SZENT ISTVÁN UNIVERSITY
FACULTY OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES
INSTITUTE OF BASIC ANIMAL SCIENCE
DEPARTMENT OF NUTRITION

STUDY ON THE EFFICIENCY OF CERTAIN PHYTOBIOTICS TO REDUCE THE DETRIMENTAL EFFECTS OF MYCOTOXINS IN POULTRY

PhD thesis

Mangesh K. Nakade

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The Doctoral School

Name: Doctoral School of Animal Science

Science: Animal husbandry

Head: Dr. Miklós Mézes

                              professor, MHAS

                              Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Basic Animal Sciences, Department of Nutrition

Supervisor: Dr. Krisztián Balogh
                              associate professor, PhD

                              Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Basic Animal Sciences, Department of Nutrition

Co-supervisor: Dr. Márta Erdélyi
                              associate professor, PhD

                              Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Basic Animal Sciences, Department of Nutrition

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Approval of the Head of the Doctoral School

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Approval of the Supervisor   Approval of the Co-supervisor

1. INTRODUCTION

Cereal grains have an important role in the nutrition of monogastric animals, and those are frequently infected with toxigenic moulds during field or storage conditions. Their secondary metabolites, the mycotoxins, are potentially toxic substances for animals and humans.

Trichothecene mycotoxins, such as deoxynivalenol (DON) and T-2 toxin, are produced by different Fusarium moulds in temperate climatic conditions. They are responsible for most of the mycotoxin problems occurring in Europe and North America, while in subtropical and tropical regions of the world the most important mycotoxins, such as aflatoxin B1 (AFB_1), and are produced by different species of Aspergillus genus.

The long-term toxic effects of the above-mentioned mycotoxins on different poultry species are well known, but there is rare information about their short-term effects.

The long-term pro-oxidant effects of T-2 toxin, DON and aflatoxin are intensively investigated, resulting sometimes confusing results, but the indicators measured show only the final stage of the process of oxidative stress, which can be in relation to increasing levels of damage or even adaptation.

The time of the manifestation of the toxic effects of mycotoxins after per os intake is an important question, including the level of the oxidative stress induced, and on the other side, the changes in the parameters of the biological antioxidant system.

These are still open questions, therefore one of the aims of the series of experiments presented in my PhD thesis was to evaluate the short-term effects of sub-lethal doses of trichothecene mycotoxins (DON or T-2 toxin) or aflatoxin on the lipid peroxidation processes in broiler chickens or laying hens, and on the amount and activity of the glutathione redox system, an important part of the biological antioxidant defence system.

Phytobiotics represent a wide range of bioactive compounds derived from different parts (leaves, shoot, root, flower and seed) of herbs and spices along with plant extracts or essential oils obtained from the plants, embedded into diets to improve livestock productivity. The importance of these phytobiotics is based on their biologically active components, such as polyphenols, flavonoids etc.

Large scale of bioactive substances has been identified in herbs and spices, with numerous effects. Amongst others, immune stimulating, digestion stimulating, bacteriostatic, antioxidant and microflora balancing activities are the most important ones and already identified in the scientific literature. However, the focus on the use of these is to improve the growth performance of farm animals. Hence, there are many phytobiotic products available as alternative growth promotes, and usually there are mixtures of herb essential oils and/or extracts.

Only a limited number of studies available about the phytobiotic supplementation on the antioxidant defence-abilities of chickens against the pro-oxidative effects as caused by

mycotoxins. In addition, the results are sometimes contradictory due to composition and dosage of bioactive components.

Therefore, the other purpose of my PhD study was to evaluate the effects of a commercially available phytobiotic feed additive (Herbamix Basic Premix™) - containing essential oils from 7 plants, namely garlic, rosemary, oregano, true cinnamon tree, common thyme, narrow-leaved paperbark and southern blue-gum, and glycerol extract of two plants, namely eastern purple coneflower and Mediterranean milk thistle - on performance traits and some parameters of lipid peroxidation and glutathione redox system of broiler chickens or laying hens.

The bioactive substances of phytobiotics have many potential applications, though the literature often lacks the important and exact information about the details of dosage.

The third aim of my work was to investigate the application of the above mentioned Herbamix Basic Premix at different concentrations.

2. OBJECTIVES

1. The main objective of my studies was to investigate the effect of mycotoxins (T-2 toxin or DON or aflatoxin) on the on initial and terminal markers of lipid peroxidation processes, and on the amount and activity of the members of glutathione redox system in the first 48-hour of mycotoxin exposure in chicken (*Gallus gallus domesticus*) with different physiological characteristics, in broiler chickens and in laying hens.

2. Investigation of the effects of a herbal mixture (Herbamix™ Basic Premix) on the parameters of lipid peroxidation and glutathione redox system of chickens. either using it parallelly with trichothecene mycotoxins (T-2 toxin, DON) or aflatoxin exposure, or using it as pre-treatment in different concentrations.

The following experiments were designed, and parameters were measured to achieve the objectives:

I. Effects of herbal mixture and short-term DON or T-2 toxin exposure on performance, lipid peroxidation and glutathione redox parameters in 3-weeks-old broiler chickens:

a) on the lipid peroxidation processes, including the markers of initiation phase, such as conjugated dienes (CD) and conjugated trienes (CT), and one of the metastable end-products of terminal phase, malondialdehyde, (MDA);

b) on the changes of parameters of the biological antioxidant system, namely glutathione peroxidase (GPx) activity and reduced glutathione (GSH) concentration.

