

**THESIS OF DOCTORATE (Ph.D.)
DISSERTATION**

**UNIVERSITY OF KAPOSVÁR
FACULTY OF AGRICULTURAL AND ENVIRONMENTAL
SCIENCES**

Mycotoxins in the Food Chain Research Group

Head of Doctoral School:

Prof. Dr. Kovács Melinda

Corresponding Member of the Hungarian Academy of Sciences

Supervisor:

Prof. Dr. Kovács Melinda

Corresponding Member of the Hungarian Academy of Sciences

Co-Supervisor:

Dr. Szabó-Fodor Judit

Ph.D., Senior research fellow

**THE DOSE AND TIME DEPENDENT, SINGLE AND COMBINED
CYTO- AND GENOTOXIC EFFECTS OF MYCOTOXINS
FUMONISIN B₁, DEOXYNIVALENOL AND ZEARALENONE**

Written by:

KACHLEK MARIAM LILIA

KAPOSVÁR

DOI: 10.17166/KE2018.003

1. BACKGROUND OF RESEARCH, OBJECTIVES

The secondary metabolites of fungi, mycotoxins, can pose serious problems to animal farming by affecting animal health both in- and directly. Around 400 metabolites have been characterised so far and the most important genera of fungi are *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*. The most frequently occurring mycotoxins worldwide are *Fusarium* mycotoxins and more specifically fumonisin B₁ (FB₁), deoxynivalenol (DON) and zearalenone (ZEN).

Mycotoxins are regulated around the world but risk assessment is based on single mycotoxin studies. Only in the last decade the focus has shifted to co-occurring mycotoxins. The co-occurrence of mycotoxins has been confirmed by several surveys conducted worldwide. Co-contamination can vary from 70%-100% of the samples. In a very recent survey, DON, FUMs and ZEN were present in at least 50% of the analysed samples. Furthermore, in Europe FB₁, DON and ZEN are the most frequently occurring mycotoxins. Despite this, the mixtures of FB₁, DON and ZEN - especially in ternary - have not been studied as thoroughly as other mycotoxins (i.e. aflatoxins, ochratoxin).

No information on the ternary combination of FB₁, DON and ZEN *in vivo* is available. On the other hand, there are only few studies *in vitro*, and their combined genotoxicity (by the means of comet assay) has never been studied.

Oxidative stress is one of the main mechanisms of action for mycotoxins. This has led researchers to use several medicinal plants in mycotoxin trials in order to investigate any possible protective effect. Apart from causing oxidative stress mycotoxins usually target specific organs like liver or kidney. Some medicinal plants can protect the liver or the kidney from malfunction after mycotoxin exposure. A plant from the Asteraceae

family, *Carduus marianus* possesses antioxidant as well as hepatoprotective properties among others.

The aims of the present work were the following:

- 1.) Investigation of the single and combined effect of low doses of FB₁, DON and ZEN in rabbit bucks focusing on the effects on reproduction.
- 2.) Determination of the effect of high dose of DON in growing rabbits on production parameters, blood indices, oxidative status, histopathology, immunity, and genotoxicity. Furthermore, investigation of the possible protective effects of the medicinal plant *Carduus marianus* against DON.
- 3.) Determination of the IC₅₀ value (half maximal inhibitory concentration) for FB₁, DON and ZEN using porcine lymphocytes. Additionally, the *in vitro* investigation of their combinations in binary and ternary mixtures on cytotoxicity and genotoxicity.

2. MATERIALS AND METHODS

In our two *in vivo* experiments, Pannon White rabbits were used. To contaminate experimental diets *Fusarium verticillioides* (for FB₁) and *Fusarium graminearum* (for DON and ZEN) (NRRL 20960 [MRC 826] and NRRL 5883, respectively) fungal culture was used for the rabbit bucks experiment. For the second trial, the *F. graminearum* IFA 77 strain was used, which produces only DON at 28°C in order to avoid co-occurrence of ZEN. In both trials, mortality and morbidity were checked daily whereas feed intake and body weight were checked weekly. The experimental protocols were authorized by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under allowance number SOI/31/1679-11/2014. To control mycotoxin and silibinin concentrations LC-MS analyses were performed by a Shimadzu Prominence UFLC separation system equipped with an LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan).

For the *in vitro* experiments, primary cultured porcine lymphocytes were used. Cytotoxicity (using cell counting kit-8) and genotoxicity (measured by comet assay) were the chosen endpoints for these studies.

2.1 Effect of FB₁, DON and ZEN in rabbit bucks

The aim of the study was to examine the single and combined effect of the mycotoxins FB₁, DON and ZEN in rabbit bucks. A special focus was given to the effects on the reproductive system.

