



HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE  
SCIENCES

**COMBINED EFFECTS OF *FUSARIUM* MYCOTOXIN EXPOSURE  
ON LIPID PEROXIDATION AND GLUTATHIONE REDOX  
SYSTEM OF LAYING HENS**

PhD thesis

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# 1 INTRODUCTION AND OBJECTIVES

## 1.1 Introduction

The occurrence of mycotoxins in feed and food is a worldwide problem, which is explained by the increasing risks to animal and human health. Based on the assessment of the mycotoxin contamination of feed materials, the occurrence of *Fusarium* mycotoxins is the most common in Europe. These monitoring studies have shown that mycotoxins mostly occur together in natural conditions. Recently, several *in vivo* short-term and long-term studies have been done with farm animals, where the interactions between mycotoxins have been investigated. Based on the research so far, it can be said that the mycotoxin mixtures have an additive or synergistic effect, which emphasizes the importance of multi-mycotoxin experiments.

We have rare information about the effects of the mentioned mycotoxins on the body of birds, including on the glutathione redox system and lipid peroxidation. For this reason, I chose the economically important broiler chicken and laying hen as a model for my experiments. Some *Fusarium* toxins have a well-known effect on activating oxidative stress and lipid peroxidation, which the glutathione redox system can neutralize. The question is that: do the T-2 toxin, DON, and FB1 mycotoxins enhance or inhibit each other's effects on the antioxidant defense mechanisms in the body of laying hens and broiler chickens?

## 1.2 Objectives

1. My research aimed to evaluate the multi-mycotoxin effect on the lipid peroxidation processes, the amount and/or activity of the elements of the glutathione redox system, and the transcription regulation of the expression of redox-sensitive and antioxidant genes in poultry. During the treatments, I determined the toxin concentrations to reflect the recommended limit values for feed established by the European Union (EU) (2006/576/EC) or double and quadruple their amounts.

2. The aim of the experiments was also to assess the direction and extent of the short-term effects of the combined application of the tested mycotoxins after a single exposure to lipid peroxidation and glutathione redox system in laying hens and broiler chickens.

The following parameters were measured to achieve the objectives:

- a) body weight, mortality, feed consumption
- b) biochemical parameters:
  - in the case of lipid peroxidation processes, the markers of the initiation stage (conjugated diene [CD] and conjugated triene [CT]), as well as the amount of the metastable end product (malondialdehyde [MDA]) of the termination stage, parameters indicating the operation of the glutathione redox system (glutathione peroxidase [GPx] activity and reduced glutathione [GSH] content
- c) gene expression studies (glutathione peroxidase-4 [*GPX4*], glutathione synthetase [*GSS*], glutathione reductase [*GSR*]), and Nrf2/Keap1-ARE (nuclear factor E2-related factor 2 /kelch-like ECH-associated protein 1/ Antioxidant response element) expression changes of some genes encoding the pathway (*Keap1* and *Nrf2*)

## 2 MATERIALS AND METHODS

### 2.1 Mycotoxin production and artificial contamination of feed

#### 2.1.1 Artificial mycotoxin contamination of the feed

The experimental feed was mixed with corn substrate with known concentrations of T-2 toxin, DON, and FB1. The mycotoxin concentrations used in the low-dose mixture corresponded to the EU recommended limit (T-2 and HT-2 toxins: 0.25 mg/kg [2013/165/EU]; DON: 5 mg/kg [2006/576/ EC]; FB1: 20 mg/kg [EC 2006/576/EC]. These contamination values reflect intermittent contamination occurring in temperate climates, while the medium- and high-dose experimental groups' feed contained more than the practical contamination level to cause easily detectable changes in a short period.

### 2.2 Experimental protocols and sampling method

For my studies, I used broiler chickens (Cobb 500 cock, 21 days old) and laying hens (Tetra SL, 49 weeks old, average daily egg production  $\cong$ 90%) as *in vivo* models. When examining the individual effects of mycotoxins (n=78), I fed the animals with artificially contaminated feed with T-2/HT-2 toxin, DON/2-AcDON/15-AcDON, and FB1 mycotoxins. On the 0th day of the experiment, samples were taken as an absolute control (n=6). The experimental groups were the follows: control group (n=18) and 3 groups treated with different mycotoxins (T-2 toxin: n=18; DON: n=18; FB1: n=18). In the multi-mycotoxin tests (n=60), after the three-day acclimatization period, on the 0th day of the experiment, 2-2 animals per experimental group (n=6) were sampled as an absolute control. The 3-day feeding experiment was performed by feeding a control (without mycotoxin supplementation, n=18) and two feeds supplemented with different amounts of mycotoxins (n=36).