II. Effects of herbal mixture and short-term aflatoxin exposure on performance, lipid peroxidation and glutathione redox parameters of laying hens:

a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);

b) on the changes of parameters of the biological antioxidant system (GPx activity and GSH concentration).

III. Effects of herbal mixture pre-treatment and short-term T-2 toxin exposure on performance, lipid peroxidation, glutathione redox and clinical biochemical parameters in broiler chickens:

a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);

b) on the changes of parameters of the biological antioxidant system (GPx activity and GSH concentration).

c) on some clinical biochemical parameters of blood plasma, such as activities of aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT), and concentrations of glucose, cholesterol, triglycerides and uric acid.

IV. Effects of herbal mixture pre-treatment and short-term aflatoxin exposure on performance, lipid peroxidation, glutathione redox and clinical biochemical parameters in broiler chickens:

a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);

b) on the changes of parameters of the biological antioxidant system (GPx activity and GSH content).

c) on some clinical biochemical parameters of blood plasma: actvities of aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT), and concentrations of glucose, cholesterol, triglycerides and uric acid.

3. MATERIALS AND METHODS

3.1 Mycotoxin production and artificial contamination of feed
3.1.1 Artificial mycotoxin contamination of the feed

DON was produced by *Fusarium graminearum* (NRRL 5883) and T-2 by *Fusarium sporotrichioides* (NRRL 3299) strains on corn substrate in collaboration with Mycotoxins in the Food Chain Research Group, Hungarian Academy of Sciences-Kaposvár University.

T-2 and HT-2 toxin concentrations were measured based on the method of Trebstein et al. (2008) and DON content according to Pussemier et al. (2006) with HPLC method after immunoaffinity cleanup.

Aflatoxins were produced in ground corn which was artificially infected with an aflatoxin producing Aspergillus flavus strain (ZT80) isolated by Dobolyi et al. (2011). Aflatoxin content of the complete feeds was analysed with AFLAPREP HPLC method after immunoaffinity clean-up (Food Analytica Ltd., Gyula).

3.2 Preparation of herbal mixture containing feed

Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest, Hungary) was added to the complete feed in powder form at the dose of 600 mg/kg. The Herbamix Basic Premix contains essential oils from 7 plants, namely garlic (*Allium sativum*), rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), true cinnamon tree (*Cinnamomum verum*), common thyme (*Thymus vulgaris*), narrow-leaved paperbark (*Melaleuca alternifolia*) and southern blue-gum (*Eucalyptus globulus*) standardized on their active substance. Glycerol extract of two plants, namely eastern purple coneflower (*Echinacea purpurea*) and Mediterranean milk thistle (*Silybum marianum*) were also components of the product.

3.3 Experimental protocols and sampling methods

3.3.1. Investigation of the effects of a medicinal herb mixture and DON or T-2 toxin exposure in broiler chickens

A total of 120 three-week old Cobb 540 broiler chickens (body weight: 749.60±90.98 g) was randomly assigned into five experimental groups of 24 chickens in each. The short-term trial lasted for 48 hours, after 12 hours of feed deprivation. The basal diet was a commercial broiler feed. The nutrient content of the diet met the requirements for broiler chickens (Hungarian Feed Code, 2004). Measured mycotoxin concentrations of the commercial diet (1 kg) were: T-2: 0.10 mg; DON: 0.25 mg. The experimentally contaminated diets contained (1 kg) 3.74 mg T-2 and 1.26 mg HT-2 or 16.12 mg DON, respectively. Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest) was added to the complete feed in powder form at the dose of 600 mg/kg.

3.3.2 Investigation of the effects of a medicinal herb mixture and short-term aflatoxin toxin exposure in laying hens

Total of 60 Bovans Goldline laying hens, being at 90% daily egg production at 49 weeks of age were divided randomly to four experimental groups (two replicates each): a control (aflatoxin content < 1.0 µg/kg) and three treated groups fed with aflatoxin (total aflatoxin content was 170.3 µg/kg; AFB2: 39.0 µg/kg; AFG1: 2.0 µg/kg; AFG2: 4.3 µg/kg), herbal mixture, and aflatoxin + herbal mixture, respectively. Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest) was added to the complete feed in powder form at the dose of 600 mg/kg. Hens were kept in deep litter condition. Because of the short-term exposure the toxin dose was much higher than the regulatory limit of 20 µg/kg for aflatoxin B1, in complete feed (Commission Regulation 574/2011).

3.3.3 Effect of short term T-2 toxin exposure in broiler chickens with medicinal herb mixture pre-treatment against the detrimental effects of high mycotoxin exposure

A total of 120 three-week old Cobb 540 broiler chickens (body weight: 749.60 ± 90.98 g) was randomly assigned into five experimental groups of 24 chickens in each. In three experimental groups Herbamix Basic Premix™ were used in three different concentrations (1 kg feed) 300 mg, 600 mg and 1500 mg as two weeks long pre-treatment, to evaluate its effect against short-term T-2 toxin exposure. The experimentally mycotoxin-contaminated diets (1kg) contained: 1.13 mg T-2 toxin and <0,1 mg HT-2 toxin. The basal diet was a commercial broiler feed. The nutrient content of the diet met the requirements for broiler chickens (Hungarian Feed Code, 2004).

3.3.4 Investigation of the effects of a medicinal herb mixture and aflatoxin exposure in broiler chickens

The purpose of present study was to investigate the short-term effect of aflatoxins (AF) (149.7 µg/kg diet) on lipid peroxidation and some glutathione redox parameters in broilers, and to evaluate the efficacy of two weeks long pre-treatment with three different doses of a herbal mixture (Herbamix Basic Premix™) for counteracting short-term (48-hours) aflatoxin exposure.