The 24-weeks old bucks (n=60) with an average weight of 4.0 ± 0.5 kg were individually caged in wire mesh cages. The rabbits were consuming the diets *ad libitum*, and they also had free access to drinking water.

The animals were divided into four different groups that received different diets. The control (C) group received toxin-free diet, while the feed of the other three groups was contaminated with FB₁ at 5 mg/kg of feed (F),

or DON + ZEN at 1 and 0.25 mg/kg, respectively (DZ) or FB₁+ DON + ZEN at the aforementioned concentrations (FDZ).

On days 30 and 60 blood and sperm was sampled (n=15/group), and a gonadotropin-releasing hormone (GnRH) test was performed (blood samplings related to GnRH test: n=6/group, 0, 15, 50, 75, 90 and 115 min after GnRH injection) for monitoring testosterone production. Blood sampling was performed also for the determination of clinical chemistry, comet assay and antioxidant status (malondialdehyde [MDA], reduced glutathione [GSH] and glutathione peroxidase [GPx]). The latter parameters were determined both in the plasma and in the red blood cell haemolysate (RBCH; day 60). Concentration of malondialdehyde and the conjugated dienes (CD) and trienes (CT) were investigated in the liver homogenate as well, referring to the antioxidant status of the animals.

Spermatological analyses covered the following parameters: pH, sperm cell concentration (improved Neubauer cell counting chamber), motility, morphology (native and stained) and acrosomal integrity (SpermacTM staining) of the spermatozoa. Motility was evaluated with a computer assisted sperm analyser (MedealabTM CASA System).

For the purposes of comet assay the sperm that was sampled on day 60 of the experiment (n=15/group) was used. The method was adapted from human spermium examination protocols with modifications. After washing the semen, sperm cells were embedded onto microscope slides. After the necessary condensation due to the high number of cells, lysis followed. Electrophoresis succeeded and then the slides were washed, dried and stained with ethidium bromide (EtBr). Consequently, the slides were investigated with a fluorescence microscope. Comets were classified into scores of '0' (no alteration), '1', '2', '3' and '4' (seriously damaged) according to the severity of DNA damage and head/tail migration.

At the end of the study (on day 65) animals were exsanguinated after stunning. The weight of liver, kidneys, testicles and spleen was measured, macroscopic changes were analysed and recorded and samples were taken for histopathological analysis.

Testosterone concentration and spermatological parameters were determined in the laboratory of University of Veterinary Medicine (Budapest, Hungary), while the antioxidant status was determined in the laboratory of SZIU Faculty of Agricultural and Environmental Sciences (Gödöllő, Hungary). The histopathological analysis was performed according to the Act/ 2011 (03.30) of the Hungarian Ministry of Agriculture and Rural Development and was carried out by the Autopsy Ltd. (Budapest, Hungary).

Statistical analyses were performed using IBM SPSS (version 20.0) software. Data processing and the mathematical-statistical calculations were performed using the compare means (independent-samples t-test, One-way ANOVA with Tukey posthoc test), interactions between groups and time (general linear model- GLM; repeated measures) and correlate and descriptive statistics modules. In the case of comet assay, crosstabs options were used for chi-square test.

The type of interaction was determined according to the characterisation of the different interactions between mycotoxins in the meta-analysis of Grenier and Oswald (2011). A synergistic effect was identified when the effect of mycotoxin combination (FDZ) was greater than expected from the sum of the individual effects of the two (F and DZ) treatments. A synergistic effect was determined also if one (F or DZ) of the treatments didn't display any effect, but the effect of their combination (FDZ) was greater than the effect of the other treatment alone. Additive effect means that the effect of combination can be calculated as the sum of the individual effects of the two treatments.

2.2 Effect of DON and *Carduus marianus* in growing rabbits

The aim of this study was to investigate the effect of dietary DON at a high concentration (10 mg/kg) and the possible protective effect of the medicinal plant *C. marianus* in growing rabbits.

The weaned 35-days old rabbits (n=72), were housed in in wire mesh cages (n=3/cage, 4 cages/group). The 6 groups formed according to the different diet received were: control (C), control supplemented with the herb at 5 g/kg (H1), control supplemented with the herb at 10 g/kg (H2), control supplemented with DON at 10 mg/kg (CT), control supplemented with the herb at 5 g/kg and DON at 10mg/kg (H1T) and control supplemented with the herb at 10 g/kg and DON at 10 mg/kg (H2T). *C. marianus* was purchased from Parceval (Pty) Ltd Pharmaceuticals, South Africa in powder form (seeds).

On day 21, blood sampling was performed from the marginal ear vein for clinical chemistry, haematology, immune response (leukocytes, phagocytic activity), comet assay and antioxidant parameters (GPx activity, GSH and MDA concentration). The latter were determined in RBCH as well.