### 2.3 Biochemical studies

CDs and CTs were determined based on the absorption spectrum (CD: 232 nm; CT: 268 nm) after the lipid content of the liver samples was extracted in 2,2,4-trimethylpentane.

The MDA concentration was determined by complex formation with 2-thiobarbituric acid in an acidic medium at high temperature. The absorbance was measured from the supernatant against the reagent blank at a wavelength of 535 nm.

The reduced glutathione concentration of the samples was determined using the method of Sedlak and Lindsay (1968). The basis of the method is the reaction of the free SH group of glutathione with a sulfhydryl-reactive compound to form a colored complex.

The basis for determining GPx activity is that GSH is oxidized to glutathione disulfide under the action of the GPx enzyme in the presence of reactive oxygen radicals. The decrease in the amount of GSH was determined by measuring the absorbance of the complex formed with 5,5-dithiobis-(2-nitrobenzoic acid) at a wavelength of 412 nm.

## **2.3 Gene expression**

### ***2.3.1 RNA purification and reverse transcription***

Total RNA was purified from liver samples of experimental animals using NucleoZOL reagent, and genomic DNA contamination was removed by DNase I treatment. Pools of RNA were generated per treatment group, with equal amounts of RNA per individual (n=6), and 1000 ng of pooled RNA was reverse transcribed into cDNA using a random nonamer (9-mer) for qPCR measurements.

### ***2.3.2 Real-time PCR measurements***

Expression of *GPX4*, *GSS*, *GSR*, *Nrf2*, and *Keap1* target genes and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control gene was determined by quantitative real-time PCR using SYBRGreen. The primers used to quantify the mRNA transcriptional levels of the target and endogenous control genes were chosen based on the literature. Measurements were performed using the Step One Plus™ Real-Time PCR systems using Maxima SYBRGreen qPCR Master Mix in 5 replicates. No-template controls were also performed for each primer pair. The PCR profile for *GPX4*, *GSS*, *GSR*, and *Keap1* genes predenaturation at 95°C for 10 min, followed by 95°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec, for 45 cycles. In the case of *Nrf2* target genes, predenaturation at 95°C for 10 minutes, followed by 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, for 45 cycles. The VIC and FAM signals were read at 72°C for 50 cycles, where the signal was detected at the end of the extension period.

### ***2.3.3 Real-time PCR evaluation and calculation***

The amplified products were verified by melting curve analysis and gel electrophoresis. The threshold cycle (Ct) of the target genes

(*gpx4a*, *gpx4b*, *nrf2*, *keap1*, *gs*, and *gr*) and the endogenous control gene (b-actin) was determined by StepOne™/StepOnePlus™ Software v2.2, the delta Ct values ( $\Delta\text{Ct}$ ), delta-delta Ct values ( $\Delta\Delta\text{Ct}$ ) and relative quantification ( $\text{RQ} = 2^{-\Delta\Delta\text{Ct}}$ ) values were calculated.

## **2.4 Statistical analysis**

The experimental results were evaluated using descriptive statistical calculations and one-way analysis of variance (ANOVA). The Kolmogorov-Smirnov test checked the normal distribution of the results, and the homogeneity of variance was checked by the Bartlett test. The calculations were performed using GraphPad Prism 7.0 software.

### 3 RESULTS AND DISCUSSION

#### 3.1 Short-term individual effect of T-2/HT-2 toxin, DON/2-AcDON/15-AcDON and fumonisin B1 in laying hens

The present study aimed to explore the effect of experimentally contaminated feeds with these mycotoxins individually during short-term oral exposure on the initiation and termination phases of lipid peroxidation in the liver, which is important in the metabolism and storage of mycotoxins and parallels with glutathione redox system activity. The feeding trial was performed with laying hens using a double dose of the EU recommendation for mycotoxin contamination (2006/576/EC, 2013/165/EU) to obtain easily measurable changes (Table 1).