A total of 120 Cobb 500 cockerels was divided in five groups: control, aflatoxin treated, aflatoxin+Herbamix (300 mg/kg), aflatoxin+Herbamix (600 mg/kg), aflatoxin+Herbamix (1500 mg/kg) of 24 chickens in each. The experimentally AF-contaminated diets (1kg) contained: 140 µg AFB1; 8.6 µg AFB2; <1.0 µg AFG1; 1.1 µg AFG2, respectively.

3.4 Sampling

All short-term trials lasted for 2 days after 12 hours of feed deprivation. Six randomly chosen birds were exterminated from each group at 12th, 24th, 36th and 48th hours of the experiment. After cervical dislocation, whole blood samples were collected into EDTA-Na$_2$ containing tubes. *Post mortem* liver and kidney samples were collected at every 12 hour.

The whole blood was separated by centrifugation (2,500g, 10 min) and blood plasma was collected. Red blood cell (RBC) hemolysates were prepared with 9-fold distilled water. Blood plasma, RBC hemolysate, liver and kidney samples were stored at −70 ºC until the biochemical analyses. Before the analyses the samples were thaw at room temperature, and liver and kidney homogenates were made with 9-fold cold (4 ºC) physiological saline (0.65% w/v NaCl).

3.5 Weighing

At all samplings, before the extermination live weight of the birds was measured. *Post mortem* the weight of liver samples was also recorded. From these data relative liver weights (g/100 g live weight) were calculated. To calculate the feed consumption of the birds during the experiment the feeders were weighed at every 4 hour in each experimental group.

3.6 Biochemical analysis

Level of conjugated dienes (CD) and conjugated trienes (CT) as markers of initial phase of lipid peroxidation, was measured in liver, according to the method of AOAC (1984). Malondialdehyde (MDA) concentration of blood plasma and red blood cell haemolysate samples was measured based on the color complex formation of malondialdehyde with 2- thiobarbituric acid in an acidic environment at high temperature (Placer et al., 1966). MDA concentrations in the native tissue (liver, kidney) homogenates were determined with colorimetric method according to Mihara et al. (1980) as modified by Matkovics et al. (1988). *Reduced glutathione (GSH) concentration* of blood plasma, red blood cell haemolysate, and 10,000 g supernatant fraction of liver and kidney homogenate samples was determined on the basis of complex formation of free non-protein SH-groups with 5,5'dithiobis-2-nitrobenzoic acid (Sedlak and Lindsay, 1968). *Glutathione peroxidase (GPx) activity* of blood plasma, red blood cell haemolysate, and 10,000 g supernatant fraction of liver and kidney homogenate samples was measured using reduced glutathione (Sigma, St. Louis) and cumene-hydroperoxide (Merck, Darmstadt) as co-substrates in an end point direct assay as described by Matkovics et al. (1988). In case of blood plasma and red blood cell haemolysate samples *protein concentration* was determined by the biuret method, using the reagent kit (No. 41951) of Diagnosticum Ltd. (Budapest, Hungary). For the determination of protein concentration in 10,000 g supernatant fraction of tissue (liver and kidney) homogenates, the method of Lowry et al. (1951) was used. For measurement of the *aspartate aminotransferase (AST, GOT) activity* of blood plasma samples a reagent kit (No. 46263) of Diagnosticum Ltd. (Budapest, Hungary) was used. To determine the *gamma-glutamyltransferase (GGT) activity* of blood plasma samples a reagent kit (No. 47263) of Diagnosticum Ltd. was used. For measurement of the *glucose concentration* of blood plasma samples a commercial reagent kit (No. 46861) of Diagnosticum Ltd. was used. To determine the *cholesterol concentration* of blood plasma samples a commercial reagent kit (No. 41031) of Diagnosticum Ltd. was used. For measurement of *triglyceride concentration* in blood plasma samples a reagent kit (No. 47163) of Diagnosticum Ltd. was used. For measurement of the *uric acid concentration* of blood plasma samples a reagent kit (No. 46763) of Diagnosticum Ltd. was used.

3.7 Statistical analysis

For statistical analysis of data the GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA) was used. After calculating the mean and standard deviation (SD) values, all data were subjected to one-way variance analysis (ANOVA), and differences between means was calculated with the Tukey test at a 95% interval of confidence ($p < 0.05$).

4. RESULTS AND DISCUSSION

4.1. Effects of a medicinal herb mixture on short-term effects of T-2 toxin or DON on some lipid peroxide and glutathione redox status parameters in broiler chicken

Experimental design are as follows.