Comet assay was performed with the lymphocytes isolated from the experimental rabbits. Tail intensity (% DNA in the tail) was determined with an Epifluorescent Microscope (B600 TiFL; optimum filter 4 and $\lambda = 302$ nm) and Comet IV (version 4.3.1.) software.

On day 21, 6 rabbits from all groups were euthanized by cervical dislocation and exsanguinated. The digestive tract was immediately removed and caecum was separated. Ceacal chyme samples were taken for pH, microbiota and short chain fatty acids (SCFA). The composition of the microbiota was determined by classical microbial culturing techniques. The concentration of SCFA was measured by gas chromatography.

Small intestinal sections were taken from the jejunum (1 cm before the Meckel's diverticulum), ileum (1 cm before the ileocaecal junction), liver,

spleen, kidneys and heart for histopathological examination. A sample from the midsection of the jejunum was taken for cytokine (IL-1, IL-2, IFN- γ) measurements using reverse transcriptase real-time polymerase chain reaction (rt-RT-PCR), detecting mRNA. Immunological parameters were determined in the University of Veterinary Medicine (Budapest).

Statistical analysis of the data was performed with IBM SPSS (version 20.0) software package using t-test and one-way analysis of variance (ANOVA). Tukey's method was used for the posthoc test. In all the cases the significance level was $p < 0.05$.

2.3 *In vitro* experiments

The aims of these studies were firstly the determination of the IC₅₀ (concentration that inhibits viability by 50%) values of FB₁, DON and ZEN and secondly the investigation of their combined effects in binary and ternary mixtures on porcine lymphocytes. The endpoints used were cytotoxicity and genotoxicity (comet assay). In these experiments pure mycotoxins were used, purchased from Sigma Aldrich. Lymphocytes isolated from blood taken from healthy adult pigs were used for all the experiments. The concentrations used were 2×10^6 cells/ml for the cytotoxicity experiments whereas $4-5 \times 10^6$ cells/ml for the comet assay.

2.3.1 Cytotoxicity testing

For the determination of the cytotoxicity, the 3-[4,5, dimethylthiazol-2, -yl]-2,5 diphenyl-tetrazolium bromide (MTT) salt is used frequently. In our studies, a water-soluble version of MTT was used [WST-8; cell counting kit-8 (CCK-8)]. CCK8 is much less toxic to the cells, exhibits higher sensitivity and eliminates the need for solubilisation.

Cells were incubated for 24, 48 and 72h in 96-well plates. After each incubation time, the CCK-8 solution was added and the cells were further

incubated for 4 hours. Then the absorbance of the wells was read at 450 and 620 nm under a Microplate Reader. To determine the IC₅₀ the concentration ranges of the single toxins used in the tests were between 50–150 µg/ml, 0.07–0.84 µg/ml and 1–50 µg/ml for FB₁, DON and ZEN, respectively.

Dose-response curves were fitted to the best linear or nonlinear models in order to determine the concentration that induced 50% loss of viability (IC₅₀). The calculations were made with the software OriginPro version 9.0.

2.3.2 Genotoxicity testing

For the genotoxicity studies, after isolation, the cell culture was subdivided (100 µl) into microcentrifuge tubes which were incubated overnight in a humidified incubator at 37°C and 5% CO₂. Then toxin or vehicle/carrier solutions were added to the tubes and were incubated for 24h, 48h and 72h. After each incubation period, the comet assay procedure described by Horvatovich et al. (2013) was followed, with slight modifications. The slides were washed with phosphate-buffered saline (PBS) and for the staining of the slides ethidium bromide (EtBr) was used. Scoring of comet slides were performed with Epifluorescent Microscope and Comet IV (version 4.3.1) software. One hundred cells/gels (i.e. 400 cells/concentration) were investigated during the analysis. The cells were classified by software analysis according to tail intensity (% tail DNA).

2.3.3 Testing combined cyto- and genotoxicity

The mycotoxins were tested in binary and ternary mixtures (n=3) at low concentrations (below the IC₅₀) to assess any interactive effects. The binary and ternary mixtures of the toxins were investigated at 5 µg/ml (FB₁ and ZEN) and 0.07 µg/ml (DON) for cytotoxicity and genotoxicity. Higher concentrations (still below the IC₅₀) were used for the second series of

genotoxicity experiments (25, 0.21 and 10 µg/ml for FB₁, DON and ZEN respectively). The combinations tested were: DON + FB₁ (DF), DON + ZEN (DZ), FB₁ + ZEN (FZ), DON + FB₁ + ZEN (DFZ).

