00	5.64^{ab} ± 0.52	4.87 ± 0.67
		Aflatoxin		6.04 ± 2.25	6.00^{b} ± 0.41	5.37 ± 0.80
		Aflatoxin + ZE		5.24 ± 1.22	5.25^{a} ± 0.27	5.08 ± 0.59

ZE: zeolite.
[a, b] Different superscripts in the same column indicate significant difference from the control at $P < 0.05$ level. *µmol/g protein content; **µmol/g 10,000 *g* supernatant protein.

Table 6. Effect of AFB_1 and AFB_1 + zeolite containing diets on glutathione peroxidase activity in blood plasma and red blood cell haemolysates (mean ± SD; $n = 6$)

Test parameter	Sample matrix	Experimental group	Day 0	Day 7	Day 14	Day 21
Glutathione peroxidase (U/g 10,000 *g* supernatant protein)	Blood plasma	Control	8.87 ± 0.72	8.49^{a} ± 1.06	10.26^{b} ± 1.41	7.67 ± 0.68
		Aflatoxin		12.10^{b} ± 2.60	8.38^{a} ± 0.32	6.48 ± 1.00
		Aflatoxin + ZE		8.65^{a} ± 1.73	9.43^{ab} ± 1.08	7.53 ± 0.89
	Red blood cell haemolysate	Control	8.51 ± 2.65	6.94 ± 1.90	6.52 ± 0.71	7.48^{b} ± 0.41
		Aflatoxin		9.35 ± 4.34	5.72 ± 0.46	6.35^{ab} ± 1.44
		Aflatoxin + ZE		7.14 ± 1.18	6.39 ± 0.78	5.06^{a} ± 0.49

ZE: zeolite.
[a, b] Different superscripts in the same column indicate significant difference from the control at $P < 0.05$ level.

Table 7. Effect of AFB_1 and AFB_1 + zeolite containing diet on the relative expression of *GPX4*, *GSR* and *GSS* genes in the liver of broiler chickens (mean ± SD; $n = 6$ in a pool, equal amounts of cDNA per individual)

Test parameter	Experimental group	Day 0	Day 7	Day 14	Day 21
Glutathione peroxidase 4 (*GPX4*) expression (RQ)	Control	1.00 ± 0.07	0.99 ± 0.28	1.30^{c} ± 0.08	0.97^{ab} ± 0.09
	Aflatoxin		0.99 ± 0.25	1.07^{b} ± 0.10	0.88^{a} ± 0.04
	Aflatoxin + ZE		0.97 ± 0.21	0.82^{a} ± 0.07	1.04^{b} ± 0.09
Glutathione synthetase (*GSS*) expression (RQ)	Control	1.04 ± 0.07	1.33^{a} ± 0.18	1.43^{a} ± 0.21	0.99^{b} ± 0.14
	Aflatoxin		1.47^{a} ± 0.19	1.68^{a} ± 0.34	0.74^{a} ± 0.17
	Aflatoxin + ZE		2.37^{b} ± 0.17	2.56^{b} ± 0.26	1.22^{c} ± 0.11
Glutathione reductase (*GSR*) expression (RQ)	Control	1.00 ± 0.06	1.16^{a} ± 0.22	0.96^{a} ± 0.22	0.75^{b} ± 0.15
	Aflatoxin		1.41^{a} ± 0.26	1.00^{a} ± 0.14	0.54^{a} ± 0.11
	Aflatoxin + ZE		2.01^{b} ± 0.30	1.68^{b} ± 0.21	1.11^{c} ± 0.17

ZE: zeolite.
[a, b, c] Different superscripts in the same column indicate significant difference from the control at $P < 0.05$ level.

Table 8. Effect of AFB_1 and AFB_1 + zeolite containing diet on the relative expression of *KEAP1* and *NRF2* genes in the liver of broiler chickens (mean ± SD; $n = 6$ in a pool, equal amounts of cDNA per individual)

Test parameter	Experimental group	Day 0	Day 7	Day 14	Day 21
Kelch-like ECH-Associated Protein 1 (*KEAP1*) expression (RQ)	Control	1.03 ± 0.06	1.18^{a} ± 0.08	1.16 ± 0.10	0.70 ± 0.07
	Aflatoxin		1.41^{b} ± 0.11	1.19 ± 0.17	0.69 ± 0.06
	Aflatoxin + ZE		1.86^{c} ± 0.16	1.14 ± 0.08	0.69 ± 0.07
Nuclear Factor-Erythroid 2 p45-Related Factor 2 (*NRF2*) expression (RQ)	Control	1.00 ± 0.03	1.20^{a} ± 0.07	1.63^{c} ± 0.20	0.92 ± 0.10
	Aflatoxin		1.53^{b} ± 0.10	1.05^{b} ± 0.09	0.91 ± 0.12
	Aflatoxin + ZE		1.52^{b} ± 0.14	0.85^{a} ± 0.12	0.96 ± 0.13