Groups	Diet	No. of birds
Control	intensive grower diet	12x2
Treated I. (**T-2 toxin treated [T]**)	intensive grower diet + [T-2 toxin (3.72 mg/kg) and HT-2 toxin (1.26 mg/kg)]	12x2
Treated II. (**DON treated [D]**)	intensive grower diet + DON (16.12 mg/kg)	12x2
Treated III. (**Herbamix treated [H]**)	intensive grower diet +Herbamix Basic premix (600 mg/kg)	12x2
Treated IV. (**T-2 toxin + Herbamix treated [TH]**)	intensive grower diet + [T-2 toxin (3.72 mg/kg) and HT-2 toxin (1.26 mg/kg)] + Herbamix Basic premix (600 mg/kg)	12x2
Treated V. (**DON + Herbamix treated [DH]**)	control diet + DON (16.12 mg/kg) + Herbamix Basic premix (600 mg/kg)	12x2

Consumption of high T-2 toxin/HT-2 toxin contaminated diet (20 times higher than the EU recommendation) or the high DON exposure (3.22 times higher than the EU recommendation) did not cause mortality during the short-term trial, and no clinical signs of toxicity were observed. Calculated feed intake showed some differences at different periods of the trial, but feed intake was measured for groups, therefore no statistical analysis could be done on this parameter. Calculated mycotoxin intake, which was calculated from feed intake and measured mycotoxin content of the particular complete feed, was almost the same between the groups fed with mycotoxin contaminated diets. Neither body weights nor absolute and relative liver showed significant changes during the trial.

Concentration of malondialdehyde (MDA), one of the end products of lipid peroxidation and reduced glutathione (GSH) concentration did not change significantly in blood plasma and RBC haemolysate. Glutathione peroxidase (GPx) activity of blood plasma showed marked changes at 12 and 48 hour of mycotoxin exposure. At hour 12 it was lower in DON + herbal mixture treated group as compared to T-2 toxin + herbal mixture group, and at hour 48 herbal mixture alone caused significantly lower enzyme activity than the control.

As effect of T-2 toxin/HT-2 toxin or DON, level of conjugated dienes and conjugated trienes, the initial phase markers of lipid peroxidation processes, did not change significantly in liver, and it was not modified by the supplementation of herbal mixture.

Concentration of MDA, the meta-stable end product of lipid peroxidation, was significantly lower in the liver homogenate of both mycotoxin loaded groups fed with herbal mixture supplemented feed at 24 hour sampling as compared to the control.

GSH concentration in liver homogenate showed differences at 24 hour sampling, out of which T-2 toxin caused significant difference, and T-2 toxin + herbal mixture combination caused markedly, but not significantly, higher values than the control. On the other hand, in the case of DON significantly higher GSH content was measured only when it was combined with herbal mixture supplementation.

In liver homogenate GPx activity changed significantly at 24 hour sampling, when higher values were found as effect of T-2 toxin, also in combination with herbal mixture, and DON in combination with herbal mixture, as compared to the control, and in case of DON, when it was used alone .

In kidney homogenates significant differences were found in MDA concentration at 12 hour sampling, when DON alone and in combination with herbal mixture revealed the highest values which were significantly higher than in T-2 toxin + herbal mixture treated group.

GSH concentration of kidney homogenates showed significant difference at 48 hour sampling, when significantly higher value was revealed in T-2 toxin + herbal mixture group as compared to the control.

GPx activity in kidney homogenates showed significant changes at 48-hour sampling, where T-2 toxin load resulted in higher activity when it was used together with herbal mixture as compared to herbal mixture supplemented group.

In conclusion, the results revealed that trichothecene mycotoxins, DON or T-2 toxin, have moderate effect on oxygen free radical formation, and consequently, but they activate the glutathione redox system in liver and kidney of broiler chicken. Addition of herbal mixture has only moderate effect against the mild oxidative stress caused by DON or T-2 toxin at the dose applied.

4.2. Effect of short-term aflatoxin exposure in combination with medicinal herb mixture (Herbamix™) on lipid peroxidation and glutathione redox system in laying hens

Experimental design are as follows.

Group	Treatment	No. of birds
Control [C]	Control layer diet	15
Treated I. (**Herbamix treated [H]**)	Control layer diet + Herbamix Basic premix (600 mg/kg)	15
Treated II. (**Aflatoxin treated [A]**)	Control layer diet + aflatoxin (125 µg AFB_1/kg)	15
Treated III. (**Aflatoxin + Herbamix treated (AH)**)	Control layer diet + aflatoxin (125µg AFB_1) + Herbamix Basic premix (600 mg/kg)	15

Consumption of high aflatoxin contaminated diet (where the AFB_1 concentration was 6.25 times higher than the EU regulation) did not cause morbidity or mortality in the experimental groups during this short-term study, and no clinical signs of aflatoxicosis were observed.

No marked change was observed in calculated feed intake of the layers , therefore the calculated total aflatoxin intake, which was calculated from feed intake and measured mycotoxin content of the complete feed, was almost the same between the groups fed with aflatoxin contaminated diet and aflatoxin contaminated and herbal mixture supplemented diet . Due to the darkness there was no measurable feed intake in the periods of 12th to 24th hour, and 36th to 48th hour , therefore aflatoxin intake was also negligible during these periods .

Body weights, absolute and relative liver weights did not vary significantly during the experimental period .

Concentration of malondialdehyde (MDA), one of the end products of lipid peroxidation, showed significantly higher values at 12th hour sampling in aflatoxin treated group and in aflatoxin + herbal mixture treated group as compared to control.

At hour 36 the aflatoxin + herbal mixture treatment also resulted significantly elevated MDA concentration as compared to control, while at hour 48 aflatoxin + herbal mixture treatment caused significantly elevated MDA concentrations as compared to the herbal mixture treated group.

No significant changes were found in reduced glutathione (GSH) concentration of blood plasma, while glutathione peroxidase (GPx) activity of blood plasma showed marked changes at hour 36. At this sampling GPx activity of all treated groups was significantly higher than that of the control.

At 12th hour sampling malondialdehyde (MDA) concentration in RBC haemolysates of aflatoxin + herbal mixture treatment group was significantly lower as compared to the other experimental groups, while at 36th hour sampling aflatoxin + herbal mixture treatment resulted significantly elevated MDA concentration as compared to control.