**Table 1:** Measured mycotoxin content of the feeds of the experimental group (mg/kg feed)

<i>Treatment group</i>	<i>T-2/HT-2</i>	<i>DON/3-AcDON/15-AcDON</i>	<i>FB1</i>
Control	<0,02	<0,02	<0,02
T-2/HT-2 toxin-treated group	0,45/0,36	<0,02	<0,02
DON-treated group	<0,02	8,68/0,11/0,05	<0,02
FB1-treated group	<0,02	<0,02	38,10

According to the results, none of the examined mycotoxins initiated measurable lipid peroxidation; possibly, the applied dose did not cause mild oxidative stress in the liver of the laying hen. On the contrary, a significant decrease in lipid peroxidation parameters, like the CD levels by all examined mycotoxins and MDA concentration as an effect of DON, was observed. This decrease may be due to a change in the fatty acid composition of liver tissue lipids due to mycotoxin exposure. Markers of the glutathione redox system, amount of GSH, and activity of GPx were also decreased at the end of the trial as an effect of FB1, but the T-2 toxin and DON did not. This difference may be due to the rapid activation of the glutathione redox system as the effect of FB1. It effectively inhibited lipid peroxidation, resulting in the rapid depletion of the glutathione redox system. However, there was not enough time to regenerate glutathione



disulphide and de novo synthesis and/or post-translational activation of GPx.

As a result of mycotoxin exposure, I found significant differences in the relative expression of genes regulating the glutathione redox system. *GPX4* induction was detectable in the group loaded with T-2/HT-2 toxin and DON/3-AcDON/15-AcDON after 24 hours of mycotoxin exposure, which was not observed in the group loaded with FB1. This result suggests that trichothecene-based mycotoxins, such as T-2 toxin and DON, rapidly activated the antioxidant defense system at the level of gene expression, thus preventing the completion of lipid peroxidation, even if the given mycotoxin is otherwise ROS induces formation. FB1 did not always activate the glutathione redox system at the gene expression level, suggesting that FB1 induces a lower ROS formation. However, the relative expression of the *GSS* gene increased in the liver of laying hens on day 2 in the FB1-loaded group and on day 3 under the influence of T-2/HT-2 toxin. This change may be related to the decrease in GSH content due to FB1 on the 3rd day, which suggests that *GSS* gene expression at the level of enzyme activity occurs later. The gene expression changes of the Keap1-Nrf2 regulatory pathway appeared on the 2nd and 3rd days in the groups exposed to mycotoxins. The observed increase in expression of *Nrf2* on the second day in all three treated groups suggests that a low amount of ROS was formed in the liver of the laying hens at the applied doses.

The results also suggested that short-term mycotoxin exposure had already reached the threshold for activation of the expression of some antioxidant genes. Still, it was not detectable at the activity level but only at the mRNA level during the 3-day trial.

### 3.2 Short-term combined effect of T-2/HT-2 toxin, DON/2-AcDON/15-AcDON and fumonisin B1 in laying hens

In the present study, the intracellular biochemical and gene expression changes were investigated in the case of multi-mycotoxin exposure, with attention to certain elements of the glutathione system. An *in vivo* study was performed in a short-term feeding trial with low and medium doses (twice the low dose) in laying hens. The mycotoxin content of the feeds is shown in Table 2, based on the results of their laboratory measurements.