Regarding combinations' experiments observed values were compared with expected values to detect any significant differences. Expected values were calculated as a mean value obtained after exposure to one substance alone plus a mean value obtained after exposure to second or third substance:

Mean % (expected for myc1+myc2) = mean % (myc1) + mean % (myc2) – 100% control

Mean % (expected for myc1+myc2+myc3) = mean % (myc1) + mean % (myc2) + mean % (myc3) – 100% control

Calculation of expected SD/SEM:

SD (expected for myc1+ myc2) = [(SD for myc1)² + (SD for myc2)²]^{1/2}

SD (expected for myc1+ myc2+ myc3) = [(SD for myc1)² + (SD for myc2)² + (SD for myc3)²]^{1/2}

The results were interpreted as follows: (i) an additive effect was recorded if the measured values were not significantly above or below the expected values; (ii) a synergistic effect was recorded if the measured values were significantly above (tail intensity) and below (cell viability) the expected values; (iii) an antagonistic effect was recorded if the measured values were significantly below (tail intensity) and above (cell viability) the expected values.

The statistical difference between observed and expected values was calculated with unpaired t-test.

3. RESULTS

3.1 Effect of FB₁, DON and ZEN in rabbit bucks

The concentrations of FB₁, DON and ZEN in the feed (5, 1 and 0.25 mg/kg feed, respectively) corresponded to 169-193 µg FB₁/kg BW, 33.7-38.7 µg DON/kg BW, and 8.5-9.7 µg ZEN/kg BW exposure. Only FB₁ could be studied individually since the *F. graminearum* strain used produced both toxins (as it is also observed in practice). Thus, DON and ZEN were studied as one treatment although the hypothetical discussion is for the two single toxins. All kinds of interactions (addition, synergism and antagonism) could be detected in our study.

The feed intake of the rabbit bucks was not different among the groups. No significant difference in body weight among groups was detected at any of the 12 timepoints, average body weight of the groups was between 4252 and 4442 g by the end of the experiment. No sign of mycotoxicosis was detected.

Among organs (testicles, liver, kidneys, spleen), only the weight of the spleen was significantly different between groups. The highest spleen weight was observed in DZ animals (1.84 ± 0.49^b), whereas the lowest in C (1.46 ± 0.38^a).

Gross hepatic and renal lesions and disorder were not induced by any of the treatments, as underscored by the unaltered ALT, AST, GGT and CREA values, the identical liver and kidney mass values and the lack of histopathological alterations in all groups. These organs were likewise tolerant towards the exposure of low dose of these mycotoxins, without providing degenerative processes.

Regarding the levels of total protein (TP), globulin (GLOB) and total cholesterol (tCHOL), slight significant differences were observed between treatments. All data were within the reference ranges and only GLOB

concentration exceeded the upper limit in the C and FDZ groups on day 30. There was no interaction regarding clinical chemical parameters with the exception of TP and GLOB. The effect of time within the same groups was observed for all groups but ALT and TP.

No differences within the same groups between the two sampling dates could be observed. There were no significant differences among the treatments on day 30. At day 60, DZ treatment resulted in significantly increased GPx activity in the red blood cells and MDA formation both in RBCH and plasma, while less GSH concentration in the blood plasma. As a result of peroxidation of dienoic and trienoic fatty acids, the concentration of CD and CT increased in case of DZ exposure, as compared to the combined effect of three mycotoxins (FDZ). Thus, FB₁ was proved to alleviate the oxidative stress caused by DZ, acting in antagonistic manner.

The testosterone concentration was significantly different due to mycotoxin exposure only at day 60 at the sampling minutes of 75, 90 and 115. Leydig cells of the animals intoxicated with the three mycotoxins in combination (FDZ) for a total of 60 days, synthesized significantly less testosterone compared to all other groups as a response to exogenous GnRH.

No interaction was observed between group and time for any of the timepoints. Furthermore, no time effect was observed for any of the time points.

No effect of the toxin treatment was detected on semen pH (ranging from 6.4 to 8.2 in each group), the quantity of the semen (on average 1 ml in each group), concentration of spermatozoa (ranging from 2.4 to 2.6 × 10⁷/ml) and sperm motility. The ratio of spermatozoa showing progressive forward motility was around 80% at the beginning of the experiment and decreased to 67% in the semen of FDZ animals showing a significant difference when compared to the other three treatment groups. The ratio of spermatozoa with normal morphology was significantly different between the groups C (80%) and DZ (66%). The most frequent morphological abnormalities were

abnormality of the tail, retention of cytoplasmic drop, absence of the acrosome and altered head.

All treatments caused DNA damage as detected by comet assay and 98.6, 91.6 and 91.8% of the treated cells could be categorized as having 1 to 3 scores in the F, DZ and FDZ group, respectively. Cells with 0 score showed the highest prevalence in group C. FB₁ resulted in significantly less 0 comets (intact cells) compared to the other treatments. The comet score 2 showed the highest prevalence in samples of F and DZ animals, while FDZ treatment resulted in significantly fewer cells of this type. This result refers to antagonism between F and DZ. No cells were found with a comet assay score of 4 in the experiment which shows that none of the toxins had a strong genotoxic effect.