Herbal mixture treatment caused significantly lower GSH concentration at hour 36 as compared to control group.

At 48th hour sampling aflatoxin + herbal mixture treatment resulted significantly higher GPx activity as compared to the control or the herbal mixture treated group .

As effect of aflatoxin, level of conjugated dienes and conjugated trienes - the initial phase markers of lipid peroxidation processes - did not change significantly in liver, and it was not modified by the supplementation of herbal mixture. In terminal phase of lipid peroxidation processes, as described with malondialdehyde concentration, marked changes were noticed in the liver at hour 36. Single medicinal herb mixture resulted significantly higher MDA concentration as compared to aflatoxin + herbal mixture treatment . Similar results were found in kidney samples at hour 48, where aflatoxin + herbal mixture treatment resulted significantly lower MDA values as compared to the herbal mixture treated group or to the control group.

No significant changes were found in reduced glutathione (GSH) concentration and in GPx activity of liver and kidney homogenates .

According to the results it can be concluded that high level of aflatoxin induced ROS formation, and possessed mild oxidative stress, which was effectively eliminated by the glutathione redox system. This result suggests that if the amount and/or activity of glutathione system is adequate, high dose of aflatoxin induces only mild oxidative stress in the liver of laying hens during a short period of time. However, during the short period of the study the positive effect of medicinal herb mixture (Herbamix™) was not confirmed at the applied dose.

4.3. Effects of a medicinal herb mixture pre-treatment and short-term T-2 toxin exposure in broiler chickens

Experimental design are as follows.

From the 7th day of age changes were made in the diet of the different groups, as it is shown below:

Groups	Diet	No. of birds
Control (C)	Intensive grower diet (from 7th day of age)	21x2
Control (C)*, * later: Treated I.	Intensive grower diet (from 7th until 21st day of age)	21x2
Treated II. (**With low dose of Herbamix treated [H1]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) from 7th day of age	21x2
Treated III. (**With medium dose of Herbamix treated [H2]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) from 7th day of age	21x2
Treated IV. (**With high dose of Herbamix treated [H5]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) from 7th day of age	21x2

From the 21st day of age the treated groups received T-2 toxin contaminated feed as shown below:

Groups	Diet	No. of birds
Control (C)	Intensive grower diet	12x2
Treated I. (**T-2 toxin treated [T]**)	Intensive grower diet + T-2 toxin (1.13 mg/kg)	12x2
Treated II. (**With low dose of Herbamix + T-2 toxin treated [H1+T]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) + T-2 toxin (1.13 mg/kg)	12x2
Treated III. (**With medium dose of Herbamix + T-2 toxin treated [H2+T]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) +T-2 toxin (1.13 mg/kg)	12x2
Treated IV. (**With high dose of Herbamix + T-2 toxin treated [H5+T]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) + T-2 toxin (1.13 mg/kg)	12x2

Consumption of high T-2/HT-2 toxin contaminated diet (which was approximately 4.52 times higher than the EU recommendation) did not cause mortality during the short-term trial, and no clinical signs of toxicity were observed.

Calculated feed intake showed some differences at different periods of the trial, but as feed intake was measured only for groups, no statistical analysis could be done on this parameter.

Calculated mycotoxin intake, which was calculated from feed intake and measured mycotoxin content of the particular complete feed, was almost the same between the groups fed with mycotoxin contaminated diet and mycotoxin contaminated and herbal mixture supplemented diets.

Neither body weights nor absolute and relative liver weights showed significant changes during the trial.

In RBC haemolysates of T-2/HT-2 toxin treated group, concentration of MDA (one of the end products of lipid peroxidation processes), increased significantly ($p < 0.05$) at 12h as compared to control. Herbamix treatment had beneficial effect resulting lower MDA concentration at 36h both in plasma and RBC as compared to T-2/HT-2 toxin alone.

As effect of T-2/HT-2 toxin treatment the GSH concentration of plasma increased moderately at 12h and 24h as compared to control, while significant increase was measured in plasma and also in RBC haemolysate at 24h in T-2/HT-2 toxin+ highest Herbamix dose group.
GPx activity of plasma was also increased ($p < 0.05$) by the T-2/HT-2 toxin treatment at 12h, while most marked ($p < 0.05$) elevations were found in T-2/HT-2 toxin+ medium Herbamix dose group in plasma at 12h and 36h , and in RBC haemolysate at 24h .

As effect of T-2/HT-2 toxin alone, level of conjugated dienes and conjugated trienes, the initial phase markers of lipid peroxidation processes, did not change significantly in liver, while in combination with herbal mixture significantly lower values were measured at 24h in T-2/HT-2 toxin+ medium Herbamix dose group as compared to control and to T-2/HT-2 toxin treated group. At 36h in T-2/HT-2 toxin+ highest Herbamix dose group also significantly lower CD and CT values were measured than in control and in T-2/HT-2 toxin treated group.

In liver homogenates, concentration of MDA, the meta-stable end product of lipid peroxidation processes, was markedly higher at 24h, and significantly higher at 48h in T-2/HT-2 toxin treated group as compared to control, while the highest dose of Herbamix treatment had beneficial effect resulting lower MDA concentration at 24h and 48h of mycotoxin exposure as compared to T-2/HT-2 toxin alone.