**Table 2:** Measured mycotoxin content of the feed of the experiment groups (mg/kg feed)

<i>Treatment group</i>	<i>T-2/HT-2</i>	<i>DON/3-AcDON/15-AcDON</i>	<i>FB1</i>
Control	<0,02	<0,02	<0,02
Low dose	0,26/0,36	4,78/0,59/1,55	19,21
Medium dose	0,43/0,31	9,11/0,09/0,06	41,20

Based on the biochemical changes that occur as a result of multi-mycotoxin exposure, it can be concluded that lipid peroxidation processes were induced in the livers of laying hens, which I proved on the first day of the experiment by the change in the amount of the metastable end product, MDA, produced in its termination phase. In the following two days, this lipid peroxidation marker decreased to control levels, indicating early activation of antioxidant defenses. This result explains early ROS formation and lipid peroxidation, especially when using contamination levels equal to the EU recommended limit values. Early ROS formation and lipid peroxidation activated the antioxidant glutathione redox system, which was supported by the increased GSH amount and GPx activity in the low-dose groups on the first day of the experiment. The changes were observed in the laying hens fed with the low mycotoxin-contaminated feed (the applied dose was the EU recommended limits), which suggests that there may be a synergistic effect between individual mycotoxins in terms of the induction of oxidative stress and activation of the glutathione redox system.

The relative expression of *GPX4*, *GSS*, and *GSR* genes was significantly higher on the first day in both the low- and medium-dose groups compared to the control. This result suggests that *Fusarium* multi-mycotoxin exposure induced redox changes in cells through ROS generation, activating genes encoding antioxidant proteins. However, the increased expression of these genes was not manifested at the protein level (GSH and GPx) in the case of the medium-dose group. Gene expression of antioxidant enzymes increased on the third day due to low-dose mycotoxin exposure, which the metabolism of mycotoxins may cause, as additional ROS formation may have occurred during this process. The mycotoxin combination did not activate the Keap1/Nrf2/ARE pathway at low or medium doses. From this, it can be concluded that the level of oxidative stress in the liver did not reach the level that would have caused the activation of the antioxidant response element during the examined period (72 hours).

These results suggested possibly synergistic toxicity interactions, including changes in the redox state of cells and activation of the antioxidant defense of multi-mycotoxin mixtures. In addition, the most changes in the antioxidant markers were found using the EU limit values, presumably due to the development of oxidative stress even at low concentrations of mycotoxins.

### 3.3 Short-term combined effect of T-2/HT-2 toxin, DON/2-AcDON/15-AcDON in laying hens

I also examined the combined effect of the two trichothecene-fusariotoxins, without FB1, on the elements of the glutathione redox system in laying hens. To assess the development of oxidative stress, I measured the markers of the initiation and termination stages of lipid peroxidation. The mycotoxin content of the feeds used for the examination is shown in Table 3 based on the laboratory measurement.

**Table 3:** Measured mycotoxin content of the feed of experimental groups (mg/kg feed)

<i>Treatment group</i>	<i>T-2/HT-2</i>	<i>DON/3-AcDON/15-AcDON</i>	<i>FB1</i>
Control	<0,02	<0,02	<0,02
Low dose	0,24/0,10	4,68/1,21/0,36	<0,02
Medium dose	0,41/0,21	9,14/0,04/0,11	<0,02

Trichothecene mycotoxins, such as T-2 toxin and DON, have a common feature in that their structure is characterized by an epoxy group, making them particularly reactive and capable of inducing ROS formation.

The markers of lipid peroxidation decreased in response to the multi-mycotoxin load on the first day of the experiment, which later was almost the same as the control value. The concentration of MDA, which indicates the termination stage of lipid peroxidation, was elevated on the 2nd day compared to the control in the low-dose group. The background of this change may be the increase in lipid peroxidation processes, which increased the MDA content on the 2nd day when using doses equal to the EU recommended limit value.

During the multi-mycotoxin study, I also measured a change in the glutathione redox state of the liver of laying hens, which I evaluated as an antioxidant response to an increase in MDA content. The GSH content was significantly higher on the first two days of the experiment due to the medium dose, as was the case with GPx activity in the same experimental group.

T-2 toxin and DON loading caused significant changes in the regulation of the glutathione redox system. *GPX4* expression decreased in the low-dose loaded group on the first day, and on the 48th hour, *GSS* gene expression decreased in both loaded groups. The expression of *Keap1* and *Nrf2* genes changed to the same degree and direction on the first and second days in response to the medium dose. At the end of the study, I measured an increased level of *Nrf2* gene expression, while no difference was observed in the case of the *Keap1*.