According to histopathology in animals consumed F, DZ and FDZ diet the intensity of spermatogenesis decreased by 43, 31 and 64%, respectively, which was reflected by the lack of differentiated spermatozoa, thinning of the germinal epithelium, the appearance of multinuclear giant cells indicative of the disturbance of meiosis and mitosis of the germinal epithelial cells and in some cases the lack of spermatogonia. These histological findings were observed to different severities in the seminiferous tubules.

In the testicles of the toxin treated animals, the cytomorphology and the proportion of the Leydig interstitial cells were not significantly different from the controls.

In the Malpighian bodies of the spleen of every DZ and FDZ animal, slight lymphocyte depletion (slight thinning of the T and B dependent zones of the Malpighian tubules) was observed. However, the cytomorphology of lymphoblast and lymphocyte cells was not different from the control.

3.2 Effect of DON and *Carduus marianus* in growing rabbits

Feed intake, body weight and body weight gain showed no significant treatment effects. The toxin exposed rabbits did not show pathologic clinical signs, the feed refusal effect was not observed, and no animals died as a result of the toxin treatment. Furthermore, no significant differences were observed when the plant was fed to the rabbits either solely or in combination with DON.

The control group had a higher neutrophil percentage compared to the other groups apart from H1. On the contrary, the percentages of monocytes (significant) and eosinophils (non-significant) were higher in the toxin-treated group as compared to the control group.

Serum total protein, albumin, glucose, triglyceride, cholesterol, urea and creatinine concentrations as well as ALT, AST and GGT activities did not differ significantly among the groups, and the mean values were within the physiological ranges. DON and the herb at the dose(s) applied had no effect on the antioxidant status of the animals.

No significant differences were observed for any of the gut cytokines (IL-1, IL-2 and INF- γ), the macrophages' number and their phagocytic activity as well.

DON did not exert a significant effect on gut and villus morphology. The morphology of the gut mucosa was normal and the epithelial layer of the villi was intact in both groups. Thickening and fusion of the villi could be observed in only one of the toxin-treated animals. Surprisingly in the gut of three animals from the H2T group villus thickening and fusion was observed. Villus height and crypt depth in duodenum and jejunum were similar for all groups. As regards the GALT, in 4 out of the 6 DON-treated rabbits the rate of proliferation and simultaneous apoptosis of the lymphoblasts shifted towards apoptosis.

The spleen was also affected by DON treatment in all animals. In the central part of the lymphoid follicles, in the Malpighian bodies, lymphocyte depletion resulting in smaller follicles (follicular atrophy) could be detected compared to the control animals. The lymphocyte depletion and follicular atrophy caused by DON was completely inhibited by the plant at both concentrations.

DON caused slight fibrosis in the liver, without degenerative alteration of the hepatocytes. *Carduus marianus* inhibited the DON-induced fibrosis, confirming thus its hepatoprotective properties.

DON did not cause any pathological changes in either the heart or the kidneys.

According to the results obtained in the comet assay, DON did not prove to be genotoxic on lymphocytes. After comparing the tail intensity and tail moment of CT group with C, H1T and H2T, no statistical differences were found.

There was no significant difference in the pH and the concentration of total short chain fatty acids (SCFA) (mmol/kg) or the ratio of the particular SCFA (acetic, propionic and butyric acid) in the caecal chyme. The composition of the caecal microbiota showed no significant differences regarding anaerobic bacteria and Bacteroides. Coliforms' numbers were very low in all the samples (colonies<100). On the other hand, there were significant differences in the number of aerobic bacteria. More specifically, groups C and H1 had significantly lower number of aerobic bacteria compared to all toxin treated groups (i.e. CT, H1T and H2T).

3.3 *In vitro* experiments

A time- and dose-dependent decrease in cell viability was observed for all three toxins. For DON and ZEN IC₅₀ values could be calculated for all incubation times. In contrast, FB₁ decreased cell viability by 50% only after

72h. The potency of cytotoxicity in an increasing order was $FB_1 < ZEN < DON$. The IC values' range depending on the length of exposure was 0.31-0.42 $\mu\text{g/ml}$ and 16.6-22.9 $\mu\text{g/ml}$ for DON and ZEN, respectively, while 101.15 $\mu\text{g/ml}$ for FB_1 (after 72h).

In the present study, combinations of FB_1 , DON and ZEN (at concentrations of 5, 0.07 and 5 $\mu\text{g/ml}$, respectively) resulted in antagonistic effects regarding cytotoxicity. The ternary mixture (DFZ) exerted significant antagonism after all incubation periods, which was most pronounced after 72h. A similar trend was observed for the rest of the mixtures which exerted interactive effects (FZ) or increased the degree of interaction (DF and DZ) in a time-dependent manner.