GSH concentration and GPx activity in liver homogenate showed differences at 24 hour sampling, out of which T-2/HT-2 toxin + medium and highest dose of herbal mixture combination caused significantly lower values than in T-2/HT-2 toxin treated group. At 36h significantly higher GSH concentrations were measured in T-2/HT-2 toxin + lowest and highest dose herbal mixture groups as compared to control.

In kidney homogenates, MDA concentration was significantly higher at 48h in T-2/HT-2 + medium dose of Herbamix treatment group as compared to the T-2/HT-2 toxin treated one.

In T-2/HT-2 + medium dose of Herbamix treatment group the GSH concentration at 12h significantly exceeded the values measured in control and in T-2/HT-2 toxin treated group.

Similarly to the changes of its co-substrate, the GPx activity showed significantly higher values in kidney homogenates in T-2/HT-2 + medium dose of Herbamix treatment group at 12h, 36h and 48h of mycotoxin exposure compared to T-2/HT-2 toxin treated group or to the control group.

In case of T-2/HT-2 toxin + highest dose herbal mixture combination aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) activities of blood plasma showed significantly lower values as compared to the control at 24h and 48h samplings, respectively. Among the measured clinical biological parameters of the blood plasma glucose concentrations were significantly higher in case of T-2/HT-2 toxin + lowest and highest dose herbal mixture combination as compared to control. Uric acid concentration measured at 24h sampling was significantly higher in control group than in the other experimental treated groups. In cholesterol concentrations of blood plasma samples no differences were found, but plasma triglycerides showed marked elevation ($p<0.05$) in T-2/HT-2 toxin treated group and in T-2/HT-2 toxin + highest dose of herbal mixture combination as compared to T-2/HT-2 toxin + lowest dose of herbal mixture group.

The results revealed that the applied trichothecene mycotoxin, T-2 toxin, had effect on oxygen free radical formation, and consequently activated the glutathione redox system redox system of broiler chickens, namely synthesis of reduced glutathione and glutathione peroxidase. Two-week pre-treatment of herbal mixture had only moderate effect against the mild oxidative stress caused by T-2 toxin at the dose applied, but it prevented liver damage and metabolic disturbances.

4.4. Effects of a medicinal herb mixture pre-treatment and short-term aflatoxin exposure in broiler chickens

Experimental design are as follows:

From the 7th day of age changes were made in the diet of the different groups, as it is shown below:

Groups	Diet	No. of birds
Control (C)	Intensive grower diet (from 7th day of age)	21x2
Control (C)*, * later: Treated I.	Intensive grower diet (from 7th until 21st day of age)	21x2
Treated II. (**With low dose of Herbamix treated [H1]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) from 7th day of age	21x2
Treated III. (**With medium dose of Herbamix treated [H2]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) from 7th day of age	21x2
Treated IV. (**With high dose of Herbamix treated [H5]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) from 7th day of age	21x2

From the 21st day of age the treated groups received aflatoxin contaminated feed as it is shown below:

Groups	Diet	No. of birds
Control (C)	Intensive grower diet	12x2
Treated I. (**Aflatoxin treated [A]**)	Intensive grower diet + aflatoxin (140 µg AFB_1/kg)	12x2
Treated II. (**With low dose of Herbamix + Aflatoxin treated [H1+A]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) + aflatoxin (140 µg AFB_1/kg)	12x2
Treated III. (**With medium dose of Herbamix + Aflatoxin treated [H2+A]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) + aflatoxin (140 µg AFB_1/kg)	12x2
Treated IV. (**With high dose of Herbamix + Aflatoxin treated [H5+A]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) + aflatoxin (140 µg AFB_1/kg)	12x2

As a result of high aflatoxin intake (7 times higher AFB_1 concentration in feed than the EU regulation) there was no morbidity and mortality in the experimental groups during this short-term study, and no clinical signs of aflatoxicosis were observed.

Calculated feed intake showed some differences at different periods of the trial, but as feed intake was measured only for groups, no statistical analysis could be done on this parameter.

Calculated mycotoxin intake, which was calculated from feed intake and measured mycotoxin content of the particular complete feed, was almost the same between the groups fed with mycotoxin contaminated diet and mycotoxin contaminated and herbal mixture supplemented diets.

Neither body weights nor absolute and relative liver weights showed significant changes during the trial, which is probably due to the short (48 hours long) period of investigation.

MDA concentration in blood plasma of aflatoxin + highest Herbamix dose resulted significantly lower MDA concentration at 48h as compared to control, while in RBC haemolysates of aflatoxin treated group it was increased ($p<0.05$) at 36h.

At 24h, GSH concentration of blood plasma increased ($p<0.05$) in all experimental groups, which suggests response of the organism to oxidative stress as provoked by aflatoxin exposure, while in RBC haemolysates the aflatoxin+ medium Herbamix dose resulted significantly lower concentrations at 24h and 48h compared to control.

GPx activity of blood plasma was significantly lower in aflatoxin treated group at 24h as compared to control, while no significant changes were found in RBC haemolysates.

As effect of aflatoxin alone, level of conjugated dienes and conjugated trienes, the initial phase markers of lipid peroxidation processes, did not change significantly in liver, while in combination with herbal mixture significantly lower conjugated triene values were measured at 48h in aflatoxin+ medium Herbamix dose group and in aflatoxin + highest Herbamix dose group as compared to control.

In liver homogenates, MDA concentration was significantly higher at 12h in aflatoxin treated group as compared to control. At 24h of mycotoxin exposure in aflatoxin + medium dose of Herbamix treatment significantly higher MDA concentration was measured as compared to control and to the other herbal mixture treated groups.