Based on the results, it can be concluded that the 3-day exposure of *Fusarium* multi-mycotoxins presumably resulted in the formation of ROS, which, through their combined effects, was manifested in the activation of the glutathione redox system. The oxidative stress that developed on the second day, which started lipid peroxidation in the liver of the laying hens, was effectively eliminated by the glutathione redox system by the 3rd day.

### 3.4 Short-term combined effect of T-2/HT-2 toxin, DON/2-AcDON/15-AcDON and fumonisin B1 in broiler chickens

I chose broiler chickens as an *in vivo* model because these animals are the largest share of poultry meat production in the European Union. The applied mycotoxin doses were the same as in the previous experiment. The measured mycotoxin content of the feeds is listed in Table 4.

**Table 4:** Measured mycotoxins content of the feeds of experimental groups (mg/kg feed)

<i>Treatment group</i>	<i>T-2/HT-2</i>	<i>DON/3-AcDON/15-AcDON</i>	<i>FB1</i>
Control	<0,02	<0,02	<0,02
Low dose	0,20/0,03	4,07/0,23/0,55	21,44
Medium dose	0,47/0,15	10,68/0,12/1,25	37,78

I measured dose-dependent, small feed rejection, a well-known effect of trichothecene mycotoxins. This effect was even measurable in combination with FB1. The amount of CT indicating the initiation stage of lipid peroxidation processes increased on the first day in response to the low multi-mycotoxin load. However, by the end of the experiment, this initially higher concentration had already decreased compared to the control values. This result indicates that lipid peroxidation processes could have been activated in the liver of broiler chickens already on the first day. Still, on the 3rd day, due to the oxidized fatty acids produced during the process and the change in the lipid-fatty acid composition of the liver membrane, lipid peroxidation decreased, presumably in the multiplicity, which is especially prone to oxidation due to a decrease in the amount of unsaturated fatty acids. Changes similar to the markers of the initiation phase of lipid peroxidation processes were also detected in the changes in the elements of the glutathione redox system. On the first day, oxidative stress developed, leading to lipid peroxidation processes. However, the MDA content did not change during this period, which suggests that the lipid peroxidation processes presumably did not reach the termination stage because the antioxidant system eliminated the oxygen-free radicals that were formed, thus preventing the further spread of the chain reaction. At the end of the experimental period, due to the changed fatty acid

composition, less lipid hydroperoxide was presumably produced, so the amount of GSH and the activity of GPx decreased due to the decreasing oxidative stress.

I also experienced significant differences in the gene expression values, which showed a similar trend for all tested genes. GPX4 gene expression was lower on the first day in the low-dose group compared to the control, indicating a change in the redox state in the liver cells. The gene expression of GSS was higher at the beginning of the experiment but not in the group treated with the low but medium dose. At the same time, there was no change in the peptide (GSH) level. According to them, the relative gene expression was also induced in the group exposed to the medium dose. Still, the level of oxidative stress in this group had not yet reached a level that would have activated the elements of the antioxidant, including the glutathione redox system, that I investigated. The GSR gene responsible for reducing glutathione disulfide (GSSG) was activated on the 3rd day in the group treated with the low dose. At the same time, I measured a decrease in the GSH content, so this increase was a response of the cell to the reduced GSH content, but this effect is an enzyme not yet manifested at the protein level. The expression of the Keap1 and Nrf2 genes partially changed to the same extent and in the same direction as a result of the applied doses. On the first day, I observed an increase in the treated groups, a decrease on the 2nd day, and an increase again on the 3rd day. However, as I mentioned earlier, the decrease measured on day 2 is related to the exceptionally high value measured in the control group in the case of the Nrf2 gene. If this change is disregarded, the expression of the Nrf2 gene increased in a trend-like manner and continuously in the groups receiving the mycotoxin load during the entire duration of the experiment. The activation of the Keap-1/Nrf2 regulatory pathway indicates a small amount of oxidative stress at both mycotoxin doses.

Based on the results, it can be concluded that the 3-day Fusarium multi-mycotoxin exposure resulted in ROS formation, to which a definite response was detected at the gene expression level in both dose-treated groups. However, gene expression changes did not correlate with the amount of GSH and the activity of GPx.