To the best of our knowledge, there are no studies addressing the combined genotoxic effects of FB_1 , DON and ZEN. Two different series of experiments were performed using a set of lower and higher concentrations, respectively.

When the lower set of concentrations (i.e. 5, 0.07 and 5 $\mu\text{g/ml}$ for FB_1 , DON and ZEN, respectively) was used, antagonism was the main interaction observed. More specifically, after 24h an antagonism was observed for all mixtures but it was statistically significant only in the case of the ternary mixture. After 48h, DZ and DFZ exerted an antagonistic effect but a statistically significant antagonism was found only for DFZ; DF displayed synergistic effect, while FZ showed no interaction. After 72h all four mixtures showed an antagonism but it was statistically significant only for DF.

In order to obtain more pronounced effects, increased concentrations of mycotoxins (25, 0.21 and 10 $\mu\text{g/ml}$ for FB_1 , DON and ZEN, respectively) were used for the further investigation of interactions. The antagonistic effect of the DFZ mixture was confirmed.

After 24h, a significant antagonism was revealed by the mixtures of DF and DFZ, while DZ demonstrated a non-significant synergism and FZ showed no interaction. After 48h the trend was the same for the mixtures of DZ and DFZ while DF showed no interaction and FZ exerted an antagonistic effect which was not significant. After 72h antagonism was expressed from the mixtures of DF and DFZ (not significant and significant respectively). On the other hand, the mixtures of DZ and FZ showed a significant synergism.

4. CONCLUSIONS

Mycotoxins can affect farm animals in an adverse manner. *Fusarium* mycotoxins are the most frequently occurring worldwide. The combined effects of FB₁, DON and ZEN have not been studied in detail *in vivo*. Additionally, although a lot of *in vitro* studies have assessed the interactive effects of FB₁, DON and ZEN, only a few have investigated their ternary mixtures.

In our multi-toxic (FB₁, DON, ZEN) exposure experiment, the obtained results indicate that a prolonged low-dose mycotoxin exposure may adversely affect male reproduction. Among the mycotoxins studied additive or less than additive effect was found in the case of spermatogenesis and sperm cell morphology, synergism in testosterone production, while FB₁ acted antagonistic against DON+ZEN on feed intake, lipid-peroxidation, and genotoxicity. All mycotoxins provoked moderate lipid-peroxidation and exerted slight genotoxicity. From our results it can be concluded that the nature of the interactions among mycotoxins depends on the endpoint used.

In our second *in vivo* experiment the medicinal plant (*Carduus marianus*) was co-administered in two levels (5 and 10 g/kg) with DON in order to assess any possible protective effect in growing rabbits. Despite its high concentration (10 mg/kg), DON did not induce adverse effects overall. Although DON is known to induce feed refusal and anorexia (already at 1mg/kg of feed) our results contradict it and thus it can be concluded that rabbits are less sensitive to DON than other species. The resistance of rabbits to DON is depicted in several parameters as it highlighted below. A lack of adverse effects on the serum biochemistry parameters (liver and kidney functions) was observed, but DON caused slight fibrosis in the liver without degenerative alteration of the hepatocytes. Gut cytokines and the phagocytic activity of the macrophages did not differ significantly, but DON caused lymphocyte depletion and follicular atrophy in the spleen and increased the

rate of apoptosis of the lymphoblasts in the GALT, indicating, that it can possess immunomodulatory properties. The liver fibrosis, lymphocyte depletion and follicular atrophy caused by DON were completely inhibited by the plant at both concentrations. In agreement with the IARC classification of DON as non-carcinogenic to humans, DON did not exhibit genotoxicity effects on lymphocytes in our study.

In conclusion, it was shown that rabbits are less sensitive to DON compared to other mammalian animal species. The most sensitive target was the immune system, but no secondary negative effect (infection) occurred. Future studies are required to explore the reason of the relative resistance of rabbits against DON. The hepatoprotective effect of *C. marianus* was confirmed and it was demonstrated that it can exert protective effects to the lymphoid organs.

In our series of *in vitro* experiments the cytotoxic and genotoxic effects of FB₁, DON and ZEN were investigated.

It was shown that DON is the most cytotoxic of the three mycotoxins and was confirmed that FB₁ requires long exposure period in order to exert its cytotoxic effects. The main interaction observed in the cytotoxicity experiments was antagonism. The antagonism observed was in a time-dependent manner for some of the binary mixtures while in the case of the ternary mixture, it was evident for all exposure times.

In the case of genotoxicity two series of experiments were conducted using a lower and a higher set of concentrations. In the first series, similarly to the cytotoxicity study, the main interaction observed was antagonism. However, at higher concentrations, the antagonism was confirmed only for the ternary mixture whereas synergism was observed for two of the binary mixtures.