At 24h sampling, GSH concentration in liver homogenates of aflatoxin treated group significantly exceeded the values measured in aflatoxin + different dose of Herbamix pre-treated groups.

Similarly, to the changes of its co-substrate, the highest GPx activity in liver homogenates were measured at 24h in aflatoxin treated group, which was significantly higher than in control.

In kidney homogenates, MDA concentration was significantly higher at 36h in aflatoxin treated group as compared to control, but 12 hours later it was significantly lower than the control. At 48h

sampling aflatoxin + medium dose of Herbamix pre-treatment resulted significantly lower MDA concentration in kidney as compared to the control.

Although aflatoxin treatment alone resulted elevation in GSH concentration of kidney homogenates at 12h sampling as compared to control, significantly higher values were measured in aflatoxin + medium and highest dose of herbal mixture pre-treatment.

GPx activity in kidney homogenates followed the changes of its co-substrate, the highest values were measured at 12h in aflatoxin + medium and highest dose of herbal mixture pre-treated groups.

In case of aflatoxin + medium dose herbal mixture combination aspartate aminotransferase (AST) activity of blood plasma showed significantly higher values as compared to the control at 48h sampling. Aflatoxin + highest dose of herbal mixture combination resulted significantly higher gamma-glutamyltransferase (GGT) activity than in control at 12h sampling, but 12 hours later it was significantly lower as compared to the aflatoxin treated one.

At 12h sampling, glucose concentrations in blood plasma were significantly lower in aflatoxin + medium and highest dose herbal mixture combination as compared to control. At 24h sampling glucose concentration in aflatoxin + highest dose of herbal mixture pre-treated group was significantly higher than in the aflatoxin treated group.

No significant changes were found in cholesterol concentrations of blood plasma,but triglycerides showed marked elevation in aflatoxin treated group at 36h sampling as compared to control and to aflatoxin + lowest dose of herbal mixture pre-treated group (p<0.05). No significant differences were found in uric acid concentrations of blood plasma.

According to the results addition of herbal mixture had moderate effect against the mild oxidative stress caused by aflatoxins at the dose applied, and it also had some effects on glucose and lipid metabolism.

5. NEW SCIENTIFIC RESULTS

1.   The investigated 'type A' trichothecene mycotoxin, the T-2 toxin has short-term effect on oxygen free radical formation, and consequently, it activates the glutathione redox system, namely synthesis of reduced glutathione and activation of glutathione peroxidase in liver and kidney of broiler chicken.

2.   Addition of herbal mixture, either using together with T-2 toxin, or after two-week pre-treatment prior to mycotoxin exposure, has only moderate effect against the mild oxidative stress caused by T-2 toxin at the dose applied.

3.   Short-term exposure of aflatoxin B_1 induces lipid peroxidation, which was confirmed by the significantly increased malondialdehyde content in blood plasma and red blood cell haemolysate, liver and kidney of broilers. These results suggest rapid activation of the antioxidant, namely glutathione redox system in tissues important for the detoxification of mycotoxins.

4.   The pre-treatment of medicinal herbal mixture (Herbamix™) prevented the pro-oxidant effect of aflatoxins, which means lowering the rate of lipid peroxidation and production of malondialdehyde in red blood cells and kidney of broilers.

6. CONCLUSIONS AND SUGGESTIONS

6.1. Conclusions

The main purpose of my dissertation was to study the effects of dietary herbal mixture supplementation on the effect of aflatoxin B1, T-2 toxin or DON on the lipid peroxidation processes and the antioxidant defence system. Initial and terminal markers of lipid peroxidation and amount or activity of the glutathione redox system were measured in three-week old broiler chickens and 49-week old laying hens in a short-term (48 hours) mycotoxin exposure.

The transit time of feed particles in the gastrointestinal tract, which may influence the time available for the absorption of mycotoxins in the intestine, was also evaluated.

Markers of lipid peroxidation, such as the markers of the initiation phase (conjugated dienes and conjugated trienes) along with the metastable end-product of terminal phase (malondiadehyde) were measured in liver and kidney. Among the parameters of the biological antioxidant system reduced glutathione content and glutathione peroxidase activity was measured in the same tissues.

It is known that broiler chicken and laying hen are less sensitive to trichothecene mycotoxins than other monogastric farm animals, but they are the most sensitive among the domesticated poultry species. However, no mortality or morbidity was observed at the applied doses during the short-term trials.

The results revealed that AFB_1 initiated lipid peroxidation even in short-term, at the dose applied. This was confirmed by the significant increase of the termination phase parameter (malondialdehyde) in the blood plasma and red blood cell haemolysate, but was not observed in the liver and kidney. These results were in contrast to some previous studies where aflatoxin B1 exposure resulted significant increase of malondialdehyde content in the liver. However, the results of the previous studies were based on long-term exposure. The results also showed that aflatoxin B1 exposure in combination with medicinal herb mixture caused less oxygen free radical formation, but only in layers. Otherwise activation of glutathione redox system was also found, but medicinal herb mixture had only minor effect on its activation. The most intensive changes were observed about 12 hours after starting feeding the mycotoxin contaminated diet, which at first can be explained with the transit time of feed particles and estimated period for absorption in poultry.

Trichothecene mycotoxins, T-2 toxin and DON also had effect on oxygen radical formation, and subsequently activated the glutathione redox system of broiler chickens. The addition of herbal mixture had only a moderate effect against the mild oxidative stress generated by DON or T-2 toxin at the dose applied. When the herbal mixture was used as a two-week pre-treatment prior to mycotoxin exposure it had only a moderate effect on the mild oxidative stress caused by T-2 toxin at the applied dose.