### 3.5 Short-term combined effect of T-2/HT-2 toxin, DON/2-AcDON/15-AcDON and fumonisin B1 in elevated doses in broiler chickens

During the feeding experiment with 21-day-old broiler chickens, I used four times the amount of the limit values recommended by the European Union to obtain greater changes than those experienced in the previous experiment. The mycotoxin content of the feeds used for the experimental contamination is shown in Table 5, based on the results of laboratory tests of the mycotoxin content of the contaminated feed.

**Table 5:** Measured mycotoxin content of the feed of experimental groups (mg/kg feed)

<i>Treatment group</i>	<i>T-2/HT-2</i>	<i>DON/3-AcDON/15-AcDON</i>	<i>FB1</i>
Control	<0,02	<0,02	<0,02
Low dose	0,23/0,12	4,56/1,89/0,40	18,59
High dose	0,78/0,09	18,68/1,05/0,98	75,16

The amount of CD was significantly lower in the 48th hour when using the EU recommended limit values compared to the control group. The background of this decrease may be a change in the lipid-fatty acid composition of the liver tissue as a result of the mycotoxin load. The concentration of MDA was also reduced compared to the control in the 72nd hour after feeding the feed contaminated with mycotoxins in the group treated with the low dose. This result also supports my hypothesis, described in the case of CD, according to which the mycotoxin load affects the fatty acid composition of the liver tissue in a dose-dependent manner. As a result, the intensity of the lipid peroxidation was also detected, which in the 48th hour was only detectable in the initiation phase of the process, while a day later, it was already detectable in the MDA concentration, indicating the termination phase.

The amount of GSH and the activity of GPx in the blood plasma were higher in the high-dose group than in the control group at the end of the experiment. This result suggests that the higher mycotoxin load caused moderate oxidative stress, which activated the glutathione



redox system, which was only detectable in the blood plasma. In the liver, however, the amount of GSH and the activity of GPx did not change, suggesting that the multi-mycotoxin load did not result in oxidative stress to a degree that would have also resulted in detectable changes in the liver.

The lack of activation of the *GPX4* gene may explain why the glutathione redox system was not activated at the protein level in the liver. However, the gene expression of *GSS* and *GSR* increased at the 48th hour of the experiment in the low-dose group, indicating a small amount of oxidative stress. This could be the explanation for the non-significant change in the GSH content in the liver, which was present at the expression level of the genes encoding the enzymes involved in the synthesis and reduction of GSH but not at the protein level. The mycotoxin combination used in high doses reduced the gene expression of *Keap1* and *Nrf2* on the second day. By the end of the experiment, the level of oxidative stress in the liver had reached the level that caused the activation of the Nrf2-induced antioxidant response element, which could not be measured at the protein level during the short duration of the experiment.

## 4 CONCLUSIONS AND SUGGESTIONS

### 4.1 Conclusions

I started my experiments by investigating the individual effect of T-2/HT-2 toxin, DON, and FB1 in laying hens in a short-term (72 hours) experiment, with a double dose of the EU recommendation limit for mycotoxin contamination (T-2/HT-2 toxin: 0.5 mg/kg; DON/3-AcDON/15-AcDON: 10 mg/kg; FB1: 40 mg/kg feed). The results showed significant changes at the gene expression level, mainly as an effect of T-2 toxin and DON. The antioxidant defense system was activated on the first day, preventing the development of lipid peroxidation processes. In the following multi-mycotoxin study, a short-term (72 hours) feeding trial was performed with laying hens in two doses: low dose (EU-recommendation doses) and high dose (twice the low dose). Changes in lipid peroxidation and an increase in the markers of the glutathione redox system were mostly observed in the group treated with the low dose, which supposes a synergistic interaction between the mycotoxins was investigated. In the other experiment, the combined effect of two trichothecene mycotoxins (T-2 toxin, DON) was investigated in laying hens. A three-day multi-mycotoxin exposure caused oxidative stress in the liver of the laying hens. The marker of the termination phase of lipid peroxidation, the malondialdehyde content, was increased in the low-dose group. However, activating the antioxidant system adequately neutralized the oxidative stress by the end of the experimental period.