It can be concluded from the data of the present study that in the case of porcine lymphocytes ZEN does not determine the interactions of the

binary mixtures as strongly as FB₁ and/or DON. Additionally, it is evident that the interactions can vary depending on the time of exposure as well as on the concentrations of the individual mycotoxins. Thus, in the future, further studies with additional endpoints should be performed (e.g. DNA fragmentation, protein synthesis) in order to elucidate the mechanisms underlying the interactions observed.

5. NEW SCIENTIFIC RESULTS

1. The interactive effects of FB₁ (5 mg/kg), DON and ZEN (1 mg/kg + 0.25 mg/kg) in the feed of rabbit bucks were investigated for the first time. Additive or less than additive effect was found in the case of spermatogenesis and sperm cell morphology, synergism in testosterone production, while FB₁ acted antagonistically against DON+ZEN in genotoxicity and lipid peroxidation.
2. It was found that rabbits are resistant to DON at a high dosage (i.e. 10 mg/kg of feed). Although no pronounced immunomodulatory effect of DON was observed, the histological findings revealed lymphocyte depletion and follicular atrophy in the spleen and the rate of lymphoblast apoptosis increased in gut associated lymphoid tissue, suggesting that DON may alter the functioning of the immune system.
3. The herb *Carduus marianus* (5 and 10 g/kg of feed) was protective against liver fibrosis, lymphocyte depletion and follicular atrophy in the spleen, while it showed no protective effect regarding apoptosis in Peyer plaque caused by 10 mg/kg of DON in the feed.
4. The IC₅₀ values of FB₁, DON and ZEN were determined by a cytotoxicity test using isolated lymphocytes from healthy pigs. The IC values' range for the three exposure periods (24, 48 and 72h) was 0.31-0.42 µg/ml and 16.6-22.9 µg/ml for DON and ZEN, respectively. For FB₁ 50% viability was reached only after 72h and thus only one IC₅₀ value -101.15 µg.ml- could be calculated. As it is depicted by the IC₅₀ values, DON was the most cytotoxic whereas FB₁ was the least.

5. The interaction of FB₁ (5 µg/ml), DON (0.07 µg/ml) and ZEN (5 µg/ml) regarding cytotoxicity on porcine lymphocytes, revealed antagonistic effects for all the mixtures in a time-dependent (24, 48 and 72h) manner.
6. The interaction of FB₁, DON and ZEN as investigated by the means of comet assay revealed antagonism in lower concentration (5 µg/ml for FB₁ and ZEN, while 0.07 µg/ml for DON) whereas for higher concentrations (25, 0.21 and 10 µg/ml for FB₁, DON and ZEN, respectively) a synergistic effect was revealed for two of the binary mixtures.

6. SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE DISSERTATION

6.1 PEER-REVIEWED PAPERS PUBLISHED IN FOREIGN SCIENTIFIC JOURNALS

Szabó-Fodor, J., **Kachlek, M.**, Cseh, S., Somoskői, B., Szabó, A., Blochné Bodnár, Zs., Tornyos, G., Mézes, M., Balogh, K., Glávits, R., Hafner, D., Kovács, M. (2015): Individual and combined effects of subchronic exposure of three Fusarium toxins (fumonisin B, deoxynivalenol and zearalenone) in rabbit bucks. *Journal of Clinical Toxicology*, 5: 264. doi:10.4172/2161-0495.1000264

Kachlek, M., Szabó-Fodor, J., Bonai, A., Bors, I., Celia, C., Gerencsér, Zs., Matics, Zs., Szendrő, Zs., Dalle Zotte, A., Kovács, M. (2015): Assessing the possible interaction between *Carduus marianus* and dietary deoxynivalenol on caecal microbiota and fermentation of growing rabbits. *Poljoprivreda*; 21(1): 186-189. doi: 10.18047/poljo.21.1.sup.44

Kachlek, M., Szabó-Fodor, J., Bonai, A., Bors, I., Celia, C., Gerencsér, Zs., Matics, Zs., Szendrő, Zs., Dalle Zotte, A., Kovács, M. (2017): Subchronic exposure to deoxynivalenol exerts slight effect on the immune system and liver morphology of growing rabbits. *Acta Veterinaria Brno*; 86, 37-44. (IF_{2015/2016}: 0.37, Q3)

6.2 PEER-REVIEWED PAPERS PUBLISHED IN HUNGARIAN SCIENTIFIC JOURNALS IN ENGLISH

Kachlek, M., Szabó-Fodor, J., Blochné Bodnár, Zs., Horvatovich, K., Kovács, M. (2017): Preliminary results on the interactive effects of deoxynivalenol, zearalenone and fumonisin B₁ on porcine lymphocytes. *Acta Veterinaria Hungarica*, 65, 3: 340-353. (IF_{2015/2016}: 1.14, Q2)

Kachlek, M., Szabó-Fodor, J., Kovács, M. (2017): Interaction of *Fusarium* mycotoxins, fumonisin B₁, deoxynivalenol and zearalenone: A review. *Állattenyésztés és Takarmányozás*, 3: 181-195.