In conclusion, the results indicated that aflatoxin B1 and the two trichothecenes, T-2 toxin or DON, induced mild oxidative stress either in broiler chicken or laying hen, which was supported by the low level of the increase, or even decrease of the markers of lipid peroxidation, but activation of the antioxidant, namely the glutathione redox system in the same period of time. The results also showed that the phytobiotic supplementation had some moderate effects, in particular it decreased the rate of lipid peroxidation, without having marked effect on the activation of the glutathione redox system, either if it was used together with mycotoxin exposure or as pre-treatment.

6.2. Suggestions

Based on the individual effect of aflatoxin and trichothecenes I recommend evaluating the effects of multiple mycotoxin exposure, as these mycotoxins are occurring in a mixture in the animal feed and those common effects might be additive, synergistic or antagonistic, which would be important information when trying to find the most effective method to decrease their detrimental effects on animal production and health.

Additionally, my results showed that elimination or attenuation of the negative effects of mycotoxins requires prevention or reduction of the oxidative stress caused by mycotoxins via action of natural antioxidants or antioxidant mixtures, such a mixture of essential oils or various extracts of medicinal plants. Thereby, these results would be provide more information about the mode of action and efficacy of other natural active substances, supporting the defence against the undesirable effects of mycotoxins in farm animals.

PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS

Publications in peer reviewed scientific journals

1. Nakade, M., Balogh, K., Szabó-Fodor, J., Zándoki, E., Mézes, M., Erdélyi, M. (2018): Effects of herbal mixture and T-2 toxin exposure on some glutathione redox and lipid peroxidation parameters of blood in broilers. Albanian J. Agric. Sci. 2018; 17(2): 99-104.

2. Erdélyi, M., Balogh, K., Pelyhe, Cs., Kövesi, B., Nakade, M., Zándoki, E., Mézes, M., Kovács, B. (2018): Changes in the regulation and activity of glutathione redox system, and lipid peroxidation processes in short-term aflatoxin B1 exposure in liver of laying hens. J. Anim. Physiol. Anim. Nutr. 2018; 1–6. (IF 2016: 1.244; Q2) https://doi.org/10.1111/jpn.12896 ISSN:1439-0396.

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4. Nakade, M., Balogh, K., Fogarasi, L., Szabó-Fodor, J., Zándoki, E., Mézes, M., Erdélyi, M. (2017): Effects of herbal mixture and DON or T-e toxin exposure on some glutathione redox and lipid peroxidation parameter of blood and liver in broiler chicken. Scientific Papers. Series D. Animal Science, Vol. LX, ISSN 2285-5750, 97-102

5. Nakade, M., Balogh, K., Zándoki, E., Cserháti, M., Mézes, M., Erdélyi, M. (2016): Effect of short-term aflatoxin exposure in combination with medicinal herb mixture on lipid peroxidation and glutathione redox system in laying hens, Columella - Journal of Agricultural and Environmental Sciences 3(1): 65-75, DOI: 10.18380/SZIE.COLUM.2016.3.1.65

Paper in conference proceeding

1. Nakade, M., Balogh, K., Szabó-Fodor, J., Zándoki, E., Mézes, M., Erdélyi, M. (2017): Effects of herbal mixture and short-term aflatoxin exposure on performance, some blood lipid peroxide and glutathione redox parameters of broiler chickens, LIX. Georgikon Napok • 2017. szeptember 28-29. • Pannon Egyetem Georgikon Kar, Hungary

Publications in Conferences and Symposiums

Oral presentations

1. <u>Nakade, M.</u>, Balogh, K., Zándoki, K., Ancsin, Zs., Bócsai, A., Erdélyi, M., Mézes, M. (2016): Effects of herbal mixture and short-term aflatoxin exposure on performance, some blood lipid peroxide and glutathione redox parameters of laying hens. International Conference on Biotechnology and Welfare in Animal Science with a session on "7th Poultry Days" • 23–24 June 2016 • Krakow, Poland

2. <u>Nakade, M.</u>, Balogh, K., Zándoki, E., Ancsin, Zs., Bócsai, M., Erdélyi, M., Mézes, M. (2017): Effects of herbal mixture and don or t-2 toxin exposure on some glutathione redox and lipid peroxidation parameter of blood and liver in broiler chickens. A4LIFE Conference • 8-10 June 2017 • Bucharest, Romania

3. <u>Nakade, M.</u>, Balogh, K., Szabó-Fodor, J., Zándoki, E., Mézes, M., Erdélyi, M. (2017): Effects of herbal mixture and short-term aflatoxin exposure on performance, some blood lipid peroxide and glutathione redox parameters of broiler chickens, LIX. Georgikon Napok • 2017. szeptember 28-29. • Pannon Egyetem Georgikon Kara, Hungary

Poster

1. <u>Nakade, M</u>., Balogh, K., Fogarasi, L., Szabó-Fodor, J., Zándoki, E., Mézes, M., Erdélyi, M. (2017): Short-term effects of herbal mixture and T-2 toxin exposure on some glutathione redox and lipid peroxidation parameters of blood in broiler chickens. THE 16th INTERNATIONAL SYMPOSIUM "PROSPECTS FOR THE 3rd MILLENNIUM AGRICULTURE" • 28-30 September 2017 • Cluj-Napoca, Romania