ZE: zeolite.
[a, b, c] Different superscripts in the same column indicate significant difference from the control at $P < 0.05$ level.

the liver among the treatment groups. In contrast, on day 21 the MDA content of the kidney was significantly lower in the AFB_1 + zeolite group than in the case of AFB_1 treatment alone. Changes in the blood plasma and kidney MDA levels suggest that the effect of mycotoxin absorbent becomes more pronounced by day 21, which suggests that less mycotoxin is absorbed from the intestine, possibly due to the longer transit time of feed particles in the intestine, resulting in more efficient binding. The decrease in MDA content in red blood cell haemolysate may be related to the changes induced by AFB_1 exposure in erythrocyte number and function (Wang et al., 2015). However, MDA concentration in the kidney increased by day 21, which may be caused by the inadequate antioxidant defence. In contrast, the MDA content of the liver did not increase, probably due to the effect of the glutathione redox system, which counterbalanced the lipid peroxidation-inducing effect of aflatoxin exposure.

The changes in lipid peroxidation processes in the liver were similar to those observed in the *KEAP1* gene but were opposite to the changes found in *NRF2* gene expression. This result suggests that the redox changes caused by the absorbed aflatoxins did not induce the same changes at the gene expression level. Zeolite addition generated a more robust Nrf2 response at the gene expression level in the 3rd week. This may be due to the decreased *KEAP1* expression detected in the 2nd week. Under normal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, ubiquitinated, and degraded in the proteasomes (Clarke et al., 2016). Under oxidative stress conditions, reactive cysteine residues in Keap1 undergo redox modification (Bryan et al., 2013), inhibiting the Keap1-mediated degradation of Nrf2, resulting in accumulation of Nrf2 in the nucleus, and increasing the transcription of genes of antioxidant responsive elements (AREs) in their promoter regions (Hayes and Dinkova-Kostova, 2014). The mode of action of zeolite on the Keap1–NrF2–ARE pathway remains unclear and requires further investigations.

GSH content in the blood plasma decreased as a result of aflatoxin exposure, which may be due to a decrease in the synthetic capacity of the liver, which is the primary site of GSH biosynthesis (Shelly and Lu, 2013), and reduced GSH efflux from the liver cells, which could be a cause of GSH depletion in the blood plasma. On the other hand, the kidney had the lowest amount of GSH due to the effect of zeolite on day 14, which may be related to decreased renal GSH synthesis and oxidation. The exact mechanism is not known yet. However, the GSH content was not correlated with the expression of *GSS* at the same time because the increase of the latter did not cause a change in the GSH content of the liver. The expression of the *GSR* gene encoding the enzyme catalysing the reduction of glutathione disulphide (GSSG) changed with the GSH content, suggesting that the trend-level increase in GSH content in the liver may have increased the reduction of glutathione disulphide by day 14. Also, our results on *GSS* expression suggest that the changes in mRNA levels do not imply a simultaneous increase in the intensity of protein synthesis

because they are influenced by other factors, such as microRNAs (Ambros, 2004). Based on our results, the changes in the expression of *GSS* and *GSR* genes are not correlated with *NRF2* expression either, suggesting that activation of the Nrf2–ARE transcription pathway presupposes the presence of Nrf2 protein. However, the alterations observed in the group with the mycotoxin adsorbent suggest that the effect of the absorbed aflatoxin on the oxidative stress processes in the liver changes with age, and zeolite affects the glutathione synthesis and/or reduction at the gene expression level, possibly by its effect on the amount of absorbed AFB_1 during the experiment.

The GSH content of the tissues is also influenced by GPx activity; however, this association was only partially detected in the blood plasma and the red blood cell haemolysate. Hepatic and renal GPx activity changed only moderately during the study period. In the case of the liver, this may be due to a decrease in *GPx4* gene expression in week 2, which is presumably related to a decrease in *Nrf2* gene expression over the same period, also at the protein synthesis level, so the Nrf2 protein did not activate ARE, including transcription of the *GPx4* gene. Subsequently, however, the expression of *GPx4* showed an increase during the entire period of aflatoxin exposure in the case of mycotoxin absorbent supplementation, although this effect was not detectable in the activity of the GPx enzyme. A relationship between plasma and renal GPx activity can also be hypothesised because plasma GPx3 is synthesised in the renal tubular cells (Avissar et al., 1994). On day 7, the effect of aflatoxin was associated with an increase in GPx activity in the kidney and the blood plasma at the same time; however, on day 14, there was no change in renal GPx activity concomitant with the decrease of GPx activity in the blood plasma.

In conclusion, the results revealed that the applied amount of aflatoxin B_1 caused only moderate oxidative stress, which was reduced further by the use of zeolite as a mycotoxin adsorbent, possibly due to the lower amount of mycotoxin absorbed. The effect of zeolite was more marked in the relative gene expressions of the *GSS*, *GSR* and *GPx* genes than at protein (GPx4 activity) or GSH level. The exact mechanism of this process is not known yet, and it requires further investigations.

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