6.3 PROCEEDINGS IN ENGLISH

Celia, C., **Kachlek, M. L.**, Gerencsér, Zs., Matics, Zs., Szendrő, Zs., Dalle Zotte, A., Giaccone, V., Kovács, M.: Effect of *Carduus marianum* herb on the productive performances of growing rabbits. The 20th International Symposium on Housing and diseases of Rabbits, Fur providing Animals and Pet Animals, Celle, Germany, 27-28 May, 2015, pp. 149-152.

Kachlek, M., Szabó-Fodor, J., Kovács, M.: Rabbits in mycotoxin research at Kaposvár University. In: Matics Zsolt (ed) 119 p. The 28th Rabbit Science Day, Kaposvár, Hungary, May 25, 2016, pp. 71-76. (ISBN:[978-615-5599-30-9](https://doi.org/10.1007/978-615-5599-30-9))

6.4 ABSTRACTS IN ENGLISH

Kachlek, M., Szabó-Fodor, J., Blochné Bodnár, Zs., Kovács, M.: Possible antagonistic effect of three *Fusarium* mycotoxins on genotoxicity of spermatozoa of breeding rabbit bucks. The International Conference of Food Contaminants 2015, Challenges in Chemical Mixtures, Lisbon, Portugal, 13-14 April, 2015.

Szabó-Fodor, J., **Kachlek, M.**, Szabó, A., Blochné Bodnár, Zs., Hafner, D., Tornyos, G., Cseh, S., Somoskői, B., Kovács, M.: Individual and combined effect of *Fusarium* toxins *in vivo*. The 37th Mycotoxin Workshop 2015, Bratislava, Slovakia, 01-03 June, 2015.

7 OTHER PUBLICATIONS

7.1 PEER-REVIEWED PAPERS PUBLISHED IN FOREIGN SCIENTIFIC JOURNALS

Szabó-Fodor, J., Dall'Asta, C., Falavigna, C., **Kachlek, M.**, Szécsi, Á., Szabó, A., Kovács, M. (2015): Determination of the amount of bioaccessible fumonisin B₁ in different matrices after *in vitro* digestion. World Mycotoxin Journal, 8 (3), 261-267.

Cullere, M., Dalle Zotte, A., Celia, C., Renteria-Monterrubio, A.L., Gerencsér, Zs., Szendrő, Zs., Kovács, M., **Kachlek, M.L.**, Matics, Zs. (2016): Effect of *Silybum marianum* herb on the productive performance, carcass traits and meat quality of growing rabbits. Livestock Science 194, 31-36. doi: 10.1016/j.livsci.2016.10.012

7.2 PROCEEDINGS IN HUNGARIAN

Bónai, A., Toldi, M., Ölbeiné Horvatovich, K., Bagóné Vántus, V., Tuboly, T., **Kachlek, M.L.**, Hafner, D., Pápai, G., Zsolnai, A., Szabó-Fodor, J., Kovács, M.: Néhány probiotikus baktérium törzs mikrobiológiai vizsgálata – *in vitro* szimulált humán emésztést megelőzően és követően – tejipari termékfejlesztés céljából. Óvári Tudományos Napok, Mosonmagyaróvár, November 13, 2014.

7.3 ABSTRACTS IN ENGLISH

Cirlini, M., Falavigna, C., **Kachlek, M.**, Szabó-Fodor, J., Kovács, M., Dall'Asta, C.: N-deoxyfructosyl-fumonisin B1 may cause DNA damage in porcine mononuclear blood cells. The 8th conference of The World Mycotoxin Forum, Vienna, Austria, 10-12 November 2014.

Szabó-Fodor, J., Dall'Asta, C., Falavigna, C., **Kachlek, M.**, Szécsi, Á., Szabó, A., Kovács M.: Determination of the proportion of matrix-associated fumonisin B₁ in different, animal feeding experiment-aided matrices after *in vitro* digestion. The 8th conference of The World Mycotoxin Forum, Vienna, Austria, 10-12 November 2014.

7.4 ABSTRACTS IN HUNGARIAN

Tornyos, G., Szabó-Fodor, J., Cseh, S., Somoskői, B., Pósa, R., Hafner, D., **Kachlek, M.**, Kovács, M.: Mikotoxinok hatása a hím szaporodási folyamataira. 21 Szaporodásbiológiai találkozó, Visegrád, Szeptember 22, 2015.