In a further experiment, I investigated the oxidative parameters of 21-day-old broiler chickens using the above-mentioned mycotoxin doses. Changes in the relative gene expression values assumed ROS formation; however, members of the glutathione redox system were synthesized to a small extent in the treated groups and only on the first day of the experiment. In the next study, the applied mycotoxin concentrations were higher due to the moderate response in the previous study. The high-dose group was used to quadruple the recommended EU recommended limit values. The glutathione redox system's amount (GSH) and activity (GPx) were higher in the blood plasma but not changed in the liver. Based on the results, it can be assumed that the high dose induced a low oxidative stress in blood plasma. Still, it was lower than the critical value to activate the antioxidant response in the liver.

According to the results, the examined mycotoxins influenced the relative expression of genes encoding the enzymes of the glutathione redox system. Still, this effect did not occur at reduced glutathione and glutathione peroxidase activity. During the three-day experiment, the development of oxidative stress depended on the applied dose and treatment time, and the glutathione redox system was mostly capable of neutralizing the generated free radicals by the end of the experiment. Most changes were measured when applying the EU-proposed limit values; therefore, a revision of the limit values is recommended due to the multitoxic effects.

## **4.2 Suggestions**

The short-term effects of T-2/HT-2 toxin, DON/3-AcDON/15-AcDON, and FB1 in poultry are partially known, and their effects on ROS formation are controversial. The most changes in the antioxidant markers were found using the EU limit values, presumably due to the development of oxidative stress even at low concentrations of mycotoxins. Therefore, it is important to continue studying mycotoxin mixtures at low concentrations and the related mechanism to understand mycotoxin exposure-related diseases better. Based on my results, I recommend continuing the research with new target genes and mycotoxins with different chemical structures.

## 5. NEW SCIENTIFIC RESULTS

Evaluating the effects of the applied doses of mycotoxins on laying hens and broiler chickens in the observed 72-hour period, I concluded that:

1. During a short-term (72 hours) feeding trial with laying hens, the T-2/HT-2 toxin, DON/3-AcDON/15-AcDON, and FB1 mycotoxins in EU-recommendation doses induced lipid peroxidation in the liver, which activated glutathione redox system. Compared to the mycotoxins' individual effect, the multi-mycotoxin effect was more pronounced, indicating a synergistic interaction.
2. The individual effects of the *Fusarium* mycotoxins are different than multi-mycotoxin exposure and suggest a synergistic effect among them, which is revealed in the change of lipid peroxidation processes and influencing the glutathione redox system.
3. The presence of FB1 in the mycotoxin combination enhanced the oxidative stress-generating effect of the two trichothecene-mycotoxins in laying hens.
4. The combined exposure of the T-2/HT-2 toxin, DON/3-AcDON/15-AcDON, and FB1 increased the relative expression of the *Nrf2* and *Keap1* genes using EU-limit values, which indicates oxidative stress in broiler chickens.
5. The amount/activity of the glutathione redox system increased as a result of the effect of T-2/HT-2 toxin, DON/3-AcDON/15-AcDON, and FB1 mycotoxins in the blood plasma of broiler chickens using quadruple the EU recommended values.

## **PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS**

### **Publications in scientific journals:**

**Kulcsár S,** Kövesi B, Balogh K, Zándoki E, Ancsin Zs, Mézes M. (2020): Együttesen előforduló *Fusarium* mikotoxinok rövidtávú terhelésének hatása a brojlercsirke glutathion redox rendszerére és lipidperoxidációs folyamataira. Állattenyésztés és Takarmányozás, 2020. 69. 4.

**Kulcsár S,** Kövesi B, Balogh K, Zándoki E, Ancsin Zs, Balláné M, Mézes M. (2021): Effects of *Fusarium* Mycotoxin Exposure on Lipid Peroxidation and Glutathione Redox System in the Liver of Laying Hens. Antioxidants (Basel). 10(8):1313.

**Kulcsár, S.;** Kövesi, B.; Balogh, K.; Zándoki, E.; Ancsin, Z.; Erdélyi, M.; Mézes, M. The Co-Occurrence of T-2 Toxin, Deoxynivalenol, and Fumonisin B1 Activated the Glutathione Redox System in the EU-Limiting Doses in Laying Hens. Toxins 2023, 15, 305.

### **Abstracts in scientific journals:**

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